Telmisartan inhibits NSCLC A549 cell proliferation and migration by regulating the PI3K/AKT signaling pathway

SUOLIN ZHANG[1] and YAYAN WANG[2]

[1]Department of Chest Surgery, The Second Hospital of Shandong University, Jinan, Shandong 250000;
[2]Department of Respiratory Medicine, Affiliated Hospital of Yanbian University, Yanji, Jilin 133000, P.R. China

Abstract. Expression of angiotensin II (Ang II), a key biological peptide in the renin-angiotensin system, is closely associated with the occurrence and development of cancer. Ang II binds two receptor subtypes, the Ang II type 1 receptor (AT1R) and the AT2R, to mediate a series of biological effects. Telmisartan, a specific AT1R blocker, has been reported to have potential as an anticancer drug for treating renal cancer. In the present study, whether telmisartan had an effect on non-small cell lung cancer (NSCLC) cell proliferation and migration was investigated. The Cell Counting kit-8 assay revealed that telmisartan significantly inhibited the growth of the NSCLC A549 cell line in a time- and dose-dependent manner. In a transwell assay, telmisartan significantly inhibited cellular invasion and migration. Furthermore, it was determined that the expression of the anti-apoptotic protein B-cell lymphoma was decreased, and that of the pro-apoptotic proteins caspase-3 and Bcl-associated X increased in the A549 cells treated with telmisartan. Additionally, levels of phosphorylated RAC serine/threonine-protein kinase (p-AKT), p-mechanistic target of rapamycin, p70-S6 kinase and cyclin D1 was decreased in the telmisartan-treated group. Therefore, the current study reveals that telmisartan-induced NSCLC apoptosis may be regulated via the phosphoinositide 3-kinase/AKT signaling pathway, which indicates that it may be a potential novel drug for clinical NSCLC treatment.

Correspondence to: Dr Yayan Wang, Department of Respiratory Medicine, Affiliated Hospital of Yanbian University, 1327 Bureau Street, Changyi, Yanji, Jilin 133000, P.R. China
E-mail: 15526770312@163.com

Abbreviations: Ang II, angiotensin II; NSCLC, non-small cell lung cancer; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; ARBs, angiotensin II receptor blockers

Introduction

Lung cancer, the most common type of cancer, has the highest morbidity rate among all types of malignancies worldwide (1,2), accounting for 30% of all incidences of cancer-associated mortality (3). In clinical practice, the two major types of lung cancer are small cell lung cancer (SCLC) and non-SCLC (NSCLC) (4), of which NSCLC accounts for ~80% of all lung cancer cases (5). Current therapies for NSCLC include chemotherapy, radiotherapy, targeted therapy and surgery, which are used alone or in combination (6). Despite the use of these therapies, the overall 5-year survival rate of NSCLC remains low, at 15% (7). Owing to the development of drug resistance and the severe side effects experienced by patients, effective and safe novel agents are required for the treatment of NSCLC (1).

Angiotensin II (Ang II), a key biological peptide in the renin-angiotensin system, is a vasoconstrictor that controls cardiovascular function and renal homeostasis (8). Ang II receptor blockers (ARBs) are extensively used as anti-hypertensive drugs (9). Studies have reported that angiogenesis is essential for tumor progression and metastasis (10,11). Furthermore, studies have demonstrated that ARBs have the potential possibility to restrain the growth of several types of cancer cells (12,13). Ang II binds two receptor subtypes, the Ang II type 1 receptor (AT1R) and the Ang II type 2 receptor (AT2R), to mediate a series of biological effects (14). AT1R mediates the main functions of Ang II, including tumor growth and angiogenesis (15). Treatment with telmisartan, a specific AT1R blocker, leads to apoptosis and the inhibition of cell cycle progression in cancer cells (16,17). It has been reported that telmisartan has anti-proliferative activity in prostate and renal cell cancer (18,19); however, there are few relevant reports on the anti-proliferative activity of telmisartan in NSCLC cells (20).

In the present study, the Cell Counting Kit-8 (CCK-8), Transwell assay and western blot analysis was used to determine whether telmisartan treatment could inhibit the proliferation and invasion of A549 NSCLC cells, which may be regulated via phosphoinositide 3-kinase (PI3K)/RAC serine/threonine-protein kinase (AKT) inhibition-induced apoptosis.

