In vivo inhibitory activity of andrographolide derivative ADN-9 against liver cancer and its mechanisms involved in inhibition of tumor angiogenesis

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Abstract: It is well known that liver cancer is a highly aggressive malignancy with poor prognosis. Andrographolide (AD), a major bioactive component of *Andrographis paniculata* (Burm. f.), is a potential anti-cancer pharmacophore and the synthesis of AD derivatives with better cytotoxicity to cancer cells has attracted considerable attentions. In the present study, we evaluated the *in vivo* inhibitory effects of ADN-9, a 15-benzylidene substituted derivative of AD, on the growth and metastasis of murine hepatoma H22 using an orthotopic xenograft model and a subcutaneous xenograft model, and we further studied the anti-angiogenic action and the related mechanisms of ADN-9 *in vivo* and *in vitro*. Importantly, ADN-9 remarkably suppressed the growth and metastasis of both orthotopic and subcutaneous xenograft tumors, and the serum AFP level in orthotopic hepatoma-bearing mice treated with 100 mg/kg ADN-9 (ig.) was decreased to the normal level. We also found that ADN-9 showed stronger abilities than AD in shrinking tumors, suppressing the invasion and metastasis of H22 cells, decreasing the MVD and promoting tumor cell apoptosis in subcutaneous xenograft of mice. Additionally, ADN-9 exhibited stronger inhibitory activity than AD against the migration and VEGF-induced capillary-like tube formation in HUVECs, which was further proved to be associated with attenuating VEGF/VEGFR2/AKT signaling pathway. The present research provides the first evidence that a 15-substituted AD derivative is more promising than the parent compound in therapeutic treatment of liver cancer.

Key words: Andrographolide; Derivative; Liver cancer; Angiogenesis

1. Introduction

Liver cancer, predominantly hepatocellular carcinoma (HCC) which is predicted to continue to be the third cause of cancer-related death by 2030 (Hoshida *et al.*, 2012), is an aggressive malignancy with poor prognosis (Jemal *et al.*, 2011; Mazzanti *et al.*, 2016). Due to the high metastatic potential, liver cancer usually cannot be surgically removed and the recurrence rate of HCC is high in patients who have received liver resection surgery (El-Serag, 2012; Teng *et al.*, 2016). Therefore, besides attenuating tumor growth, it is absolutely crucial to inhibit the invasion and metastasis of liver cancer effectively.

Angiogenesis is not only associated with tumor growth as well as metastasis but also one of the dominant features in HCC. The inhibition of tumor-derived angiogenesis is considered to be a promising therapeutic strategy for HCC (Dong *et al.*, 2009). Recently, andrographolide (AD) extracts from the plant *Andrographis paniculata* (Burm. f.), a traditional herbal medicine with multiple pharmacological functions, has attracted considerable attentions owing to the moderate cytotoxic effects on several cancer cell lines, as well as its inhibitory activity against migration and invasion in a variety of human cancer cells (Tzeng *et al.*, 2012; Lai *et al.*, 2013; Preet *et al.*, 2014; Wong *et al.*, 2016). Furthermore, it is proved that AD is effective in inhibiting tumor growth and attenuating tumor-associated neoangiogenesis *in vivo* (Nateewattana *et al.*, 2014; Shen *et al.*, 2014; Ji *et al.*, 2015).

Although some researchers have committed themselves to designing and synthesizing AD derivatives with stronger cytotoxicity to tumor cell lines, and several derivatives of AD, having ester side chain(s) at C-14 and/or C-3, C-19, show better cytotoxic activity than the parent molecule (Dai *et al.*, 2006; Wei *et al.*, 2013; Song *et al.*, 2015), more valuable derivatives, especially with satisfactory *in vivo* anti-cancer

activities are still needed to be developed.

We have previously reported that several 15-benzylidene substituted analogues of AD exhibit much stronger inhibitory activities than the parent compound against migration and invasion in various cancer cells (Wu *et al.*, 2013). Therefore, it is very necessary and important to explore the *in vivo* anti-tumor activity, especially against tumor metastasis of this kind of compounds and to understand the related mechanisms.

In the present work, an orthotopic mouse xenograft model of H22 was used to evaluate the *in vivo* inhibitory effects of ADN-9 (one of the 15-benzylidene substituted analogues of AD), which is much more effective in inhibition of migration and invasion in cancer cell lines than AD (Wu *et al.*, 2013), and of the growth and metastasis of hepatoma. In addition, its mechanisms contributed to the better inhibitory activities against the growth and metastasis of hepatoma were studied by a subcutaneous mouse xenograft model and human umbilical vein endothelial cells (HUVECs), emphasizing on its inhibition of angiogenesis and metastasis, compared with that of AD.

