

An integrin $\alpha_v\beta_3$ antagonistic modified peptide inhibits tumor growth through inhibition of the ERK and AKT signaling pathways

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Abstract. HM-3, an RGD (Arg-Gly-Asp)-modified antitumor polypeptide designed independently, has been demonstrated for its robust inhibitory effects on tumors. However, the intravenous administration and short half-life *in vivo* are inconvenient to its clinical application. To solve these issues, PEGylated HM-3 (mPEG-SC_{20k}-HM-3) with prolonged half-time *in vivo* and subcutaneous administration was obtained after repeated screening of different types of PEG and numerous efficacy assays. The present study aimed to evaluate the antitumor activity and investigate the mechanism of the modified peptide to interpret the antitumor properties of mPEG-SC_{20k}-HM-3 comprehensively and clearly. The results of the antitumor activity assays *in vitro* indicated that mPEG-SC_{20k}-HM-3 exhibited a marked inhibitory activity on tumor metastasis and angiogenesis. mPEG-SC_{20k}-HM-3 (73.4 mg/kg, sc) achieved a tumor inhibitory rate of 70.1% in an H460 (human non-small cell lung cancer) xenograft model with scarce cytotoxicity, compared with a rate of 72.2% for Avastin (10.0 mg/kg, iv). The mechanistic study showed that mPEG-SC_{20k}-HM-3 could target integrin $\alpha_v\beta_3$ to block the downstream ERK and Akt pathways, as the expression levels of VEGF, Akt1, p-Akt1, ERK1/2, p-ERK1/2, MEK1, p-MEK1, integrin α_v and β_3 were reduced after HUVECs were incubated with mPEG-SC_{20k}-HM-3 for 24 h. In conclusion, the antitumor activity of mPEG-SC_{20k}-HM-3 *in vitro* and *in vivo* is promising and the mechanism was clearly reflected in the present study.

Introduction

As the theory of tumor angiogenesis proposed by Folkman in 1971 gained a firm foothold, studies concerning the inhibition of tumor angiogenesis mushroomed worldwide. Angiogenesis is the process by which new blood vessels are formed from preexisting vasculature and is an essential step in normal tissue growth and regeneration, immunity and nutrition (1). Based on the treatment strategy for this theory, tumor growth and metastasis are suppressed due to the lack of nutrients and oxygen following treatment with inhibitors of tumor angiogenesis (2). In recent years, several angiogenesis inhibitor agents such as bevacizumab (monoclonal anti-VEGF antibody drug) and sunitinib (second-generation multivariate receptor tyrosine kinase inhibitor drug) have already 'hit' the market of antitumor drugs (3,4). As a branch of the angiogenesis theory, integrin receptors are attracting increased attention in the field of cancer research. According to the theory, the expression levels of integrin receptors in tumor tissues are relatively higher than levels in normal tissues, which stimulate the over-expression of angiogenesis-related proteins in downstream pathways leading to tumor metastasis and angiogenesis.

HM-3, designed and synthesized with an (Arg-Gly-Asp) RGD integrin ligand sequence, is a type of integrin blocker. A preliminary study demonstrated that HM-3 significantly inhibited the migration of endothelial cells *in vitro*, as well as achieved a high inhibitory rate in SMMC-7721 (human hepatoma), MGC-803 (human gastric) and H460 (human non-small cell lung cancer) xenograft models (5). However, similar to most polypeptide compounds, susceptibility to the degradation of proteolytic enzymes and the lower stability *in vivo* are two main defects associated with HM-3. Particularly in pharmacokinetic studies *in vivo*, HM-3 has a relatively short half-life (27.66±7.37 min in SD rats) (6), which bring inconvenience to its clinical application. Therefore, prolonging the half-life of HM-3 *in vivo* by modification has been a significant topic in our laboratory.

In the past several years, numerous covalent modifications with polyethylene glycol (PEG) to HM-3 have been carried out in our research. Four different types of PEG [mPEG-ALD_{5k}, mPEG-ALD_{10k} (7), mPEG-SC_{10k} and mPEG-SC_{20k}] were

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obtained to modify HM-3, and mPEG-SC_{20k} was determined to be the optimal molecule after taking the reaction conditions (8), the antitumor activity of the modified products and half-life *in vivo* into consideration. The preliminary antitumor activity *in vivo* of mPEG-SC_{20k}-HM-3 in a human liver cancer SMMC-7721 xenograft model showed that mPEG-SC_{20k}-HM-3 inhibited tumor (SMMC-7721 xenograft) growth better than HM-3. Pharmacokinetic assays *in vivo* indicated that the elimination half-life *in vivo* of mPEG-SC_{20k}-HM-3 subcutaneously administered was 20.13±0.64 h, which was 43.76 times compared to HM-3. The primary target research confirmed that integrin $\alpha_v\beta_3$ was the main target of mPEG-SC_{20k}-HM-3 in tumor cells (9).

To better evaluate the antitumor property of mPEG-SC_{20k}-HM-3, the present study aimed to systematically investigate the antiangiogenesis and antitumor metastasis activity *in vitro*, the antitumor activity *in vivo* and explore the antitumor mechanism. The research provides a good foundation for the medicinal development and application of mPEG-SC_{20k}-HM-3.

Materials and methods

Reagents. HM-3 [sequence: IVRRADRAAVPGGGGRGD; synthesized by GL Biochem Ltd. (Shanghai, China)] and mPEG-SC_{20k}-HM-3 were prepared and purified (with a purity of >98.5% as analyzed by analytical high-performance liquid chromatography) as previously described (8). Oxaliplatin (Lot no. 10702428) and docetaxel (Lot no. 12072811) were purchased from Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China) and Avastin (Lot no. B3451) was purchased from Genentech Inc. (South San Francisco, CA, USA), and were stored at 4°C.

Cell culture. The following human cell lines were used in the present study: human umbilical vein endothelial cells (HUVECs) obtained from American ScienCell Research Laboratory (Carlsbad, CA, USA), human hepatoma cells (SMMC-7721), human gastric cancer cells (MGC-803), and human non-small cell lung cancer cells (H460) all purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HUVECs were maintained in endothelial cell medium (ECM) supplemented according to the instructions of the manufacturer. SMMC-7721, MGC-803 and H460 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) purchased from Gibco (Grand Island, NY, USA).