Materials and methods

Cell lines and cell culture. The NSCLC A549 cell line was obtained from the American Type Culture Collection

(Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA). All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. When cells entered into the logarithmic growth phase, they were washed three times with PBS and digested with 0.25% trypsin (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China). When the cells detached, DMEM was re-added to terminate digestion and resuspended the cells into single cell suspension, then the cells (100 μl, 1x10^5) were seeded into a 6-well plate for subsequent experiments.

Cell Counting Kit-8 (CCK-8) proliferation assay. A 100 μl suspension of A549 cells was seeded into 96-well plate with the standard 1,000 cells/well. In this assay, 5 groups were used, which were control (DMEM only), vehicle [0.1% dimethyl sulfoxide (DMSO); Ameresco, Inc., Framingham, MA, USA)] and 0.2, 2 and 20 μM telmisartan (MedChem Express, Princeton, NJ, USA). Following this, cells were cultured in a CO_2 incubator and the cell viability was detected at 24, 48, 72 h. A total of 10 μl CCK-8 (Beijing Solarbio Science & Technology, Co., Ltd.) reagent was added into each well and the plates were incubated at 37°C for 1.5 h. The optical density value was measured at a wavelength of 450 nm using a microplate reader and a proliferation curve was plotted. The assay was performed in triplicate. In the following experiments, the negative control (NC) group was treated with 0.1% DMSO, and the treated group with 20 μM telmisartan.

Cell invasion and migration assessed via transwell assay. A total of 100 μl Matrigel (diluted 1:6 in serum-free DMEM; BD Biosciences, Franklin Lakes, NJ, USA), was melted overnight and then added to the upper chamber of a 24-well transwell plate (EMD Millipore, Billerica, MA, USA). Following shaking, the Matrigel was placed into 37°C CO_2 incubator cultivating for 4-6 h until the gel set, and then the culture medium was dried. The cells, which had been treated with 20 μM telmisartan for 24 h, were prepared using serum-free DMEM in a cell suspension. A total of 100 μl (1x10^5) cell suspension were added to the upper chamber of the transwell. Following this, 500 μl complete DMEM (containing 10% FBS) was mixed into the bottom chamber of the transwell. Cells were incubated for 24 h, then the transwell was taken out and the remaining cells in the upper chamber were removed with a cotton swab. After washing with PBS, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, and stained with 0.1% crystal violet for 20 min at room temperature. Following washing with PBS, five clear fields of vision were selected random using a light microscope (magnification, x200), and images were captured to observe the cell count.

The migration experiment procedure was similar to the invasion assay; however, the Matrigel was not applied and the 5,000 cells were seeded in the upper chambers.

Cell apoptosis assay. Apoptotic A549 cells were evaluated using an Annexin V-fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) Apoptosis Detection kit (4A Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. The NC group was treated with 0.1% DMSO. Following the treatment of A549 cells with 20 μM telmisartan and digested with 0.25% trypsin (without EDTA), collected in a centrifuge tube, centrifuged at 800 x g at 4°C for 5 min, resuspended in 4°C pre-cooled PBS and finally centrifuged once again at 800 x g at 4°C for 5 min. The supernatant was aspirated and the cells were resuspended in 1x Binding Buffer (Biomiga Inc., San Diego, CA, USA). Cell density was regulated to ~1x10^6/ml. A total of 100 μl cell suspension was added into a 5-ml flow tube and stained with 5 μl Annexin V-FITC (Apoptosis Detection kit) for 5 min at room temperature in the dark. Next, cells were stained with 10 μl propidium iodide (PI) and incubated at room temperature in the dark for 5 min, and 400 μl PBS was added to detect cells. The cells were then assessed using a flow cytometer and results were analyzed using FlowJo software (version 7.6.3; FlowJo, LLC, Ashland, OR, USA). The assay was performed in triplicate.

Western blot analysis. When the cell confluence was ~80%, the cells were treated with 20 μM telmisartan or 0.1% DMSO. After 24 h, the 6-well plate was placed on ice. Next, Radioimmunoprecipitation Assay lysis buffer (including protease inhibitor; Beijing ComWin Biotech Co., Ltd., Beijing, China) was added to extract protein. The protein concentration was measured using the BCA Protein Assay kit (Beijing ComWin Biotech Co., Ltd.). Next, the protein was heated at 95°C for 5 min. A total of ~20 μg protein per lane was separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked using 5% skimmed milk for 1 h at room temperature. Next, the membrane was incubated with the primary antibodies listed below at 4°C overnight. The membrane was then washed three times for 5 min with 0.1% Tween 20-TBS, then incubated with the appropriate secondary antibodies, listed below, for 1 h at room temperature. Following washing of the membrane (as aforementioned), bands were visualized using an enhanced chemiluminescence reagent (PerkinElmer, Inc., Waltham, MA, USA). Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to perform densitometry analysis.

