Anti-tumor and anti-angiogenic effect of metronomic cyclic NGR-modified liposomes containing paclitaxel

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1. Introduction

Tumor angiogenesis is recognized as a major therapeutic target in the fight against cancer [1]. Inhibitors of angiogenesis can suppress tumor growth in preclinical models and are being used clinically as anti-tumor therapies [2]. However, two animal studies have shown that VEGF-targeted anti-angiogenic drugs, while inhibiting primary tumor growth, can promote tumor invasion and metastasis [3,4]. An alternative to these angiogenesis inhibitors that is generating clinical interest is metronomic chemotherapy [5]. Metronomic chemotherapy, which is also intended to prevent tumor angiogenesis, involves the chronic administration of comparatively low doses of cytotoxic drugs at close, regular intervals, with no prolonged drug-free interruptions compared with conventional maximum tolerated dose (MTD) chemotherapy [5].

Using this approach shortens the time between cycles, preventing effective recovery of the damaged tumor vasculature. This suggests that activated tumor vascular endothelial cells may be more sensitive to lower doses of cytotoxic drugs compared with normal or cancer cells when exposed frequently or in a continuous manner [6]. It has been reported that metronomic delivery of cytotoxic drugs produces anti-angiogenic effects and may be more effective [7,8]. Among the currently used cytotoxic drugs, paclitaxel appears to be a strong candidate for metronomic chemotherapy given its ability to inhibit endothelial cell functions relevant to angiogenesis in vitro at extraordinarily low concentrations and its broad-spectrum anti-tumor activity [9–11].

It is well known that proliferation of endothelial cells is an important factor in tumor angiogenesis [12]. Endothelial cells in the angiogenic vessels within solid tumors express several proteins that are absent or barely detectable in established blood vessels, including αv integrins, receptors for angiogenic growth factors, and other types of membrane spanning molecules such as aminopeptidase N (APN) [13,14]. APN is a membrane-bound, zinc-dependent...
metalloproteinase that plays a key role in tumor invasion and angiogenesis. A peptide containing the Asn-Gly-Arg (NGR) motif which can recognize a specific isoform of APN has been identified as a potent targeting ligand for the delivery of chemotherapeutic drugs [15,16]. The angiogenic tumor vasculature is estimated to have an average pore size of 100–600 nm [17,18]. These pores are significantly larger than the gaps found in the normal endothelium, which are typically <6 nm wide. After intravenous administration, nano-size drug delivery systems are small enough to passively infiltrate the tumor endothelium but large enough to be excluded from the normal endothelium. Nontargeted sterically stabilized liposomes (SSL) have been extensively used for delivering chemotherapeutic drugs to tumors due to their enhanced permeability and retention mechanism (EPR effects) [19]. Although liposomal delivery of cytotoxic drugs can improve anti-tumor activity, targeted delivery of these particles represents a potential approach to further enhance efficacy and minimize toxicity. Recent studies have described the design of liposomes that target the tumor endothelium to improve the delivery of therapeutic agents to solid tumors [20,21]. In addition, the use of vascular targeting approaches in combination with tumor targeting therapies has been reported [22]. The anti-angiogenic effect is indicated by the combination administration of liposomal formulations of doxorubicin targeted against tumor cells via anti-GD2 monoclonal antibodies (which toward to tumor cell over-expressed disialoganglioside GD2) and against the tumor vasculature via the NGR peptide have improved therapeutic effects relative to each therapy used individually, showing the enhanced anti-tumor effect of this combination of dual targeting treatment strategy.

In view of the anti-angiogenic and anti-tumor effect of metronomic chemotherapy, the APN isofrom over-expressed on tumor endothelial cells and some tumor cells, and using NGR peptide as a tumor vasculature and tumor cell targeting moietry in addition to the anti-angiogenic effect of paclitaxel, the main objective of this study was to demonstrate the proof-of-principle for the hypothesis that NGR-modified sterically stabilized liposomes containing paclitaxel (NGR-SSL-PTX), which mediated by NGR-ligand to target both endothelial cell and tumor cell, can have an increased anti-tumor effect when given as metronomic chemotherapy compared with that of MTD dosing. To test this hypothesis, NGR-SSL-PTX was investigated in vitro and in vivo. HUVEC (human umbilical vein endothelial cells, intermediate level of expression of APN [23]), and HT1080 cells (human fibrosarcoma cell line, high level of expression of APN [23,24]) and MCF-7 cells (human breast adenocarcinoma cell line, low level of expression of APN [23,24]) were chosen as the model for tumor endothelial cells and tumor cells, respectively. The in vitro targeting and anti-angiogenic characteristics of NGR-modified SSL were investigated. Furthermore, the anti-tumor activity of NGR-SSL-PTX was also evaluated in HT1080 tumor-bearing BALB/c nude mice in vivo.

