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ABSTRACT:

The recently reported inhibitory effects of angiotensin 1-7 (Ang-(1-7)) on various cancers indicate its potential use as a therapeutic agent for primary and metastatic cancers. However, its extremely short half-life in the circulation greatly compromises its potential applications. Here, we reported an Ang-(1-7) analogue peptide with the amino and carboxy terminal protected by acetylation and amination. The *in vitro* and *in vivo* degradation of the resulting analogue, Ang-AA, were determined using high-performance liquid chromatography (HPLC). At the same time, small RNA interference and competition studies were performed to evaluate the specific capacity of Ang-AA to bind to the cell surface Mas receptor. Cell Counting Kit-8 (CCK8), wound-healing and Boyden chamber assays were performed to investigate the inhibitory effects of Ang-AA on A549 cells. Finally, the synergistic inhibitory effects of Ang-AA and paclitaxel (PTX) on A549 xenografts in mice were observed using animal imaging systems and survival observations. The toxicity of Ang-AA in mice was evaluated. Our results showed that acetylation and amination significantly inhibited the hydrolyzation of Ang-(1-7) *in vitro* and *in vivo*. The half-life of Ang-(1-7) in rats was prolonged from 2.4±0.6 min to 238.7±61.3 min ($p<0.001$). The specific

binding of Ang-AA to the Mas receptor was well preserved, and Ang-AA exerted significantly greater inhibitory effects on the proliferation, migration and invasion of A549 cells than Ang-(1-7). The combination of Ang-AA and PTX exhibited a significantly greater synergistic inhibitory effect on A549 xenografts than the combination of Ang-(1-7) and PTX. Ang-AA did not display obvious toxicity in mice. Our findings indicate acetylation and amination is a simple and effective method for producing Ang-(1-7) as a bioactive peptide.

KEY WORDS: Angiotensin 1-7, Acetylation, Amination, Hydrolyzation, Analogue

ABBREVIATIONS:

Ang-(1-7), angiotensin 1-7; ACE, angiotensin converting enzyme; NEP, neutral-endopeptidase; LAP, leucine aminopeptidase; PEP, prolyl-endopeptidase; PCP, prolyl-carboxypeptidase; PTX, paclitaxel; COX2, cyclooxygenase-2; APs, aminopeptidase; SD, Sprague-Dawley; DIEA, ethyldiisopropylamine; DCM, dichloromethane; HPLC, High Performance Liquid Chromatography; siRNAs, small interfering RNAs (siRNAs); siCON, control siRNA (siCON); qRT PCR, quantitative real-time PCR; PBS, phosphate-buffered saline; GAPDH, housekeeping gene; PFA, paraformaldehyde; RT, room temperature; CCK8, Cell Counting Kit-8; NS, normal saline; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; CREA, creatinine; BUN, blood urea nitrogen.

INTRODUCTION

Ang-(1-7), an endogenous, seven amino acid peptide hormone, is a critical component of the renin-angiotensin system. Endogenous Ang-(1-7) is mainly generated from angiotensin I (Ang I) and angiotensin II (Ang II) degraded by ACE, PEP, NEP and PCP *in vivo* [1]. The most well-known function of Ang-(1-7) is to regulate cardiovascular efficiency, which consists of anti-fibrosis, anti-hypertension, anti-hypertrophic and anti-arrhythmia activities [2-8]. However, based on accumulating evidence from recent experimental and clinical studies, Ang-(1-7) makes an important contribution to the inhibition of tumour progression in a variety of cancers [9-16]. For instance, Ang-(1-7) reduces the expression of COX2 to inhibit the proliferation of human lung adenocarcinoma cells [10, 11]. Ang-(1-7) up-regulates E-cadherin expression in human lung and

colorectal cancer cells and subsequently decreases the migration and invasion of cancer cells [12, 13]. More importantly, Ang-(1-7) is the sole ligand of a unique G-protein-coupled receptor encoded by the MAS gene [14-16]. The inhibitory effects of Ang-(1-7) on cancer cells are primarily mediated through the cell surface Mas receptor. All of these studies suggest promise in the potential use of Ang-(1-7) as a therapeutic agent for primary and metastatic cancers [15, 16].

However, Ang-(1-7) has an extremely short half-life (3-15 min) in the circulation because of its physiological hydrolyzation by ACE, NEP and APs (as shown in Fig. 1A) [17-19]. Because Ang-(1-7) is rapidly degraded, very little peptide arrives in cancer tissues when it is used as an anti-tumour agent. Additionally, the low efficiency will eventually result in the administration of repeated doses, which definitely produces more side effects on patients. Therefore, new Ang-(1-7)-derived analogues that are not degraded by ACE, NEP and APs represent potentially useful and promising improved therapeutic agents.

In the present study, we performed amination at the carboxy terminus and acetylation at the amino terminus of Ang-(1-7) to inhibit its physiological hydrolyzation, and we observed the *in vitro* and *in vivo* degradation of this resulting Ang-(1-7) analogue, hereafter named Ang-AA. Moreover, the ability of Ang-AA to bind the Mas receptor was investigated, and the anti-tumour effects of Ang-AA on lung cancer cells and athymic mice were evaluated. Our results show that Ang-AA displayed significantly enhanced stability *in vitro* and *in vivo* compared to Ang-(1-7). The ability of Ang-AA to bind the Mas receptor was well preserved. Ang-AA exerted significantly greater inhibitory effects on the proliferation, migration, and invasion of lung cancer A549 cells than Ang-(1-7) in the presence of ACE, NEP and LAP. When combined with paclitaxel, Ang-AA exhibited significantly enhanced anti-tumour effects on A549 xenografts compared to Ang-(1-7). In addition, Ang-AA did not display obvious toxicity in mice. Based on our results, acetylation and amination at the N- and C-terminal of Ang-(1-7) effectively protected it from physiological hydrolyzation and therefore enhanced its anti-tumour effects. The analogue of Ang-(1-7) described in this study represents a potential application of Ang-(1-7) in cancer treatment. Moreover, acetylation- and amination-mediated protection represents an effective method for the production of small bioactive peptides.

MATERIALS AND METHODS

Cell cultures

Human lung adenocarcinoma cells A549 were purchased from the American Tissue Culture Collection (Manassas, VA, USA) and were maintained in Ham's F12 medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS, Gibco) and antibiotics. In all experiments, cells were cultured in a 5% CO_2 atmosphere at 37°C.

Animals

Sixty Male SD rats (150-250 g, 6-9 weeks of age), sixty male Balb/c mice (20-25 g, 4 weeks of age) and 192 athymic nude mice (20-25 g, 3-5 weeks of age) were purchased from the Experimental Animal Center of The Fourth Military Medical University. All animals were maintained in a temperature-controlled barrier facility on a 12 h light/dark cycle, which conformed to the guidelines of the Chinese Public Health Service Policy on Human Care and Use of Laboratory Animals. Every experiment in this article was performed in accordance with the protocols approved by the Fourth Military Medical University Committee on Animal Care.

Peptide synthesis

All peptides were produced using standard Fmoc-mediated solid-phase synthesis. We chose rink amide MBHA resin with amino group protection at the C-terminus to synthesize the Ang-AA analogue peptide. At the end of the synthesis reaction, acetic anhydride was added along DIEA and DCM to protect the N-terminus. The final purity of the peptides was confirmed by HPLC (AKTA Purifier 10 Plus, GE Healthcare, Buckinghamshire, UK) with an analytical C18-column (Hypersil-ODS2, GE Healthcare). Further characterization was performed with ESI mass spectrometry (Life Technologies Corporation, Gaithersburg, MD, USA). The process used to synthesize FITC-labelled peptides was same as for the unlabelled peptides. Upon complete assembly of the peptide, resin-bound peptides were incubated with FITC in DCM for 3 h. The completion of the reactions was monitored using a qualitative ninhydrin test.