The following antibodies were used. Primary antibodies: AKT (dilution, 1:1,000; cat. no. 4691; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-AKT (dilution, 1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), mechanistic target of rapamycin (mTOR) (dilution, 1:1,000; cat. no. 2983; Cell Signaling Technology, Inc.), p-mTOR (dilution, 1:1,000; cat. no. 5536; Cell Signaling Technology, Inc.), Bcl-2 (dilution, 1:1,000; cat. no. 12789-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), Bax (dilution 1:1,000; cat. no. 23931-1-AP; ProteinTech Group), caspase-3 (dilution, 1:1,000; cat. no. 25546-1-AP; ProteinTech Group), cyclin D1 (dilution, 1:1,000; cat. no. 60186-1-Ig; ProteinTech Group), p70 S6 kinase (p70S6K) (dilution, 1:1,000; cat. no. 14485-1-AP; ProteinTech Group), Tubulin (dilution, 1:5,000; cat. no. 10759-1-AP; ProteinTech Group); Secondary antibodies: horseradish peroxidase-labeled goat anti-rabbit (dilution 1:5,000; cat. no. SA00001-2; ProteinTech Group). The relative expression of each protein was normalized to tubulin levels in each sample.

Results

Telmisartan inhibits A549 cell proliferation. To evaluate the effect of telmisartan on NSCLC, the NSCLC A549 cell line was treated with 0.2, 2 and 20 μM telmisartan to detect cell proliferation rates. In this experiment, the cells were measured at 24, 48, 72 h. The results of the CCK-8 proliferation assay indicated that only the 20 μM telmisartan treatment group exhibited a significant reduction in the proliferation of A549 cells following 72 h (P<0.05; Fig. 1). These results demonstrated that telmisartan could effectively inhibit A549 proliferation in a dose- and time-dependent manner; therefore, 20 μM telmisartan was used in the following experiments.

Telmisartan inhibits A549 cell invasion and migration. A transwell assay was used to analyze the effects of telmisartan on the invasive and migratory ability of A549. After crystal violet staining the A549 cells were reduced via the telmisartan treatment in the invasion experiment. Additionally, the cells were also reduced in the migration experiment. The number of invaded cells in the telmisartan-expressing group was less than control group (8±2 vs. 46±4 cells invaded, respectively) and indicated a significant difference (P<0.05; Fig. 2A). The migratory capacity of cells was also inhibited in the telmisartan-expressing group compared with the control group (29±3 vs. 74±5 cells migrated, respectively), indicating the negative effect of telmisartan on the migration ability of A549 cells (P<0.05; Fig. 2B), compared with the control group. These results indicated that telmisartan could significantly inhibit the invasion and migration of A549.

Telmisartan promotes A549 cell apoptosis. An annexin V-FITC/PI double-staining assay was used to determine the effect of telmisartan on A549 cell apoptosis. Following the induction of serum-free apoptosis, the apoptosis rate in the telmisartan-treated group was significantly higher than that in the control group (21.85 vs. 5.81%, respectively; P<0.05). In addition, it was determined that telmisartan had a greater effect on early apoptosis (17.8%) compared with late apoptosis (4.05%) (Fig. 3A). Furthermore, western blot analysis was used to analyze the protein expression of apoptosis regulators, including the anti-apoptotic protein Bcl-2, and the pro-apoptotic proteins caspase-3 and Bax (Fig. 3B). The expression of the pro-apoptotic proteins caspase-3 and Bax increased in the telmisartan-treated group compared with the control group, whereas the expression of the anti-apoptotic protein Bcl-2 decreased (P<0.05; Fig. 3C-E). These results indicated that telmisartan could promote A549 cell apoptosis.