2. Materials and methods

2.1. Materials

Soyabean phosphatidylcholine (SFC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). 1,2-Distearoyl-snglycero-3-phosphoethanolamine [methoxy (polyethylene glycol)-2000] (mPEG-DSPE) and N-hydroxysuccinimydil-PEG2000-DSPE (NHDS-PG-DSPE) were provided by Nof Co. (Tokyo, Japan). Cholesterol, sulfo-horhodamin B (SRB), and tris base were obtained from Sigma–Aldrich (St. Louis, MO, USA). Coumarin-6, Hoechst 33258 and FITC goat anti-rabbit secondary antibody (1:1000) were supplied by Molecular Probes Inc. (Eugene, OR, USA). Near infrared lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiR) and the fluorescent probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylrhodaminecarbocyanine iodide (DiO) were obtained from Biotium, Inc. (Hayward, CA, USA). Rabbit polyclonal to CD31 (10 μg/ml) was obtained from Abcam Inc. (Cambridge, Massachusetts, USA). The cyclic NGR peptide, GCCNGRC, was obtained from SciLight Biotechnology (Beijing, China). NGR-PEG-DSPE was synthesized in our laboratory according to a previously reported method [16]. Paclitaxel (PTX) was purchased from Yunnan Hande Bio-Tech Co., Ltd (Kunming, Yunnan, China). Tanol (Bristol-Myers Squibb Co., Princeton, NJ) was obtained from a local Beijing hospital. All other solvents and reagents were of analytical grade.

2.2. Cell lines

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords (Hadian Maternal & Child Health Hospital, Beijing, China) as previously reported [25]. Briefly, the culture medium consisted of M 199, containing 20% (v/v) newborn calf serum and 5% (v/v) pooled human serum with 2 mM l-glutamine. Cells were plated onto plates pre-coated with 0.02% (w/v) gelatin with a medium change after 24 h and every 2 days thereafter until confluent. Primary cultures of HUVEC were harvested by incubation with 0.05% trypsin, 0.02% EDTA, and the cells were collected. The HT1080 human fibrosarcoma cell line and the MCF-7 human breast adenocarcinoma cell line were obtained from the Institute of Basic Medical Sciences (Chinese Academy of Medical Sciences, Beijing, China) and cultivated under the recommended conditions.

2.3. Animals

Sprague–Dawley (SD) rats weighing 200 ± 20 g were obtained from the Experimental Animal Center of Peking University Health Science Center. Female BALB/c nude mice were purchased from the Academy of Military Medical Sciences (Beijing, China) at 6 weeks of age (initially weighing 20–25 g). All care and handling of animals were performed in accordance with the Institutional Authority for Laboratory Animal Care of Peking University.

2.4. Preparation of NGR-modified sterically stabilized liposomes containing PTX (NGR-SSL-PTX)

The NGR-modified sterically stabilized liposomes containing PTX (NGR-SSL-PTX) were prepared by the thin-film hydration method, as described previously [11]. Briefly, the mixture of PTX, SPC, cholesterol, mPEG-DSPE and NGR-PEG-DsPE was dissolved in chloroform and dried in a RES2 rotary evaporator (Shanghai Yangro Biochemistry Instrument Company, China) in a round-bottom flask at 40 °C. The lipid film was flushed with nitrogen gas for 5 min and maintained overnight in a desiccator to remove traces of chloroform. The resulting thin-film was then hydrated in phosphate buffered saline (PBS, pH 7.4) by vortexing and sonicated using a bath type sonicator at 50 °C for 30 min to produce liposome suspensions. The liposome suspensions were purified by centrifuging at 1000 rpm for 10 min and then they were passed through a 0.2 μm pore size membrane to remove any non-entrapped PTX, giving the final NGR-SSL-PTX.

For the preparation of sterically stabilized liposomes containing PTX (SSL-PTX), an identical procedure was carried out except that the equivalent molar NGR-PEG-DsPE was replaced by mPEG-DsPE.

The preparation of coumarin-6, DiR or DiI loaded liposomes (NGR-SSL-coumarin-6 and SSL-coumarin-6, NGR-SSL-DiR and SSL-DiR, NGR-SSL-DiI and SSL-DiI) was exactly the same as for PTX-loaded liposomes, except that PTX was replaced by coumarin-6, DiR or DiI.

2.5. Characterization of NGR-SSL-PTX

The particle size and zeta potential of NGR-SSL-PTX were measured by photon correlation spectroscopy using a Malvern Zeta sizer 3000 HS (Malvern, UK) at 25 °C. The encapsulation efficiency was estimated from the following formula:

\[
\text{Encapsulation Efficiency} = \frac{\text{PTX concentration in the filtered liposomes}}{\text{PTX concentration in the un-filtered liposomes}} \times 100\%
\]

2.6. In vitro release of PTX from NGR-SSL-PTX

The release of PTX from NGR-SSL-PTX was investigated using dialysis. Briefly, a volume of NGR-SSL-PTX (0.2 ml, 1 mg/ml) was placed in a dialysis tube (MWCO 7000) and tightly sealed. Then, the dialysis tube was immersed in 200 ml release medium (PBS pH 7.4) containing 0.1% (v/v) Tween 80 and incubated in an orbital shaker for 24 h at room temperature. Samples (0.5 ml) were taken at predetermined time intervals from the release medium for 24 h, and replaced by a similar volume of fresh medium. The concentration of PTX was determined by HPLC after appropriate dilution with acetonitrile without further treatment.

2.7. Flow cytometry analysis

HUVEC, HT1080 or MCF-7 cells were seeded at a density of 1 × 10⁵ cells/well in 6-well plates and incubated at 37 °C for 24 h to allow cell attachment. After 24 h, the medium was replaced with coumarin-6 solution or coumarin-6 loaded liposomes.
incubated for another 2 h at 37 °C. The cells were then harvested by trypsinization and centrifuged at 1000 rpm for 5 min and resuspended in 500 μl α-Hank’s medium and examined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA). Cell-associated coumarin-6 was excited with an argon laser (487 nm) and fluorescence was detected at 502 nm.

