Introducción

El osteosarcoma (OS) es un tipo de cáncer óseo primario común en humanos con alta incidencia y mortalidad en ambos niños y jóvenes adultos, normalmente presentado como osteogénesis por células malignas y caracterizado por alta agresividad local y metástasis óseas y orgánicas distantes [1, 2]. A pesar de los muchos avances en terapias, incluyendo métodos quirúrgicos y quimioterapia neoadyuvante, la cirugía que conlleva la amputación del miembro y la quimioterapia como método terapéutico común también traen múltiples efectos secundarios. Además, una notable proporción de recurrencia o metástasis sigue siendo altamente probable [3-5]. Por lo tanto, son urgentes las terapias efectivas sin efectos secundarios en el OS.

Telmisartán es uno de los bloqueadores del receptor de angiotensina II tipo 1 (AT1R) (ARB). Se conoce que AT1 y AT1R tienen actividades estímuladoras de tumores que involucran la promoción de la proliferación celular, migración, neoangiogénesis y antianiquilamiento durante el desarrollo del cáncer [6-9]. Los ARBs inhiben estas acciones al antagonizar el AT1R en diferentes tumores [10-12]. El telmisartán es un ARB que se ha reportado como un agente antitumor activo, incluyendo la inducción de apoptosis en células de cáncer de próstata humana [13], el control de la ginebra del cáncer gástrico [14], la mejora del aclaramiento autómático de riñones poliquísticos hereditarios [15], la inhibición del crecimiento del cáncer de esófago [16], la prevención de la proliferación y la promoción de la apoptosis del cáncer ovárico humano [17] y así sucesivamente. No obstante, no se han realizado estudios con telmisartán en OS. Lo más importante, las últimas publicaciones exponen que los riesgos de cánceres, incluyendo los cuatro cánceres más comunes de pulmón, mama, próstata y cáncer colorectal, no aumentan con el uso del telmisartán en comparación con otros ARBs con base en el estudio de población [18]. Estos datos proporcionan apoyo sólido a la teoría de que el telmisartán tiene un efecto protector en el tratamiento del cáncer sin efectos adversos. Sin embargo, los mecanismos de acción antitumor del telmisartán en OS son aún inciertos, en particular en OS.
Thus, anticarcinogenic activity of telmisartan on OS and involved mechanisms are urgent to be explored.

In the present study, we demonstrate that telmisartan suppressed growth, invasiveness and migration and promoted apoptosis of OS cells. Furthermore, telmisartan treatment inhibited mTOR activation.

2 Materials and methods

2.1 Chemicals and antibodies

Telmisartan was purchased from MedChemExpress Biotechnology (Shanghai, China). Primary antibodies against the serine/threonine protein kinase B (AKT), phospho-(p-) AKT, mTOR, p-mTOR, B-cell lymphoma 2 (Bcl-2), Bcl-associated X (Bax), Cyclin D1, Cleaved Caspase-3, phospho-p70 ribosomal S6 kinase (p-P70S6K), Tubulin and anti-rabbit/mouse peroxidase conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The enhanced chemiluminescence (ECL) detection system was from Proteintech Group, Inc (Wuhan, China).

2.2 Cell culture

Human OS cell lines (Shanghai Institute of Cell Biology, China) were grown in RPMI-1640 (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), and penicillin-streptomycin sulfate (100 mg/ml; Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO₂. Cells in logarithmic growth phase (approximately 1 × 10⁶ cells/ml) were used in the following experiment.