2. Materials and methods

2.1. Chemicals and reagents

ADN-9 was synthesized by us from AD and the purity of both AD and ADN-9 was higher than 99.8%. Cyclophosphamide (CTX) and Thalidomide (THD) were purchased from Zhejiang Medicine Co., Ltd. Xinchang Pharmaceutical Factory (Zhejiang, China) and Changzhou Pharmaceutical Factory (Jiangsu, China), respectively. Rabbit polyclonal antibodies, anti-CD34, anti-MMP-2, anti-MMP-9, anti-Bax and anti-Bcl-2, were purchased from Wuhan Boster Biological Technology Co., Ltd. (Hubei, China). Rabbit polyclonal antibodies, anti-VEGF and anti-TIMP-1

were purchased from Proteintech Group, Inc. (Hubei, China). Rabbit polyclonal antibodies including anti-NF- κ B p65, anti-phospho-NF- κ B p65 (Ser536), anti-phospho-VEGFR2 (Tyr1175), anti-STAT3, anti-phospho-STAT3 (Ser727) and anti-GAPDH were supplied by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). AKT1 and phospho-AKT1 (Ser473) antibodies were obtained from Bioss Inc. (Beijing, China). The streptavidin/horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was supplied by Beijing Kangwei Century Biology Co., Ltd. (Beijing, China). Donkey anti-Rabbit IgG (H+L) Alexa Fluor 594® conjugate secondary antibody was purchased from Thermo Fisher Scientific Inc. (MA, US). Recombinant VEGF was obtained from Sino Biological Inc. (Beijing, China). Other reagents were purchased from Sigma-Aldrich (MO, US) unless otherwise specified.

2.2. Cell lines and cell culture

HUVECs and murine hepatoma cell line H22 were kindly presented by professor Xiukun Lin of the Institute of Oceanology, Chinese Academy of Sciences and associate professor Yan Zhang of Henan Academy of Medical and Pharmaceutical Sciences, Zhengzhou University, respectively. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, US) supplemented with 10% fetal bovine serum (FBS, Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China), penicillin (100 U/ml) and streptomycin (100 mg/ml) in a 5% CO₂ humidified atmosphere at 37 °C.

2.3. Animals

Balb/c mice (for the orthotopic mouse xenograft model) and Kunming mice (for the subcutaneous mouse xenograft model) of male aged 4–6 weeks and weighing 18–22g were supplied by the Hunan Silaikejingda Experimental Animal Co., Ltd. (Hunan, China). All animals were kept under a 12-h light-dark cycle at constant

temperature ($25\pm 2^{\circ}\text{C}$) and relative humidity (60%–70%). Experiments were performed in accordance with the ethical guidelines of the Henan Province Experimental Animal Management Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.4. Animal models and drug-treatment

Orthotopic mouse xenograft model was established to reference the previous reports by Yang et al. (Yang *et al.*, 2017). Briefly, H22 cells (0.1 ml, 5×10^5 per animal) were subcutaneously inoculated into the right axilla of the animals. When the diameter of tumor became about 1.5 cm, it was removed into pre-cooled normal saline and cut into small fragments (1 mm^3), then one of them was implanted into the left lobe of liver of each mouse. After 48h the surgery mice were randomized into groups, administrated intragastrically (ig.) once a day with ADN-9 (30 mg/kg, 70 mg/kg or 100 mg/kg), 50 mg/kg THD dispersed in carboxymethylcellulose (0.5% v/v), or the vehicle along in the control and the sham-operated mice. On the 28th day after implantation, blood samples were taken for ELISA assay, livers and lungs were removed from mice, and also the megascopic lesions were recorded and the tumor sizes in livers and lungs were measured in details. The left lobe of livers and the whole lungs were fixed in 4% paraformaldehyde for the histopathological examination (as shown in 2.5).

Moreover, a subcutaneous xenograft model of H22 was used to study the *in vivo* anti-hepatoma activity of ADN-9 compared with AD. After inoculation, the mice were randomly divided into five groups, and each group of mice was administrated (ig.) once a day for 7d with 0.5% carboxymethylcellulose (control), standard controls CTX (30 mg/kg) or THD (50 mg/kg), AD as well as ADN-9 at the tested dosages. On day 8, the mice were sacrificed and tumors were immediately removed and weighed. Each

tumor was cut into two sections in which one was fixed in 4% paraformaldehyde for histologic examination and immunohistochemistry, whereas the other was snap-frozen in liquid nitrogen and stored at -80°C for the analysis of western blot and DNA fragmentation.