***In vitro* stability analysis**

Firstly, the stabilities of Ang-(1-7) and Ang-AA in ACE, NEP or LAP solutions were evaluated. Two peptides were dissolved in PBS containing 0.5 IU/mL of ACE, NEP or LAP (Sigma-Aldrich,

St. Louis, MO, USA) at a concentration of 0.1 mM. After a 30 min incubation at 37°C, 0.4% acetonitrile phosphate was added to stop the degradation reaction. The quantity of the residual peptides in each solution was determined using HPLC (GE Healthcare). Then, the stabilities of Ang-(1-7) and Ang-AA in an ACE, NEP and LAP mixed solutions were determined using the same methods. The starting concentrations of Ang-(1-7) and Ang-AA in the mixed solutions were 100 μM. ACE, NEP and LAP were added at 0.03 IU/mL. Afterwards, the stabilities of Ang-(1-7) and Ang-AA in rat serum were evaluated. Rat blood was collected by cardiac puncture followed by CO_2 euthanasia. The serum was separated by centrifugation at 3500rpm for 10 min. A 10-fold diluted rat serum sample was incubated with 100 μM Ang-(1-7) or Ang-AA for 15 h at 37°C. The quantity of the residual peptides in the rat serum was determined using HPLC.

***In vivo* stability analysis**

Twelve SD rats were divided into groups and administered a single intravenous (i.v.) injection of 400 μg/kg Ang-(1-7) or Ang-AA (n=6). At 2, 4, 6, 8, 10, 15, 50, 120, 240, 480, 720 and 960 min after the injection, 100 μL of blood were sampled from each animal through the caudal vein. The serum was separated by centrifugation at 3500 rpm for 10 min. The residual amounts of Ang-(1-7) and Ang-AA in serum were determined by HPLC using a reverse-phase column. After the experiments, the rats were euthanized with an overdose of CO_2. The pharmacokinetic parameters were analysed using WinNonlin 5.2 (Pharsight, Mountain View, CA, USA).

RNA interference

siRNAs targeting the MAS gene and a siCON were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The siRNAs and siCON sequences were: siRNA1: sense 5' GCUUCCGGAUGAGAAGAAATT 3', anti-sense 5' UUUCUUCUCAUCCGGAAGCTT 3'; siRNA2: sense 5' GCUACAACACGGGCCUCUATT 3', anti-sense 5' UAGAGGCCCGUGUUGUAGCTT 3' and siCON: sense 5' UUCUCCGAACGUGUCACGUTT 3', anti-sense 5' ACGUGACACGUUCGGAGAATT 3'. Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect the nucleic acids into A549 cells according to the manufacturer's instructions. Mas expression was determined by qRT-PCR and western blotting within 72 h.

qRT PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher) and then reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The resulting cDNAs were analysed with the iTaq Universal SYBR Green Supermix (Bio-Rad) on a Fast Real-Time PCR System (7900HT, Applied Biosystems, Foster City, CA, USA) using the MAS-specific primers 5' GCCTGTCAGTCCTTTACCCC 3' and 5' AGCTTGGAGGAATGGGAAGC 3'. The comparative 2-ΔΔCt method was performed by calculating the delta Ct values for the gene of interest and the GAPDH and then calculating the difference between both of the delta Ct values.

Western blot

Cells were washed with cold PBS three times. Total protein samples were prepared by lysing the cells with RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitor cocktail tablets (Merck Millipore, Billerica, MA, USA). After equal amounts of total protein samples were separated using SDS-PAGE, proteins were transferred to PVDF membranes (Merck Millipore). Membranes were blocked with skim milk and incubated with the corresponding primary antibodies against Mas (#NBP1-78444, Novus Biologicals, Littleton, CO, USA), COX2 (#12282, Cell Signaling Technology, Danvers, MA, USA), and E-cadherin (#14472, Cell Signaling Technology). After an incubation with an HRP-conjugated secondary antibody, protein levels were visualized in an UVP Imaging System (ChemiDoc-IT 510, UVP, Upland, CA, USA) using a Chemiluminescent HRP Substrate (Merck Millipore). α-Tubulin (#2144, Cell Signaling Technology) or GAPDH (#ab8245, Abcam) was used as the loading control.

Confocal fluorescence microscopy

A549 cells were grown in 4-well chamber slides (Merck Millipore) to 80% confluence. Next, cells were transfected with siRNAs for 48 h, fixed with 4% PFA, blocked with 20% goat serum (Life Technologies, Gaithersburg, MD, USA) and incubated with 10 μM FITC-labelled Ang-AA or Ang-(1-7) at RT for 10 min. After three washes with PBS, cells were stained with DAPI and mounted with antifade mounting medium (Beyotime). All images were collected with a confocal fluorescence microscope (Fv1000, OLYMPUS, Tokyo, Japan). The fluorescence intensities from 3

independent images were quantified using custom MATLAB software. Regions with saturated intensities were avoided.

A799 (Asp-Arg-Val-Tyr-Ile-His-D-Ala), a well-known peptide with affinity for the Mas receptor [17], was used for the competition assay. Ang-AA-FITC was incubated with the A799 peptide at a concentration of 10 μM and then applied to cells. The cell staining and imaging processes were performed using the same methods described above.

Flow cytometry

The competition between the A799 peptide and Ang-AA-FITC for binding to the cell surface Mas receptor was confirmed by flow cytometry. A549 cells were detached with trypsin, blocked with 20% goat serum and then incubated with 10 μM Ang-AA-FITC and A799 peptide together for 30 min. After three washes with PBS, cells were analysed (10,000 cells/sample) by flow cytometry (FACSCalibur, Beckton Dickinson, Franklin Lakes, NJ). In another competition assay, Ang-AA-FITC was incubated with 10, 50, 100, 150 or 200 μM unlabelled Ang-AA before application to cells. Simultaneously, an unrelated peptide (GGGAGGGAGGGK-FITC) was used as a control and incubated with Ang-AA at 150 or 200 μM. Ang-AA-FITC or Ang-(1-7)-FITC with concentration from 0-200 nM was respectively incubated with A549 cells to determine the k_d value. The fluorescence staining and the calculations were performed using the same methods described above for flow cytometry.

Cell proliferation analysis

A549 cells were grown in 96-well plates at a density of 2,000 cells/well. Ang-(1-7) or Ang-AA were applied to the cells at 0.01, 0.1 and 1 μM in the presence of 0.03 IU/mL ACE, LAP and NEP, and 48 h later, cell proliferation was determined using the CCK8 (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm using a 96-well plate reader (ELx800; BioTek Instruments, Winooski, VT, USA).

Wound-healing assay

A total of 1×10^5 A549 cells were grown to confluence in wells (35 mm) carrying a strip-like insert (ibidi, Munich, Germany). After 12 hours of serum deprivation, the insert was removed.

Cells were then incubated with serum free medium supplemented with 0.01, 0.1 and 1 μM Ang-AA or Ang-(1-7). ACE, LAP and NEP were added to each well at 0.03 IU/mL. 24 h later, images were taken at a 10-fold magnification (Olympus, Tokyo, Japan), and the wound area was calculated (ImageJ 1.43u, NIH, USA).

