Therapeutic Targeting of Angiogenesis with a Recombinant CTT Peptide–Endostatin Mimic–Kringle 5 Protein

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Abstract

Angiogenesis is required for tumor growth and metastasis, and targeting angiogenesis is a novel anticancer strategy. However, cancer development is a complex multistep process, and single antiangiogenic agents have limited therapeutic efficacy. Here, we report a triple fusion protein, namely CTT peptide–endostatin mimic–kringle 5 (AARP), consisting of MMP-2/9–selective inhibitory peptide (CTT peptide) and well-known endogenous antiangiogenic agents (endostatin mimic and kringle 5), which can simultaneously target matrix metalloproteinases (MMP) and endothelial cells, blocking their actions. AARP was bacterially expressed, and biologic activity of purified AARP was assessed. AARP could significantly inhibit the enzymatic activity of MMP-2/9, proliferation, migration, and tube formation of endothelial cells in vitro. The antitumor activity of AARP was shown in a concentration-dependent manner when injected i.p. into immunodeficient mice bearing multidrug-resistant human epidermoid carcinomas (KB), and AARP is superior to clinical grade endostatin in inhibiting KB xenograft growth. In mouse models of Lewis lung carcinoma (LLC) and hepatoma H22, when given as a single dose, AARP is highly effective for reducing tumor growth, angiogenesis, and metastasis, and increasing survival time. AARP possessed significantly greater antiangiogenic activity than endostatin mimic, CTT peptide–kringle 5 (RK5) both in vitro and in vivo. Compared with conventional chemotherapeutic agents (cyclophosphamide and paclitaxel), AARP is also effective. More importantly, AARP is cytocompatible and no tissue toxicity could be observed after large dose administration. Taken together, our findings suggest AARP is a highly effective, safe, and more potent antiangiogenic agent for blocking tumor angiogenesis and metastasis, and warrants further testing for clinical applications.

Introduction

Angiogenesis is a program of deliberately orchestrated cellular events that includes endothelial cell proliferation, migration and differentiation, and remodeling of the extracellular matrix (ECM; refs. 1, 2). Under normal physiologic circumstances, angiogenesis is tightly regulated by a balance between angiogenic activators and inhibitors (1). However, persistent and upregulated angiogenesis is often found to be a critical causal factor in certain pathologic conditions such as cancer, atherosclerosis, and diabetic retinopathy (2, 3). In case of tumors, angiogenesis is a rate-limiting step in the development and progression of tumors because tumor growth is generally limited to 1 to 2 mm³ in the absence of a blood supply (3). In the tumor microenvironment, when angiogenic stimulators outbalance angiogenic inhibitors, generating a proangiogenic response and an increased blood vessel density to allow the tumor to progress (3, 4). Thus, interfering with angiogenesis is a promising strategy in cancer therapy to delay tumor growth by preventing a tumor from developing its own blood supply system (3, 5).

Since 1980, at least 28 endogenous angiogenesis inhibitors have been identified (5). Two of the most potent endogenous antiangiogenic inhibitors, angiotatin and endostatin are the most well-studied in the angiogenesis literature (5). Angiostatin (kringles 1–4), an endogenous angiogenesis inhibitor of 38 kDa internal fragment of plasminogen (contains kringles 1–5), which was originally identified from mice bearing a Lewis lung carcinoma (LLC) because of its ability to inhibit the growth of established metastases (6). Kringle 5 is a proteolytic fragment of plasminogen, consisting of 80 amino acids (7). On the basis of in vitro assays, kringle 5 has more potent antiangiogenic activity than angiostatin (7, 8). Kringle 5 has been shown to inhibit angiogenesis by inducing the cell-cycle arrest, autophagy, and apoptosis of proliferating endothelial cells (8, 9). Endostatin, a 20-kDa C-terminal proteolytic fragment of collagen XVIII, inhibits endothelial cell
findings indicate that this triple fusion protein AARP may be solubilized in 8 M urea, and the target proteins a, b, c, of the molecule (12). Clinical studies showed that human endostatin was unstable (with a halflife less than 2 hours) and truncated, limiting its clinical efficacy (13–15). Matrix metalloproteinases (MMP), especially MMP-2 and MMP-9, are responsible for ECM remodeling during angiogenesis, and MMP inhibitors have also been shown to effectively inhibit angiogenesis (16, 17).

Because endothelial cells (EC) play a central role in the angiogenic process, they naturally constitute a primary target for therapeutic antiangiogenesis (18–20). Antiangiogenic tumor therapies with endogenous inhibitors have attracted intense interest because of their broad spectrum of action, low toxicity, and absence of drug resistance (4, 3, 21). However, tumor angiogenesis is a complex process resulting from numerous signaling molecules and pathways from the tumor microenvironment, and inhibition of a tumor angiogenesis pathway by a single drug alone is not sufficient to block redundancies in tumor angiogenesis regulators (21, 22). Therefore, the development of multitargeted antiangiogenic agent is needed for tackling these challenges and arises as an attractive therapeutic approach to treat cancer and other angiogenesis-dependent diseases (22).