2.5. Histologic examination and immunohistochemistry

After fixation, tissue samples were embedded with paraffin, sectioned into 3.5 μm sections, dewaxed, and rehydrated using xylene and successive ethanol baths. For histologic examination, sections were stained with hematoxylin and eosin (H&E); whereas for the immunohistochemical staining, tissue sections were incubated with specific primary antibody overnight at 4°C in a humidified atmosphere, then were incubated with HRP-conjugated goat anti-rabbit IgG for 1 h at 37°C , following by visualizing the immunoreactivity by the DAB detection kit (Wuhan Boster Biological Technology Co., Ltd., Hubei, China). The sections were analyzed using a Leica DM3000 (Leica Biosystems, Germany) microscope, and the images were captured and processed.

2.6. ELISA

Concentrations of interleukin-6 (IL-6) and alpha-fetoprotein (APF) in serum of mice were measured using commercially available ELISA kits (Shanghai Guangrui Biological Technology Co. Ltd., Shanghai, China) in accordance with the manufacturer's instructions.

2.7. Western blot analysis

Cells or tumor tissues were lysed in RIPA buffer containing PMSF and phosphatase inhibitor. Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific Inc., MA, US). The proteins were fractionated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was

blocked with 2% BSA and then incubated with the primary antibody. After washing, blots were incubated with the secondary antibody and detected using BeyoECL Plus kit (Beyotime Institute of Biotechnology, Jiangsu, China). The gray value of bands was quantified by using Quantity One software (Bio-Rad, CA, US).

2.8. DNA fragmentation assay

The genomic DNA in tumor tissues was extracted and purified using the Apoptotic DNA Ladder Extraction Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China), and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA). Genomic DNA (5 µg per sample) was separated by agarose gel (1.0%) electrophoresis and photographed with an automatic digital gel imaging analysis system (Tanon-4100, Tanon Science & Technology Co., Ltd., Shanghai, China).

2.9. Wound-healing assay

Briefly, HUVECs were allowed to grow to full confluence in a 96-well plate and then starved with medium containing 1% FBS overnight. Twenty-four hours later, scrape wounds were made and cells were incubated with compounds at the concentrations indicated. The scratch was monitored by taking the images at 0 and 24 h using a microscope respectively (XD-101, Nanjing Jiangnan Novel Optics Co., Ltd, Nanjing, China) and the areas of cell migration into the scrape wound were analyzed by the Image J software.

2.10. Transwell migration assay

HUVECs (5.0×10^4) were added into the upper chamber (8-µm pore size, Corning Life Sciences, MA, US) of the transwell, while the culture medium containing 10% FBS was added into the lower chamber. Two hours later, medium in the upper chamber was replaced by serum-free medium with or without compound and cultured for another 24 h. Cells that migrated to the lower wells were then fixed by methanol,

stained by 0.1% (w/v) crystal violet, and photographed. The number of cells on the lower surface of the filters was quantified.

2.11. Matrigel assay (tube formation)

Matrigel™ basement membrane matrix (BD Biosciences, Ca, US) was loaded in each well (100 µl) of a 96-well plate and allowed to polymerize for 0.5 h at 37°C. HUVECs were added to the top of the gel at density of 3×10^5 /well in the presence or absence of the compound; and then incubated for 8 h at 37°C in a CO₂ incubator. The formation of tube-like structure by HUVECs was photographed and the number of branching point per field was measured by counting 5 random fields per well.

2.12. Immunofluorescence staining

HUVECs were seeded onto cover slips at the bottom of a 24-well plate at density of 6.0×10^4 /well. Following treatments, cells were fixed in cold 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 and blocked in PBS containing 2% BSA for 2 h at room temperature. The cells were incubated with the primary antibody overnight at 4°C followed by incubation with Alexa 594 conjugated secondary antibody for 2 h at room temperature within a moist chamber. After washing with PBS, the cell nuclei were stained with DAPI. Images were acquired using an Olympus BX53 microscope coupled with an Olympus DP70 digital camera.