2.8. Confocal laser microscopy

Following incubation of HUVEC, HT1080 or MCF-7 cells on glass-bottomed dishes containing culture medium at 37 °C for 24 h, coumarin-6 solution and coumarin-6 loaded liposomes were added (final drug concentration 50 ng/ml) to each dish and incubated for another 2 h at 37 °C. The medium was then removed and cells were washed with ice cold PBS followed by fixing with 4% paraformaldehyde in PBS. After fixing, cells were treated with Hoechst 33258 for 5 min. The fluorescent images of the cells were analyzed using a TCS SP2 confocal microscope (Leica, Germany).

2.9. Endothelial cell proliferation

HUVEC cells were seeded into 96-well plates at 5 × 10^3 cells/well and allowed to attach for 48 h. Then, HUVEC cells were exposed to a series of concentrations of PTX in NGR-SSL-PTX (0.5–10 nM). After incubation for 48 h at 37 °C, cells were fixed with trichloroacetic acid, washed and stained with SRB [26]. After shaking, the absorbance at 540 nm was recorded using a 96-well plate reader (Bio-rad, 680, America). The percentage survival of HUVEC cells was calculated using the following formula:

\[
\text{Survival} = \left( \frac{A_{540} \text{nm for the treated cells}}{A_{540} \text{nm for the control cells}} \right) \times 100
\]

where \( A_{540} \text{nm} \) is the absorbance. Each assay was repeated a minimum of three times with quadruplicate determinations per dose level.

2.10. Migration assay

The confluent HUVEC cells in 24-well plates (Costar, America) were mechanically “wounded” by scraping away a swath of cells with a pipette tip and denuding a strip of the monolayer 300 μm wide. The variation in the wound width within experiments was approximately 5%. Endothelial monolayers were washed twice with D-Hank’s solution to remove wound-derived loose and dislodged cells and incubated in medium supplemented with NGR-SSL-PTX (0.01–10 nM). Control HUVEC cultures only received medium. The extent of wound closure was observed and photographed after a 24 h incubation. The effect of NGR-SSL-PTX on the progression of endothelial cell migration was represented by the wound width which was measured in six areas and compared with the untreated wound width at 0 h [27].

2.11. Pharmacokinetic studies

SD rats were randomly divided into three groups of five rats each. All the animals were fasted for 12 h prior to the experiments. NGR-SSL-PTX, SSL-PTX or Taxol was diluted with 5% glucose at a concentration of 1 mg/ml. These PTX formulations were all injected into the tail vein at a single dose of 5 mg/kg. Blood samples (0.5 ml) were collected via the orbital venous plexus at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 h after injection. After centrifugation at 3000 g for 5 min, the obtained plasma was stored at −20 °C until required for analysis.

2.12. In vivo anti-tumor efficacy

HT1080 cells were re-suspended in serum-free cell culture medium. Approximately 4 × 10^6 HT1080 cells (100 μl) were subcutaneously injected into the armpits of female BALB/c nude mice. Once the tumor masses in xenografts reached 150–200 mm^3 in volume, the nude mice were randomly assigned to six groups (5–6 animals per group). Each group was treated with physiological saline, MTD Taxol

| Table 1 Characteristics of NGR-SSL-PTX (n = 3). |
|-----------------|-----------------|-----------------|
|                | SSL-PTX         | NGR-SSL-PTX     |
| Average particle size (nm) | 87.92 ± 8.05 | 90.55 ± 9.34 |
| Polydispersity    | 0.20 ± 0.01    | 0.19 ± 0.04    |
| Zeta Potential (mV) | −1.73 ± 0.55 | −1.66 ± 0.79 |
| Entrapment efficiency (%) | 90.66 ± 3.07% | 87.49 ± 2.60% |

Fig. 1. The release of PTX from NGR-SSL-PTX, SSL-PTX or Taxol at room temperature. The release medium was PBS (pH 7.4) containing 0.1% (v/v) of Tween 80. Each data represents the mean ± standard deviation (n = 3).

Fig. 2. The flow cytometric measurement of coumarin-6 uptake from NGR-SSL-coumarin-6 and SSL-coumarin-6 by HUVEC, HT1080 and MCF-7 cells at the time point of 1 h. Cells were incubated with NGR-SSL-coumarin-6 and SSL-coumarin-6 with the final coumarin-6 concentration of 20 ng/ml. And at the time point of 1 h, the cells were trypsinized, washed and analyzed using flow cytometry. Black represents control, green represents incubated with SSL-coumarin-6, blue represents incubated with NGR-SSL-coumarin-6, peak represents pre-incubated with free NGR + SSL-coumarin-6, and brown represents coumarin-6, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
(15 mg/kg, i.v., q4d), MTD SSL-PTX (15 mg/kg, i.v., q4d), MTD NGR-SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), metronomic SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), respectively. For each administration, formulations were given to mice in each group via the tail vein. The total dose of PTX in all treatment groups was 60 mg/kg. Throughout the study, mice were weighed and tumors were measured with calipers every two days. Tumor volumes were calculated from the formula: $V = \text{length(cm)} \times \text{width(cm)}^2 \times 0.5236$. Once animals in any group seemed moribund, all animals in the experiment were sacrificed, and then tumors were harvested and weighed. The tumor weight inhibition (TWI) was calculated from the formula (TWI = $[WC - WD]/WC$, where WC and WD represent the tumor weight of animals from physiological saline and treatment groups, respectively [28]). The harvested tumors were then fixed in formalin solution and paraffin-embedded for immunohistochemical analysis.