Telmisartan suppresses the PI3K pathway in A549 cells. In tumors the PI3K/AKT pathway is extremely important.
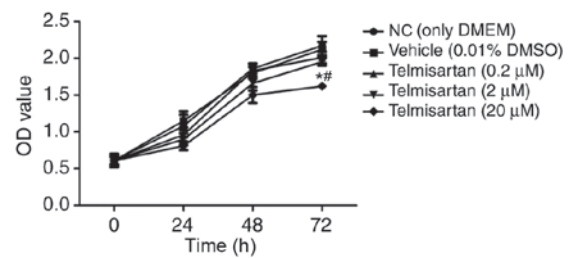

Figure 1. Telmisartan inhibited A549 cell proliferation using the Cell Counting Kit-8 assay. *P<0.05, compared with the vehicle group; #P<0.05, compared with NC group. OD, optical density; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; NC, negative control.
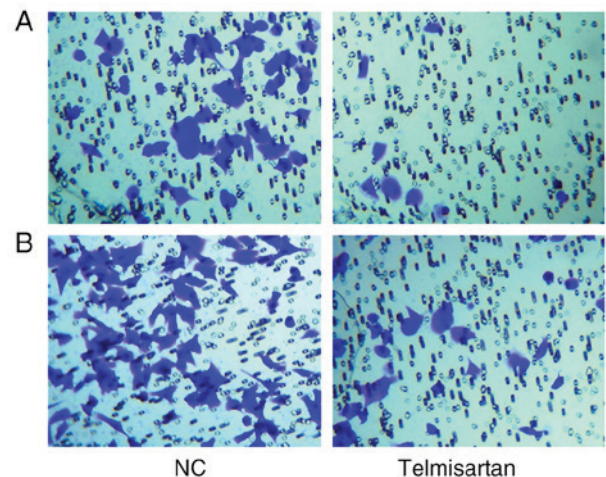

Figure 2. Telmisartan inhibits A549 cell invasion and migration. (A) Images are representative of crystal violet-stained A549 invaded cells. (B) Images representative of crystal violet-stained A549 migrated cells. Images were captured using an inverted microscope at a magnification of x100. NC, negative control.

Following telmisartan treatment, mTOR, p70S6K and cyclin D1 proteins were selected as indicators for the evaluation of the activity of the PI3K/AKT signaling pathway. The results of western blot analysis indicated that the phosphorylation level of mTOR decreased significantly in telmisartan-treated A549 cells (P<0.05). Similarly, the expression levels of p70S6K and cyclin D1 were decreased following telmisartan treatment (P<0.05; Fig. 4). These results indicated that telmisartan-induced inhibition of A549 cell growth might be regulated via the PI3/AKT pathway.

Discussion

In the present study, a preliminary investigation into the effects and potential underlying molecular mechanisms of telmisartan was conducted. Telmisartan inhibited the proliferation, invasion and migration of A549 cells. Furthermore, it was demonstrated that cell apoptosis was significantly promoted via telmisartan. These effects mediated by telmisartan on A549 cells may be associated with the inhibition of the PI3K/AKT signaling pathway.