Here, we report a novel triple fusion antiangiogenic protein, AARP, consisting of endostatin mimic (NH2-terminal 25-amino acid peptide of endostatin), an MMP-2/9–selective inhibitory peptide (CTTHWGFTLC), and kringle 5 fragment of human plasminogen (7, 12, 23). We reasoned that this triple fusion protein would increase the half-life of endostatin mimic peptide, enhance antiangiogenic activity of RK5 (CTT peptide-kringle 5) or endostatin mimic peptide. We produced this novel fusion protein in Escherichia coli and described the antitumor activity in vitro and in vivo. We showed that this triple fusion protein have significantly enhanced antiangiogenic activity and antitumor efficacy in several cancer models. More importantly, compared with conventional chemotherapeutic agents such as paclitaxel and cyclophosphamide, AARP is also effective without detectable toxicity. Our findings indicate that this triple fusion protein AARP may be a promising agent for antiangiogenic therapy of cancer.

Materials and Methods

Detailed methodology is described in the Supplementary Data.

Cell lines and animals

Lewis lung cancer cell line LL/2, murine hepatoma cell line H22, nonendothelial cells (293T, HepG2, HT1080, HeLa, HCT116, RAW264.7, THP-1, U937) were purchased from the ATCC. Theses cell lines were authenticated using PCR fingerprinting by the provider. Human multidrug-resistant KB epidermoid carcinoma cells were kindly provided by professor L.-W. Fu from the Sun Yat-sen University (GuangZhou, China). The human KB cells were authenticated by short tandem repeat profiling (Promega Powerplex 16 System). KB cells, 293T, HepG2, HT1080, HeLa, and HCT116 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (GIBCO) and 1% penicillin-streptomycin (GIBCO). LLC cells, H22 cells, RAW264.7, THP-1, and U937 cells were grown in complete RPMI-1640 (Invitrogen) supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Jincai Luo (PeKing University, China), which were isolated from umbilical cord vein following collagenase digestion (24). HUVECs were maintained in M199 with 2 mmol/L L-glutamine, 20% FBS, 3 ng/mL basic fibroblast growth factor (bFGF), 1% penicillin-streptomycin, were used at passage 2 to 6. No authentication of the HUVECs was done by our authors. All cells were maintained and propagated at 37°C in a humidified 5% CO2 incubator. All cells were passaged for less than 6 months in our laboratory after receipt or resuscitation.

Male athymic Balb/c nude mice (nu/nu), Balb/c mice, Kunming mice, C57BL/6 mice (6–8 weeks) were purchased from Beijing Vital River Laboratories. All animal experiments were done in compliance with the guidelines approved by the Institutional Animal Care and Use Committees of PeKing University and the mice were maintained in accordance with the guidelines for the care and use of laboratory animals.

Construction, expression, and characterization of RK5, AARP

RK5 was expressed and purified according to Zou and colleagues (25). cDNA encoding triple fusion protein AARPα and isoform AARPβ were amplified with overlap extension PCR. cDNA sequence encoding 25 aa of N-terminal endostatin and CTT peptide (10 aa) were connected to kringle 5 DNA, 25aa/10aa/kirngle 5 were connected with a flexible peptide linker. AARPα and AARPβ were expressed in E. coli BL21 (DE3) cells by 1 mmol/L isopropyl-1-thio-β-galactopyranoside (IPTG) induction 16 hours at 25°C. The isolated inclusion bodies are solubilized in 8 mol/L urea, and the target proteins were purified and refolded.

Cell proliferation analysis

HUVECs were maintained in M199 containing 20% heat-inactivated FBS (GIBCO) and recombinant human bFGF (3 ng/mL). Cells were dispersed in 0.05% trypsin solution, resuspended with M199 containing 1% FBS, and seeded in each well of 24-well plates (1 × 104 cells in 0.5 mL/well, counted with a hemocytometer). After adherence (4–6 hours), the medium was replaced with 0.1% FBS containing test proteins (25 aa, RK5, AARPα, 

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and AARPβ). Seventy-two hours later, HUVEC proliferation was measured by MTT colorimetric assay (n = 4 independent experiments).

**Cell migration assay**

Cell migration assay was performed using a Transwell system (Costar), which allows cells to migrate throughout an 8-mm pore size polycarbonate membrane of millicell (Matrigel-coated Transwells). HUVECs were plated (10⁶ cells/well) into upper chamber in M199 containing 0.1% FBS, and 1.5 μmol/L of 25aa, RK5, 25aa+RK5, AARPα, AARPβ or phosphate buffered saline (PBS) were added in the lower chamber of a 24-well plate containing 20% FBS, 20 ng/mL VEGF, 20 ng/mL bFGF. After 48 hours, cells remaining on the upper surface of the membrane were scraped and the cells on the lower surface of the membrane were fixed with cold methanol for 30 minutes and stained with 0.2% crystal violet. Cells that had migrated to the bottom of the membrane were visualized and counted (n = 4 independent experiments).