Boyden chamber assay

A Boyden chamber assay was performed using 24-well transwell units with 8 μm pore size polycarbonate inserts (Corning Life Sciences, NY, USA). The insert chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. A549 cells were seeded onto the Matrigel™ coated chambers at a density of $4x10^4$ cells/well in serum-free medium. Ang-AA or Ang-(1-7) were applied to the lower chamber of the transwell units at 0.01, 0.1 and 1 μM. Simultaneously, ACE, LAP and NEP were added to each well at 0.03 IU/mL. After 48 h of incubation at 37°C, the Matrigel™ coating was carefully removed with cotton swabs. Cells on the bottom of the insert chambers were fixed with 20% ethanol, stained with crystal violet and counted under a microscope.

Analysis of the anti-tumour effects

A549 cells were infected with luciferase lentiviral particles (#LPP-FLUC-LV105-025, Genecopoeia, Rockville, MD, USA) according to the manufacturer's instructions to construct an *in vivo* imaging system compatible with xenograft models. Cells stably infected with the luciferase lentivirus were acquired by puromycin (1 μg/mL) selection and re-suspended in HBSS/Matrigel™ (1:1) (BD Biosciences) at a density of $1x10^6$ cells/mL. One hundred microliters of Matrigel™ containing cells were subcutaneously (s.c.) injected into the lower flank of athymic mice, and the tumour volumes were measured using callipers every two days. Seven days later, 24 mice bearing similarly sized tumours (~100 mm^3) were randomly allocated into groups (n=6) and administered NS (normal saline), PTX (paclitaxel), PTX-Ang (paclitaxel plus Ang-(1-7)) or PTX-AA (paclitaxel plus Ang-AA). PTX (PTX-Cremophor EL, Sigma-Aldrich) was administered at a dose of 15 mg/kg by an intraperitoneal injection on days 6, 10, 14 and 18. Ang-(1-7) and Ang-AA were administered for 5 consecutive days at a dose of 400 μg/kg by an intravenous injection followed by a 2 day interval for 4 cycles. On day 30, all mice were anaesthetized, injected with luciferin

and imaged using a Xenogen IVIS 100 *in vivo* imaging system (PerkinElmer Inc., Waltham, MA, USA). Light emission from the animals' tissues was measured using the software provided by the vendor. After imaging, the mice were euthanized with an overdose of CO_2, and the tumours were excised for Ki67, COX2 and E-cadherin immunohistochemical staining. The overall survival rates were observed in another cohort of the four groups of animals receiving the same treatments as above described.

Toxicity evaluation

Ang-(1-7) and Ang-AA toxicities were evaluated in 20 Balb/c mice (n=10). Briefly, Ang-(1-7) or Ang-AA was administered using the same procedure as described for the study of the anti-tumour effects. The body weights, general conditions and behaviours of all animals were monitored daily. On day 30, all mice were euthanized with an overdose of CO_2 for necropsy. Simultaneously, blood samples were submitted to the Experimental Animal Center of the University to determine the AST, ALT, ALP, CREA and BUN levels.

Statistical analysis

For the *in vitro* studies, all experiments were performed in triplicate. The data were analysed using GraphPad Prism 5 software. Normality tests were performed to ensure that the dataset was normally distributed. Data from two groups were statistically compared using an unpaired Student's t-test. A one-way ANOVA with Bonferroni's correction was used for multiple comparisons. The pharmacokinetic parameters were analysed using WinNonlin 5.2 software. Kaplan-Meier survival curves and log rank tests were used to examine the OS of each treatment group. The data are presented as means and error bars depict the standard errors of the means. P values ≤ 0.05 were considered significant for all analyses.

RESULTS

Characterization of Ang-(1-7) and Ang-AA

We acetylated and aminated the N- and C-terminal of Ang-(1-7), respectively, to inhibit its degradation and enhance its half-life. The Chemical structures of Ang-(1-7) and the resulting Ang-(1-7) analogue, hereafter named Ang-AA, are shown in Fig. 1A and B. Based on the HPLC

data, both Ang-(1-7) and Ang-AA displayed >98% purity (Fig. 1C). The actual molecular weight of Ang-AA detected by mass spectrometry was 940.30 Da, consistent with its theoretical molecular weight (Fig. 1D).

Ang-AA exhibited significantly enhanced stability *in vitro* and *in vivo*

We initially incubated Ang-(1-7) or Ang-AA in PBS, ACE, LAP or NEP solutions to compare their stabilities. LAP was chosen for its critical role in APs and cleaved functions for various N-terminal residues from proteins and peptides [20]. After a 30 min incubation, the residual concentrations of Ang-AA in each solution were significantly higher than that of Ang-(1-7), as measured by HPLC (Fig. 2A and B). Next, we incubated Ang-(1-7) or Ang-AA with a solution containing all three hydrolytic enzymes, ACE, LAP and NEP. According to the HPLC data, the half-life of Ang-AA in this mixed solution was 135.7±37.7 min, which was significantly higher than the half-life of Ang-(1-7) at 9.2±0.5 min ($p<0.001$, Fig. 2C). Then, we incubated Ang-(1-7) or Ang-AA with 10% rat serum and evaluated the half-life again. The half-life of Ang-AA in rat serum was 171.1±40.7 min, whereas Ang-(1-7) exhibited a half-life of 13.8±5.7 min ($p<0.001$, Fig. 2D). Finally, the stabilities of the peptides were evaluated in rats. The half-life of Ang-AA in rats was 238.7±61.3 min, whereas Ang-(1-7) exhibited a half-life of 2.4±0.6 min ($p<0.001$, Fig. 2E). Thus, the protections on terminals by acetylation and amination effectively inhibited Ang-(1-7) degradation *in vitro* and *in vivo*.

The ability of Ang-AA to bind the cell surface Mas receptor did not change

We labelled Ang-(1-7) and Ang-AA with FITC to compare the binding abilities of Ang-AA and Ang-(1-7) to the Mas receptor. Mas-overexpressing A549 lung cancer cells were transfected with Mas-targeted siRNAs (siRNA1 and siRNA2) or a control sequence (siCON) to knock down Mas expression (Fig. 3A). As shown in the confocal fluorescence microscopy images, both siRNA1 and siRNA2, but not siCON, significantly decreased the binding of Ang-(1-7)-FITC and Ang-AA-FITC to the A549 cells (Fig. 3B).

Next, we performed two competition assays to reveal the specific binding of Ang-AA to A549 cells and the Mas receptor. Firstly, we used unlabelled Ang-AA to compete with Ang-AA-FITC for binding to A549 cells. The FITC intensities determined using flow cytometry showed that the

binding of Ang-AA-FITC to A549 cells was inhibited by the unlabelled Ang-AA in a dose-dependent manner. A scrambled sequence did not show any inhibitory effect (GGG*, Fig. 3C). Secondly, we used A799, a well-known peptide with affinity for the Mas receptor [21], to compete with Ang-AA-FITC for binding to the A549 cells. According to the flow cytometry and confocal microscopy results, the addition of A799 (10 μM) significantly inhibited the binding of Ang-AA-FITC to A549 cells. Similar results were observed for Ang-(1-7)-FITC (Fig. 3D, E and F). Finally, the k_d values of Ang-AA-FITC and Ang-(1-7) in A549 cells were calculated using the intensities acquired from flow cytometry. The nonlinear fit of intensities obtained from the binding of various concentrations of Ang-AA or Ang-(1-7) to A549 cells revealed their equilibrium k_d were 65 nM ($R^2 = 0.99$) and 69nM ($R^2 = 0.99$), respectively (Fig. 3G). All of these results demonstrate acetylation and amination did not change the ability of Ang-AA to bind the cell surface Mas receptor.