2.13. Statistical analysis

Data were presented as mean $\pm$ standard deviations (SD) or mean $\pm$ standard errors (SE). Statistical analysis was performed using SPSS® 15.0 for Windows (SPSS Inc, IL, US), and one-way analysis of variance (ANOVA) test followed by Dunnett's test were applied. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of ADN-9 on the growth and metastasis of hepatoma in tumor-bearing

mice

Twenty-eight days after implantation, it was found that all of the animals administrated (ig.) with ADN-9 at the middle (70 mg/kg) or at the high (100 mg/kg) dose, and those in the sham-operation group were still survival; while four of sixty one, in which two were control mice received vehicle along and the other two were respectively administrated with THD and 30 mg/kg ADN-9, died from the metastatic spread of hepatoma (Table 1).

Though it has no obviously inhibitory activity against cell proliferation in various human hepatoma cell lines (results were not listed) *in vitro* at concentration of no higher than 15 μ M, ADN-9 lowered the mean size of primary lesions of the livers. Compared with that in the control mice, the primary tumor volume in orthotopic hepatoma-bearing mice treated with high-dose ADN-9 was significantly decreased by 93.92% ($P < 0.05$; Fig.1A and B).

Furthermore, the *in vivo* anti-metastatic activity of ADN-9 was appraised by the anatomical and histological observations on the livers and lungs of mice. Showed by the results (Table 1 and Fig.1), that besides the primary tumors, metastatic lesions were found in both livers and lungs of the mice including the two dead treated with the vehicle individual; whereas those were observed on livers only in two of ten, and on lungs in four of ten mice, respectively, with 100 mg/kg ADN-9.

In addition, the invasive ability of hepatoma cells transplanted into livers were remarkably reduced by treatment with ADN-9, especially at middle or high dose, and the interface between tumor mass and normal tissue was much clearer, than those of the control mice showed by the histological sections of livers (Fig.1C).

Consistently, ADN-9 dose-dependently reduced the serum levels of AFP, an important liver carcinoma marker, in orthotopic hepatoma-bearing mice ($P < 0.01$),

with the highest inhibition of 92.91% by 100 mg/kg-treatment, nearly the same as that in the sham-operated mice (Fig.2A). Moreover, remarkably lowered serum IL-6 levels were observed in the animals administrated with ADN-9 at dose of 70 mg/kg or 100 mg/kg, resulting in respective reduction of 56.09% and 62.79% (Fig.2B), than that in control mice ($P < 0.01$).

3.2. Inhibitory activity of ADN-9 against the growth and metastasis of hepatoma in subcutaneous tumor-bearing mice

To further confirm the *in vivo* anti-hepatoma effect of ADN-9 compared to that of AD and to make it easy to carry out the related-mechanism research, a subcutaneous mouse xenograft tumor model was used. ADN-9 induced reduction in mean tumor weight was remarkably higher than that obtained with AD ($P < 0.05$) as ADN-9 resulted in 50.25% (300mg/kg; ig.) and 35.38% (200mg/kg; ig.) reduction, while AD caused only 13.32% reduction. Moreover, the hepatoma xenographs in four of seven mice treated with the vehicle along spread from the inoculation site into their ribs and/or chests; whereas all the tumors in mice with ADN-9-treated (300 mg/kg) were confined on the inoculation sites (Fig.3).

3.3. ADN-9 induced tumor cell apoptosis *in vivo*

Histopathological examination of resected tumor tissue (Fig.4A) showed that the density of tumor cells in hepatoma tissue of AD-treated mice was reduced to a certain degree and the cells were arranged relatively regular in small nest-like structures, with less bleeding, but no visible change on lymphocyte infiltration, compared with those obtained from the control mice. But in the ADN-9-treated mice, especially in those treated with high-dose, hepatoma cell density was greatly decreased, hemorrhage and leukocyte infiltration in tumors were significantly reduced, and also varying degrees of patchy necrosis, as well as DNA fragmentation in hepatoma cells were observed.

Showed by the gel electrophoresis assay of total cellular DNA from tumors (Fig. 4B), obvious bands of DNA ladder appeared in ADN-9-treated mice, as well as in those with the two standard control drugs CTX and THD. Correspondingly, ADN-9 lowered the Bcl-2 expressions in tumor cells with marked promotion of Bax ($P < 0.05$), compared to AD or the vehicle along, suggesting that ADN-9 could induce the apoptosis in tumor cells, and the effect was stronger than AD (Fig.4C–F).

3.4. ADN-9 suppressed tumor angiogenesis *in vivo*

In view of the key role of angiogenesis in the growth, invasion and metastasis of liver cancer, we detected the microvessel density (MVD) of tumors in mice through immunohistochemical staining method (Fig.5).

