Superior antimetastatic effect of pemetrexed-loaded gelatinase-responsive nanoparticles in a mouse metastasis model

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Novel pemetrexed-loaded gelatinase-responsive nanoparticles were prepared as a targeted delivery system to determine its potential for clinical therapy of malignant melanoma. The pemetrexed-loaded poly(ethylene glycol)(PEG)–peptide–poly(ε-caprolactone) (PCL) nanoparticles included a gelatinase-cleavage peptide and a PEG–PCL-based structure. The pemetrexed-loaded PEG–peptide–PCL nanoparticles have shown the best antimetastatic effect in experimental lung metastasis models. The expressions of CD133 and thymidylate synthetase of metastatic tumors were also evaluated in our studies. Our results showed that pemetrexed-loaded gelatinase-responsive nanoparticles may represent a potent drug delivery system for inhibiting pulmonary metastasis and our preclinical results can provide new avenues for clinical therapy of malignant melanoma. *Anti-Cancer Drugs* 23:1078–1088 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The targeting delivery system for anticancer therapy has shown considerable potential in the target-to-nontarget ratio, drug residence at the target site, and improved cellular uptake and intracellular stability. Active targeting plays an important role in the ultimate localization of therapeutic nanoparticles.

Matrix metalloproteases (MMPs), a zinc-dependent family of endopeptidase, modulate cell–cell and cell–extracelluar matrix interactions and affect the cell phenotype by regulating the expression of E-cadherin and integrin [1]. Most importantly, MMPs play a major role in cancer progression, including tumor growth, invasion, metastasis, and angiogenesis [2]. In particular, MMP2 and MMP9 have been found in most tumor cells and to be involved in degradation of the extracellular matrix, cancer cell proliferation and apoptosis, tumor angiogenesis and vasculogenesis, epithelial to mesenchymal transition, etc. [3]. Nano-drug-targeted MMPs have been reported in some studies and the MMP-targeted drug delivery system has shown antitumor superiority [4–5].

Pemetrexed disodium is a novel anticancer agent and has a wide range of applications in many cancers, including non-small-cell lung carcinomas and malignant mesotheliomas. Pemetrexed has also been reported for use in the treatment of other cancers in clinical trials, such as carcinomas of the head and neck, ovary, prostate, breast, and uterine cervix [6–12]. However, adverse effects have been reported, such as myelosuppression, hepatic enzyme elevations, maculopapular rash, emesis, fatigue, mucositis, and diarrhea [13]. Some studies have reported a correlation between the folic acid status of patients and the incidence of toxicity with pemetrexed [14,15]. A recent report of a clinical trial has indicated that toxicities are markedly decreased with folic acid supplementation in patients with advanced gastric cancer [16]. A preliminary comparative toxicity analysis of patients treated in different trials with or without vitamin supplementation has also shown that the addition of folic acid and vitamin B_{12} markedly reduced the incidence of adverse events, such as neutropenia, thrombocytopenia, and mucositis [17]. Some clinical studies have indicated that patients could benefit from pemetrexed treatment combined with vitamin supplementation [18,19]. Daily oral folic acid and vitamin B_{12} supplementation has been included as part of the treatment with pemetrexed of all patients undergoing clinical therapy. However, the targeted drug delivery system for pemetrexed has the potential to significantly improve the therapeutic outcome of cancer treatments while minimizing the side effects. There is no report on the use of therapeutic nanoparticles for the delivery of pemetrexed, and the use of a targeted drug delivery system for this is very important.

Both poly(ethylene glycol)(PEG) and poly(ε-caprolactone) (PCL) are FDA-approved biodegradable and biocompatible materials and have been used widely in the biomedical field [20,21]. In our previous work, biodegradable amphiphilic copolymer mPEG–PCL and gelatinase-responsive copolymer mPEG–peptide–PCL were synthesized for the local delivery of docetaxel [22]. The nanoparticles consisted of gelatinase-cleavable peptide and an mPEG–PCL-based structure. Characterization of nanoparticles, in-vitro cellular uptake, real-time biodistribution of nanoparticles, and in-vivo antitumor efficacy of docetaxel-loaded nanoparticles were studied systemically [22]. Compared with mPEG–PCL nanoparticles without the gelatinase-cleavable peptide, the PEG–peptide–PCL nanoparticles showed higher intracellular uptake, higher local tumor accumulation and retention in the long run, and better in-vivo antitumor efficacy [22]. In this paper, we further evaluated the pharmacologic activity of the PEG–peptide–PCL nanoparticles on lung metastasis of melanoma. We synthesized pemetrexed-loaded PEG–peptide–PCL nanoparticles. The characterizations, drug-loading content, and encapsulation efficiency, security and release of nanoparticles were studied in this paper. Especially, we determined the antimetastatic effect of nanoparticles and examined the expressions of CD133 and thymidylate synthetase (TS) of metastatic tumors in experimental lung metastasis models.