Ang-AA exhibited a greater inhibitory effect on the proliferation of A549 cells than Ang-(1-7)

The effects of Ang-AA and Ang-(1-7) on the proliferation of A549 cells were evaluated using the CCK8 assay. ACE, LAP and NEP were added to the culture medium at 0.03 IU/mL. Both Ang-(1-7) and Ang-AA significantly inhibited the proliferation of A549 cells. Ang-AA exhibited a greater inhibitory effect than Ang-(1-7) at each applied concentration (Fig. 4A). COX2 is a molecule responsible for the anti-tumour effect of Ang-(1-7) on A549 cells. Therefore, COX2 expression was evaluated in A549 cells treated with Ang-(1-7) or Ang-AA. According to the western blot and qRT-PCR data, Ang-AA exerted a significantly stronger inhibitory effect on COX2 expression than Ang-(1-7) at 0.1 μM (Fig. 4B). Thus, Ang-AA inhibited the proliferation of A549 cells to a significantly greater extent than Ang-(1-7) in the presence of hydrolytic enzymes.

Ang-AA inhibited the migration and invasion of A549 cells to a greater extent than Ang-(1-7)

In addition to proliferation, Ang-(1-7) inhibits the migration and invasion of tumour cells. Therefore, the effects of Ang-AA on the migration and invasion of A549 cells were evaluated using a scratch wound-healing assay and a Boyden chamber assay, respectively. ACE, LAP and NEP were added to the culture medium at 0.03 IU/mL. In the Boyden chamber assay, significantly fewer cells passed through the membrane insert after Ang-AA treatment compared with that after

Ang-(1-7) treatment (Fig. 4C and 4E). In the scratch wound-healing assay, a significantly greater wound area was present in the wells containing Ang-AA-treated A549 cells than in the wells containing Ang-(1-7)-treated cells at each applied concentration (Fig. 4D and 4F). We evaluated E-cadherin expression to further confirm the inhibitory effect of Ang-AA on the migration and invasion of A549 cells. As expected, the Ang-AA-treated cells displayed a significant increase in E-cadherin expression compared with the Ang-(1-7)-treated cells (Fig. 4G). Thus, Ang-AA inhibited the migration and invasion of A549 cells to a greater extent than Ang-(1-7) in the presence of hydrolytic enzymes.

Ang-AA exerted a stronger anti-tumour effect on mice than Ang-(1-7)

A549 cells stably infected with a luciferase lentivirus were subcutaneously injected into the lower flank of athymic mice to construct xenograft tumour model and determine the anti-tumour effects of Ang-AA *in vivo*. Twenty-four mice bearing similarly sized tumours (~100 mm^3) were randomly allocated into four groups (n=6) to receive NS, PTX, PTX-Ang or PTX-AA. After treatment, the quantification of the bioluminescence revealed a significant decrease in the tumour growth of the PTX-AA- or PTX-Ang-treated mice compared with that of the PTX-treated mice (Fig. 5A). The average tumour weights and volumes were significantly reduced in the PTX-AA treated mice compared with those in the PTX-Ang-treated mice (Fig. 5B). Moreover, as shown in the images of the immunohistochemical staining, the PTX-AA treated mice exhibited the lowest level of Ki67 expression in tumour tissues compared to the PTX-Ang-, PTX- or NS-treated mice. COX2 was expressed at significantly lower levels in the tumours of the PTX-AA-treated mice than in the tumours of the PTX-Ang-, PTX- or NS-treated mice. E-cadherin was expressed at significantly higher levels in the tumours of the PTX-AA-treated mice than in the tumours of the PTX-Ang-, PTX- or NS-treated mice (Fig. 5C). Forty additional xenograft-bearing mice were divided into groups (n=10) and treated as described above to observe the overall survival (OS) and further illustrate the anti-tumour effects of PTX-AA. The PTX-AA-treated mice displayed a significantly longer OS than the PTX-Ang-, PTX- or NS-treated mice (Fig. 5D). Based on these results, Ang-AA exerted significantly greater anti-tumour effects on mice than Ang-(1-7) when combined with paclitaxel.

Ang-AA did not exhibit significant toxicity in mice

Finally, the toxicity of Ang-AA and Ang-(1-7) was evaluated in mice. After the administration of 400 μg/kg peptides, hyperaemia, necrosis, and treatment-related clinical or histopathological findings were not observed in the main internal organs of any mice. Symptoms of an allergic response were not observed. The body weights of the Ang-AA- and Ang-(1-7)-treated mice were not significantly different (Fig. 5E). AST, ALP, ALT, CREA and BUN levels in all mice fell within the normal range (Table 1). Thus, neither Ang-AA nor Ang-(1-7) exhibited significant toxicity in mice.

DISCUSSION

Previous studies have documented the effectiveness of Ang-(1-7) in reducing the progression, migration and invasion of various tumours [8-15]. These pleiotropic features suggest the potential utility of Ang-(1-7) as a therapeutic agent for cancers. However, its rapid degradation in the circulation has prevented the use of the natural Ang-(1-7) peptide as a drug [19]. In the present study, the C- and N-terminal of Ang-(1-7) were protected by acetylation and amination, respectively. These two simple modifications substantially increased the stability of Ang-(1-7). The half-life of Ang-AA was more than ten times higher than Ang-(1-7). Moreover, these modifications did not disturb the ability of Ang-AA to bind its receptor, Mas. Ang-AA exerted greater inhibitory effects on tumour cell proliferation, migration and even angiogenesis than natural Ang-(1-7).

Actually, several Ang-(1-7) analogues and pharmaceutical methods have been reported to prolong the half-life of natural Ang-(1-7), with the expectation of developing these molecules into practical drugs [22-27]. For example, AVE 0991 is a non-peptide mimic of Ang-(1-7) that exhibits anti-fibrosis, anti-proliferative and anti-inflammatory activities [22, 23]. CGEN-856S is a computer designed Ang-(1-7) mimic with cardio-protective functions [24]. Other methods include beta-cyclodextrin encapsulation, D-amino acid replacement and thioether bridge modification [25-27]. However, none of these methods are sufficiently simple and may interfere with the structure of natural Ang-(1-7). In contrast, the protection of the peptide terminal through acetylation and amination in the present study is a simple, economical and convenient method for industrial-scale peptide synthesis. Moreover, acetylation and amination effectively inhibit enzyme-mediated hydrolysis. We compared the half-life of our Ang-AA analogue to that of some previously reported

Ang-(1-7) analogues. The *in vitro* and *in vivo* half-lives of Ang-AA were both higher than other reported analogues [24].

More importantly, terminal acetylation and amination effectively preserved the bioactivities of natural Ang-(1-7). Actually, in addition to prolonging the half-life, the ability of the analogue to preserve the bioactivity of the natural peptide is an important aspect to consider during peptide reconstruction. As shown in Fig. 1A, natural Ang-(1-7) is specifically hydrolysed by NEP at the Tyr-Ile peptide bond, by ACE at the Ile-His peptide bond and by LAP at the amino terminus. Initially, we synthesized analogues that simultaneously contained protective groups at the terminal and D-amino acid replacement at the Tyr, Ile and His hydrolysis sites. However, the D-amino acid replacements substantially impaired the bioactivities, although the half-life was prolonged (data not shown). Thus, we only used terminal acetylation and amination in this study. Terminal acetylation and amination did not change the ability of Ang-(1-7) to bind the Mas receptor and therefore preserved the bioactivities of Ang-(1-7) very well. Interestingly, terminal acetylation and amination also protected Ang-AA from hydrolysis by NEP and ACE. The mechanisms by which terminal acetylation and amination enable the peptide to resist hydrolysis by NEP and ACE remain to be explained. We speculate that this protective effect is likely derived from the reduced exposure of ACE and NEP hydrolysis sites. However, this hypothesis requires further investigation.