HUVEC migration assay. The HUVEC migration assay was performed to investigate the effect of mPEG-SC_{20k}-HM-3 on antitumor metastasis *in vitro* assays. Matrigel (purchased from BD Biosciences) was coated uniformly on the surface of the Transwell bottom after being diluted 1:2 with serum-free medium and cells were seeded into 24-well culture plates and grown to 90% confluency. Subsequently, the Transwell chamber and medium were discarded after 24 h of incubation and migrated cells were stained with 0.1% crystal violet for 10 min after being fixed with ethanol for 30 min. Images of the cells were captured using a Zeiss Axio Vert.A1 inverted microscope (Zeiss, Oberkochen, Germany) to calculate the migration inhibitory rate.

Anti-angiogenesis assays *in vitro*. Tube formation, chick embryo chorioallantoic membrane and the rat aortic ring assays were performed to investigate the effect of mPEG-SC_{20k}-HM-3 on anti-angiogenesis *in vitro*. In the tube formation assay, HUVECs were seeded on a 96-well plate coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Concerning the chick embryo chorioallantoic membrane assay, 21 SPF chick embryos were purchased and stored in an aseptic incubator at 37°C with 70% humidity after disinfection with iodine tincture and 75% ethanol. A 1x1 cm window was opened on the top of the embryo on the following day and sealed with tapes after the upper air chamber membrane was turned on, with subsequent incubation for 24 h. A piece of neutral filter paper of 1 cm diameter was settled in the embryo as a sample carrier on the third day. Stationary liquid (identical volume of formaldehyde and acetone mixed) was added into the chick chamber after administration. With regard to the rat aortic ring assay, extravascular fibrous and fatty tissues of the artery obtained from rats were removed after being cleaned with phosphate-buffered saline (PBS) containing high concentrations of antibiotics. Condensing Matrigel was dropped into 96-well culture plates to immerse the aortic rings. Samples, dissolved with DMEM, were dosed into the ring when the Matrigel was curdled. Images were captured by a Zeiss Axio Vert.A1 inverted microscope.

Xenograft experiments. Animal experiments were authorized by the Ethics Committee of China Pharmaceutical University and carried out according to the Guidelines for the Care and Use of Laboratory Animals of China Pharmaceutical University and according to principles of laboratory animal care and protection. Seventy-two five-week-old SPF female BALB/c nude mice were purchased from SLRC Animals Laboratory Co., Ltd. (Shanghai, China). Before the experiment, eight animals/cage were kept in a pathogen-free environment and every procedure was carried out in a laminar airflow cabinet. Human non-small cell lung cancer H460 cells were selected to construct the xenograft model. Cells (1x10⁶) were subcutaneously injected into the right flank of each mouse. When tumor nodules reached a mean size of 80 mm³, the animals were treated with 10 mg sample/kg body weight using different modes of administration (dosing regimen is shown in Table I). Animals in the negative control group subcutaneously received the NS solvent. The experiment was terminated after 23 days from transplantation by cervical dislocation. The following parameters were evaluated: daily body weight, tumor growth (assessed daily by caliper measurement), and final tumor weight. Tumor volume was calculated using the formula: length x width²/2.

Mechanistic study. With regard to the mechanism of mPEG-SC_{20k}-HM-3 involved in its antitumor activity, we performed two assays at different levels, including the genetic and the protein levels. Originally, Tumor Metastasis PCR array, Angiogenesis PCR array and Human Cancer PathwayFinder PCR array (SABiosciences: <http://www.sabiosciences.com>) were performed to identify key genes that were upregulated or downregulated after incubation with effective dose of mPEG-SC_{20k}-HM-3, by which a possible mechanistic pathway could be predicted. All PCR array assays were operated by

Table I. Groups and administration in the H460 xenograft models.

Groups	Drugs	Days, dosage and administration
1	Control (NS)	0-23 days, 0.2 ml, subcutaneous
2	PEG	0-23 days, 67.4 mg/kg/2 days, subcutaneous, 0.2 ml
3	Avastin	0-23 days, 5 mg/kg, tail vein injection, 0.2 ml
4	mPEG-SC _{20k} -HM-3	0-23 days, 73.4 mg/kg/2 days, subcutaneous, 0.2 ml
5	mPEG-SC _{20k} -HM-3	0-23 days, 36.7 mg/kg/2 days, subcutaneous, 0.2 ml
6	mPEG-SC _{20k} -HM-3	0-23 days, 18.35 mg/kg/2 days, subcutaneous, 0.2 ml
7	mPEG-SC _{20k} -HM-3	0-23 days, 36.7 mg/kg/2 days, tail vein injection, 0.2 ml
8	HM-3	0-23 days, 3 mg/kg/days, tail vein injection, 0.2 ml

Kangchen Biological Engineering Co., Ltd. (Shanghai, China) and negative HUVECs and testing HUVECs were provided by our laboratory. Based on these findings, western blot assay was performed to verify the expression variation of key proteins in the pathways previously predicted. HUVECs were seeded in 6-well culture plates and treated with different doses of mPEG-SC_{20k}-HM-3 for 24 h, and resuspended in 100 μ l of mammalian protein extraction reagent (Shanghai Generay Biotech Co. Ltd., Shanghai, China). Concentrated protein was separated on 12% SDS-polyacrylamide gel (SDS-PAGE) for each protein, and then the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Briefly, the PVDF membrane was incubated with a 1:1,000 dilution of VEGF, Akt1, p-Akt1, ERK1/2, p-ERK1/2, MEK1, p-MEK1, integrin α_v , β_3 , α_5 , β_1 and β -actin antibodies (Cell Signaling Technology, Danvers, MA, USA). Following incubation with a 1:2,000 dilution of secondary antibodies, the blots were incubated with ECL reagents (Beyotime, Jiangsu, China) and exposed to Tanon 5200-Multi to detect protein expression. However, the HUVECs were first treated with α_v , β_3 antibodies and then incubated with mPEG-SC_{20k}-HM-3 to detect the expression variation of VEGF.

Statistical analysis. Statistical analysis was carried out using the Student's t-test to evaluate the significance of differences between different groups. $P < 0.05$ was considered significant for all tests.