Materials and methods
Materials
The pemetrexed disodium was provided by Eli Lilly Company (Indianapolis, Indiana, USA). Methoxy-poly-ethyleneglycol-NHS was custom-made from Beijing Jiankai Technology Co. (Beijing, China). The gelatinase-cleavable peptide (PVGLIG) was custom-made from Shanghai HD Biosciences Co. (Shanghai, China). Gelatinases were purchased from Sigma-Aldrich (St Louis, Missouri, USA). ε-Caprolactone (ε-CL, Sigma-Aldrich) and dimethyl formamide were purified by dehydration over CaH_2 at room temperature and extracted under reduced pressure. DMEM culture was obtained from Gibco (Grand Island, New York, USA). Cell Counting Kit-8 was obtained from Dojindo (Kumamoto, Japan). All other chemicals were of analytical grade and were used without further purification. Murine melanoma cell line B16 was obtained from the Shanghai Institute of Cell Biology (Shanghai, China). Male C57/B6 mice were purchased from the animal center of Drum Tower Hospital (Nanjing, China).

Synthesis of nanoparticles
Synthesis of PEG–peptide–PCL and PEG–PCL copolymers
The PEG–peptide–PCL copolymers were synthesized as described previously [32]. The PEG–PCL copolymers were synthesized for pemetrexed-loaded PEG–PCL nanoparticles using the method previously described [23].

Preparation of pemetrexed-loaded nanoparticles
Pemetrexed-loaded nanoparticles were prepared according to a previously described method [23]. Briefly, a certain amount of vehicle and pemetrexed was dissolved in 1 ml of dichloromethane. The mixture was emulsified in 3 ml of a 5% polyvinyl alcohol solution (w/v) by sonication (XL2000, Misonix, Farmingdale, New York, USA) for 30 s and was converted into an oil/water emulsion. The emulsion obtained was added to 8 ml of a 1% polyvinyl alcohol solution (w/v) and subjected to sonication again, and then stirred to remove dichloromethane at room temperature in a fume cupboard. The resulting solution was filtered through a filter membrane to remove nonincorporated drugs and copolymer aggregates. The nanoparticle solution was freeze dried with 3% mannitol and stored at 4°C. Drug-free nanoparticles were produced in a similar manner, but without the addition of pemetrexed.

Characterization of nanoparticles
The particle size and polydispersity of the pemetrexed-loaded PEG–PCL nanoparticles and the pemetrexed-loaded PEG–peptide–PCL nanoparticles were measured by dynamic light scattering (DLS; Brookhaven Instruments Corporation, Holtsville, New York, USA). Particle size was the average of triplicate measurements for a single sample. The morphological characteristics of the nanoparticles were examined by transmission electron microscopy (TEM; JEM-100S; NEC Corporation, Tokyo, Japan). One drop of the pemetrexed-loaded PEG–peptide–PCL nanoparticle suspension was placed on a copper grid overlaid with a nitrocellulose membrane and air-dried before observation.

Drug-loading content and encapsulation efficiency
A predetermined amount of freeze-dried nanoparticles was weighed and dissolved in the mobile phase. The drug-loading content and encapsulation efficiency were measured by high-performance liquid chromatography (C18 column; Agilent Technologies Ltd, Santa Clara, California, USA). The mobile phase consisted of $0.01 \, mol/l \, NaH_2PO_4$ solution (pH 3.0) and methyl cyanides (82 : 18, v/v); the column was eluted at a flow rate of 1.0 ml/min at room temperature with a wavelength of 227 nm for the detection of pemetrexed.