**Tube formation assay**

A 48-well plate coated with 0.2 mL Matrigel (10 mg/mL, BD Biosciences) per well was allowed to solidify at 37°C for 1 hour. Each well was seeded with 1 x 10⁴ previously starved HUVEC and cultured in M199 containing 20 ng/mL VEGF+20 ng/mL bFGF in the presence of tested substances (1.5 μmol/L of 25aa, RK5, 25aa+RK5, AARPα, AARPβ, or PBS) for 48 hours. The networks of tubes were photographed and relative lengths were compared (n = 4 independent experiments).

**In vitro β-casein degradation assay**

MMP-2 (50–100 ng; Sigma) was preincubated for 60 minutes with RK5, AARPα, and AARPβ at concentrations indicated in the text. The substrates, a 21 kDa β-casein (52 μmol/L) was incubated for 1 or 2 hours at 22°C (the total reaction volume was 10–15μL). Degradation of β-casein was analyzed by SDS gel electrophoresis essentially as described previously (23).

**Hemolytic assay**

Erythrocytes were washed with PBS until the supernatant was clear and then resuspended in PBS. Erythrocyte suspensions (1 x 10⁷ cells/mL) were incubated with 25 μmol/L of different proteins (kringle 5, RK5, AARPα, and AARPβ) at 37°C for 30 minutes and then centrifuged at 1,000 x g for 5 minutes at 4°C to precipitate intact erythrocytes and debris. The supernatants were assayed for absorbance at 570 nm to determine the amount of hemoglobin released from the lysed erythrocytes. The absorbance of a supernatant obtained using 0.1% Triton X-100 was used as positive control. The supernatant of an untreated erythrocyte suspension in PBS was used as a spectrophotometric blank.

**Pharmacokinetics and toxicity analysis of AARPα**

For the in vivo pharmacokinetics study, 21 Balb/c mice (7 mice each group) were i.v. injected with 125I-AARPα, 125I-25aa, 125I-RK5 at a single dose of 5 mg/kg. The γ-counter was used to detect the radioactivities of all samples. The concentration of AARPα at each time points was used to determine its blood clearance rates. The distribution data were expressed as the percentage of the injected dose per gram of tissue.

The hemolytic activity of purified proteins (kringle 5, RK5, AARPα, and AARPβ) was determined using human erythrocytes, as previously reported (26).

To evaluate the toxicity effect of AARPα, mice were treated with normal saline or AARPα (250 mg/kg, i.p) for continuous 32 days, and their body weights were recorded. By the end of treatment, histologic analysis (hematoxylin and eosin, H&E) of liver, lung, and kidneys was conducted (randomly selected at least 5 mice, 5–8 sections/mice, 7 μm).

**Mouse tumor models**

**Human xenograft model.** Multidrug-resistant human KB epidermoid carcinoma cells were grown and maintained in DMEM medium supplemented with 10% FBS and antibiotics. The KB cell concentration was adjusted to 1 x 10⁶ cells/mL. A suspension of 1 x 10⁶ tumor cells in 0.1 mL DMEM containing thawed Matrigel (1:1) was injected s.c. into the dorsal back of athymic Balb/c nude mice (n = 12–14 per group) at the proximal midline. At the completion of this experiment, the mice were euthanized with CO₂ inhalation.

**Syngeneic xenograft models.** LLC (syngeneic with the C57BL/6 mouse strain) cells were propagated by sequential subcutaneous transplantation in C57BL/6 mice. Tumor cells were prepared by mincing the tumor, cells were sieved through a 300 mesh stainless steel, then thoroughly washed with PBS to remove erythrocytes. The viability (>85%) of the tumor cells was determined by trypan blue exclusion. Then 1 x 10⁶ LLC cells in 0.1 mL of PBS via a 29-guage needle were injected s.c. into the dorsal back of each mouse (mice were shaved 1 day before injection, n = 20–23 per group).

Murine H22 hepatoma cells (syngeneic with the Balb/c mouse strain) were passaged in the ascites of Kunning mice. After 8 days, the intraperitoneal tumor cells were collected from the mouse with ascites tumor. Viable tumor cells were counted by trypan blue staining (>85% viability); next, 5 x 10⁶ murine H22 hepatoma cells in 0.1 mL of PBS via a 29-guage needle were injected s.c. into the dorsal back of each mouse (mice were shaved 1 day before injection, n = 20–23 per group).

For treatment of tumor-bearing mice, when solid tumors were visible (tumor volumes allowed to grow to 15–30 mm³, average tumor volume >20 mm³), and mice were randomized into indicated groups, and the treatments were carried out. The mice were given...
intrapertitoneal injections of clinical grade endostatin, endostatin mimic (25 aa), RK5, AARPα, AARPβ, clinical grade cyclophosphamide (KB human xenograft), or paclitaxel (LLC and H22 syngeneic xenograft; indicated dose, frequency, and duration). As a control, an equal volume of saline was injected in the same manner. The tumor weight, tumor volume of each mouse (KB xenograft) was recorded 3 to 6 days. The body weight, survival rate of each mouse (LLC and H22) was recorded, and longitudinal tumor volume was calculated from caliper measurements using volume = (length) \times (width)^2 \times 0.5. The representative xenograft tumor tissues were dissected (LLC and H22) and fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analysis. The unpaired Student t test was used for statistical analysis.