The values of MVD of mice treated by ADN-9 at 200 mg/kg and 300 mg/kg were reduced to 34.73 and 26.63 respectively, which were significantly lower than those obtained by cyclophosphamide (42.63; $P < 0.01$), AD (51.92; $P < 0.01$), as well as that in the control mice (62.83; $P < 0.01$).

3.5. ADN-9 down-regulated VEGF and MMPs but up-regulated TIMP-1 expressions

The mean relative protein level of VEGF in hepatoma tissues of animals ADN-9-treated with 300 mg/kg was just 0.14 fold of that of drug-untreated mice, which was much lower than that obtained by treated with AD or CTX, but similar to that by THD (Fig.6A and B).

According to the results of matrix metalloproteinases (MMPs) detected by immunohistochemistry method (Fig.6C–F), compared to the vehicle along, ADN-9 at both the high-dose and the low-dose significantly suppressed the protein expressions of MMP-2 and MMP-9 in the tumor tissues ($P < 0.05$). But no obvious reduction of MMPs expression was found in AD-treated mice ($P > 0.05$). The inhibitory effect of ADN-9 on MMP-9 expression was stronger than it on MMP-2's. With regard to the

expression of tissue inhibitor of metalloproteinases 1 (TIMP-1), ADN-9 and AD, as well as the two standard drugs significantly increased the levels of TIMP-1 in tumors, with the highest relative expression of 8.60 fold caused by 200 mg/kg ADN-9 treatment.

3.6. ADN-9 inhibited the migration and VEGF-induced capillary-like tube formation of HUVECs

To better understand the anti-angiogenic effect of ADN-9, we further appraised its inhibitory activities against the ability of migration, by wound-healing and transwell assays, as well as the tube formation of HUVECs. As shown in Fig.7A and B, ADN-9 significantly inhibited HUVEC's migration by wound-healing assay, in a dose-dependent manner ranging from 1.25 μ M to 5.00 μ M within the nontoxic concentration, resulting in inhibition from 42.70% to 60.16%, accordingly. The number of cells transferred to the below chamber was obviously and dose-dependently decreased by ADN-9 (1.25 μ M, 2.50 μ M, 5.00 μ M), with the highest inhibition of 57.04% caused by 5.00 μ M treatment (Fig.7C and D).

Though the cytotoxicity tested by MTT assay, caused by AD was stronger than that of ADN-9 (results were not listed; $P < 0.01$), the inhibition of migration in HUVECs by AD was not remarkably stronger than that of ADN-9 at 5.00 μ M.

Both of ADN-9 and AD, within the concentration ranging from 1.25 μ M to 5.00 μ M, could dose-dependently and remarkably reduce the number of the vascular cavity formed by HUVECs clearly ($P < 0.01$; Fig.7C and E), and the number of tube branch points formed in HUVECs was significantly diminished by ADN-9, compared to AD, at the same concentration of 5.00 μ M ($P < 0.01$; Fig.7D and E).

3.7. Associated anti-angiogenic mechanisms of ADN-9 *in vitro*

Considering the importance of activation of NF- κ B and VEGF/VEGFR2

signaling pathways to tumor angiogenesis, and the differences of expression levels of MMPs, VEGF and IL-6 between AD and ADN-9 probably being the key factors for exhibiting the improvement of *in vivo* anti-tumor activity by ADN-9, we compared the effects of AD and ADN-9 on protein expression levels of VEGF, NF- κ B p-65/p-p65, p-VEGFR2, STAT3/p-STAT3, and AKT1/p-AKT1 in HUVECs using western blot method (Fig.8), to better understand if the increased inhibition showed by ADN-9 on tumor growth, invasion and metastasis, as well as on angiogenesis was associated with attenuating the activation of NF- κ B as well as the VEGF/VEGFR2 signaling pathways.

Results were the same as we expected, with the increase of drug concentration from 1.25 μ M to 5.00 μ M, it was found that ADN-9 remarkably decreased the VEGF protein expression levels, in a dose-dependent manner, and the protein level was much lower than that obtained by AD treatment at the same concentration of 5.00 μ M. Compared with the control and AD, ADN-9 (5.00 μ M) substantially down-regulated the expression levels of p65 and p-p65, as well as STAT3 and p-STAT3 in HUVECs induced with VEGF (10 ng/ml). Accordingly, we observed that the protein expression levels of pVEGFR2 and p-AKT1 in HUVECs treated with ADN-9 were greatly lower than that with AD. What interesting was that p-STAT3 level in HUVECs treated with AD at 2.50 μ M or 5.00 μ M was notably higher than that in control cells, very different from that with ADN-9.