PTX is a frequently used chemotherapy for lung cancer that works by interfering with the normal function of microtubules during cell division [28, 29]. However, when used alone, PTX has limited tumour-suppressive effects and always induces serious drug resistance [30, 31]. How to amplify the effects of PTX and reduce resistance is a problem that needs to be solved urgently. In previous studies, the anti-tumour effects of Ang-(1-7) have typically been evaluated on lung cancer because of the high Mas expression [9-12]. In the present study, terminal protections did not disturb the ability of Ang-AA to bind its receptor, Mas. Therefore, we combined PTX with Ang-(1-7) or Ang-AA with the purpose of enhancing the anti-tumour effects of PTX. Compared with Ang-(1-7), Ang-AA effectively enhanced the inhibitory effects of PTX on A549 xenograft models. COX2 expression is closely related to PTX sensitivity, and a selective COX2 inhibitor enhances the response of patients with NSCLC to PTX [32, 33]. Thus, we speculate that the synergistic interaction between PTX and Ang-AA mainly depends on the Ang-AA-induced

decrease in COX2 expression. In addition, we postulate that the Ang-AA-mediated up-regulation of E-cadherin also contributes to the anti-tumour effects of PTX, since epithelial-to-mesenchymal transition-related chemotherapy resistance is rescued by E-cadherin expression [34].

CONCLUSION

To our knowledge, this is the first study to use terminal acetylation and amination to improve the half-life of Ang-(1-7). Based on our results, acetylation and amination at the C- and N-terminal of Ang-(1-7) effectively protected Ang-(1-7) from physiological hydrolysis and therefore enhanced its stability *in vitro* and *in vivo*. More importantly, the ability of the resulting Ang-AA analogue to bind the cell surface Mas receptor was well preserved. Ang-AA also exhibited enhanced anti-tumour effects compared to natural Ang-(1-7) *in vitro* and *in vivo*. The Ang-AA analogue described in this study represents a potential application for Ang-(1-7) in cancer treatment. Moreover, the combination of acetylation and amination represents an effective method for producing small bioactive peptides.

ACKNOWLEDGEMENTS

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AUTHOR INFORMATION

Xiaowen Ma, Zhijun Pang and Jiming Zhou contributed equally to this work.

Conception and design: Meng Li, Cun Zhang. Development of methodology: Xiaowen Ma, Zhijun Pang, Jiming Zhou. Acquisition of data: Xiaowen Ma, Jiming Zhou, Lei He, Qiang Hao. Analysis and interpretation of data: Xiaowen Ma, Lei He, Jiming Zhou, Zhijun Pang, Weina Li, Kuo Zhang. Writing, review, and/or revision of the manuscript: Meng Li, Cun Zhang. Administrative, technical, or material support: Shuning Wang, Wangqi Zhang, Xiaochang Xue. Study supervision: Wei Zhang, Yingqi Zhang.

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Tables

Table 1 Hepatic and renal toxicities of free PTX, PTX-AA and PTX-Ang.

	PTX	PTX-AA	PTX-Ang	Normal Range
CREA (mg/dL)	0.34±0.14	0.29±0.11	0.31±0.16	0.2-0.5
AST (IU/L)	126.8±27.66	93.4±23.85	102.2±29.56	55-381
ALT (IU/L)	86.1±16.82	78.9±20.11	85.5±22.13	40-170
BUN (mg/dL)	19.8±6.9	24.63±5.47	22.78±4.32	7-31
ALP (IU/L)	80±9.33	74.5±6.99	82.7±7.56	55-100

Figure Captions

Figure 1. Characterization of Ang-(1-7) and Ang-AA. (A) Ang-(1-7) is degraded at the peptide bond into Ang-(1-4), Ang-(1-5) or nonfunctional peptides by NEP, ACE and APs. Ang-AA was designed with protective amino and acyl groups at the termini to suppress degradation. (B) The chemical structures of Ang-(1-7) and Ang-AA are shown. The Ang-AA is labelled with acyl group (red) and the amino group (blue). (C) The purities of Ang-(1-7) and Ang-AA were 98.66% and 98.45%, as determined using HPLC. (D) Mass spectrometry reveals the quality of the Ang-(1-7) and Ang-AA peptides.

Figure 2. Enhanced stability of Ang-AA *in vitro* and *in vivo*. (A and B) The degradation of Ang-AA and Ang-(1-7) incubated with PBS, LAP, NEP or ACE was detected using HPLC ($p \leq 0.001$). (C) The half-life of Ang-AA following degradation by both ACE, LAP and NEP, was 135.7±37.7 min, with the 9.2±0.5 min half-life of Ang-(1-7). (D) The half-life of Ang-(1-7) degraded in 10% rat serum was 13.8±5.7 min, higher than Ang-AA ($T_{1/2}$=171.1±40.7 min) detected by HPLC ($p < 0.001$). (E) HPLC analysis showed that the amount of intact Ang-(1-7) peptides was 2.4±0.6 min in SD rat plasma *in vivo*. Ang-AA was much more stable *in vivo* ($T_{1/2}$=238.7±61.3 min) ($p < 0.001$). All pharmacokinetic parameters were analysed by WinNonlin 5.2 software. All experiments were performed in triplicate, and data were expressed as mean ±SD, with t-test where ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

Figure 3. The ability of Ang-AA to bind the Mas receptor. (A) The expression reduction of the Mas receptor in A 549 cells infected with siRNA1 or siRNA2 was analysed by qRT-PCR and Western Blot. (B) The confocal fluorescence microscopy images showed stronger binding of the Ang-AA peptide or Ang-(1-7) to the siCON-transfected A549 cells than to the A549 cells when the expression of the Mas receptor was silenced with either siRNA1 or siRNA2. (C) The relative fluorescence intensity of Ang-AA-FITC bound to A549 cells detected by flow cytometry decreased in a dose-dependent manner and relied on the addition of the unlabelled Ang-AA peptide. The addition of a high concentration of unlabelled GGG* showed little change. (D, E and F) Flow cytometry and confocal microscopy data showed the similar rates of competition for

binding to A549 cells between Ang-AA and A 799 or between Ang-(1-7) and A 799. (G) The k_d for Ang-AA-FITC to the A549 cells was 65 nM (R^2=0.99) and the K_d value of Ang-(1-7)-FITC in A549 cells was 69 nM (R^2 = 0.99) measured by flow cytometry. Results for each measurement are representative of three independent experiments. Data were expressed as mean ±SD, with a one-way ANOVA where ***p < 0.001, **p < 0.01 and *p < 0.05. Bar = 20 μm.

Figure 4. Anti-tumour functions of Ang-AA. (A) The CCK8 assay measured more substantial inhibition effects of A549 cell proliferation treated with Ang-AA at different doses than A549 cells with Ang-(1-7). (B) qRT-PCR and Western Blot detected the reduction of COX2 expression in A 549 cells with Ang-AA or Ang-(1-7) addition. (C and E) Ang-AA inhibited cancer cell invasion to a greater extent than Ang-(1-7), as determined using a Boyden chamber ssay. Bar=50 μm. (D and F) Based on the results of the scratch wound-healing assays, a significantly larger wound area was observed in the cultures of Ang-AA-treated A549 cells than cultures of cells treated with Ang-(1-7). The migration is shown by red lines. Bar=100 μm. (G) Ang-AA increased E-cadherin expression in A549 cells detected by qRT-PCR and Western Blot. Results for each measurement are representative of three independent experiments, and data were expressed as mean ±SD, with a one-way ANOVA where ***p < 0.001, **p < 0.01 and *p < 0.05.

