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Original Article

Potential anti-tumor mechanisms of renin angiotensin system inhibitors through inhibiting angiogenesis and influencing angiotensin II actions

Bei Zhao[1], Hong Sun[2], Xiao-Dan Zhang[1], Guang-Hui Li[1], Yong-Hong Zhao[1], Bin Wang[1,3]

[1]Department of Pharmacy, Jing'an District Center Hospital of Shanghai, Fudan University, Shanghai, China; [2]Department of Clinical Pharmacy and Pharmaceutical Management, School of Pharmacy, Fudan University, Shanghai, China; [3]Department of Pharmacy, Hua Shan Hospital of Fudan University, Shanghai, China

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Abstract: Recent studies have shown that renin angiotensin system (RAS) inhibitors improve the survival of patients with several types of cancer such as pancreatic cancer. However, the anti-tumor mechanisms of RAS inhibitors remain unclear. The objective of this study was to investigate the potential mechanisms of the improvement of survival by RAS inhibitors. MTT and scratch wound healing assays were conducted using pancreatic cancer cells treated with RAS inhibitors or paclitaxel as a single agent or in combination to investigate the influence of RAS inhibitors on pancreatic cancer cell proliferation and migration. Rat aortic ring assays were conducted to investigate the influence of RAS inhibitors on angiogenesis. Cell proliferation assays were conducted using human umbilical vein endothelial cells (HUVECs) treated with RAS inhibitors or angiotensin II as a single agent or in combination to investigate the influence of RAS inhibitors on angiotensin II-mediated cellular effects. Our results showed that a high concentration (100 μM) of RAS inhibitors suppressed the proliferation of pancreatic cancer cell lines (Panc-1, Bxpc-3, and CFPAC-1) when used alone or in combination with paclitaxel, but did not influence cell migration. RAS inhibitors significantly inhibited angiogenesis in rat thoracic aorta rings. In addition, a low concentration of angiotensin II promoted the growth of HUVEC cells, whereas the high concentration suppressed cell growth. ARBs inhibited the cellular effects caused by angiotensin II in HUVEC cells, whereas ACEIs did not inhibit such effects. In conclusion, RAS inhibitors may improve survival of cancer patients by inhibition of angiogenesis and influencing angiotensin II.

Keywords: RAS inhibitors, survival, mechanisms, angiogenesis, angiotensin II

Introduction

The renin angiotensin system (RAS) is mainly associated with the maintenance of blood pressure and electrolyte balance [1-4]. Angiotensin II type 1 receptor blockers (ARBs) and angiotensin I-converting enzyme inhibitors (ACEIs) are the two major classes of RAS inhibitors [5, 6], which are widely used for the treatment of hypertension, diabetic nephropathy, and congestive heart failure [7-9]. In recent years, there has been accumulating evidence showing that ARBs and ACEIs can not only treat cardiovascular diseases, but are also associated with disease progression and survival of patients with various types of cancer, including pancreatic cancer, renal cell carcinoma, gastric cancer, and hepatocellular carcinoma [10-13].

For example, Nakai*et al*. [10] investigated the association between ACEIs/ARBs and survival outcomes of advanced pancreatic cancer patients. Their results showed that the use of ACEIs/ARBs significantly improved both progression-free survival (PFS) and overall survival (OS). Subsequently, they updated their study and suggested that the use of RAS inhibitors might improve clinical outcomes of patients with advanced pancreatic cancer [14]. McKay *et al.* [15] found that use of RAS inhibitors significantly improved survival outcomes (PFS and OS) of renal cell carcinoma patients who received targeted therapies. Kim *et al.* [12] reported that ACEI/ARB in combination with platinum-based chemotherapy might improve survival of patients with advanced gastric cancer.