Experimental lung metastasis assay

LLC cells (1 \times 10^7) were injected directly into the tail vein of 6- to 8-week-old male C57BL/6 mice (n = 8 per group; ref. 27). Six days later, mice were randomized into four groups, and treatments initiated. The mice were given intraperitoneal injections of RK5, AARPα, and AARPβ (indicated dose, frequency, and duration). As a control, an equal volume of saline was injected in the same manner. Mice were sacrificed by CO2 inhalation for examination at 28 days, after tumor inoculation. Metastases were assessed by gross necropsy, macroscopic pictures of lungs were taken and metastatic nodules (>200 \mu m) were counted for each lobe using a dissecting microscope at \times 4 magnification.

Immunohistochemistry analysis

Following the standard antigen retrieval protocol, CD31 antibody (Abcam) was used to stain formalin-fixed, paraffin-embedded sections at a 1:500 dilution for 1 hour at room temperature. Slides were again rinsed and then incubated with horseradish peroxidase conjugate of goat anti-mouse IgG (Santa Cruz Biotechnology) at a 1:200 dilution for 30 minutes at room temperature. Slides were then rinsed and incubated with DAB (Dako Cytomation K3468). Slides were rinsed and dehydrated through a series of ascending concentrations of ethanol and xylene and then coverslipped. The CD31/MVD expression was determined in photomicrographs of tumor tissues (n = 5) with total \times 100 magnification in three fields from area of highest vascular density in the tumor per slide. Vessels up to 50 \mu m in diameter were considered for counting.

Statistical analyses

Results are depicted as the mean ± SEM. Statistical analyses were performed using the Student t test. GraphPad Prism (v. 5, GraphPad) was used for statistical analysis. Multiparameter statistics for the Kaplan–Meier survival curves were performed by a log-rank test. All statistical tests were two-sided, with P < 0.05 considered significant.

Results

Characterization and preparation of AARPα and AARPβ

The chimeric protein AARP (Fig. 1A) is composed of CTT peptide (an MMP-2/9 inhibitory peptide, CTTHWGFTLC), endostatin mimic (N terminal 25 aa of the native molecule), and kringle 5 fragment of plasminogen (7, 12, 23). AARP represents antiangiogenic recombinant protein. RK5 is a double fusion protein we previously developed, which contains CTT peptide and kringle 5 (25). There could be two variants of triple fusion protein when 25 aa fused with RK5 as illustrated in Fig. 1A. One is named AARPα, another AARPβ according to the position of 25aa fused with RK5. The recombinant cDNA was cloned in a T7 promoter–based E. coli expression vector (pet 22b) equipped with a hexahistidine affinity tag for purification. The recombinant plasmid was transformed into E. coli, and the IPTG-induced target proteins, mainly existing in inclusion bodies, were solubilized with 8 mol/L urea and purified by Ni²⁺ chelate affinity chromatography under denaturing conditions. The proteins were then refolded by dialysis against gradually decreased urea concentrations (from 4 to 2 mol/L) and finally in PBS (pH 7.4; refs. 10, 28, 29). RK5 showed an apparent molecular weight of 12 kDa, AARPα and AARPβ showed an apparent molecular weight of 15 kDa, matching the calculated molecular weight based on the sequence (Fig. 1B). The purity of the target proteins reached >90%, and the integrity of RK5, AARPα, and AARPβ were further determined with N-terminal sequencing.

AARP is more potent in inhibiting endothelial cell proliferation, migration, and tube formation

The CTT peptide (CTTHWGFTLC) is a potent and selective inhibitor for MMP-2 and MMP-9, which was found by phage-display technology (23). Importantly, synthetic and recombinant CTT peptides showed nearly identical inhibitory activities against MMP-2 and MMP-9 (25, 30–33). In our previous study, we found recombinant CTT peptide, when fused with kringle 5, could inhibit MMP-9 caseinolytic activity in a concentration-dependent manner (25). In this report, we initially investigated whether recombinant CTT peptide, when fused with 25 aa and kringle 5, could inhibit potent MMP-2/9 inhibitory activity. The inhibitory activities of CTT peptide–endostatin mimic (25 aa)–kringle 5 (namely AARPα) and endostatin mimic–CTT peptide–kringle 5 (namely AARPβ) toward MMP-2/9 were examined by cascin degradation assay. As shown in Fig. 1C and Supplementary Fig. S1, the degradation of cascin by MMP-2/9 was prevented in the presence of RK5, AARPα, and AARPβ at a dose-dependent manner; the results of the MMP-2/9 inhibition study were consistent with those previously described (23).

Next, we evaluated the effect of AARPα and AARPβ on the proliferation of endothelial cells. MTT assay showed that both AARPα and AARPβ are much more potent in inhibiting HUVEC proliferation than RK5 alone and RK5+25aa in a dose-dependent manner (P < 0.01).

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indicating that the triple fusion protein (AARPα/AARPβ) is more effective than the additive inhibitory efficacy of 25aa + RK5 (Fig. 1D). RK5, AARPα/AARPβ appeared to be endothelial cell specific because macrophage-derived cells and tumor cells were completely insensitive to RK5, AARPα/AARPβ treatments at the concentration required for maximal inhibition of HUVEC cell proliferation (>1.5 μmol/L) as compared with nontreated cells (Supplementary Fig. S3).