Results

HUVEC migration assay. As for the antitumor metastasis activity *in vitro* of mPEG-SC_{20k}-HM-3, HUVEC migration assay was performed. Compared with the negative control, mPEG-SC_{20k}-HM-3 exhibited an obvious inhibitory effect on HUVEC migration in a dose-dependent manner at concentrations ranging from 2.0 to 4.0 μ M and from 4.0 to 10.0 μ M, as shown in Fig. 1, in which 4.0 μ M (65.80%) was the optimal concentration for mPEG-SC_{20k}-HM-3 to inhibit the migration of HUVECs compared with 10.0 mg/kg Avastin (70.85%). The above results indicated that effective doses of mPEG-SC_{20k}-HM-3 significantly inhibited HUVEC migration *in vitro*.

Anti-angiogenesis assays *in vitro*. Three independent assays were carried out to study the anti-angiogenesis activity

of mPEGSC_{20k}-HM-3: tube formation, rat aortic ring and chick embryo chorioallantoic membrane assays. In the tube formation and rat aortic ring assays, the optimum inhibitory concentration was 4.0 μ M and the inhibitory rates were 76.34% (Fig. 2A) and 58.9% (Fig. 2B), compared with 86.02 and 80.6% for 10 mg/kg Avastin, respectively. As for the chick embryo chorioallantoic membrane assay, the angiogenesis inhibitory rate reached the highest level (71.8%) when the concentration of mPEG-SC_{20k}-HM-3 was 1.5 μ M (Fig. 2C), compared with 51.3% for 10 mg/kg Avastin. The assays *in vitro* above indicated that effective doses of mPEG-SC_{20k}-HM-3 significantly inhibited angiogenesis.

Xenograft experiments. The antitumor activities *in vivo* of mPEG-SC_{20k}-HM-3 were validated in a human non-small cell lung cancer (H460) xenograft model. The results (Fig. 3) indicated that the T/Cs of each group of mPEG-SC_{20k}-HM-3 against NCI-H460 nude xenografts were 62.7% (18.35 mg/kg, sc), 43.8% (36.7 mg/kg, sc), 30.6% (73.4 mg/kg, sc) and 30.0% (36.7 mg/kg, iv). The inhibitory rates were 35.8, 56.6, 70.1 and 72.3%, respectively. The T/Cs of HM-3 (3 mg/kg, iv), Avastin (10 mg/kg, iv) and PEG (67.4 mg/kg, sc) were 43.7, 29.2 and 91.6%, while the inhibitory rates were 62.9, 72.2 and 4.2%, respectively. To assess the effects of the treatments on toxicity, body weight and animal behavior were monitored throughout the study. None of the mPEG-SC_{20k}-HM-3 groups was associated with any apparent signs of toxicity such as reduced food and fluid consumption, fatigue (data not shown) or body weight alterations (Fig. 3). In contrast, the weight of the mice increased evidently after injection of different dose of mPEG-SC_{20k}-HM-3 during the 23 days of administration. Taken together, the data obtained from the *in vivo* experiments further confirmed the potential of mPEG-SC_{20k}-HM-3 administered subcutaneously against human non-small cell lung cancer with a high inhibitory rate.

Mechanistic study. To better understand the antitumor property of mPEG-SC_{20k}-HM-3, the antitumor mechanism of mPEG-SC_{20k}-HM-3 was determined at the gene level (RT² Profiler PCR assays) and protein level (western blot assays). According to the results of three PCR arrays (Fig. 4A-C), 12 genes involved in the three PCR arrays were downregulated in the test group when compared with the negative group, and 27 genes were upregulated. We classified these upregulated or downregulated genes into groups with Gene Ontology (GO)

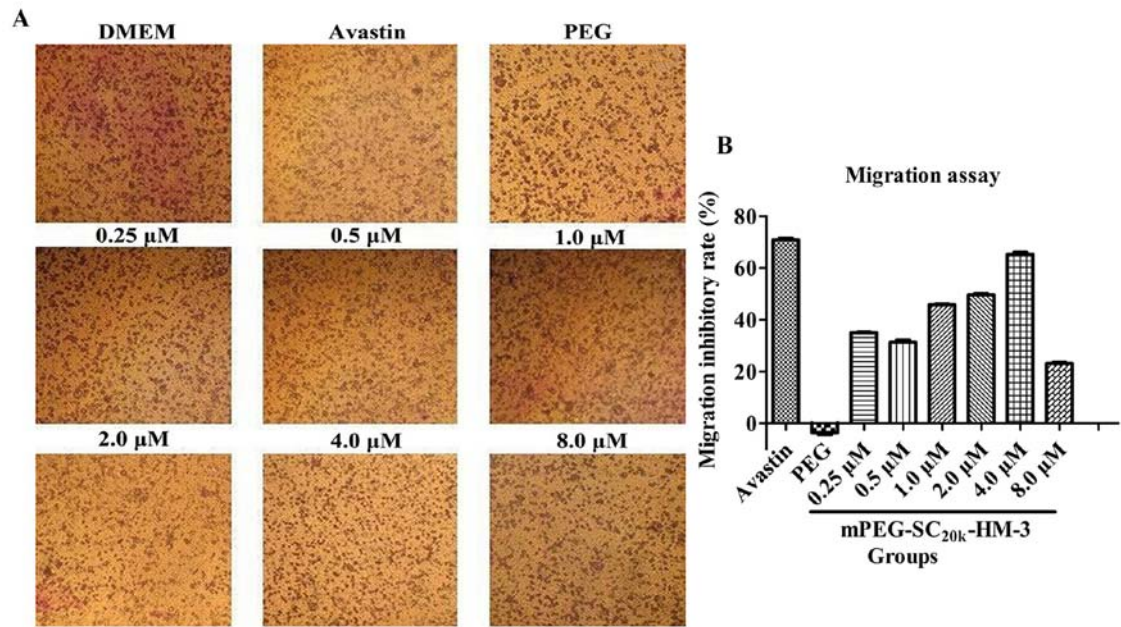


Figure 1. HUVEC migration assay. (A) Images of mPEG-SC_{20k}-HM-3 against HUVEC migration. (B) Inhibitory rates of each treatent group against HUVEC migration. Three independent experiments were performed.