Table 1. The antiproliferative profile of ARBs/ACEIs and paclitaxel on various pancreatic cancer cell lines

Drugs	Cell lines	IC_{50}, mean ± SD
Candesartan	Panc-1	>100 ìM
	Bxpc-3	>100 ìM
	CFPAC-1	>100 ìM
Telmisartan	Panc-1	>100 ìM
	Bxpc-3	>100 ìM
	CFPAC-1	>100 ìM
Valsartan	Panc-1	87.25±11.28 ìM
	Bxpc-3	91.30±14.45 ìM
	CFPAC-1	>100 ìM
Benazepril	Panc-1	>100 ìM
	Bxpc-3	81.91±18.91 ìM
	CFPAC-1	>100 ìM
Captopril	Panc-1	>100 ìM
	Bxpc-3	64.93±21.65 ìM
	CFPAC-1	>100 ìM
Enalapril	Panc-1	>100 ìM
	Bxpc-3	>100 ìM
	CFPAC-1	>100 ìM
Moexipril	Panc-1	>100 ìM
	Bxpc-3	>100 ìM
	CFPAC-1	>100 ìM
Paclitaxe	Panc-1	29.41±4.21 nM
	Bxpc-3	6.47±2.91 nM
	CFPAC-1	127.78±24.03 nM

Several studies were performed to investigate the mechanisms of the anti-cancer effects exerted by RAS inhibitors [16-21]. For example, Kosugi *et al*. [19] indicated that candesartan enhanced cis-dichlorodiammine platinum-induced cytotoxicity in mice with bladder cancer. Alhusban *et al.* [20] found that clinically relevant doses of candesartan significantly inhibited the growth of prostate tumor xenografts in mice. However, the mechanisms are not completely understood. Thus, the objective of this study was to explore the potential mechanisms involved in the influence of RAS inhibitors on cancer patient survival. Because tumor cell proliferation, tumor cell migration, angiogenesis, and cytokine effects are essential for tumor progression and metastasis, we tested the following hypotheses. Whether RAS inhibitors directly influence cancer cell proliferation, whether RAS inhibitors directly influence cancer cell migration, whether RAS inhibitors influence angiogenesis, and whether RAS inhibitors influence the effect of some cytokines such as angiotensin II. In the present study, we investigated the anti-proliferation, anti-migration, anti-angiogenic effects and the interference of the role of cytokines by RAS inhibitors.

Materials and methods

Drugs and reagents

A panel of RAS inhibitors, including candesartan, telmisartan, valsartan, benazepril, captopril, enalapril, and moexipril, were dissolved in DMSO to prepare a 50 mM stock solution. Chemotherapeutic agent paclitaxel was purchased from the National Institutes for Food and Drug Control (Beijing, China) and dissolved in DMSO to prepare a 20 mM stock solution. Angiotensin II was purchased from Sigma-Aldrich Co. LLC and dissolved in water to prepare a 20 mM stock solution.

Cells and culture

Human pancreatic cancer cell lines Bxpc-3 and CFPAC-1 were purchased from the Chinese Academy of Sciences (Shanghai, China). Panc-1 cells were kindly provided by Professor Ke Yu (Fudan University, Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were kindly provided by Professor Weiyue Lu (Fudan University).

Panc-1 cells and HUVECs were maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich) and 1% (v/v) penicillin-streptomycin (10,000 U/mL penicillin and 10,000 μg/mL streptomycin; Gibco). Bxpc-3 cells were maintained in RPMI 1640 culture medium (Hyclone) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin. CFPAC-1 cells were cultured in Iscove's modified Dulbecco's medium (Hyclone) with 10% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin. All cells were cultured in an incubator (Thermo Scientific Forma) at 37°C in a humidified atmosphere with 5% CO_2. The cells were routinely checked for mycoplasma contamination.

MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to

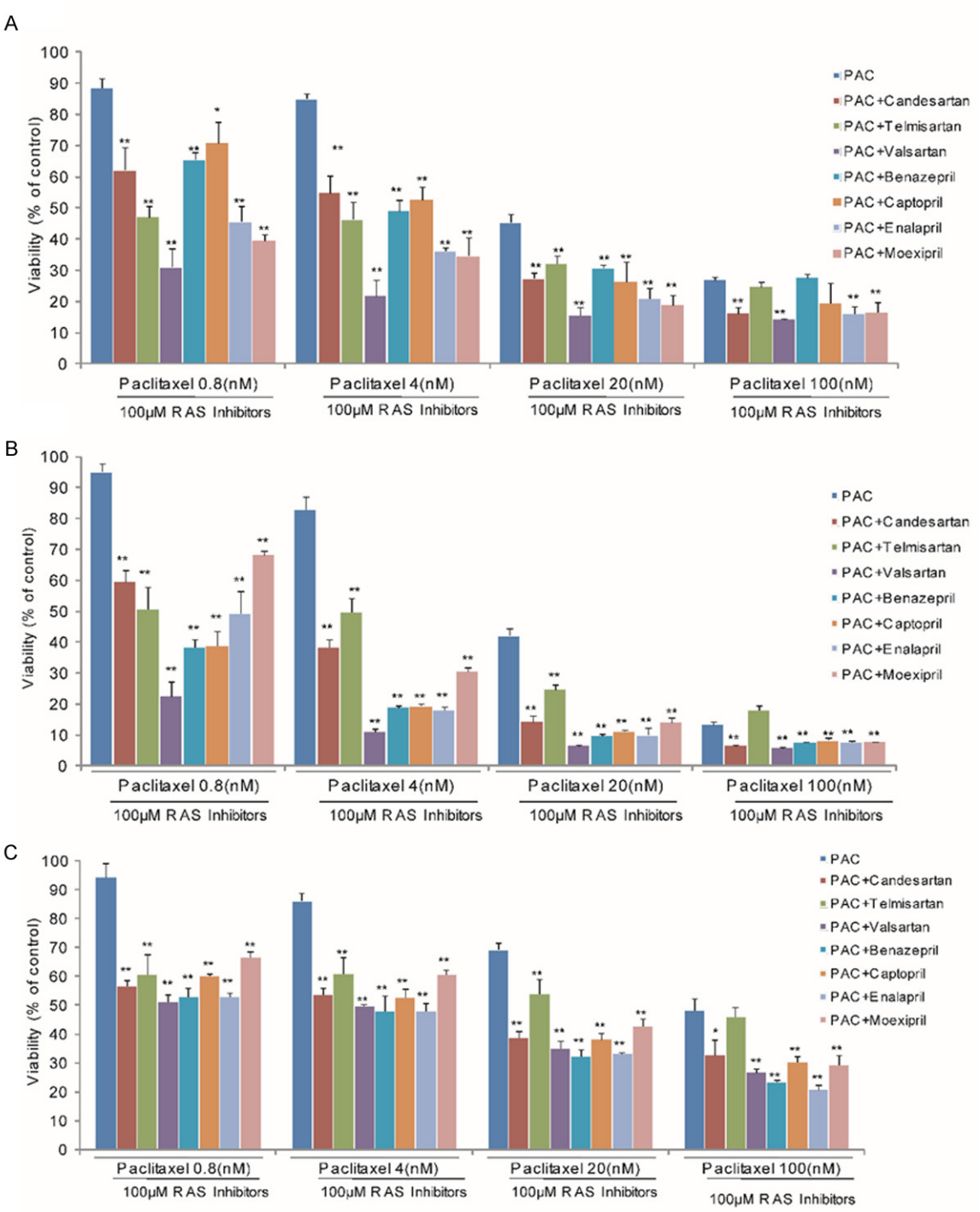

Figure 1. The influence of RAS inhibitors combined with paclitaxel on pancreatic cancer cell proliferation in three cell lines (A, Panc-1; B, Bxpc-3; C, CFPAC-1). The cells were incubated with different concentrations of RAS inhibitors (1, 10, 50, 100 μM) and paclitaxel (0.8, 4, 20, 100 nM) for 72 h. Cell viability was measured with an MTT assay. The data shown are the mean ± SD of triplicate experiments. *P<0.05, **P<0.01 compared with the same concentration of paclitaxel alone group.

evaluate cell viability [22]. Cells were cultured in 96-well plates at appropriate densities. The densities of Panc-1, Bxpc-3, and CFPAC-1 cells were 2.5×10^3, 3×10^3, and 1×10^4 cells per well, respectively. Then, the cells were treated with the vehicle (DMSO, 1‰) or various concentra-