Furthermore, the much stronger inhibitory activity by ADN-9 against VEGF-induced nuclear translocation of NF- κ B p-p65 and p-STAT3 than that obtained by AD was confirmed through immunofluorescence analysis (Fig.9).

Therefore, the stronger anti-angiogenesis of ADN-9 than AD probably is associated with attenuating the activation of STAT3 and NF- κ B, weakening the

expression of VEGF, and then inhibiting the activation of VEGFR2.

4. Discussion

Orthotopic murine xenograft models take advantage of the local tumor microenvironment and can more closely mimic the clinical scenario (Bibby, 2004). The present study provides the first evidence that ADN-9, a promising semi-synthetic derivative of AD, with stronger anti-migrative activities than AD against several human cancer cell lines *in vitro* (Wu et al., 2013) potently suppresses growth and metastasis of orthotopic xenograft tumors of hepatoma H22 *in vivo*. Correspondingly, serum level of AFP, an important prognostic indicator of liver carcinoma, in orthotopic hepatoma-bearing mice treated with 100 mg/kg ADN-9 was remarkably lower than that in control mice, and even nearly similar to the normal.

Importantly, our results indicated that modifying AD at C15 position is successful to enhance its *in vivo* anti-cancer effect. Compared with AD, ADN-9 at the same dose (300 mg/kg; ig.) significantly lowered the tumor size and reduced the invasive and metastatic abilities of H22, though it showed less cytotoxic to various human cancer cell lines (Wu *et al.*, 2013). Gong et al. reported that a semi-synthetic analogue A5 (19-N-hexylamino-8-methylandrograpanin) of AD decreased tumor weight and size of hepatoma in a subcutaneous mouse xenograft model through intraperitoneal injection (Gong *et al.*, 2013). As we have known that AD exerts hepatoprotective effect through its anti-inflammatory and antioxidant activities. Tang et al. reported that an AD derivative, modified at C3 and C9 positions, significantly prevented acute liver damage induced CCl₄ in mice (Tang et al., 2014). However, to our knowledge, the present work is the first demonstration that oral administration of a 15-benzylidene substituted derivative of AD causes remarkably improved *in vivo* inhibitions on both growth and metastasis of liver cancer, compared with AD.

Several previous works proved that AD possessed inhibitory activity against proliferation, and triggered apoptosis via increasing the Bax/Bcl-2 ratio in various human cancer cell lines (Zhou *et al.*, 2006; Pratheeshkumar *et al.*, 2012; Banerjee *et al.*, 2016). Our results suggested that down-regulation of Bcl-2 but up-regulation of Bax proteins caused by ADN-9 much greatly than AD was probably one of the mechanisms behind apoptosis induction in hepatoma H22 cells, resulting in significant tumor shrinking *in vivo*.

It is well known that suppression of MMP-9, MMP-7 or MMP-2 could reduce the invasive and metastatic abilities of tumor cells (Verma and Hansch, 2007; Kim *et al.*, 2009). Recently, AD was reported to have anti-migratory and anti-invasive activities in human cancer cell lines, which was related to the down-regulation of MMP-7 expression (Shi *et al.*, 2009; Lee *et al.*, 2010). AD (1-50 μ M) was found to suppress the expression of MMP-9 messenger RNA that was induced by either tumor necrosis factor- α (TNF- α) or lipopolysaccharide (LPS) in human monocytic THP-1 cells. The main mechanism behind this action was speculated to involve the NF- κ B signaling pathway (Lee *et al.*, 2012). In the present work, we also observed that protein expression of MMP-2 and MMP-9 in tumor tissues were significantly suppressed by ADN-9 compared with AD, which was consistent with our previous results in SGC7901 cells (Wu *et al.*, 2013).

Considering that the MMPs have been strongly linked to the angiogenic switch, we wonder if the improved anti-tumor function of ADN-9 compared with AD is associated with enhanced inhibition on tumor angiogenesis. Just as we have expected, much stronger reduction of MVD and inhibition on VEGF expression in tumor tissues in mice treated with ADN-9 was observed. Moreover, ADN-9 proved more effective in inhibition of migration and capillary-like tube formation in HUVECs than AD.

Therefore, we speculated that at least one of the keys to exhibiting good activities against hepatoma growth and metastasis was the enhanced anti-angiogenic effect of ADN-9.