### 2.13. Assay of in vivo angiogenesis

The microvessel density in the tumor tissues was examined using a CD31 staining method. Briefly, after paraffin embedding, the tissue sections (5 μm thick) were deparaffinized in xylene and rehydrated in alcohol, and sections were incubated in 0.3% H$_2$O$_2$ to block endogeneous peroxidase activity. Each slide was incubated with normal goat serum for 20 min at room temperature, and then incubated with the primary antibody at 4°C overnight. After incubation with the secondary antibody, biotinylated for 30 min at 37°C, each slide was rinsed in PBS and then incubated in the avidin-biotin peroxidase complex for 30 min at 37°C. The peroxidase was visualized with 3-3′-diamino-benzidinetetrahydrochloride (DAB) solution and then counterstained with hematoxylin. MVD was assessed according to the international consensus report. Immuno-stained slides were scanned at ×100 magnification to identify the areas with the highest number of vessels (so called ‘hot spots’). Counts were performed on 10 fields in the hot spot by two independent pathologists at ×400 magnification and the mean was calculated.


The bio-distribution of NGR-SSL-DiR in HT1080 tumor-bearing female BALB/c nude mice following intravenous administration was investigated using an in vivo
imaging system. HT1080 tumor-bearing nude mice were prepared by injecting a suspension of HT1080 cells (4 × 10^6) into the subcutaneous in the right armpits of nude female BALB/c mice. Once the tumor masses in the xenografts reached 200 mm^3 in volume, 200 µl 5% glucose injection, SSL-DiR or NGR-SSL-DiR was intravenously injected via the tail vein of the tumor-bearing mice at a dose of 800 ng/mouse. Mice were anaesthetized by isoflurane (1.5%), and scanned at 2, 6, 12 and 24 h after administration using a Kodak In Vivo Imaging System FX PRO (Carestream Health, Inc., USA) with an excitation bandpass filter at 730 nm and an emission at 790 nm. The exposure time was 30 s per image. The fluorescent signal intensities in the tumor-bearing mice were analyzed using Carestream MI SE software. For each NIR image, a corresponding X-ray image was taken to identify the anatomical location of the tumor. After in vivo imaging, the mice were sacrificed at 24 h, and the major organs, including heart, liver, spleen, lungs, kidneys and tumors were excised. The near-infrared fluorescence signal intensities in different tissues were measured.

2.15 Confocal immunofluorescence microscopy study

In order to show the microcosmic characteristic distribution difference of our liposome, we employed DiI as tracer and prepared the NGR-SSL-DiI and SSL-DiI. Female BALB/nude mice were inoculated subcutaneously in the right armpits with 0.2 ml HT-1080 cell suspension (4 × 10^6) or MCF-7 cell suspension (4 × 10^6), respectively. Once the tumor masses reached approximately 200 mm^3 in volume, the mice received intravenous injection, via the tail vein, of 5% glucose infusion, NGR-SSL-DiI or SSL-DiI at a dose of 800 ng per mouse. At the time of sacrifice (2 and

Fig. 5. A. Migration assay. Wound assay was done to determine whether NGR-SSL-PTX inhibits HUVEC migration. After treatment with various concentrations of NGR-SSL-PTX, HUVEC were allowed to migrate into the denuded area for 24 h. HUVEC migration was visualized by light microscopy. Typical photomicrographs (final magnification, ×25) were shown in unwounded (A1), untreated in 0 h (A2), untreated in 24 h (A3) and NGR-SSL-PTX treated in 24 h at the concentration of 10 nM (A4). B. Percentage of wound width which measured in denuded areas at 24 h compared with the untreated wound width at 0 h time point versus NGR-SSL-PTX concentration. **, p < 0.01, vs untreated as control.
of NGR-SSL-PTX, SSL-PTX or Taxol at 5 mg/kg PTX in SD rats (means ± S.D., n = 5).

6 h after administration, tumor tissues were harvested, and frozen in optimal cutting temperature (OCT) embedding medium. For immunostaining, tumor sections (8 μm) were first incubated with 10% BSA for 1 h at room temperature followed by incubation with the primary antibody (rabbit polyclonal to CD31) overnight at 4 °C (5 μg/ml). The primary antibodies were detected with FITC goat anti-rabbit secondary antibodies. Nuclei were counterstained with Hoechst 33342 (5 μg/ml). The sections were mounted in gel/mount mounting medium (Biomeda, Foster City, California) and visualized under a confocal microscope (Leica, Germany).

2.16. HPLC analysis of paclitaxel

The concentration of PTX in NGR-SSL-PTX was determined by HPLC using a Waters HPLC system consisting of a 1525-pump, and a 2487-ultraviolet detector (Waters Co. Inc., Westerville, OH, USA). The mobile phase consisted of methanol-water-tetrahydrofuran (70:27.5:2.5, v/v/v) delivered at a flow rate of 1 ml/min. Chromatographic separation was performed on a Phenomenex ODS3 column (250 × 4.6 mm, 5 μm, Torrance, CA, USA) and the detector wavelength was 230 nm. PTX was measured in plasma according to a previous report [29]. Briefly, an aliquot of 100 μl plasma and 2.5 ml acetonitrile were mixed in a vortex mixer for 30 s. The mixture was centrifuged at 3000 g for 10 min, then 2.0 ml supernatant was collected, and dried under a gentle stream of nitrogen gas at 50 °C in a water bath. The residue was reconstituted using the mobile phase below, and assayed by HPLC using a Waters system consisting of a 1525-pump, and a 2487-ultraviolet detector (Waters Co., Inc., Westerville, OH, USA). The mobile phase consisted of methanol-water-tetrahydrofuran (70:27.5:2.5, v/v/v) delivered at a flow rate of 1 ml/min. Chromatographic separation was performed on a Phenomenex ODS3 column (250 × 4.6 mm, 5 μm, Torrance, CA, USA), at a detector wavelength of 230 nm. The peak areas of PTX(Ap) and docetaxel (Ad) were recorded, and the concentration of PTX was calculated from the ratio of Ap/Ad.