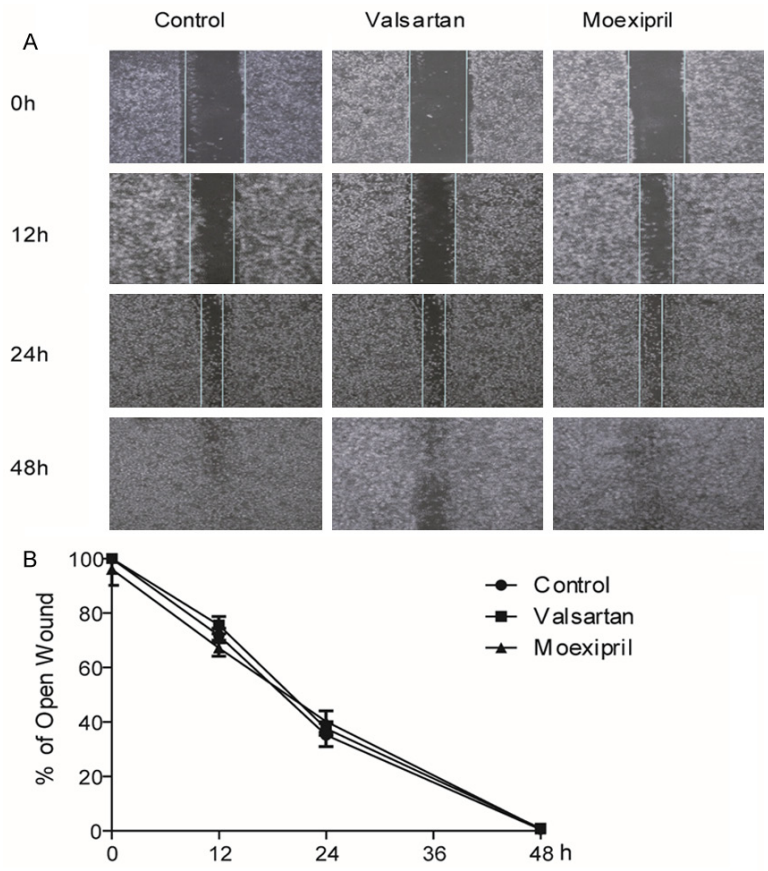

Figure 2. The influence of RAS inhibitors on pancreatic cancer cell migration. The microscopic images of scratch wound migration assay (A) and the ratio of the remaining width in Panc-1 cells (B) exposed to 1‰ DMSO (control group), 10 μM valsartan or 10 μM moexipril at 0, 12, 24 and 48 h. Each width in different time points was averaged (n=3, means ± SD). *P<0.05, **P<0.01 compared with control group.

tions of RAS inhibitors (0.1, 1, 10, and 100 μM) or paclitaxel (0.8, 4, 20, and 100 nM) overnight. After 72 h, MTT was added to each well, and the cells were cultured at 37°C for 4 h. The supernatant was removed and 150 μL DMSO was added per well to dissolve the formazan. Absorption values were measured at 570 nm by a microplate reader (Thermo Fisher Scientific).

Scratch wound healing assay

Cell migration was evaluated by a scratch wound healing assay [23]. Panc-1 cells were seeded in a 24-well plate. At confluency, a scratch wound was manually made in each well by a 200 μL pipette tip and photographed immediately (0h). Cells were treated with 0.1% DMSO (Control group) or different concentrations of RAS inhibitors (10 and 50 μM) and incubated at 37°C. Then, the scratch area was imaged after 12, 24, and 48 h. The distance between the two cell edges was measured by Image J software (National Institutes of Health, Bethesda, MD).

Rat aortic ring assay

A rat aortic ring assay was performed in accordance with the guidelines defined by Fudan University. The study was approved by the Animal Care and Ethics Committee of Fudan University.

The 48-well plates were prepared by adding 50 μL Matrigel (BectonDickinson, Bedford, MA) to each well and placing the plate on ice. Thoracic aortas were dissected from 48 Wistar rats (140-150 g), which were sterilized with 75% alcohol. After removing the fat layer and blood, the thoracic aortas were sectioned into 1 mm rings. The aortic rings were then seeded into the 48-well plates (one ring per well) and covered with another 50 μL Matrigel. Forty minutes later, all aortic rings were cultured in EBM-2 Basal Medium (Lonza) supplemented with 1% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin with or without RAS inhibitors at 37°C in a humidified environment for 1 week. The control groups received 0.1% DMSO alone. Images of microvessels were obtained on day 7 and analyzed by Image J software.