Ang II, as a vasoconstrictor, is a multifunctional and bioactive octapeptide of the renin-angiotensin system, which controls cardiovascular function and kidney homeostasis (21). The prior
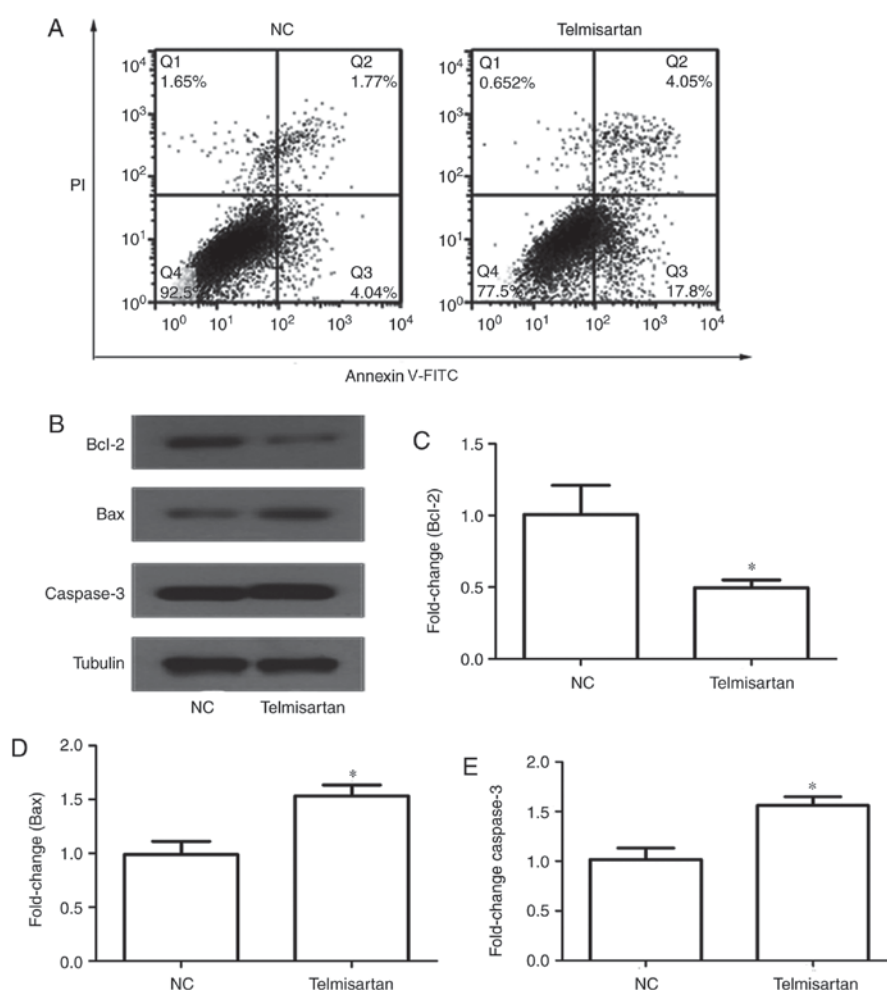
Figure 3. Telmisartan induces A549 cell apoptosis. (A) A549 cells were treated with 20 μM telmisartan for 24 h and labeled with Annexin V-FITC and PI, followed via flow cytometric analysis. (B) Western blot analysis of A549 cells treated with telmisartan for 24 h. The band intensities were quantified. The results were normalized to the GAPDH loading control. (C-E) Relative levels of the indicated apoptosis-associated proteins. *P<0.05, compared with NC group. NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate.
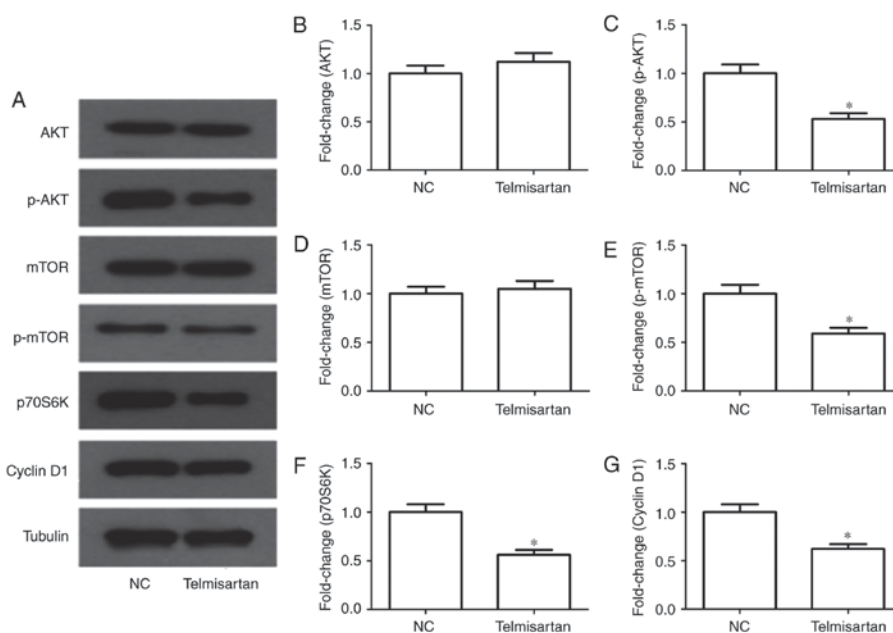

Figure 4. Effects of telmisartan on the PI3K signaling pathway in A549 cells. (A) Expression levels of AKT, p-AKT, mTOR, p-mTOR, p70S6K and cyclin D1 were measured in A549 cells treated with 20 μM telmisartan by western blot analysis. (B-G) The relative protein levels of AKT, p-AKT, mTOR, p-mTOR, p70S6K and cyclin D1 compared with the control group. *P<0.05, compared with NC group. PI3K, phosphoinositide 3-kinase; p-AKT, phosphorylated RAC serine/threonine-protein kinase; mTOR, mechanistic target of rapamycin; p70S6K, p70-S6 kinase; NC, negative control.