The drug-loading content and encapsulation efficiency were calculated using the following equations:

$$\text{Drug-loading content}(\%) = \text{Weight of the drug in nanoparticles}/\text{weight of the nanoparticles} \times 100\%, \quad (1)$$

$$\text{Encapsulation efficiency}(\%) = \text{Weight of the drug in nanoparticles}/\text{weight of the feeding drug} \times 100\%. \quad (2)$$

In-vitro release of pemetrexed-loaded nanoparticles

The lyophilized pemetrexed-loaded PEG–PCL nanoparticles and pemetrexed-loaded PEG–peptide–PCL nanoparticles were suspended in PBS, pH 7.4, with or without 1 mg/ml gelatinases and the pemetrexed concentration of the solutions was 100 μg/ml. The pemetrexed solution was placed in a dialysis bag with a 12 kDa molecular weight cutoff (Sigma-Aldrich). The dialysis bags were immersed into PBS at 37°C. Periodically, the total release medium was withdrawn and replaced with fresh PBS, pH 7.4. The amount of pemetrexed released was measured by high-performance liquid chromatography. The experiments were repeated three times.

Zymography

B16 cells (1×10^7) were cultured in Dulbecco's modified Eagle medium (DMEM) medium without blood serum for 24 h and the culture solution was concentrated using centrifugal concentrators (Vivaspin, Sartorius, Goettingen, Germany). The concentration of the loaded protein was 10 μg as determined using the BCA protein assay kit (Beyotime, Shanghai, China). Gelatinolytic activity was analyzed using the method described previously [24]. For zymography, a 5% spacer gel and 10% separation gel containing 0.1% gelatin (Sigma-Aldrich) were used. After electrophoresis, the gel was washed with washing buffer (2.5% TritonX-100) to remove the SDS for 1 h. After incubation with incubation buffer (20 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 5 mmol/l $CaCl_2$) overnight at 37°C, the gels were stained with Coomassie blue R250 for 4 h, and then destained with 30% methanol and glacial acetic acid. The destaining process was not interrupted until the clear band appeared.

Cell viability assay

B16 cells (5×10^3) were placed in a 96-well plate and cultured by DMEM medium at 37°C. When the cells adhered, they were treated with several doses of free pemetrexed, pemetrexed-loaded PEG–PCL nanoparticles, and pemetrexed-loaded PEG–peptide–PCL nanoparticles. After incubation for 48 h, 20 μl of the Cell Counting Kit-8 (Dojindo) was added to each well for an additional 2 h. The absorbance at 450 nm was read by a microplate reader (Bio-Rad, Hercules, California, USA). The entire experiment was repeated three times.

Antimetastatic effect in experimental lung metastasis models

The animal experiments were performed following the guidelines in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985), and was approved by the Animal Care Committee at Drum Tower Hospital, Nanjing, China.

B16 cells (1×10^5) resuspended in 200 μl PBS were injected into the tail vein of six-week-old C57/B6 mouse and the day was designated as 'Day 0'. At day 11, mice were randomized into five groups and each group included five mice. The mice were treated intravenously with pemetrexed-loaded PEG–peptide–PCL nanoparticles, pemetrexed-loaded PEG–PCL nanoparticles, free pemetrexed, empty nanoparticles, and saline on days 11, 14, 17, and 20, respectively. The pemetrexed solution was administered at a dose of 4 mg/kg. The pemetrexed-loaded nanoparticles were administered as a saline solution at the equivalent pemetrexed dose of 4 mg/kg. The weight of each mouse was measured every day throughout the experiment. On day 21, the mice were euthanized and the lung tissues were collected for further study. After necropsy, lung metastasis was evaluated qualitatively by macroscopic photographs and quantitatively by determining lung weight [25].

Hematoxylin and eosin staining

The mice were killed and the tissue samples of the lung were instantly fixed in buffered formalin overnight, followed by dehydration with a tissue processor for 16 h. The tissues were embedded by paraffin. The 7 μm sections were prepared for H&E staining and microscopic examination.