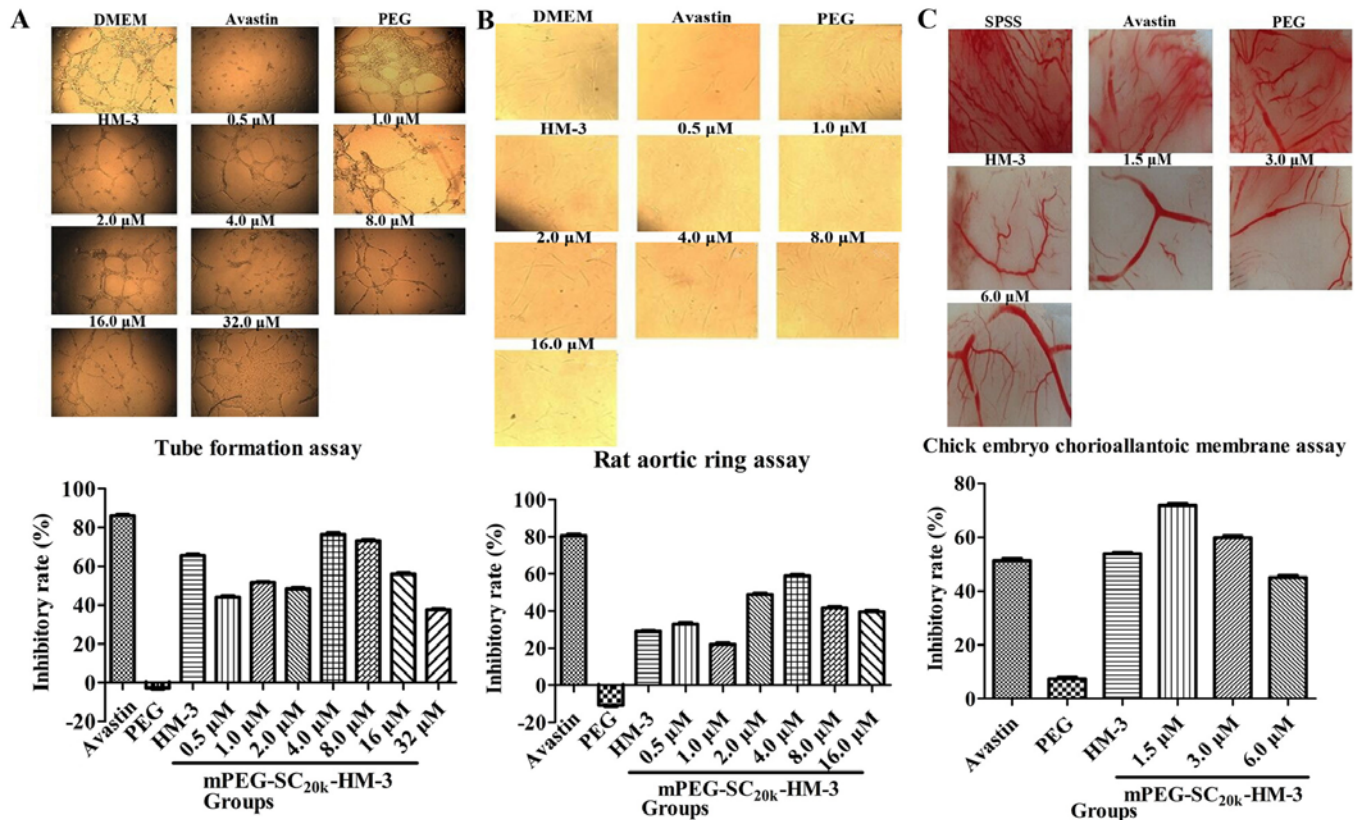


Figure 2. Anti-angiogenesis assays. (A) Images of HUVECs in the tube formation assay following the different treatments for 6 h and the inhibitory rates of each group. (B) Images of rat arterial ring growth and the inhibitory rates of each group. (C) Images of the chorioallantoic membrane (CAM) following the different treatments and the inhibitory rates of each group. Three independent experiments were performed.

analysis system (Table II), and found that pathways of angiogenesis and tumor metastasis were involved. The previous study indicated that the main target of mPEG-SC_{20k}-HM-3 in cells was integrin $\alpha_v\beta_3$. Based on the results of RT² Profiler PCR assays and references on the integrin-related pathways together, expression divergences of various key proteins of the