2.17. Statistical analysis

Data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post-hoc tests with the Bonferroni correction were used for comparison between individual groups. Statistical significance was established at p < 0.05.

3. Results

3.1. Characterization of NGR-SSL-PTX

The particle size of NGR-SSL-PTX was approximately 91 ± 9 nm and the polydispersity was 0.19 ± 0.04. The value of the zeta potential of NGR-SSL-PTX had a slightly negative charge (−1.66 ± 0.79 mV). The entrapment efficiency of NGR-SSL-PTX was 87.5 ± 2.6% (n = 3), as shown in Table 1. The typical particle size and distribution of NGR-SSL-PTX is shown in Fig. S1.

In the in vitro release study, PTX in Taxol was released rapidly and this was almost complete within 24 h. However, the PTX release from NGR-SSL-PTX was only 22% within 24 h at room temperature (Fig. 1). A similar release behavior of PTX was observed in SSL-PTX.

3.2. Flow cytometry analysis and confocal microscopy studies

Flow cytometry was used to quantify the total coumarin-6 uptake by HUVEC, HT1080 or MCF-7 cells for different coumarin-6 formulations. As shown in Fig. 2, the cellular coumarin-6 level for NGR-SSL-coumarin-6 in the HUVEC and HT1080 cell lines was about 2.9- and 2.3-fold higher than that for SSL-coumarin-6. When the HUVEC cells were pre-incubated with free NGR for 30 min, the cellular coumarin-6 level of coumarin-6 in the NGR-SSL-coumarin-6 groups was only 1.1-fold higher than that for SSL-coumarin-6. For MCF-7 cells, the intense fluorescence of coumarin-6 in SSL-coumarin-6, NGR-SSL-coumarin-6 or NGR pre-incubated groups was similar.

Fig. 3 shows the confocal microscopic images of HUVEC, HT1080 and MCF-7 cells after incubation with SSL-coumarin-6, NGR-SSL-coumarin-6, NGR pre-incubated and coumarin-6 at 37 °C. Because of its highly hydrophobic nature, free coumarin-6 readily partitioned into the lipid membranes and then diffused into the three types of cells leading to a greater cellular accumulation, which was taken as the positive control group. For NGR-SSL-coumarin-6, the images showed a more intense fluorescence of coumarin-6 in the cells than that of the SSL-coumarin-6 group in both HUVEC and HT1080 cells. When the HUVEC and HT1080 cells were pre-incubated with free NGR for 30 min, the intense fluorescence of coumarin-6 in NGR-SSL-coumarin-6 groups was reduced. For MCF-7 cells, the intense fluorescence of coumarin-6 in NGR-SSL-coumarin-6, SSL-coumarin-6 or NGR pre-incubated groups was similar.

3.3. Endothelial cell proliferation and migration

HUVEC proliferation was determined by the SRB method. The results showed that HUVEC proliferation was significantly inhibited by NGR-SSL-PTX (p < 0.01), as shown in Fig. 4. The value of the anti-proliferative ratio (100% - variability %) at 5 and 10 nM was 27% and 32%, respectively. Similar results of HUVEC proliferation inhibited by PTX were shown in our previous report [11].

The effect of NGR-SSL-PTX on endothelial cell migration was analyzed in the wound assay. Many HUVEC cells migrated into the denuded areas in the untreated group. In the NGR-SSL-PTX treatment group, the migration of HUVEC cells into the denuded areas was inhibited. As shown in Fig. 5A, the scratch wound was clearly observed in NGR-SSL-PTX treatment groups, indicating the inhibitory effect of NGR-SSL-PTX on HUVEC cell migration. Fig. 5B shows the wound closure at 24 h after treatment as a percentage of the control wound width (untreated group at 0 h). The wound closure in the NGR-SSL-PTX treatment groups was significantly lower than that in the untreated group and was concentration-dependent (p < 0.01). At a concentration of 0.01 nM, the wound closure was 62.2 ± 1.6%. At the highest concentration (10 nM), the value was 19.6 ± 6.2%. Similar results of HUVEC migration inhibited by PTX were shown in our previous report [11].

3.4. Pharmacokinetics of the NGR-SSL-PTX after intravenous administration

The plasma concentration-time profiles of PTX after intravenous administration of the PTX formulations (5 mg/kg) were...
consecutively), respectively. For each administration, formulations were given to mice metronomic NGR-SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), metronomic SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), respectively. The clearance of PTX in the SSL-PTX and NGR-SSL-PTX treatment groups was significantly lower than that in the Taxol treatment group (p < 0.01). The values of AUC in the SSL-PTX and NGR-SSL-PTX treatment groups increased to 27.54 and 25.01 µg h/ml, respectively, compared with those in the Taxol treatment group (10.06 µg h/ml). The bioavailability of PTX in the SSL-PTX and NGR-SSL-PTX treatment groups compared with that in the Taxol group was 273.7% and 248.6%, respectively.