Angiotensin II-mediated cellular effects and the influence exerted by RAS inhibitors

MTT assays were used to evaluate the viability of HUVECs treated with 0.1% DMSO (Control group) or various concentrations of angiotensin II (0.01, 0.1, 1, 10, 100, 1000, and 10000 nM) and/or RAS inhibitors (0.1, 1, 10, and 50 μM) and incubated at 37°C. After 72 h, the measurements were performed.

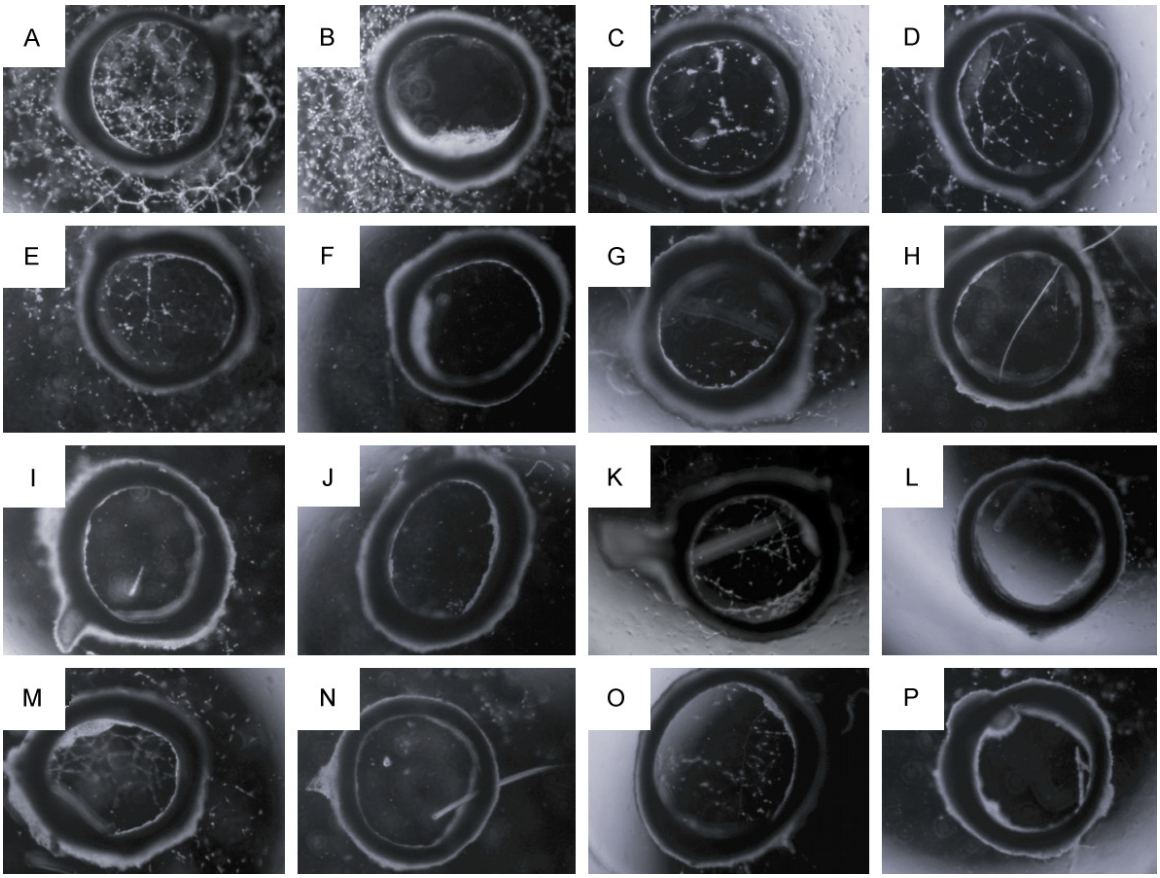

Figure 3. The influence of RAS inhibitors on angiogenesis in mice. Phase contrast microscopic images of rat aortic rings exposed to 1‰ DMSO (Control group), 10 μM and 50 μM RAS inhibitors (candesartan, telmisartan, valsartan, benazepril, captopril, enalapril, and moexipril) for 7 days. A and B: Control group; C and D: 10 μM and 50 μM candesartan; E and F: 10 μM and 50 μM telmisartan; G and H: 10 μM and 50 μM valsartan; I and J: 10 μM and 50 μM benazepril; K and L: 10 μM and 50 μM captopril; M and N: 10 μM and 50 μM enalapril; O and P: 10 μM and 50 μM moexipril.