To determine whether AARPα/AARPβ induces endothelial cell apoptosis, 1.5 μmol/L RK5, AARPα, and AARPβ were incubated with proliferating HUVECs. After 48-hour incubation, a significant proportion of HUVECs became apoptotic as detected by Hoechst 33258 staining and DNA fragmentation analysis (Supplementary Fig. S2). Moreover, apoptotic quantification analysis demonstrated that AARPα and AARPβ were more potent than RK5 (Supplementary Fig. S2E).

To further compare the antiangiogenic efficacy of AARPα/AARPβ with that of RK5, we next tested the effect of these recombinant proteins on HUVEC migration and tube formation. AARPα and AARPβ inhibited...
the HUVEC migration and tube formation much more effectively than RK5 at the concentration of 1.5 μmol/L. However, an additive inhibitory efficacy of RK5 + 25 aa, was detected when RK5 and 25 aa (1.5 μmol/L) were added together to HUVECs. Interestingly, this additive inhibitory efficacy is inferior to that produced by AARPα or AARPβ alone, and no statistically significant difference has been found between AARPα and AARPβ (Fig. 2A and B). We chose AARPα, hereafter referred to as AARP, as the best candidate.

**Pharmacokinetics and potential toxicity of AARP**

The pharmacokinetic profiles of 125I labeled 25 aa, RK5, and AARPα (5 mg/kg) were evaluated in Balb/c mice following single intravenous administration. After harvesting blood samples, drug concentration was quantified with a survey meter (Geiger counter). The pharmacokinetic profiles showed that the half-life of AARPα (≈13.1 hours) was longer than that of RK5 (≈9.4 hours) and 25 aa (2 hours; Fig. 3A).

Hemolysis of red blood cells (RBC) is another problem associated with the bioincompatibility of a material. We examined the potential hemolytic activity of 25aa, RK5, AARPα, and AARPβ (25 μmol/L) by measuring supernatant absorbance at 570 nm, positive control was Triton X-100 (0.1% v/v), a known hemolytic agent. As shown in Fig. 3B, 25aa, RK5, AARPα, and AARPβ exhibited negligible hemolysis degrees in comparison with the control Triton X-100.

Furthermore, we examined the potential toxicity of high dosage of AARPα in Balb/c mice (250 mg/kg of
body weight, i.p. administrated every day for 32 days), PBS served as normal control. During the treatment period, no significant differences in body weight change were observed in saline- and AARPα-treated groups (Fig. 3C). Mice treated with saline and AARPα were sacrificed at the end of the 32 days treatment period, blood samples were collected. Serum biochemical parameters, including aspartate aminotransferase (AST), aspartate aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) were evaluated. Kidneys, lungs, and livers were collected for pathologic examination. AST, ALT, BUN, and creatinine levels in AARPα-treated mice were all within the reference range (Supplementary Fig. S4). No gross histologic signs of toxicity were observed in AARPα-treated mice (Fig. 3D), indicating that treatment with AARPα produced virtually no toxicity or non-detectable toxicity in mice.

**AARP has significant in vivo antitumor activity against KB xenografts**

To explore the value of AARPα as an antiangiogenic therapeutic molecule, we first evaluated the effect of AARPα on tumor growth in vivo. We implanted 1 × 10^6 multidrug-resistant human epidermoid carcinoma KB cells subcutaneously into dorsal back of immunodeficient mice, the mice were left for 6 days, allowing a small nodule (>20 mm^3) to grow and mice were randomly grouped into 6 groups. The mice received intraperitoneal injection of 10, 20, and 40 mg/kg of AARPα, 10 mg/kg clinical grade endostatin (produced and approved in China), equivalent amount of saline (as a control) every other day, 100 mg/kg clinical grade cyclophosphamide (i.p injected every 5 days) for the subsequent 30 days, and the tumor growth was measured every 4 to 6 days. Compared with normal saline and endostatin treatment, tumor growth was markedly attenuated with AARPα and...
cyclophosphamide treatment (Fig. 4A). Treatment with AARPα significantly reduced tumor growth and tumor weight in mouse xenograft models of KB in a concentration-dependent manner, whereas no distinct differences were observed between 10 mg/kg AARPα- and endostatin-treated groups (Fig. 4A). Because 20 to 40 mg/kg AARPα administration exerted strong inhibitory effect in tumor growth, we chose this dosage range for subsequent experiments.

AARP is highly effective in reducing tumor growth, metastasis, and extending mouse survival in the LLC implantation model

We generated an LLC model by implanting 1 × 10⁶ LLC cells into subcutaneous dorsal back of C57BL/6J mice. Six days later, the mice received intraperitoneal injections of 25 mg/kg RK5, AARPα, AARPβ, equal amount of normal saline, and 10 mg/kg of paclitaxel every day for the subsequent 22 days and the tumors were sampled. In the LLC tumor model, the tumor growths were significantly attenuated by AARPα and AARPβ compared with RK5 treatment (Fig. 5A and B). Kaplan–Meier survival analysis showed that mice treated with AARPα and AARPβ lived significantly longer than RK5-treated mice (P < 0.01; Fig. 5C). The toxicity of RK5, AARPα, and AARPβ treatment was evaluated by observing the body weight, and no apparent weight loss was observed. However, paclitaxel treatment resulted in significant toxic side effects evidenced by significant weight loss (Supplementary Fig. S5). Then, we tested the effects of RK5, AARPα, and AARPβ on the histologic structure of LLC tumors. In comparison with normal saline treatment,
RK5, AARPα, and AARPβ treatment significantly reduced tumor blood vessel densities as demonstrated by CD-31 immunostaining. Importantly, AARPα- and AARPβ-treated tumors showed less tumor blood vessel densities than RK5 (Fig. 5D; **, P < 0.01).