Figure 5. Ang-AA inhibits tumour growth *in vivo*. (A) Micro-PET imaging demonstrated the combined Ang-AA and PTX treatment significantly decreased tumour growth compared to PBS, free PTX and Ang-(1-7). (B) The tumour volumes and tumour weights were reduced in the group treated with the combination of Ang-AA and PTX, revealing an effective anti-tumour effect. (C) Immunohistochemical staining (n = 24) for Ki67, COX2, and E-cadherin was consistent with the results obtained from A549 cells treated with Ang-AA. PTX showed little effect on COX2 and E-cadherin expression. Bar=200 μm. (D) Both PTX, PTX-Ang, and PTX-AA prolonged the survival of mice bearing xenografts. The PTX-AA group survived for a longer period than the PTX group and Ang-(1-7) group ($p<0.05$). (E) Weight loss was similar in each group and was mainly caused by PTX. No obvious changes were observed after Ang-AA treatment. Data were expressed as mean ±SD, with a one-way ANOVA where ***p < 0.001, **p < 0.01 and *p < 0.05.

Table of contents graphic

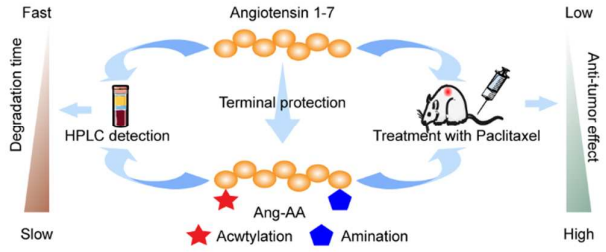
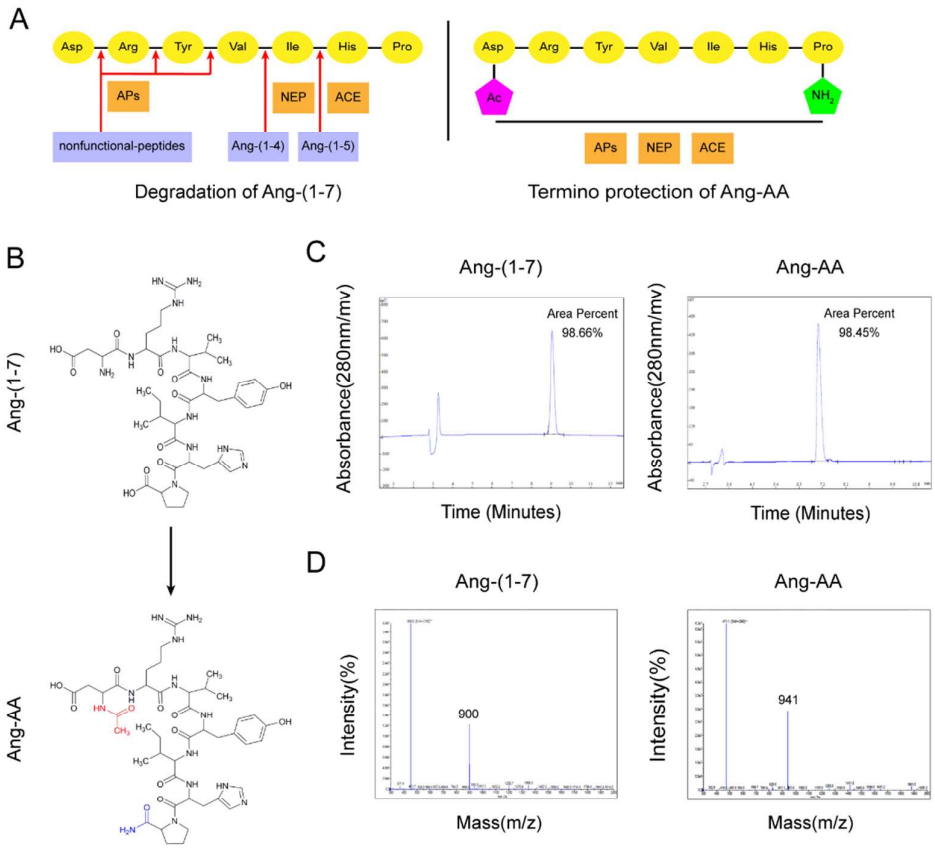


Figure 1.


A
Asp
Arg
Tyr
Val
Ile
His
Pro
APs
NEP
ACE
nonfunctional-peptides
Ang-(1-4)
Ang-(1-5)
Degradation of Ang-(1-7)
Ac
NH2
Termino protection of Ang-AA
B
Ang-(1-7)
Ang-AA
C
Ang-(1-7)
Ang-AA
Absorbance(280nm/mv)
Area Percent
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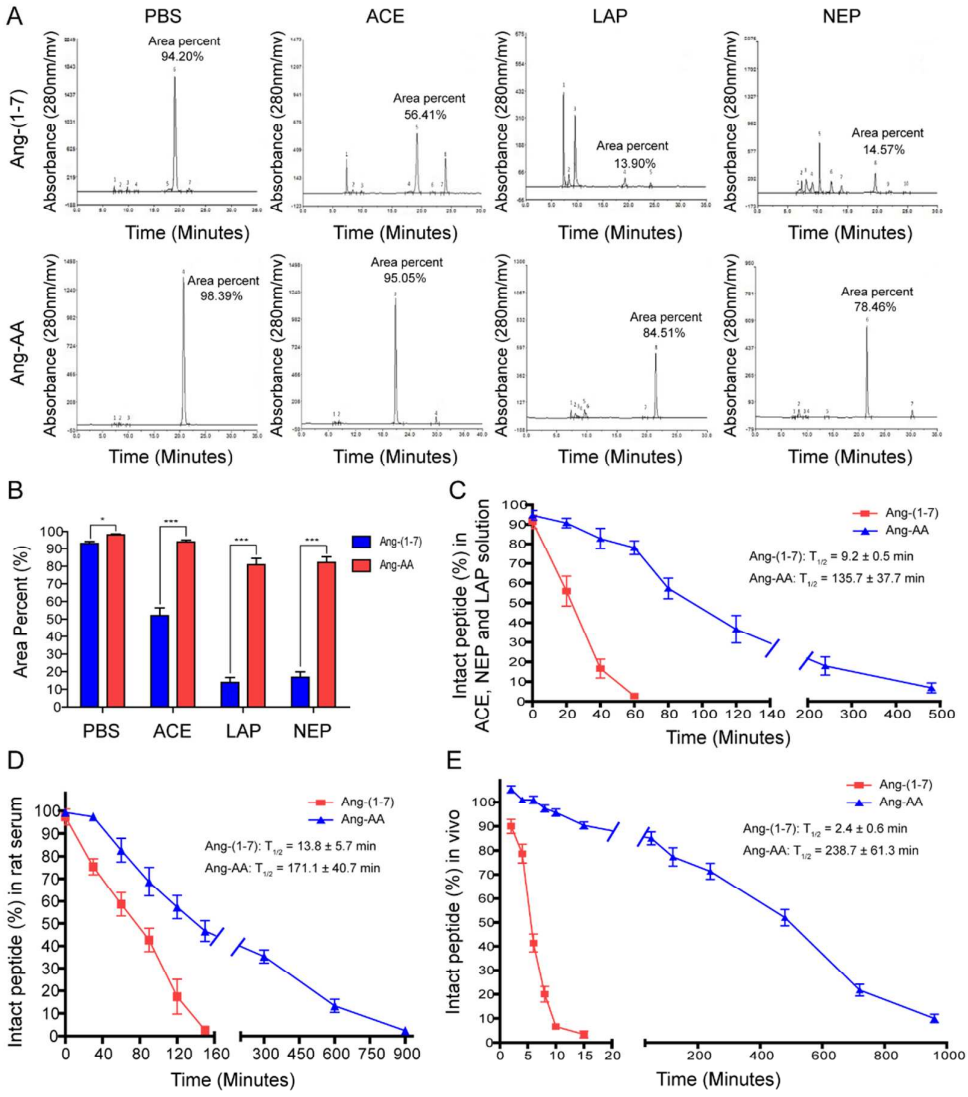
98.45%
Time (Minutes)
D
Intensity(%)
900
941
Mass(m/z)