Statistical analysis

All *in vitro* experiments were performed in triplicate and repeated three times. All values are presented as the mean ± standard deviation (SD). The two-tailed Student's t-test for comparison of two groups or analysis of variance for comparison of more than three groups were conducted using SPSS software (version 20.0; IBM SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $P<0.05$.

Results

Influence of RAS inhibitors on pancreatic cancer cell proliferation

The growth-inhibiting effect of RAS inhibitors and paclitaxel on various pancreatic cancer cell lines was investigated by MTT assays and the results are shown in **Table 1**. The results indicated that the inhibitory effects of RAS inhibitors on pancreatic cancer cell proliferation were weak. IC_{50} values of the majority of RAS inhibitors were greater than 100 μM. In addition, we performed a further experiment to investigate the growth-inhibiting effect of the combination of RAS inhibitors and paclitaxel and the results are shown in **Figure 1**. The results suggested that RAS inhibitors improved the anti-cancer effect of paclitaxel on pancreatic cancer cells.

Influence of RAS inhibitors on pancreatic cancer cell migration

The scratch wound healing assay was performed to investigate the effect of RAS inhibitors on the migration of pancreatic cancer cells. The effects of 10 μM valsartan and moexipril

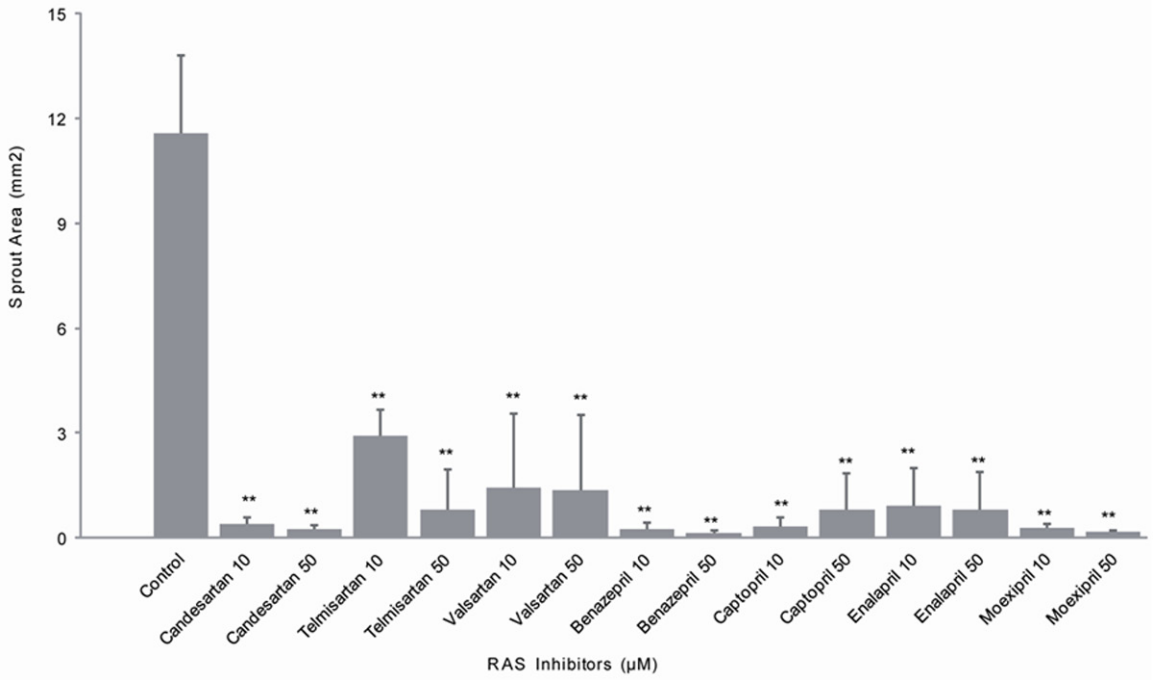

Figure 4. Sprout area (mm^2) of the rat aortic rings after exposed to 1‰ DMSO (Control group), 10 μM and 50 μM RAS inhibitors (candesartan, telmisartan, valsartan, benazepril, captopril, enalapril, and moexipril) for 7 days, as assessed by Image J software. The data were quantified from 3 aortic rings per group. Means ± SD. *P<0.05, **P<0.01 compared with control group.