As tumor metastasis is also angiogenesis dependent, an experimental lung metastasis model was generated to evaluate whether RK5, AARPα, and AARPβ could also inhibit growth of metastatic nodules. LLC cells were i.v. injected into the tail vein and allowed to circulate for 6 days, then treatments were initiated. After 22 days of treatments, mice were autopsied for metastatic analysis. RK5, AARPα, and AARPβ treatment dramatically reduced development of lung metastatic nodules. All saline-treated mice had enlarged lung and macroscopic malignant nodules. In contrast, the median numbers of macroscopic metastases (>200 μm) in lung were significantly lower in AARPα- and AARPβ-treated mice than in the RK5 treatment group (Fig. 5E; **, P < 0.01).

AARP exerts markedly greater effects than RK5 and 25aa in the implanted H22 hepatocarcinoma model

We performed additional experiments to assess the effects of 25aa, RK5, AARPα, and AARPβ on tumor growth in vivo. We implanted about 1 × 10⁶ H22 cells into subcutaneous dorsa of Balb/c mice, then mice were treated with intraperitoneal injections of 6.25 mg/kg 25aa, 25 mg/kg RK5, AARPα, and AARPβ from day 8 to 56 (every other day). Control mice were treated with equivalent amount of normal saline. AARPα and
AARPβ treatment significantly slowed H22 xenograft tumor growth compared with the RK5 and 25 aa treatment. Roughly, 25aa inhibited H22 carcinoma by 16.1%, RK5 inhibited H22 carcinoma by 38.4%, AARPα inhibited H22 carcinoma by 60.4%, AARPβ inhibited H22 carcinoma by 62.1% (Fig. 6A). Corresponding survival curves showed significantly increased median survival in the RK5, AARPα, and AARPβ treatment groups when compared with controls, and groups AARPα and AARPβ significantly prolonged overall survival than groups RK5 and 25aa (Fig. 6B; **, P < 0.01). During the treatment course, no significant (P > 0.05) difference in body weight was observed between the 25 aa, RK5, AARPα, AARPβ, and control groups, whereas paclitaxel treatment caused significant weight loss (Fig. 6C; **, P < 0.01). The tumors of saline-treated mice had a net organization of large vessels with an apparent lumen structure as demonstrated by CD31 immunostaining. In contrast, tumor microvessel density in RK5-, AARPα-, and AARPβ-treated mice were significantly decreased and the decrease of tumor microvessel density was more pronounced in AARPα/AARPβ than in RK5 (Fig. 6D; **, P < 0.01). Furthermore, the toxic effects of RK5, AARPα, and AARPβ were examined by conventional histologic H&E staining of main organ tissue slices (kidney, lung, liver, and spleen). No tissue abnormalities were detected by histologic analyses (H&E) of liver, kidney, lung, and spleen in RK5-, AARPα-, and AARPβ-treated mice compared with normal control (Fig. 7).

Discussion

The angiogenic switch, a rate-limiting step in tumor progression, has already occurred by the time most human tumors are detectable. The angiogenic switch is not limited at earliest stages, but occurs also at different stages of tumor progression (2). Antiangiogenic therapy is a promising alternative for treatment of cancer, and may also be used as a maintenance therapy to prevent the metastasis or recurrence (4). Therapy with endogenous angiogenic inhibitors such as endostatin and angiotatin may reverse the angiogenic switch by preventing growth of tumor vasculature. Angiotatin can maintain metastasis in a dormant state in laboratory animals when administered exogenously (34). In transgenic mice overexpressing endostatin, a small increase of circulating endostatin (approximately 1.6-fold) is sufficient to confer dramatic protection against tumor growth (11). In individuals with Down syndrome, a similar small increase of circulating endostatin is associated with a remarkably low incidence of solid tumors (35). These suggest that modest but sustained increases in angiogenic inhibitors (angiotatin or endostatin) may be capable of significantly inhibiting tumor growth.
Peptides have emerged as important therapeutics that is rigorously tested in angiogenesis-dependent diseases due to their low toxicity and high specificity (36). Since the discovery of endogenous proteins and protein fragments that inhibit microvessel formation (thrombospondin and endostatin), several peptides (including endostatin mimics and gelatinase inhibitory peptide CTTHWGFTLC) have shown promise in preclinical and clinical studies for cancer (23, 37). Angiogenesis inhibitors, particularly polypeptides or endogenous peptides, may become the safest and least toxic therapy for diseases associated with abnormal angiogenesis (36, 37). However, the use of peptides in vivo has largely been limited by their extremely short half-life. Therefore, increasing the in vivo residence times of peptide therapeutics could decrease their dosing frequencies. Several strategies have been used to improve the half-life of biologic peptides, including fusion or conjugation to immunoglobulins and serum proteins, incorporation into the drug delivery vehicles, and conjugating to natural or synthetic macromolecules (38).