published data have demonstrated an association between Ang II and cancer development (22,23). Penafuerte *et al* (24) revealed that Ang II is a major upstream regulator of cancer cachexia. Recently, studies have reported that Ang II stimulates angiogenesis and tumor growth, particularly in breast and pancreatic cancer (25,26). The local production of Ang II in gastric cancer has been indicated to promote lymph node metastasis and cancer progression (27). By contrast, ARBs have been demonstrated to be effective in reducing tumor growth, angiogenesis and metastasis in mouse models *in vivo* (28,29). Ang II binds two receptor subtypes to mediate its biological effects, AT1R and AT2R (30). Studies have demonstrated that the main functions of Ang II in tumor growth are mediated by the AT1R (31). ARBs block the activation of AT1R, thus inhibiting the renin-angiotensin system (32).

In the present study, NSCLC A549 cells were treated with telmisartan. The results of the CCK-8 proliferation assay indicated that telmisartan could inhibit A549 proliferation in a dose- and time-dependent manner. In transwell assays, telmisartan significantly inhibited the invasion and migration of A549. Telmisartan is a specific AT1R blocker, which has the strongest binding affinity for AT1R in ARBs (16,33). It has previously been reported that Ang II can potentiate breast cancer cell migration (25). Godugu *et al* (7) revealed that telmisartan could enhance the anticancer effect on A549 cells; however, whether telmisartan can inhibit proliferation, promote migration, invasion and apoptosis in lung cancer cells has not, to the best of our knowledge, been studied. The results of the present study demonstrated that telmisartan could inhibit A549 cell proliferation and migration.

The results of the apoptosis assay indicated that telmisartan had a greater effect in early apoptosis, compared with late apoptosis, indicating that the Bcl-2 intrinsic mitochondrial apoptosis pathway was activated. The results of western blot analysis demonstrated that the expression of the pro-apoptotic proteins caspase-3 and Bax increased in the telmisartan-treated groups whereas that of the anti-apoptotic protein Bcl-2 decreased. Bcl-2 is a negative regulator of apoptosis, whereas Bax and caspase-3 are positive regulators of apoptosis (34). Apoptosis is an important antitumor target, and a number of antitumor drugs function by inducing apoptosis (35). The activation of apoptosis is regulated via multiple signaling pathways, of which PI3K/AKT is one of the more important (36). The results of western blot analysis indicated that levels of p-AKT, p-mTOR, p70S6k and cyclin D1 were significantly decreased in the telmisartan-treated group, which indicated that telmisartan may exert an antitumor effect through the regulation of apoptosis via the PI3K/AKT pathway. The PI3K/AKT pathway can mediate cell survival signals via the Bcl-2 family (37). AKT has direct effects on the effectors of the apoptotic pathway, including Bcl-2 (38). Bcl-2 activity is regulated via p-AKT in various cell types (39). AKT phosphorylates Bax, disrupting its interaction with Bcl-XL to protect cells from apoptosis (40). In addition, AKT can phosphorylate Bcl-2, making it resistant to protein degradation and ultimately protecting the cell from apoptosis (41). The effect of mTOR on Bcl-2 is similar to that of AKT (42). Additionally, p70S6K and cyclin D1 may also be involved in pathways that telmisartan effects in cancer cells. Cyclin D1 and p70S6K are located downstream of the PI3K/AKT pathway, which is closely

associated with cell apoptosis (43,44). Cyclin D1 is a member of the cyclin D family of proteins that serve a pivotal role in facilitating the cell cycle (45). The expression of cyclin D1 is increased in different types of tumor tissues (46). The present results were consistent with these studies (41-46), which indicated that telmisartan inhibited signaling via the PI3K/AKT pathway in A549 cells, and reduced the phosphorylation of mTOR, p70S6K and cyclin D1. In summary, the downregulation of the PI3K/AKT pathway may have contributed to the inhibitory effect of telmisartan on A549 cells.

In conclusion, the present study demonstrated that telmisartan could inhibit the proliferation, invasion and migration of lung cancer cells and promote their apoptosis via the PI3K/AKT signaling pathway. The present study provides a prospect for further clinical research of telmisartan in lung cancer treatment.

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