on Panc-1 cell migration are shown in **Figure 2**. We found that 10 μM valsartan and moexipril did not affect Panc-1 cell migration compared with the control group. Furthermore, a similar effect was observed for 50 μM valsartan and moexipril (data not shown).

Influence of RAS inhibitors on angiogenesis

Rat aortic ring assays were performed to demonstrate the anti-angiogenic effect of RAS inhibitors. After 7 days of incubation, angiogenic processes were suppressed by RAS inhibitors (**Figure 3**). Compared with control groups, aortic rings exposed to RAS inhibitors showed a significantly reduced sprout area (**Figure 4**).

Influence of RAS inhibitors on angiotensin II-mediated cellular effects

To investigate the influence of RAS inhibitors on the effect of angiotensin II, we performed a further MTT assays. As shown in **Figure 5**, angiotensin II at low concentrations (0.01, 0.1, 1, and 10 nM) promoted the growth of HUVECs. Compared with each angiotensin II alone, ARBs (0.1, 1, 10, and 50 μM) inhibited the cellular effects caused by angiotensin II on HUVECs, whereas ACEIs did not inhibit such effects (data not shown).

Discussion

In recent years, RAS inhibitors have been shown to influence the progression and prognosis of patients with various types of cancer, such as pancreatic cancer, renal cell carcinoma, hepatocellular carcinoma, and localized upper tract urothelial carcinoma [11, 13-15, 24]. Although these studies appear to be controversial, our previous study conducted by meta-analysis [25] confirmed that RAS inhibitors improve the survival of cancer patients. Because the mechanism is unclear, in the present study, we hypothesized that RAS inhibitors may improve the survival of patients with some types of cancer by influencing cancer cell proliferation, migration, angiogenesis, and the effect of some cytokines such as angiotensin II. The results confirmed our hypotheses that RAS inhibitors influence the proliferation of three pancreatic cancer cell lines, sprouting of rat aortic rings, and angiotensin II-mediated cellular effects.