Figure 2.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PBS
ACE
LAP
NEP
Ang-(1-7)
Ang-AA
Absorbance (280nm/mv)
Time (Minutes)
Area percent
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13.90%
14.57%
98.39%
95.05%
84.51%
78.46%
B
Area Percent (%)
Ang-(1-7)
Ang-AA
PBS
ACE
LAP
NEP
C
Intact peptide (%) in ACE, NEP and LAP solution
Ang-(1-7): T1/2 = 9.2 ± 0.5 min
Ang-AA: T1/2 = 135.7 ± 37.7 min
D
Intact peptide (%) in rat serum
Ang-(1-7): T1/2 = 13.8 ± 5.7 min
Ang-AA: T1/2 = 171.1 ± 40.7 min
E
Intact peptide (%) in vivo
Ang-(1-7): T1/2 = 2.4 ± 0.6 min
Ang-AA: T1/2 = 238.7 ± 61.3 min

Figure 3.

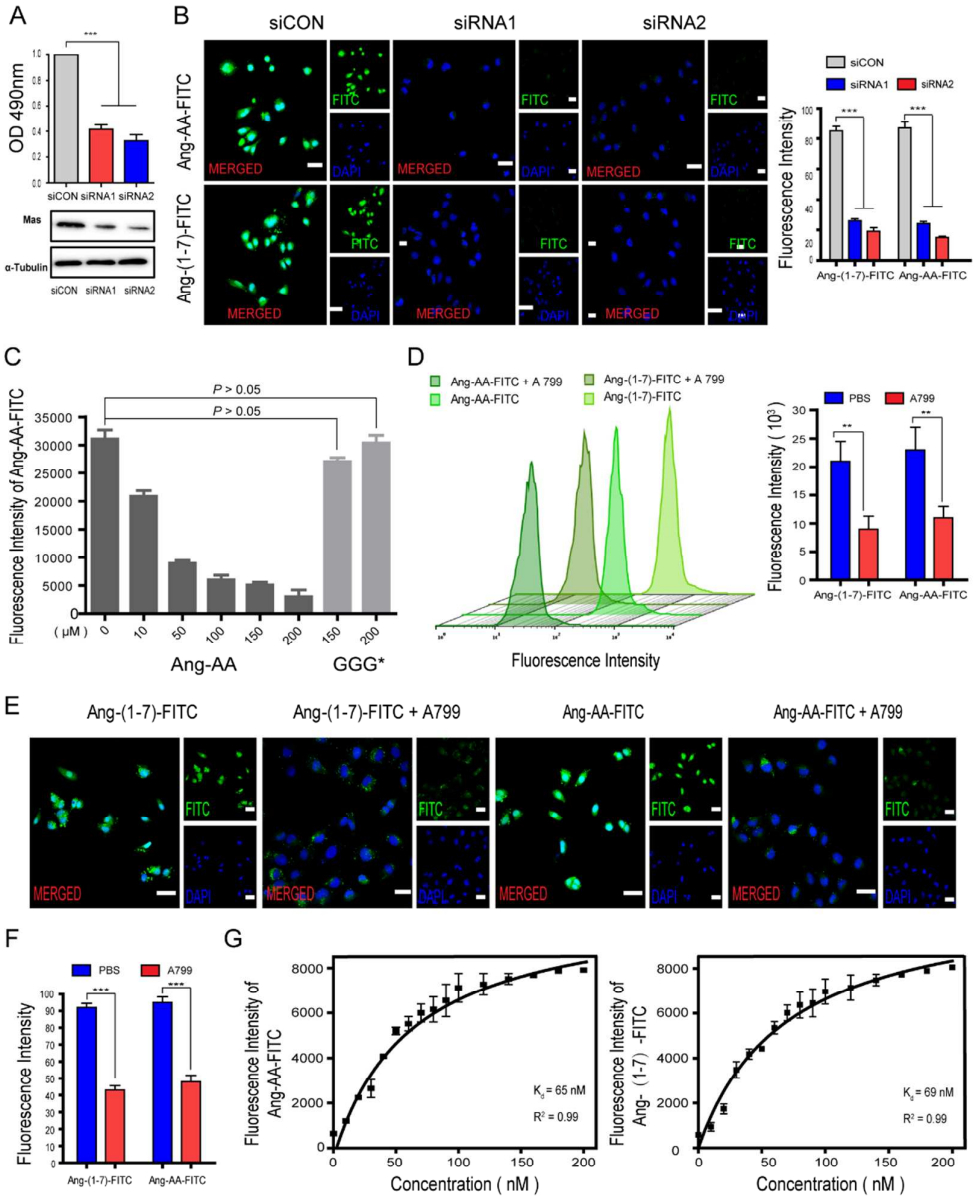

A
OD 490nm
siCON siRNA1 siRNA2
Mas
α-Tubulin
B
siCON
siRNA1
siRNA2
Ang-AA-FITC
Ang-(1-7)-FITC
MERGED
FITC
DAPI
Fluorescence Intensity
C
P > 0.05
P > 0.05
Fluorescence Intensity of Ang-AA-FITC
(μM) 0 10 50 100 150 200 150 200
Ang-AA
GGG*
D
Ang-AA-FITC + A 799
Ang-AA-FITC
Ang-(1-7)-FITC + A 799
Ang-(1-7)-FITC
Fluorescence Intensity
PBS
A799
Fluorescence Intensity (10³)
Ang-(1-7)-FITC
Ang-AA-FITC
E
Ang-(1-7)-FITC
Ang-(1-7)-FITC + A799
Ang-AA-FITC
Ang-AA-FITC + A799
F
PBS
A799
Fluorescence Intensity
G
Fluorescence Intensity of Ang-AA-FITC
K_d = 65 nM
R² = 0.99
Concentration (nM)
Fluorescence Intensity of Ang-(1-7)-FITC
K_d = 69 nM
R² = 0.99
Concentration (nM)

Figure 4.