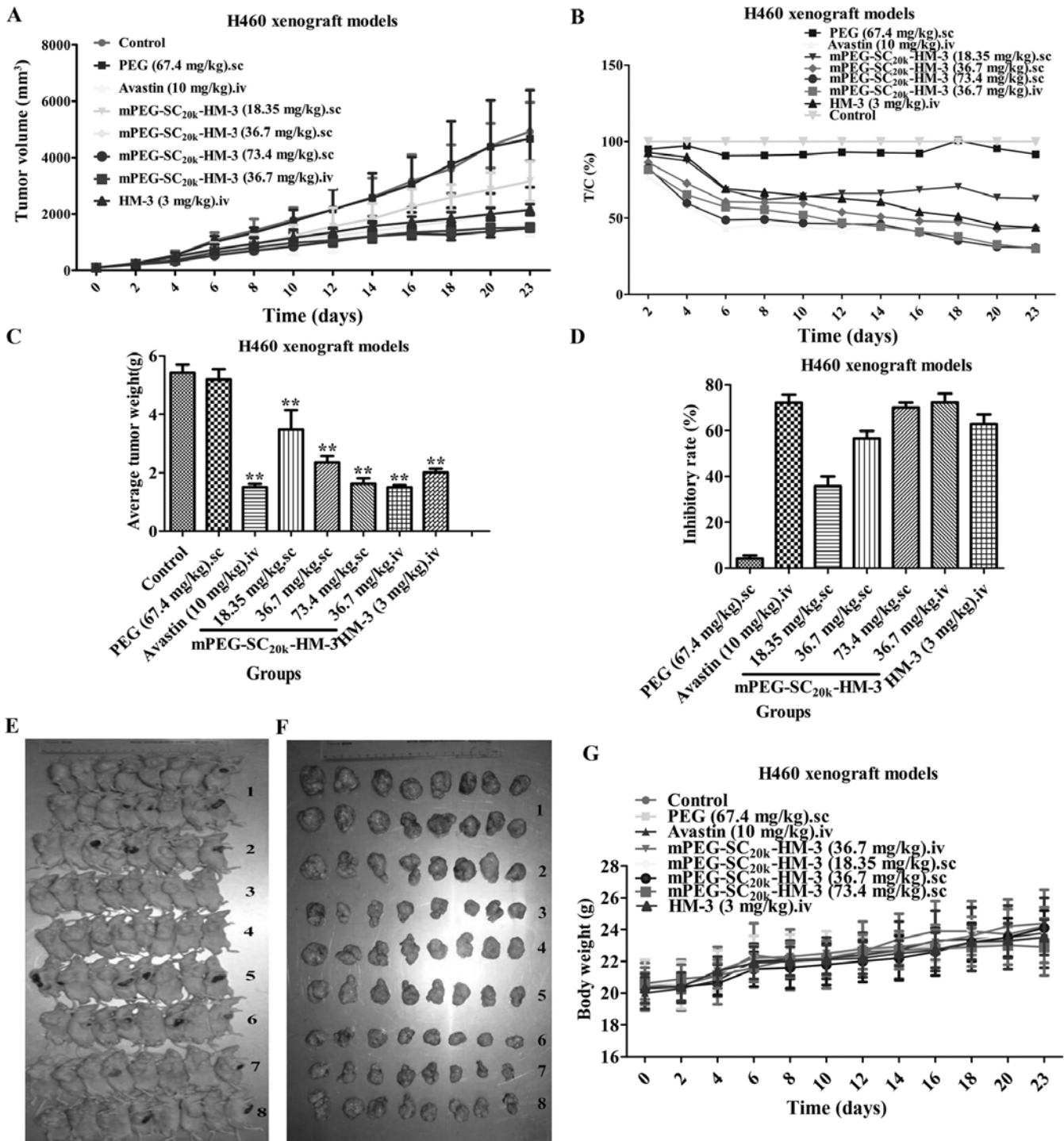


Figure 3. H460 xenograft model. (A) Curves of tumor volume vs. administration time. (B) Curves of T/C vs. administration time. (C) Average tumor weight in mice. (D) Dynamic inhibitory rate of tumor growth by the different treatment strategies. (E) Images of mice used in the experiment. (F) Images of tumors at the end of the study indicating the therapeutic effects of mPEG-SC_{20k}-HM-3 in mice. (G) Mouse weight curves. 1, control group; 2, 67.4 mg/kg PEG group sc; 3, 10 mg/kg Avastin iv; 4, 18.35 mg/kg mPEG-SC_{20k}-HM-3 group sc; 5, 36.7 mg/kg mPEG-SC_{20k}-HM-3 group sc; 6, 73.4 mg/kg mPEG-SC_{20k}-HM-3 group sc; 7, 36.7 mg/kg mPEG-SC_{20k}-HM-3 group iv; 8, 3 mg/kg HM-3 group iv. *P < 0.05, **P < 0.01. Three independent experiments were performed.

ERK and Akt signaling pathways were detected by western blot assay before and after cells were incubated with mPEG-SC_{20k}-HM-3. The results shown in Fig. 5 revealed that the expression levels of Akt1, p-Akt1, ERK1/2, p-ERK1/2, MEK1, p-MEK1, integrin α_v and integrin β_3 were reduced when the concentration of mPEG-SC_{20k}-HM-3 was 4.0 μ M. For integrin α_5 and integrin β_1 , the expression levels did not change as the

concentration of mPEG-SC_{20k}-HM-3 changed. With regard to the impact of mPEG-SC_{20k}-HM-3 on the expression of VEGF, we carried out two independent assays. The expression of VEGF was reduced when the concentration of mPEG-SC_{20k}-HM-3 reached 2.0 μ M; while the expression of VEGF did not change as the concentration of mPEG-SC_{20k}-HM-3 changed when the antibody of integrin $\alpha_v\beta_3$ was administered