### 3.5. In vivo anti-tumor activity

The anti-tumor effect of PTX formulations was evaluated in HT1080 tumor-bearing mice after cell implantation. As shown in Fig. 7A, the tumor growth was significantly inhibited in all treatment groups compared with the control group given physiological saline (p < 0.01), but the effect obtained varied. Metronomic NGR-SSL-PTX or SSL-PTX significantly inhibited the growth of HT1080 tumors compared with that in the MTD (NGR-SSL-PTX or SSL-PTX) treatment group (p < 0.01). In addition, the MTD NGR-SSL-PTX treatment was significantly more effective than MTD Taxol and MTD SSL-PTX treatment in inhibiting tumor growth (p < 0.05 or p < 0.01). The tumor growth inhibition in the metronomic NGR-SSL-PTX treatment group was significantly more effective than that in the metronomic SSL-PTX treatment group (p < 0.05). In contrast, metronomic NGR-SSL-PTX produced a greater anti-tumor response than in the other PTX formulation treatment groups (p < 0.01 or p < 0.05).

The average tumor weight in the physiological saline, MTD Taxol treatment, MTD SSL-PTX, MTD NGR-SSL-PTX, metronomic SSL-PTX and metronomic NGR-SSL-PTX treatment groups at day 20 after HT1080 cell implantation was 2169, 356, 581, 140, 136 and 32 mg, respectively (Fig. 7B). The value of TWI (%) in the MTD Taxol treatment, MTD SSL-PTX treatment, MTD NGR-SSL-PTX treatment, metronomic SSL-PTX treatment and metronomic NGR-SSL-PTX treatment groups compared with the control group was ~83.6, ~73.2, ~94.6, ~93.7 and ~98.8%, respectively. In addition, no significant weight loss was observed between the treatment groups and the control group (data not shown).

### 3.6. In vivo angiogenesis

To evaluate the anti-angiogenic activity of metronomic NGR-SSL-PTX treatment in vivo, the microvessel density was assessed by immunohistochemistry. As shown in Fig. 8A, microvessels were clearly observed by CD31 staining. Very few microvessels were observed in the metronomic NGR-SSL-PTX treatment group (Fig. 8A6). The MVD in the PTX treatment groups was significantly less than that in the control group (p < 0.01), as shown in Fig. 8B. There

Fig. 7. In vivo anti-tumor activity of NGR-SSL-PTX by metronomic administration. BALB/c nude mice were inoculated s.c. with HT1080 cells and treated with physiological saline, MTD Taxol (15 mg/kg, i.v., q4d), MTD SSL-PTX (15 mg/kg, i.v., q4d), MTD NGR-SSL-PTX (15 mg/kg, i.v., q4d), metronomic SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), metronomic NGR-SSL-PTX (5 mg/kg, i.v., from day 6 to day 17 consecutively), respectively. For each administration, formulations were given to mice characterized in rats and illustrated in Fig. 6. The main pharmacokinetic parameters of PTX are summarized in Table 2. As shown in Fig. 6, PTX in Taxol was quickly eliminated after intravenous administration. However, PTX in NGR-SSL-PTX and SSL-PTX was more slowly eliminated from the circulation. The values of MRT and t1/2 in the SSL-PTX and NGR-SSL-PTX treatment groups significantly increased to 11.78 h and 13.67 h, and 9.15 h and 13.62 h, respectively, compared with those in the Taxol treatment group (2.46 h and 3.11 h, respectively). The clearance of PTX in the SSL-PTX and NGR-SSL-PTX treatment groups was significantly lower than that in the Taxol treatment group (p < 0.01). The values of AUC in the SSL-PTX and NGR-SSL-PTX treatment groups significantly increased to 27.54 and 25.01 µg h/ml, respectively, compared with those in the Taxol treatment group (10.06 µg h/ml). The bioavailability of PTX in the SSL-PTX and NGR-SSL-PTX treatment groups compared with that in the Taxol group was 273.7% and 248.6%, respectively.
was significantly less MVD in the metronomic treatment groups (SSL-PTX or NGR-SSL-PTX) compared with the MTD treatment groups \( (p < 0.01) \). For MTD treatment, the NGR-SSL-PTX treatment was slightly less effective than the Taxol treatment in inhibiting MVD, but the differences failed to reach statistical significance. In fact, the metronomic NGR-SSL-PTX group reduced MVD more markedly compared with the other treatment groups \( (p < 0.05 \text{ or } p < 0.01) \).

### 3.7. Bio-distribution of NGR-SSL-DiR in tumor-bearing mice

Fig. 9 shows the distribution and tumor accumulation of fluorescent DiR in the HT1080 tumor-bearing mice. The DiR fluorescence signal of the tumor site was stronger for the mice treated with NGR-SSL-DiR than those treated with SSL-DiR \( (\text{Fig. 9A and B}) \) at all observed time points. The major organs (heart, liver, spleen, lung, kidney) and tumor tissues were isolated and the \textit{ex vivo} images were studied. The results showed that the stronger fluorescence intensity was found in tumor tissue after administration of NGR-SSL-DiR compared with that in the SSL-DiR group \( (\text{Fig. 9C and D}) \).