VEGF/VEGFR2 signaling is considered as an important therapeutic target for the treatment of pathogenic angiogenesis because it is a predominant mediator of vasculogenesis and angiogenesis by induction of multiple signaling cascades (Olsson *et al.*, 2006; Meissner *et al.*, 2009). It has been reported that AD decreases VEGFD expression in hepatoma cancer cells (Ji *et al.*, 2015) and both AD and its derivative A5 have been revealed to inhibit tumor angiogenesis via blocking VEGF-induced activation of VEGFR2 and its downstream MAPKs signaling pathways (Gong *et al.*, 2013; Shen *et al.*, 2014). Here, we found that ADN-9 was more powerful than AD in down-regulating VEGF expression and blocking VEGF-induced activation of VEGFR2, resulting in enhanced inactivation of multiple intracellular signaling cascades in HUVECs. Furthermore, ADN-9 not only maintained the suppression of PI3K/AKT and NF- κ B pathways showed by AD (Lee *et al.*, 2010; Luo *et al.*, 2013), but also greatly improved the inhibitory effect. This probably was a reason why ADN-9 (70, 100 mg/kg) significantly reduced the serum levels of IL-6 in tumor-bearing mice, because PI3K/AKT and NF- κ B pathway play an important role in activating expression of IL-6 (Lin *et al.*, 2007; Hou *et al.*, 2013; McFarland *et al.*, 2013). Intriguingly, AD (5.00 μ M) did not down-regulate the increased p-STAT3 expression induced by VEGF in HUVECs, whereas ADN-9 (5.00 μ M) decreased it strongly. It has been reported that AD exhibits distinct regulatory effect on the expression and phosphorylation of STAT3 in different cell lines. In human prostate cancer cells AD suppresses both IL-6 autocrine-paracrine loop induced cell signalings including STAT3 and Erk phosphorylation (Chun *et al.*, 2010), while in the lung of

cigarette smoke-exposed mice with AD administration the phosphorylation of STAT3 was enhanced (Yang *et al.*, 2013).

As we known that aberrant STAT3 signaling promotes initiation and progression of human cancers by either inhibiting apoptosis or inducing cell proliferation, angiogenesis, invasion, and metastasis (Siveen *et al.*, 2014), what's more, there is evidence that activated STAT3 is required for the survival of HCC cells (He *et al.*, 2010). Therefore, inhibition of STAT3 expression and its phosphorylation should be another contribution to the great improved activities of ADN-9 against angiogenesis, tumor growth and metastasis.

Taken together, in our opinion, there is no doubt that ADN-9 is a potential candidate therapeutic drug for liver cancer and the present results can provide useful informations for new drug development from this kind compounds, though the targets still need us to identify and the other multiple mechanisms, besides that showed in the present paper, need to be revealed. Moreover, evaluation of ADN-9 on the growth and metastasis of hepatocellular carcinoma xenografts in nude mice has been going on.

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Figure legends

Fig.1. Effects of ADN-9 on orthotopic hepatoma progression and metastasis in mice. (A) Representative images of livers (tumor nodules indicated as circle) from mice in each experimental group. (B) At experimental endpoint, orthotopic tumor volume was measured and calculated using the formula: tumor volume (mm^3) = $0.5 \times \text{length} \times \text{width}^2$. Data were shown in mean $\pm$ SE, $n=9$ or 10 per group, $^{\#}P < 0.05$ vs control. (C and D) Representative images of H&E stained sections of liver (C) and lung (D) from mice of each group (200 $\times$).

Fig.2. ADN-9 decreased serum levels of AFP and IL-6 in orthotopic hepatoma-bearing mice. Concentrations of AFP (A) and IL-6 (B) in serum of mice were determined using ELISA ($n=9$ or 10 per group). Data were mean $\pm$ SD; $^{\#\#}P < 0.01$ vs control; $^{\Delta\Delta}P < 0.01$ vs sham; $^{\&\&}P < 0.01$ vs THD;