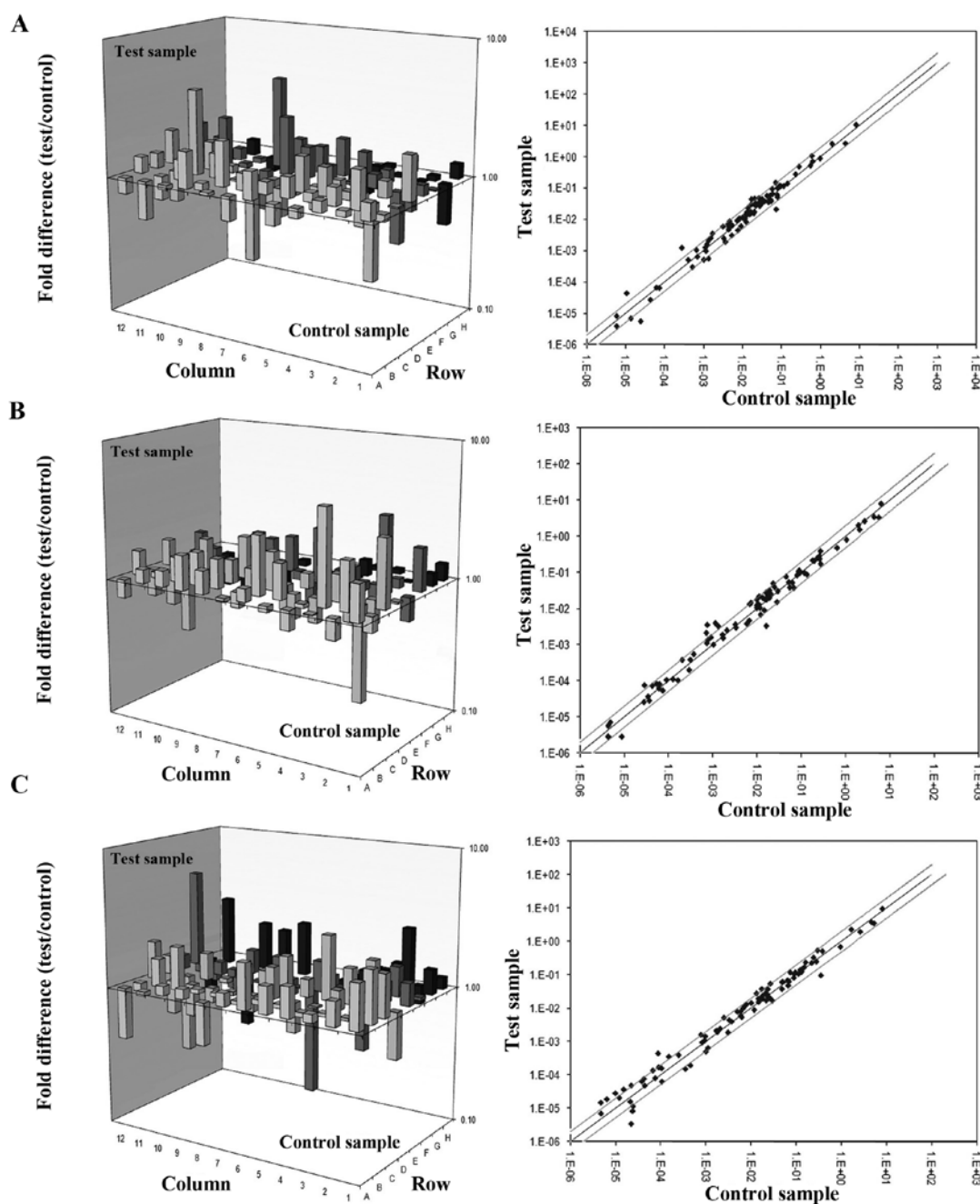


Figure 4. Analysis of the RT² Profiler PCR array results. (A) The 3D profile and scatter plot of Human Cancer Pathway Finder PCR array. (B) The 3D profile and scatter plot of Human Angiogenesis PCR array. (C) The 3D profile and scatter plot of Human Tumor Metastasis PCR array.

before mPEG-SC_{20k}-HM-3 (Fig. 6A). In summary, mPEG-SC_{20k}-HM-3 can target integrin $\alpha_v\beta_3$ to reduce the expression of key proteins in the ERK and Akt signaling pathways, by which tumor metastasis and angiogenesis are inhibited and the expression of VEGF is downregulated.

Discussion

The aim of the present study was to investigate the effects of mPEG-SC_{20k}-HM-3, a modified product of the antitumor peptide HM-3, which was found to overcome the short half-life and high immunogenicity of HM-3. mPEG-SC_{20k}-HM-3 can be developed into antitumor drugs with promising prospects. Preliminary research was performed in regards to the

reaction conditions of the modification, the pharmacokinetics and pharmacodynamics (SMMC-7721 xenograft model) of mPEG-SC_{20k}-HM-3. However, systematic research on the antitumor metastasis and anti-angiogenesis activities *in vitro*, antitumor activity *in vivo* and a thorough exploration of the antitumor mechanism of mPEG-SC_{20k}-HM-3 were not yet carried out, which were the main goals of the present study.

In the pharmacodynamic trials *in vitro* of mPEG-SC_{20k}-HM-3, apart from tumor metastasis and angiogenesis-related assays, MTT cytotoxicity, cell cycle and apoptosis assays (data not shown) were also performed. According to the results, mPEG-SC_{20k}-HM-3 did not show any cytotoxicity, cell cycle arrest or cell apoptosis inhibition. In the xenograft pharmacodynamical experiments, however in the H460

Table II. Gene Ontology analysis of the downregulated and upregulated genes identified from the PCR arrays.

Categories	Downregulated	Upregulated
Angiogenesis	LECT1	CXCL1, CXCL10, CXCL9, ERBB2, ITGB3, MMP14, PLAUG, FASLG, TIMP4, TNFSF10
Apoptosis		CDC20, MKI67, TP53
Cell cycle		
Cellular senescence	IGFBP5	
DNA damage and repair	DDIT3	
Metabolism	LPL	PFKL, SNAI2
Angiogenic factors	F3	NOS3
Cell adhesion	CDH6, CST7I, TGA7	CDH1, APC, ITGB8
ECM	MMP10, MMP3	
Cell proliferation	RORB, TRPM1	CTBP1, FGFR4, KISS1R, SRC, SYK, TSHR
Transcription factors and regulators		ETV4