Thus, when developing therapeutic antiangiogenic proteins, the efficacy, half-life, bioavailability, molecular size, stability, and effectiveness should be considered. To fulfill these considerations, we developed an elegant triple fusion protein, AARPα. AARPα is a chimeric protein consisting of an MMP-2/9–selective inhibitory peptide (CTTHWGFTLC), endostatin mimic (NH₂ terminal 25 aa of endostatin), and kringle 5 fragment of human plasminogen. Our results showed that AARPα has relatively high bioavailability and a longer half-life (half-life = 13.1 hours), raising the possibility that AARPα could be a superior therapeutic protein to endostatin (half-life = 2 hours), angiotatin (half-life = 15 minutes), and RK5 (half-life = 9.4 hours), assuming that it retained its ability to simultaneously inhibit the growth and migration of endothelial cells (12, 39). The results of hemolytic effect on human erythrocytes showed that AARPα and AARPβ are cytocompatible. Furthermore, repeated administration of large amount of AARPα (250 mg/kg, once daily for 32 days) did not induce anticipated toxicities such as weight loss, abnormal serum biochemical parameters, and main organ damage, indicating that AARPα is safe without any detectable toxicity.

In this research, we used three preclinical cancer models, multidrug-resistant human epidermoid carcinoma xenograft model, and two murine allografts (LLC and H22 allografts), which are angiogenesis dependent and have been widely used to test the activity of anticancer agents. In the multidrug-resistant human epidermoid KB carcinoma xenograft model, i.p. administered AARPα showed more potent antitumor effects. The effect on KB xenograft growth was dose-dependent, a dose of 10 mg/kg AARPα was more effective than 10 mg/kg of clinical grade endostatin every other day, and 40 mg/kg (every other day) was comparable with 100 mg/kg cyclophosphamide (given every 5 days). Endostatin therapy alone did not have a significant antitumor effect on multidrug-resistant human epidermoid carcinoma xenografts, its
antitumor effect might be compromised by lower molar concentration and a short half-life. The theoretical molar concentration of 10 mg/kg AARP\(\alpha\) (Mw = 15 kDa) is 16.66 \(\mu\)mol/L, whereas for 10 mg/kg clinical grade endostatin (Mw = 21 kDa), the theoretical molar concentration is 9.09 \(\mu\)mol/L. The half-life of clinical grade endostatin is less than 2 hours, whereas the half-life of AARP\(\alpha\) is 13.1 hours. Thus, it seems plausible that AARP\(\alpha\) has more pronounced antitumor effect than endostatin.

China accounts for almost 50% of the world’s liver cancer cases, accounts for one-third of global deaths from lung cancer, and developing drugs in cancer therapy is imperative than ever before. We further evaluated the antitumor effect of AARP in two murine syngeneic models (LLC and H22 hepatoma xenograft). Superior antitumor effect and survival-promoting effect of AARP were observed with LLC and H22 tumors, confirming the broad biologic effect of AARP. Immunohistochemical studies carried out on LLC and H22 tumor tissues showed AARP\(\alpha\) and its isoform AARPB could significantly reduce tumor angiogenesis. One of the most important effects of AARP is the prevention of lung metastasis in mice. Metastatic tumors are very common in the late stages of cancer and are often associated with a poor prognosis and survival rate. Expansion of metastatic lesions is also angiogenesis dependent. Our results showed that AARP is more potent in suppressing lung metastasis than RK5. In this report, we also compared the antitumor effects of paclitaxel and RK5, AARP\(\alpha\) and AARPB. Our results showed that paclitaxel was more effective than antiangiogenic inhibitors (RK5, AARP\(\alpha\)/AARPB). The paclitaxel dose was 10 mg/kg, the doses for RK5, AARP\(\alpha\)/AARPB were 25 mg/kg. But when doses were converted to molar concentration, we found the molar concentration for paclitaxel was 117.1 \(\mu\)mol/L, whereas the molar concentration for RK5, AARP\(\alpha\)/AARPB was 16.66 \(\mu\)mol/L. It is perhaps not surprising that the antitumor effect of paclitaxel is more pronounced than that for RK5, AARP\(\alpha\)/AARPB. But compared with saline treatment, the RK5, AARP\(\alpha\)/AARPB still exerted very significant antitumor effects.

