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

Bei Zhao¹, Hong Sun², Xiao-Dan Zhang³, Guang-Hui Li³, Yong-Hong Zhao¹, Bin Wang¹,³

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

Received December 1, 2017; Accepted September 5, 2018; Epub October 15, 2018; Published October 30, 2018

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.
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% CO₂. 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...
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 $2.5 \times 10^5$, $3 \times 10^3$, and $1 \times 10^4$ cells per well, respectively. Then, the cells were treated with the vehicle (DMSO, 1%) or various concentra-
Anti-tumor mechanisms of renin angiotensin system 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.

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

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.
Anti-tumor mechanisms of renin angiotensin system inhibitors

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*<sub>50</sub> 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
Anti-tumor mechanisms of renin angiotensin system inhibitors

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).

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).

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.
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 neo-vascularization 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.

Acknowledgements

The study was supported by the Jing’an District Health and Family Planning Commission (Grant: 2017MS03). We wish to thank Mitchell Arico from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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

References


Anti-tumor mechanisms of renin angiotensin system inhibitors