Fig.3. ADN-9 suppressed hepatoma growth and metastasis in subcutaneous hepatoma-bearing mice. Upper panel, tumor weight; middle panel, representative images of hepatoma tumors dissected from mice; Lower panel, incidence of metastasis in mice implanted with H22 cells which spread from the inoculation site into ribs and/or chests. Values were mean $\pm$ SD; $n=6$ or 7 per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.4. ADN-9 treatment induced apoptosis in tumor tissues. (A) H&E-stained tumor sections (200 $\times$). (B) DNA fragmentation analysis of tumor tissues by DNA agarose gel electrophoresis. Lane 1: marker; lane 2, 3: control; lane 4, 5: THD; lane 6, 7: CTX; lane 8, 9: AD; lane 10, 11: ADN-9 (200 mg/kg); lane 12, 13: ADN-9 (300 mg/kg). (C–F) Immunohistochemical staining of Bax (C) and Bcl-2 (E) in tumor tissues (200 $\times$). The integrated optical density (IOD) was evaluated by Image-Pro Plus image analysis software (D and F). Data were carried out on multiple sections from three mice per group and presented as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.5. Microvessel density (MVD) in tumor tissues. (A) Photomicrograph of immunohistochemical staining of CD34 (400 $\times$). (B) Microvessel density (MVD) was quantified by counting CD34-positive blood vessels from five fields in each tumor section. Data were presented as mean $\pm$ SD from three mice per group. $^{\#\#}P < 0.01$ vs control; $^{\&}P < 0.01$ vs CTX; $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.6. Effect of ADN-9 on the expression of angiogenic and metastasis-associated proteins in tumor tissues. (A and B) TIMP-1 and VEGF expressions were determined by western blot. The results were normalized against GAPDH and presented as fold of Control. (C–F) MMP-2 (C) and MMP-9 (E) expressions were detected by immunohistochemical staining (400 $\times$). The IOD (D and F) was evaluated by Image-Pro Plus image analysis software. Data were carried out on multiple sections from three mice per group and presented as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs THD; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.7. ADN-9 inhibited migration and tube formation of HUVECs. (A) Migration of HUVECs treated with AD or ADN-9 at indicated concentration in wound-healing assay (40 $\times$). (B) Percentages of wound closure at 24 h after scratch. (C) Representative images of transwell assay (upper panel, 200 $\times$) and tube formation assay (lower panel,

100×) of HUVECs in the presence of indicated concentration of AD or ADN-9. (D and E) Both the number of cells migrated through transwell (D) and the branching points per field (E) were measured by counting in five random fields/well. Each experiment was performed in triplicate and the values represent as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.8. ADN-9 decreased VEGF level and attenuated VEGF/VEGFR-2/AKT signaling pathway in HUVECs. (A and B) HUVECs were treated with vehicle (Control), AD or ADN-9 for 48 h then VEGF protein level in HUVECs was determined by western blot analysis. (C-H) The serum-starved HUVECs were pretreated with indicated concentration of ADN-9 or AD for 12 h before exposure to VEGF(10 ng/mL) for 20 min, and then protein levels of phosphorylated VEGFR2, p65, STAT3, AKT, the total p65, STAT3 and AKT were assessed using western blot analysis (C, G). Relative quantities of these protein levels were normalized against GAPDH and presented as fold of control (B, D–F and H). Values were presented as mean $\pm$ SD from three independent experiments. $^{\&\&}P < 0.01$ vs negative control (-VEGF); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs positive control (+VEGF); $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs AD.

Fig.9. ADN-9 inhibited NF- κ B p65 and STAT3 nuclear translocation induced by VEGF. HUVECs were pre-incubated in RPMI 1640 medium with indicated concentration of ADN-9 or AD for 12h, then treated with VEGF (10ng/ml) for an additional 40 min, followed by immunofluorescently stained with DAPI (blue) and anti-NF- κ B p65 (red) or anti-STAT3 (red). Images were representative of three independent experiments (400×). White arrows identified cells with NF- κ B p65 or STAT3 nuclear localization.

Tables and Figures

Table 1

Effects of ADN-9 on mortality and tumor metastasis in orthotopic H22 tumor-bearing mice

Group	Dose (mg/kg)	Number (n)	Mortality	Metastasis percentage	
				Live	Lung
Sham	/	10	0%(0/10)	0%(0/10)	0%(0/10)
Control	/	11	18%(2/11)	64%(7/9)	100%(9/9)
THD	50	10	10%(1/10)	56%(5/9)	78%(7/9)
	30	10	10%(1/10)	56%(5/9)	56%(5/9)
ADN-9	70	10	0%(0/10)	30%(3/10)	50%(5/10)
	100	10	0%(0/10)	20%(2/10)	40%(4/10)

^aMice were observed daily for mortality during experiment.

^bThe presence of metastasis was investigated by anatomical and histological observation

Fig.1.

Fig.2.

Fig.3.

Fig.4.

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Fig.6.

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Fig.9.

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