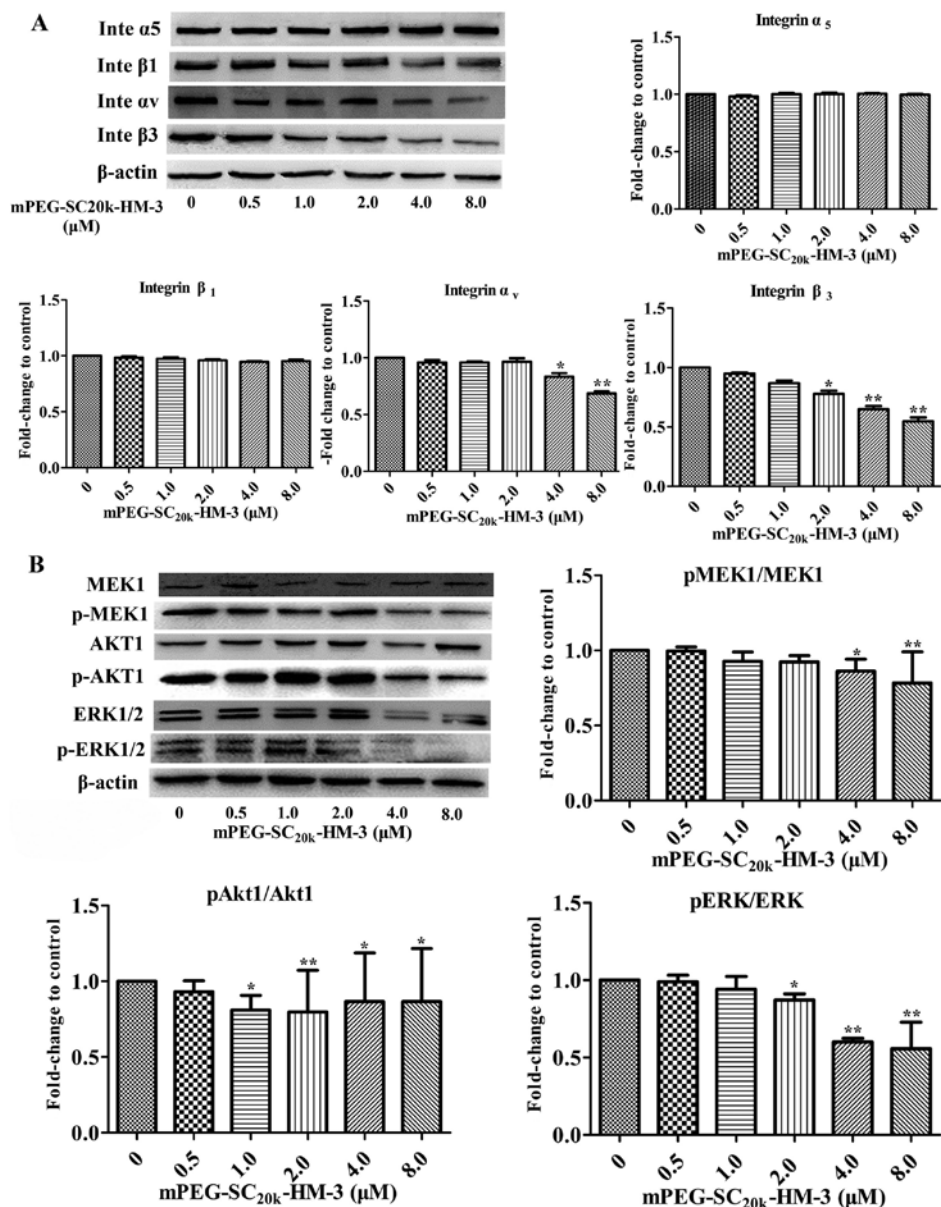


Figure 5. Western blot analysis of the expression levels of key proteins in the HUVECs after treatment. (A) Western blot analysis of the expression levels of integrin α_v , β_3 , α_5 and β_1 in HUVECs following treatment with mPEG-SC_{20k}-HM-3. (B) Western blot analysis of the expression levels of MEK1, p-MEK1, AKT1, p-AKT1, ERK1/2 and p-ERK1/2 in HUVECs following treatment with mPEG-SC_{20k}-HM-3. *P<0.05, **P<0.01. Three independent experiments were performed.

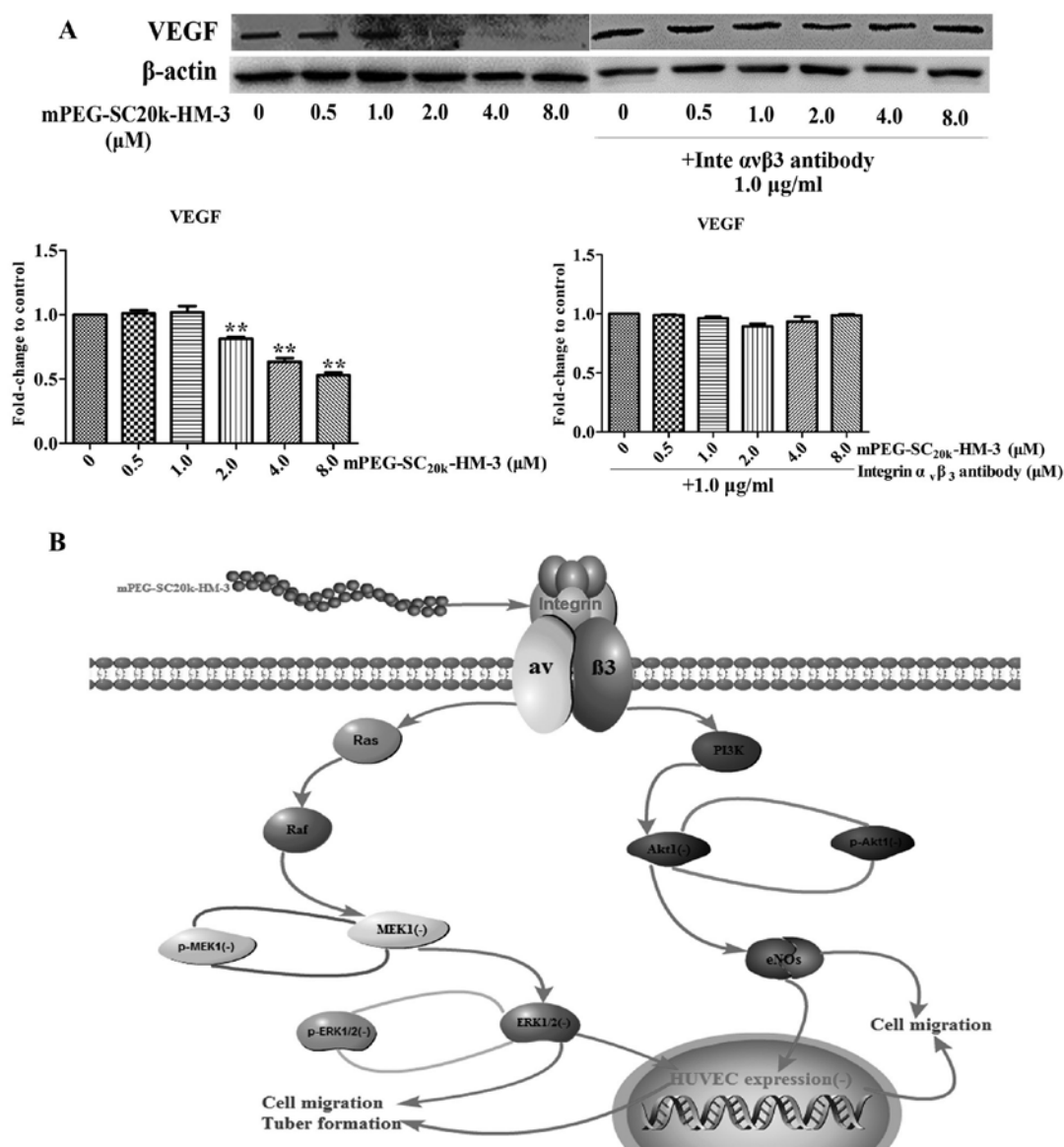


Figure 6. Inhibitory effects of mPEG-SC_{20k}-HM-3 on VEGF in HUVECs and a scheme of the possible mechanism of mPEG-SC_{20k}-HM-3 explaining its antitumor activity. (A) Western blot analysis of the expression level of VEGF in the HUVECs following treatment with mPEG-SC_{20k}-HM-3 and after treatment with the integrin $\alpha_v\beta_3$ antibody and mPEG-SC_{20k}-HM-3. *P<0.05, **P<0.01. Three independent experiments were performed. (B) mPEG-SC_{20k}-HM-3 may execute its biological activity through integrin $\alpha_v\beta_3$.