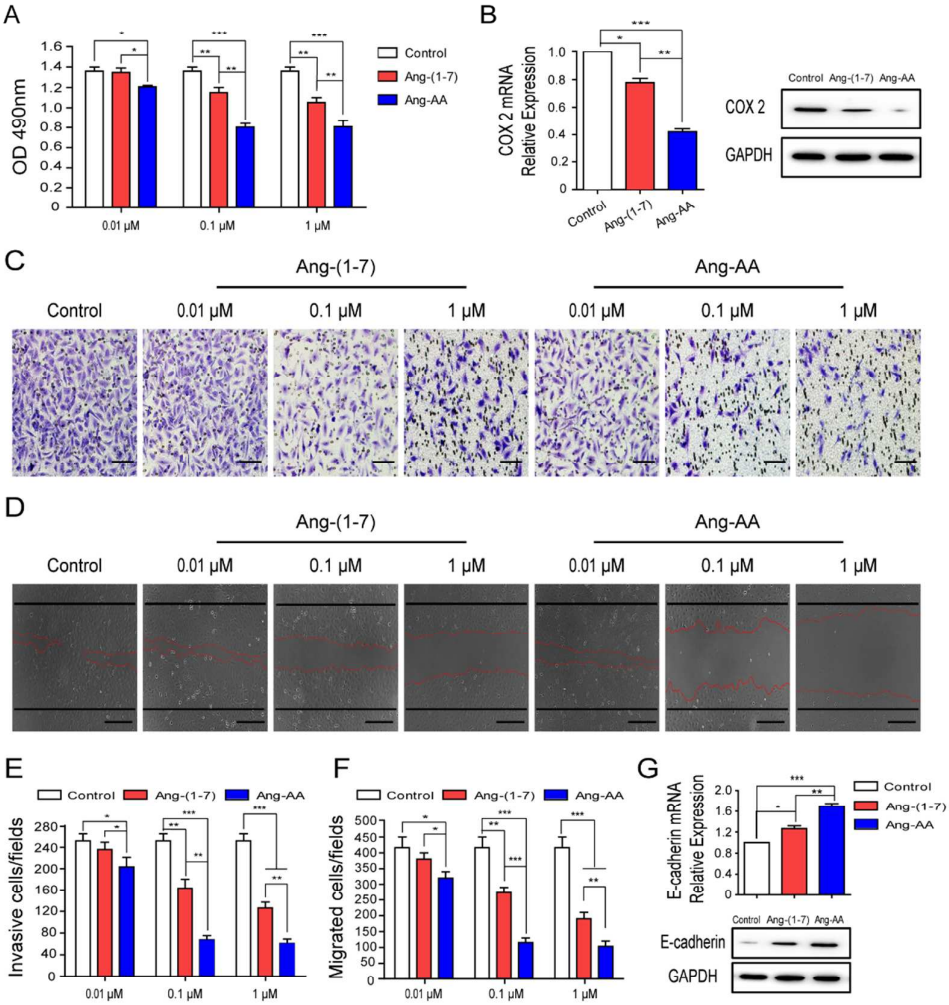
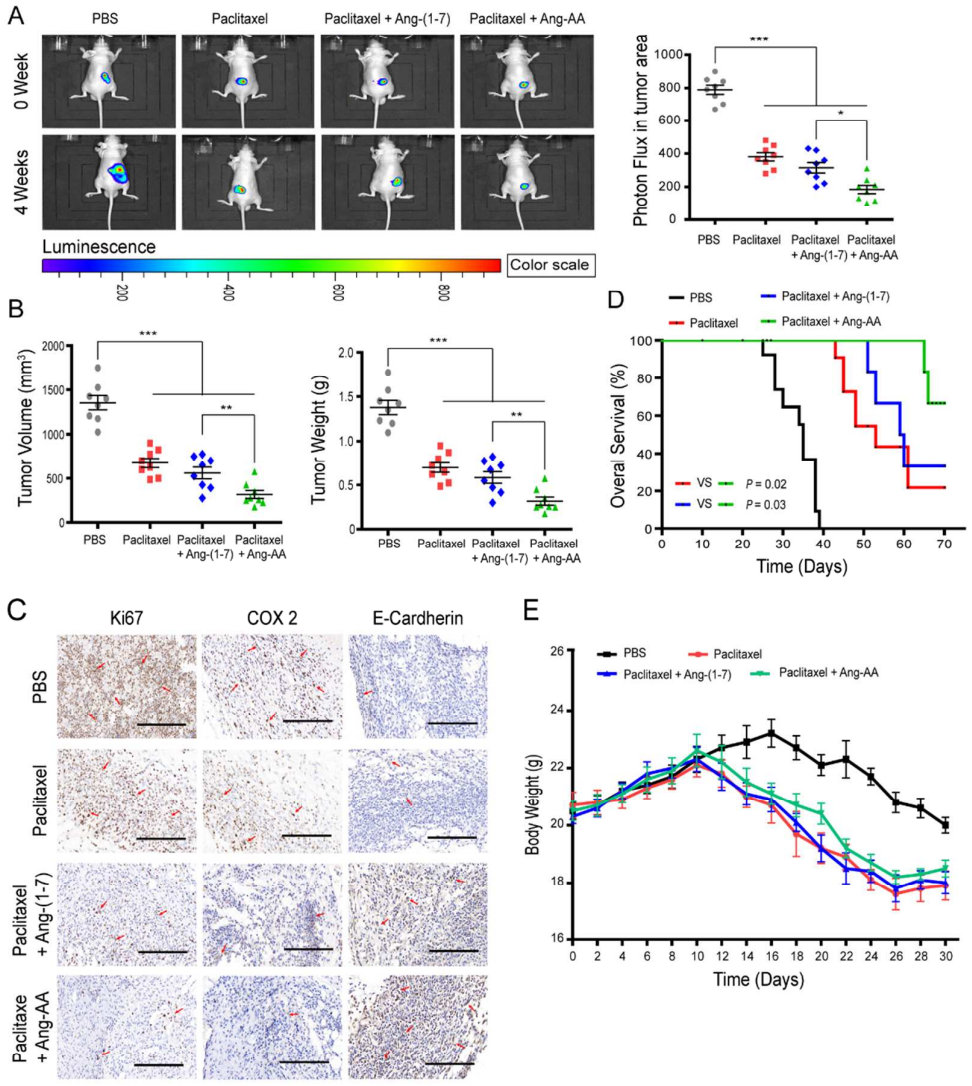

A
OD 490nm
Control
Ang-(1-7)
Ang-AA
0.01 μM
0.1 μM
1 μM
B
COX 2 mRNA Relative Expression
Control
Ang-(1-7)
Ang-AA
COX 2
GAPDH
C
Ang-(1-7)
Ang-AA
Control
0.01 μM
0.1 μM
1 μM
D
E
Invasive cells/fields
F
Migrated cells/fields
G
E-cadherin mRNA Relative Expression
E-cadherin
GAPDH

Figure 5.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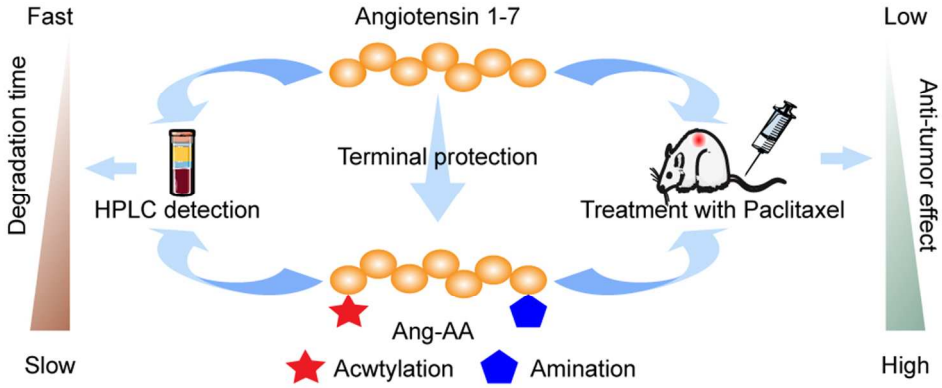
Paclitaxel
Paclitaxel + Ang-(1-7)
Paclitaxel + Ang-AA
0 Week
4 Weeks
Luminescence
Color scale
Photon Flux in tumor area
B
Tumor Volume (mm3)
Tumor Weight (g)
C
Ki67
COX 2
E-Cardherin
PBS
Paclitaxel
Paclitaxel + Ang-(1-7)
Paclitaxe + Ang-AA
D
Overal Servival (%)
VS P = 0.02
VS P = 0.03
Time (Days)
E
Body Weight (g)
Time (Days)



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89x36mm (300 x 300 DPI)