Immunohistochemistry

After B16 tumor samples were fixed by formalin and embedded using embedding equipment, 4 μm sections were prepared. The sections were baked at 60°C for 60 min, followed by 10-min washes with xylene. The rehydration of tissues was performed by 5-min washes in 100, 95, 85, and 70% ethanol and distilled water, respectively. Antigen retrieval was performed by heating the samples at 100°C for 20 min in 10 mmol/l sodium citrate (pH 6.0). Endogenous peroxidase activity of the tissue was blocked by incubation in 3% hydrogen peroxide in methylalcohol for 15 min. The sections were incubated with rabbit anti-mouse polyclonal antibody (Wuhan Boster Biotechnology, Wuhan, China) at 37°C for 2 h. The sections were then incubated for 30 min with the secondary antibody (EliVision plus polyer HRP IHC Kit, Fuzhou Maixin Biotechnology, Fuzhou, China). Subsequently, the samples were redyed by hematoxylin. Dehydration was then performed following a standard procedure, and the slides were sealed with cover slips.

RNA extraction and reverse transcription polymerase chain reaction analysis

The RNA of metastatic tumor of lungs was extracted according to a previously described method [26]. The samples were resuspended in 300 μl RNA lysate [10 mmol/l Tris-HCl (pH 8.0), 0.1 mmol/l EDTA (pH 8.0), 2% SDS (pH 7.3), and 500 μg/ml proteinase K] for 16 h at 60°C. The RNA was extracted by chloroform and phenol. The isopropanol and sodium acetate were used for precipitation at -20°C for 1 h. The RNA was washed

Table 1 Diameters and polydispersity of the nanoparticles

Nanoparticles	Diameter (nm)[a]	Polydispersity[a]
PEG–PCL	171.1±7.0	0.112±0.057
PEG–peptide–PCL	231.3±5.3	0.187±0.139

PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol).
[a]The SD value was for the mean particle size obtained from the three measurements.

with 70% ethanol and dissolved in 50 μl of RNase-free water. The RNA was reverse-transcribed to cDNA with reverse-transcriptase (Takara, Kyoto, Japan). Quantitative PCR was performed using the SYBR green qPCR kit (Takara) by a fluorescent temperature cycler (Mx3000P Real-Time PCR System, Stratagene; Agilent Technologies Inc.). The target cDNA sequences were amplified with the following primers: GAPDH 5′-AAATGGTGA AGGTCGGTGTG-3′and 5′-TGAAGGGGTCGTTGATG G-3′; thymidylate synthase 5′-GGAGTAGACCAGCTGA AAAGTG-3′ and 5′-GATATGTGCAATCATGTAGGTGA G-3′. CD133: 5′-ACCAACACCAAGAACAAGGC-3′ and 5′-GGAGCTGACTTGAATTGAGG-3′. The following PCR conditions were used: denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, and annealing and extension at 60°C for 1 min. Each real-time PCR was repeated three times and the levels of mRNA expressions were calculated according to the comparative C_t method using GAPDH as an internal standard. The results were analyzed using the $2^{-\Delta\Delta C_t}$ method [27].

Statistical analysis
Analysis was presented as means ± SD. Differences within the treatment groups were analyzed by two-factor analysis of variance methods and the t-test. Differences were considered significant when P was less than 0.05.

Results
Characterization of the nanoparticles
The size of pemetrexed-loaded nanoparticles in an aqueous solution was measured by DLS (Table 1). Figure 1 shows the TEM micrographs of pemetrexed-loaded PEG–peptide–PCL nanoparticles. TEM images show that the nanoparticles were almost spheroid with a size of 200–250 nm each, which was consistent with the data from DLS (around 230 nm).

Drug-loading content and encapsulation efficiency
Table 2 shows the encapsulation efficiency and the drug-loading content of pemetrexed-loaded PEG–PCL nanoparticles under the different feeding ratios of copolymer and pemetrexed. With increased feeding, the encapsulation efficiency and the drug-loading content increased. The P3 nanoparticles had the highest encapsulation efficiency and drug-loading content: 98 and 37.5%, respectively. The yield of P3 nanoparticles was higher than 95%, indicating no obvious loss of material during