xenograft model in the present study, mPEG-SC_{20k}-HM-3 also showed a more marked inhibitory effect than HM-3 in hepatic carcinoma (SMMC-7721), gastric (MGC-803) and colon cancer (HCT-116) nude xenograft models (data not shown). A previous study indicated that the mouse survival rate of the mPEG-SC_{20k}-HM-3 (90.0 mg/kg) group was 90% after 15 days of therapy in a DBA/2 mouse lymphatic metastasis model (P388D1), compared with a 50% survival rate of 3.0 mg/kg HM-3 (data not shown). In addition, the number of surviving mice and body weight data after therapy suggest that the modified peptide did not have any cytotoxicity compared with conventional chemical drugs, which are consistent with the results of the pharmacodynamic trials *in vitro*. For combination therapy consideration, the pharmacodynamic trails *in vivo* (data not shown) of the modified peptide combined with other antitumor chemical drugs (docetaxel, oxaliplatin

and capecitabine) have already been launched and we obtained considerable inhibitory rate data with these combination therapies.

All the results above indicated that the mPEG-SC_{20k}-HM-3 has enhanced antitumor activities and further study should be carried out to elucidate the mechanism of the pharmacodynamical effects.

Since Judah Folkman proposed the theory of tumor angiogenesis in 1971, tumor angiogenesis has been investigated during the past 30 years and many achievements and new ideas based on this theory have emerged. Integrins and their receptors are derivatives of this theory and they have already been studied for many years. Among all the receptors of the integrin family, $\alpha_v\beta_3$ is considered as one of the most significant factors with regard to its significance to tumor angiogenesis (10,11) and it is the main target focused on by current antitumor drug

designers. Its expression in endothelial cells in dormancy and other normal tissues is relatively low compared with its expression in many types of cancer and endothelial cells in tumor angiogenesis, which makes integrin $\alpha_v\beta_3$ special and a hotspot in terms of tumor angiogenesis and cancer treatment (12). In our preliminary study, flow cytometric assay was performed to demonstrate that integrin $\alpha_v\beta_3$ is the main target of mPEG-SC_{20k}-HM-3 (data not shown).

Current research indicates that the antitumor property of mPEG-SC_{20k}-HM-3 is closely related to the target and its downstream pathways. Several signaling pathways are switched on when the ligands bind with the integrin receptors, including MAPK and Akt signaling pathways. The MAPK signaling pathway is an important pipeline that transports signals from the surface of cells to the nucleus. A variety of physiological processes in cells, such as growth, development, differentiation, division and apoptosis, are related to the regulation of the MAPK signaling pathway. Sufficient evidence indicates that the MAPK family participates in the process of cell adhesion, proteolysis of extracellular matrix and cell migration. Members of the MAPK family in mammals include extracellular signal regulated protein kinase (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 signaling pathways (13). Among these members, the ERK signaling pathway has been thoroughly studied, and was found to be activated by Ras-Raf-MEK-ERK1/2 and participates in the migration of different types of cells (14). Abnormal PKB/Akt signaling is usually detected in human tumor tissues and can be closely related to the process of tumor development. The PKB/Akt signaling pathway participates in the regulation of apoptosis, proliferation, differentiation and metabolism of cells and plays an important role in angiogenesis. PKB/Akt can be phosphorylated and activates endothelial nitric oxide synthase (eNOS), which leads to angiogenesis and cell migration (15). Another factor connected with angiogenesis and cell migration is VEGF, with a molecular weight of between 34 and 45 kb (16) and its sequences are highly conserved. VEGF is a member of the platelet-derived growth factor (PDGF) family isolated from the bovine pituitary follicle stellate cell culture medium and can promote cell division, proliferation, invasion and metastasis via binding to a specific receptor (17). It is reported that the specific receptor of VEGF is highly expressed in vascular endothelial cells of tumor tissues compared with normal ones.

Based on the results of RT² Profiler PCR assays performed in the present study, mPEG-SC_{20k}-HM-3 significantly downregulated the expression of LECT1, F3, CDH6, CST7I and TGA7, which are factors related to angiogenesis and tumor metastasis. Concurrently, the results of the western blot assay indicated that mPEG-SC_{20k}-HM-3 inhibited the expression of MEK1, p-MEK1, AKT1, p-AKT1, ERK1/2 and p-ERK1/2 in the ERK and Akt signaling pathways. Therefore, the scheme of the mechanism of action (Fig. 6B) based on the data above can clearly explain the antitumor properties of mPEG-SC_{20k}-HM-3. Our previous research conducted on the mechanism of HM-3 also involved the two signaling pathways mentioned in the present study. However, in addition to the expression of non-phosphorylated forms of the key proteins, the expression divergences of phosphorylated forms of these proteins were not taken into consideration, which was not rigorous (9). In addition, the explanation of the relationship between HM-3

and VEGF is unreasonable as no trails have demonstrated that HM-3 targets VEGF directly. Therefore, the study on the mechanism of mPEG-SC_{20k}-HM-3 is more systematic and thorough. According to the results of RT² Profiler PCR assays, the expression levels of MMP3 and MMP10 which belong to the matrix metalloproteinase family were also downregulated by mPEG-SC_{20k}-HM-3. The MMP family are zinc and calcium-dependent peptide chain endonucleases, which can degradate various protein components in ECM to damage the histological barrier of tumor cell invasion and as a result, tumor metastasis can be inhibited (18). The results that mPEG-SC_{20k}-HM-3 downregulated MMP3 and MMP10 expression indicated that mPEG-SC_{20k}-HM-3 may also target certain members of the MMP family. Certainly, the hypothesis can be further demonstrated by a series of pharmacodynamic *in vitro* studies and research regarding the related mechanisms.

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