Tumor-induced sprouting angiogenesis involves remodeling of cell–cell junctions and ECM (in part by MMPs), endothelial cell proliferation, migration, and lumen formation (2). The specialization of quiescent endothelial cells into migrating tip cells and proliferating stalk cells is a key event during sprouting angiogenesis (40). From a therapeutic viewpoint, strategies targeting endothelial cells (tip and stalk cells) that line tumor infiltrating blood vessels would prevent neoangiogenesis, and cause vessel regression (41). On the basis of our results, AARP not only retains the biologic activity of kringle 5, CTT peptide, and endostatin, but is more potent than each single agent alone in inhibiting endothelial cell growth, migration, tube formation, and tumor angiogenesis. Therefore, we hypothesize that AARP is a versatile, broad-spectrum antiangiogenic molecule that could target both endothelial phenotypes. But the exact downstream signaling of AARP in endothelial cell proliferation, migration, tube formation remains to be further elucidated, then clinical trials can be done in the correct context, the right tumor types, and thus in patients that will benefit the most.

Because Folkman (42) first proposed the hypothesis that tumor growth is angiogenesis dependent and suggested that tumor dormancy could be maintained by preventing neovascularization of microscopic cancers, antiangiogenic therapy has been an attractive target for cancer therapy and increasingly validated in various preclinical cancer models. However, to date, preclinical and clinical studies evaluating the therapeutic potential of various targeted antiangiogenic agents have reported conflicting results, generating controversy (43, 44). The crux of clinical validation of antiangiogenic therapy lies in the fact that cancer is still usually treated at advanced, often metastatic, disease stages (45). In the setting of heavy disease burden, most patients treated with antiangiogenic agents eventually experience disease progression due to evolution of biologic escape mechanisms by tumors (46, 47). What has emerged from our work is that the utility of tumor angiogenesis inhibition in the clinic should be tumor context dependent. We propose that tumor type, stage, microenvironment, and maybe even age of the patient will be major contributors to the outcome of therapy. It could be argued that the clinical benefit and impact would be greater if the therapy was initiated at earlier stages of malignancy, with the aim of keeping them in a dormant state by preventing the angiogenic switch. This would result in a substantial reduction in the cancer incidence or cancer recurrence risk. However, lengthy clinical trials will be required to test this hypothesis.

The use of multitargeted antiangiogenic agent such as AARP is an appealing approach targeting nonmalignant endothelial cells that form the tumor vasculature and indirectly affects tumor cells, thus minimizing the risk of toxicity (22). A gram of tumor has been estimated to contain approximately \(10^8\) to \(10^9\) tumor cells, and the ratio of endothelial cells to tumor cell is in the range of 1/10 to 1/100 (19). This means that endothelial cell–targeted therapies have a built-in amplification in that a relatively small insult to the vascular endothelium induces vascular failure and extensive ischemic necrosis results. The problem of drug resistance associated with conventional chemotherapy agents is avoided because normal endothelial are genetically stable unlike tumor cells (4). Endothelial cells form a continuum throughout the circulation, and all endothelial cells are in direct contact with the blood, which makes them readily accessible by therapeutic drugs delivered i.v. (18, 19). More importantly, endothelial cells in new vessels are vulnerable to therapeutic attack compared with mature, quiescent, and protected ECs in the established vasculature (18–20). In addition, as a multi-target angiogenesis inhibitor, chimeric protein AARP can be more efficacious and can act synergistically with current chemotherapies, radio-, and gene therapies (48).

There could be possible shortcomings of AARP. The clinical relevancy of AARP may be limited because of the
use of xenograft models. Because subcutaneous xenograft models do not faithfully recapitulate the clinical, pathologic, genetic, and molecular aspects of human tumors, which may affect the results when comparing therapy responses between preclinical mouse models and patients with cancer (49). However, to overcome the limitations of the xenograft model, we used the syngeneic mouse model (H22, LLC carcinoma) to evaluate the angiogenic efficacy of AARP, the immune system is not compromised, and thus it may more closely mimic the tumor microenvironment in patients. In addition, in three tumor models, we used conventional chemotherapeutic agents (cyclophosphamide and paclitaxel) to test the reliability, robustness of established tumor xenograft models and evaluate the angiogenic efficacy of AARP. Certain tumor types can also use nonsprouting modes of angiogenesis, including intussusceptive angiogenesis, vasculogenic mimicry, vessel cooption, recruitment of endothelial progenitor cells, and cancer stem-like cell–derived vasculogenesis (40, 50). Nonsprouting angiogenesis may operate in parallel with sprouting angiogenesis. These noncanonical mechanisms of angiogenesis may provide blood supply before induction of the angiogenic switch, or occur as an adaptive response to changes in the tumor environment. However, we believe our findings clearly indicate that tumor angiogenesis is mainly dependent on sprouting angiogenesis in the tumors we studied. Therefore, the application of AARP deserves to be diversified to warranting data, constructing databases): Z. Yang

In summary, AARP shows promising therapeutic properties in the treatment of solid tumors and metastasis in mice; however, possess no side effects. Because recombinant AARP protein is fully humanized, stable, and selectively cytotoxic to tumor associated endothelial cells and can be produced in large amounts. We propose that the simultaneous inhibition of MMP-2/9, endothelial cell growth, and migration by AARP can be a reasonable candidate therapeutic protein for cancer maintenance therapy, metastasis, and further preclinical studies are warranted to explore additional applications of AARP for the treatment of cancer.

Disclosed Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Yang, J. Gu
Development of methodology: H. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Wang
Writing, review, and/or revision of the manuscript: H. Wang, J. Gu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Yang
Study supervision: J. Gu

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References
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