Fig. 1
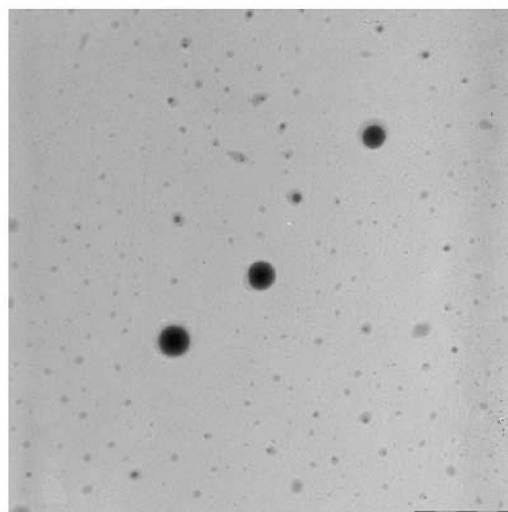
Transmission electron microscopy micrographs of PEG–peptide–PCL nanoparticles.

the preparation process. The PEG–peptide–PCL nanoparticles were formed by the feeding ratio of P3, and the encapsulation efficiency and the drug-loading content were calculated to be 86.8 and 37.45%. In the following evaluation *in vitro* and *in vivo*, we used the pemetrexed-loaded nanoparticles formed by the feeding ratio of P3 because of its relatively higher encapsulation efficiency and drug loading.

In-vitro release of pemetrexed-loaded nanoparticles
Figure 2a shows the release profile of the pemetrexed-loaded nanoparticles and both nanoparticles showed similar release profiles in the absence of gelatinases. An initial burst of more than 30% release in 3 h and a sustained release from these nanoparticles in the following period were observed as shown in Fig. 2a. Actually, 24 h of incubation with PBS caused approximately a total 60% release. Figure 2b shows the release profile of the pemetrexed-loaded nanoparticles in gelatinases solution. The release of pemetrexed from pemetrexed-loaded PEG–peptide–PCL nanoparticles was faster than that of pemetrexed-loaded PEG–PCL nanoparticles in gelatinases solution (26.7 vs. 20% at 1 h, and 73.4 vs. 61.8% at 96 h). These results suggest that the gelatinases can accelerate the release of pemetrexed from pemetrexed-loaded PEG–peptide–PCL nanoparticles, and the pemetrexed-loaded nanoparticles may be used as a controlled-release system for the following in-vitro and in-vivo evaluations.
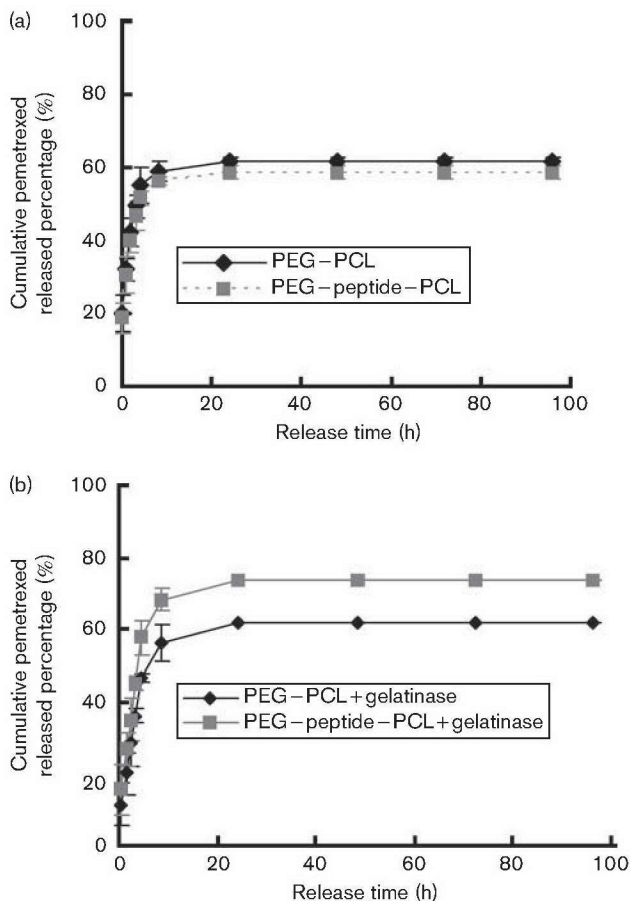
Zymography
Zymography was performed to measure the MMPs activities in melanoma cells. Figure 3a shows the results of zymography and indicates that melanoma cells secreted