### 3.8. Confocal immunofluorescence microscopy study

To investigate the intratumoral distribution of NGR-SSL-DiI after systemic delivery, confocal immunofluorescence microscopy were used to examine cryosections of subcutaneous HT1080 tumors excised from mice 2 and 6 h after tail-vein injection with NGR-SSL-DiI or SSL-DiI. As shown in \textit{Fig. 10}, the blood vessels stained with CD31 exhibited a green fluorescence while those stained with DiI exhibited a red fluorescence. NGR-SSL-DiI accumulated in the blood vessels as shown by its co-localization with CD31 staining \( (\text{Fig. 10C and D}) \). In addition, the accumulation effect of NGR-SSL-DiI on HT1080 tumor cells was also observed \( (\text{Fig. 10C and D}) \). These results showed that the targeting effect of NGR-modified liposomes to APN over-expression tumor cells. In contrast, the accumulation effect of SSL-DiI on HT1080 tumor cells, but not in the blood vessels, was observed \( (\text{Fig. 10A and B}) \), due to the EPR effect. As shown in \textit{Fig. 10E, F and G, H}, for APN negative expression MCF-7 tumor-bearing model, the tumor cell accumulation effect was not observed in NGR-SSL-DiI group \( (\text{Fig. 10G and H}) \). However, the NGR-SSL-DiI showed the accumulation effect in the blood vessels \( (\text{Fig. 10G and H}) \).

### 4. Discussion

The anti-angiogenic therapy strategies that have been investigated involve targeting APN receptors using drug delivery systems modified by an NGR ligand \([30–35]\) and metronomic administration \([36–39]\). In the present study, we prepared a NGR-SSL-PTX with the aim of evaluating its potential targeting of APN receptors both expressed in tumor endothelial cells and on the tumor cell surface as well as the anti-angiogenic activity obtained following metronomic administration. The detailed scheme for the preparation and targeting effect of NGR-SSL-PTX is illustrated in \textit{Fig. 11}.

It has been reported that tumors treated with anti-angiogenic targeting liposomes containing doxorubicin have a markedly reduced tumor vessel density and more potent inhibited tumor growth than tumors treated with non-targeting liposomes \([18]\). This tumor site-specific target therapy may overcome the adverse effects caused by the current use of systemic therapy using anti-angiogenic drugs and increase the therapeutic index. In the present study, the targeting activity of the NGR-modified drug delivery systems to endothelial cells and blood vessels was confirmed in our \textit{in vitro} and \textit{in vivo} experiments. Our current
in vitro results of flow cytometry and confocal microscopy indicated specific binding of NGR-modified liposomes in APN-expressing endothelial cells, demonstrating the anti-angiogenic targeting characteristics of these NGR-modified liposomes (Figs. 2 and 3). In addition, the targeting effect of the NGR-modified liposomes in blood vessels was also observed in our

Fig. 9. Bio-distribution of NGR-SSL-DiR in tumor-bearing mice. In vivo whole body imaging of HT1080 tumor-bearing mice after SSL-DiR (A), NGR-SSL-DiR (B) administration at 2, 6, 12 and 24 h time point, respectively. The ex vivo optical images of tumors and organs of HT1080 tumor-bearing mice sacrificed at 24 h after SSL-DiR (C) or NGR-SSL-DiR (D) administration.

in vivo confocal immunofluorescence microscopy study experiments (Fig. 10C,D and G,H). The NGR-modified liposomes could target the blood vessels in both of APN over-expression HT1080 and APN negative expression MCF-7 bearing-tumor (Fig. 10C,D and G,H), showing the anti-angiogenic targeting effect independent of the tumor cells.
Fig. 10. Localization of NGR-SSL-Dil in tumor tissues. BALB/C nude mice bearing HT1080 tumors (~200 mm³) were intravenously injected with SSL-Dil or NGR-SSL-Dil at a dose of 800 ng per mouse. At 2 or 6 h time point, the mice in the experiment were sacrificed, and the tumors were harvested and tumor sections were examined for fluorescence. A: 2 h after given SSL-Dil; B: 6 h after given SSL-Dil; C: 2 h after given NGR-SSL-Dil; D: 6 h after given NGR-SSL-Dil; BALB/C nude mice bearing MCF-7 tumors (~200 mm³) were intravenously injected with SSL-Dil or NGR-SSL-Dil at a dose of 800 ng per mouse. At 2 or 6 h time point, the mice in the experiment were sacrificed, and the tumors were harvested and tumor sections were examined for fluorescence. E: 2 h after given SSL-Dil; F: 6 h after given SSL-Dil; G: 2 h after given NGR-SSL-Dil; H: 6 h after given NGR-SSL-Dil; Nuclei were counterstained with Hoechst 33258 (blue, A1, B1, C1, D1, E1, F1, G1 and H1); Dil exhibited a red fluorescence (red, A2, B2, C2, D2, E2, F2, G2 and H2); tumor blood vessels exhibited a green fluorescence (anti-CD31, green, A3, B3, C3, D3, E3, F3, G3 and H3); Merge (A4, B4, C4, D4, E4, F4, G4 and H4); A5, B5, C5 and D5 were the amplificatory figures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Although the vascular APN expression was independent of the tumor cells, some tumor cells also over-expressed APN receptors, such as HT1080 cells. Our in vitro results of flow cytometry and confocal microscopy also indicated specific binding of NGR-modified liposomes in APN-expressing HT1080 cells, unlike the APN negative expression MCF-7 cells (Figs. 2 and 3), indicating the targeting characteristics of these NGR-modified liposomes to tumor cells fed by tumor blood vessels [23,40]. The tumor cell targeting
effect was also observed in our in vivo confocal immunofluorescence microscopy study experiments, showing that the NGR-modified liposomes could accumulate to the APN-expressing HT1080 cells, not to APN negative expression MCF-7 cells (Fig. 10). Our in vivo biodistribution results demonstrated the targeting activity of these NGR-modified liposomes to the APN over-expression HT1080 bearing-tumor (Fig. 9). Therefore, considering the results described above, we concluded that these NGR-modified liposomes could target both APN over-expression tumor endothelial cells and the tumor cells producing both the anti-angiogenic and anti-tumor effect. With both neovascularization and tumor cells being targeted, this will help increase the drug therapeutic index.