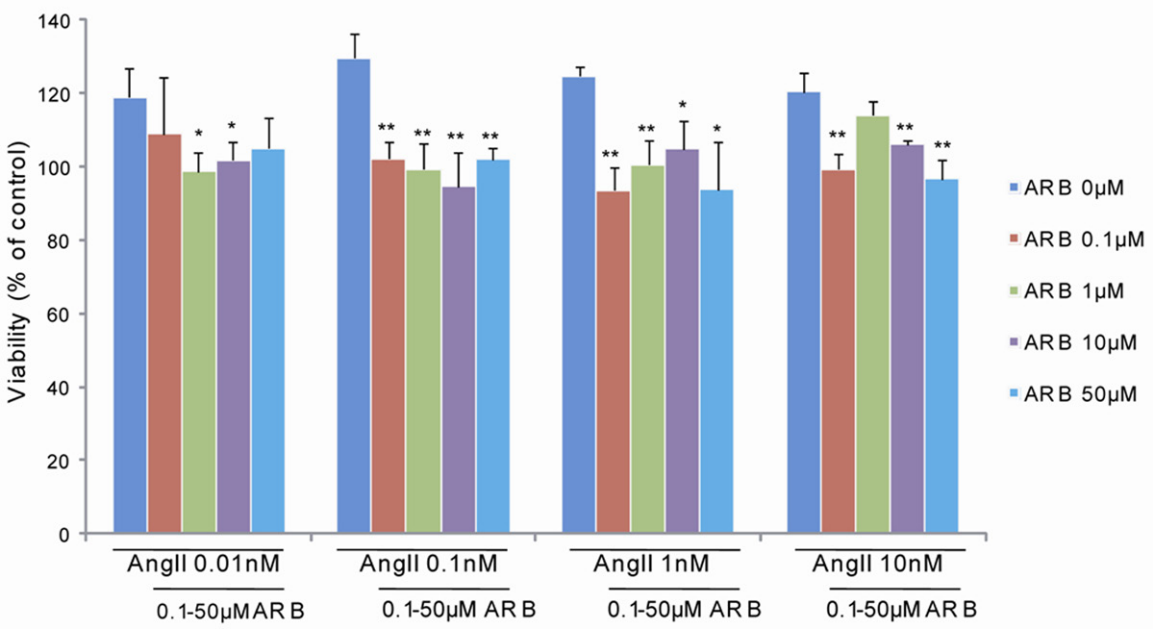

Figure 5. The influence of RAS inhibitors on angiotensin II-mediated cell effect. HUVEC cells were incubated with different concentrations of angiotensin II (0.01, 0.1, 1, 10) or/and RAS inhibitors (0.1, 1, 10, 50 μM) for 72 h. Cell viability was measured with an MTT assay. The data shown are the mean ± SD of triplicate experiments. *P<0.05, **P<0.01 compared with the same concentration of angiotensin II alone group.

Although our results indicated that RAS inhibitors suppressed the proliferation of three pancreatic cancer cells, the suppression was only significant when the concentration of RAS inhibitors was high. However, RAS inhibitors cannot reach such high concentrations in the human body via route doses [26-30]. Thus, we believe that the direct cell inhibitory effect of RAS inhibitors is weak.

Angiogenesis, the growth of new blood vessels, is thought to be one of the most crucial processes in the pathogenesis and metastasis of tumors, which involves a multi-step process mediated by many factors such as growth factors [31-33]. Since 1982, when Nicosia et al [34] first reported their findings that explants of the rat aorta generate vessels *ex vivo*, the aortic ring sprouting model has become one of the most widely used assays to investigate angiogenesis [35-40]. In the present study, we performed the rat aortic ring assay to demonstrate the anti-angiogenic effect of RAS inhibitors. Our results showed that RAS inhibitors significantly inhibited rat aortic ring sprouting, which may be a potential mechanism of the improvement of cancer patient survival by RAS inhibitors.

As a growth factor, angiotensin II promotes neovascularization of tumors, which is important for tumor growth [41]. Two different types of RAS inhibitors (ARBs and ACEIs) affect the functions of angiotensin II by different mechanisms. ARBs selectively block the action of angiotensin II type 1 receptors, whereas ACEIs reduce the production of angiotensin II to suppress the RAS [42]. In the present study, we showed that angiotensin II influenced the cell proliferation of HUVECs. Low concentrations of angiotensin II promoted the growth of HUVECs and high concentrations of angiotensin II suppressed the growth of HUVECs. However, the concentration of angiotensin II in the normal physiological state of humans is very low, and an elevated serum concentration of angiotensin II is no more than 1 nM even under pathological conditions [43]. Thus, it is important to consider promotion of HUVEC growth by angiotensin II. Our results showed that ARBs inhibited the cellular effects caused by angiotensin II in HUVECs, which may explain the anti-angiogenesis effect of ARBs. In terms of ACEIs, although no inhibition of the cellular effects caused by angiotensin II in HUVECs was found, we believe that ACEIs inhibit angiogenesis by reducing the production of angiotensin II *in vivo*.

In conclusion, our results suggest that RAS Inhibitors may improve cancer patient survival

by inhibiting angiogenesis and influencing the actions of angiotensin II. Considering that our study is *in vitro*, which may not reflect the *in vivo* situation, *in vivo* studies are needed to explore the potential mechanisms of the improvement of cancer patient survival by RAS inhibitors.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bin Wang, Department of Pharmacy, Jing'an District Center Hospital of Shanghai, Fudan University, 259 Xikang Road, Shanghai 200040, China. Tel: +8621 61578133; E-mail: wangbin@huashan.org.cn

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