Feeding ratio (pemetrexed/copolymer)	P1 (2 mg/10 mg)	P2 (4 mg/10 mg)	P3 (6 mg/10 mg)
DLC (%)	6.39	30.3	37.5
EE (%)	22.7	80.9	98

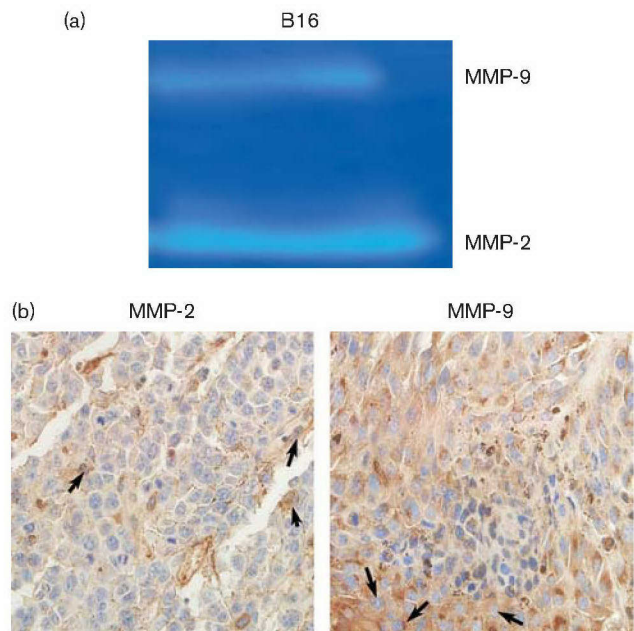DLC, drug-loading content; EE, encapsulation efficiency.

Fig. 2

(a)

(b)

The gelatinase-stimuli characterization of PEG–peptide–PCL nanoparticles. (a) Cumulative in-vitro release profile of pemetrexed from nanoparticles in the absence of gelatinases. Data are presented with SD. (b) In-vitro release of pemetrexed from nanoparticles in the presence of gelatinases. Data are shown with mean±SD. PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol).

Fig. 3

(a) B16

MMP-9

MMP-2

(b) MMP-2   MMP-9

Detection of gelatinase (MMP2/9) expression (a) MMP2 and MMP9 activities was carried out by zymography. (b) Immunohistochemical analysis of the gelatinases (MMP2/9) expression in B16 tumors (× 400). MMP, matrix metalloprotease.

MMP2 and MMP9 proteins. The previous studies also obtained consistent results [24,28,29].

Immunohistochemical analysis

We observed MMP2/9 expression by immunohistochemical analysis and MMP2/9 expression was considered to be positive when the cytoplasm of B16 cells turned brown. Our results showed that B16 cells expressed MMP2 and MMP9, and consistent results were obtained from zymography (Fig. 3b).

In-vitro cytotoxicity

Figure 4a shows the cytotoxicity of the free pemetrexed, two kinds of pemetrexed-loaded nanoparticles, and PBS. The treated groups showed similar concentration-dependent cytotoxicity. There was no significant difference in cytotoxicity between free pemetrexed and the two kinds of pemetrexed-loaded nanoparticles.

Inhibition of pulmonary metastasis after treatment with PEG–peptide–PCL nanoparticles

Figure 4b shows the experimental design. B16 cells were administered by a tail vein injection to enable lung metastasis during a 10-day period. The metastasis burden refers to the relative increase in the total lung weight. Lung metastatic burden in mice treated with the pemetrexed-loaded PEG–peptide–PCL nanoparticle group was the smallest among all the groups (Fig. 4c, the lung of a normal mouse expressed 1.0±0.00095% of body weight). We did not observe apparent changes in body weight in any of the groups; no toxicity or adverse effects of empty nanoparticles were observed during the entire treatment period (Fig. 4d). Macroscopic figures of lung metastasis from five treatment groups are shown and the black arrowheads indicate metastatic nodules (Fig. 5). Both free pemetrexed and the two kinds of pemetrexed-loaded nanoparticles effectively inhibited tumor metastasis. Pemetrexed-loaded PEG–peptide–PCL nanoparticles showed the best anti-metastatic effect among all the treatment groups. The lung tissues in five groups were observed in the H&E-stained

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