Paclitaxel is a strong candidate for metronomic chemotherapy given its ability to inhibit endothelial cell functions associated with angiogenesis in vitro at extraordinarily low concentrations and because of its broad-spectrum anti-tumor activity [10]. However, clinically relevant concentrations of the formulation vehicle CrEL in Taxol have previously been reported to nullify the anti-angiogenic activity of paclitaxel. We previously reported that metronomic chemotherapy with SSL-PTX exhibits potent anti-angiogenic activity in vivo [11]. In addition, low dose metronomic chemotherapy with PTX has been reported to display a stronger antitumor activity in vivo compared with primary and metastatic breast tumors with a stronger antiangiogenic and antilymphohangiogenic activities than MTD PTX therapy [36]. Also, low-dose metronomic chemotherapy of PTX results in a more potent antitumor effect against colon carcinoma tumors and a reduced microvessel density in tumors as compared with MTD PTX [41]. Our current in vitro endothelial cell proliferation and migration assay results show that the anti-angiogenic activity of NGR-SSL-PTX is similar with that in SSL-PTX (Figs. 4 and 5), indicating the potential in vivo anti-angiogenic activity of NGR-SSL-PTX administrated by metronomic therapy.

The results of the immunohistochemistry study confirm the anti-angiogenic effect of metronomic NGR-SSL-PTX in vivo (Fig. 8). We also observed anti-angiogenic effects in the SSL-PTX MTD or NGR-SSL-PTX MTD treatment group, but this effect was much lower than that in the metronomic treatment group (p < 0.01), as shown by the microvessel density evaluation. These results indicate that frequent administration of SSL-PTX or NGR-SSL-PTX, at doses lower than MTD, produces anti-angiogenic effects to block the blood supply and this may be more effective in suppressing tumor growth in vivo. Our data on the anti-angiogenic effect also demonstrate that the metronomic NGR-SSL-PTX group reduced MVD more markedly compared with the metronomic SSL-PTX treatment groups (p < 0.05). We suggested that the anti-angiogenic effect produced by NGR-modified active targeting was superior to that produced by EPR effect of passive targeting for metronomic therapy.

PEGylated liposomes, regarded as having great potential as a drug delivery system, have a longer half-life in the blood [42]. Our pharmacokinetic results indicate that the sustained circulation of PEGylated liposomes was not abrogated by NGR modification. It has been reported that PEGylated liposomes can spontaneously accumulate in solid tumors because of enhanced permeability and retention (EPR) effects through a passive targeting mechanism [43,44]. However, the efficiency of passive targeting of tumors by the EPR effect is limited [20]. Therefore, to increase tumor-specific drug accumulation, much attention has been given to unique active-targeting ligands that are specifically over-expressed in cancerous tissues. In the present work, we selected NGR-peptide as a ligand for APN and developed NGR-modified liposomes. Our in vivo confocal immunofluorescence microscopy results indicated that specific binding effect producing by NGR-SSL-Dil was significant higher than that producing by SSL-Dil in the APN over-expressing HT1080 cells. The results of the in vivo bio-distribution demonstrated that the targeting activity of the NGR-modified liposomes was significantly higher than that of PEGylated liposomes.

To further verify the anti-tumor activity of NGR-SSL-PTX in vivo, an APN over-expressed HT1080-bearing animal model was established and the animals were treated with PTX formulations. It has been reported that the anti-tumor activity in NGR-targeted DOX liposome MTD treatment group was superior to that in metronomic treatment group [20]. The author suggested that these results, which apparently contradict the metronomic chemotherapy, might be explained by the fact that liposomes behave as a “metronomic dosing system,” because they are long-circulating and have sustained release properties. The half-life for release of DXR for liposomes of the composition used in these experiments is 315 h. In the current study, the anti-tumor activity and anti-angiogenic effect produced by NGR-SSL-PTX (MDT or metronomic) were higher than that produced by SSL-PTX (MDT or metronomic), as shown in Figs. 7 and 8, indicating the effect of NGR-modified active targeting. Our results also showed that the tumor growth inhibition as well as the tumor weight in the NGR-SSL-PTX metronomic treatment group was significantly higher than that in the other PTX formulation treatment groups (p < 0.05 or p < 0.01), confirming the anti-tumor activity of this anti-angiogenic targeting and drug delivery system administrated by metronomic therapy.

5. Conclusion

We prepared a NGR-SSL-PTX with the aim of evaluating its potential targeting of APN receptors expressed on tumor endothelial cells as well as the tumor cell surface and examined its anti-angiogenic activity following metronomic administration. The targeting activity of the NGR-modified liposomes was demonstrated by in vitro flow cytometry and confocal microscopy as well as in vivo confocal immunofluorescence microscopy and bio-distribution experiments. The results of endothelial cell proliferation and migration and MVD confirmed the anti-angiogenic activity of NGR-SSL-PTX in vitro and in vivo. The sustained circulation of NGR-SSL-PTX was shown in the pharmacokinetic study. NGR-SSL-PTX is able to improve treatment efficacy producing the most significant anti-tumor activity and anti-angiogenic following metronomic administration.

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Appendix A. Supplementary data

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References