2.3 Assessment of Cell Proliferation

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8) (Beijing solarbio science & technology co. Ltd., Beijing, China) according to the manufacturer’s instructions. Briefly, 5 × 10⁴ cells per well were seeded into 96-well plate and cultured in 100 μL of RPMI-1640 medium supplemented with 10% FBS for 24 h. Telmisartan (0, 1, 10, 20, 30, 40, 50 μM) was added to each well and cells were cultured for an additional 72 h, CCK-8 reagent (10 μL) was then added to each well, and the plates were incubated at 37°C for 1.5 h. The Optical density (OD) was measured at a wave-length of 450 nm using a microplate reader. Judging by results with concentration gradient, the action concentration of telmisartan treated in following experiment was identified, telmisartan at this concentration or vehicle (DMSO, 1‰ in culture media) was added to each well for 24 h, 48 h, 72 h. CCK-8 detection steps were the same as above. The following experimental group was treated with telmisartan at this concentration, and negative control group (NC) was cultured with 1‰ DMSO in culture media.

2.4 Cell migration and invasion assay

24-well transwell chamber with membrane pore size of 8.0 μm (Coring Costar, Cambridge, MA, USA) with or without Matrigel matrix (BD Sciences, San Jose, CA, USA) were used to assess cell invasive and migratory abilities, according to the protocol provided by the manufacture. In brief, Approximately 1 × 10⁵ cells were suspended in 100 μl serum-free medium and plated in the upper chamber, while the lower chamber was filled with 500 μl of complete medium (medium containing 10% FBS). After incubation for 24 h at 37°C, 5% CO₂, non-invading cells on the top chamber were scraped off with cotton-tipped swabs, invaded cells were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet for 20 min. Subsequently, the number of invaded cells was counted under a microscope in five random fields for each well.

For cell migration detection, the procedures resembled detection of cells invasion, but Matrigel was not applied.

2.5 Detection of apoptosis

A apoptotic U2OS cells were evaluated by an Annexin V-fluorescein isothiocyanate ( Annexin V-FITC)/propidium iodide (PI) Apoptosis Detection kit (Beijing 4A Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instruction. The U2OS cells were treated with telmisartan and harvested by trypsinization without EDTA, washed twice with cold PBS, centrifuged at 3000 r/min for 5 min and the supernatant was discarded, the pellet was resuspended in 500 μl 1 × binding buffer. Then, 5 μl FITC-conjugated Annexin V and 10 μl PI were added. After incubation for 5 min at room temperature in the dark, the cells were analyzed with a FACScan instrument (FACS Calibur, BD Biosciences, CA).
2.6 Western blot analysis

Total proteins were isolated from cells with RIPA lysis buffer (cwbio, Beijing, China). After lysis for 30 min on ice, the lysates were centrifuged at 12,000 rpm at 4°C for 15 min. Total protein concentration was determined by BCA assay (cwbio, Beijing, China). Equal amounts of total protein was loaded and separated on 8%-10% Tris-glycine gradient gels via dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to poly vinylidene difluoride membranes for 2 h and blocked with 5% non-fat milk in TBST buffer (pH7.4 tris-buffered saline buffer containing 0.1% Tween-20) for 1.5 h. Then, the membranes were incubated with primary antibodies (1 : 1000 dilution) against AKT, p-AKT, mTOR, p-mTOR, Cyclin D1, p-P70S6K, Bcl-2, Bax, Cleaved Caspase-3, and against Tubulin (1 : 10000 dilution) at 4°C overnight, and were detected using anti-rabbit/mouse horseradish peroxidase -conjugated secondary antibodies (1 : 5000 dilution). Western blot bands were detected by ECL kit and were measured with Image-Pro Plus6.0 software.

2.7 Statistical analysis

The Student’s t-test was utilized to determine significant differences. SPSS (International Business Machines Corporation, New York, USA) 22.0 software and GraphPad Prism 5 (San Diego, CA, USA) were used to perform statistical analysis. Images were quantified by Image-Pro Plus6.0 software (Media Cybernetics, Maryland, USA). All values were expressed as the mean ± standard deviation (SD) and evaluated by an analysis of Student’s t-test. The differences between two groups were considered to be significant at a **p < 0.01. All assays were conducted in triplicate.

3 Results

3.1 Effect of telmisartan on the proliferation of OS cell lines in vitro

To investigate the influence of telmisartan on OS cell proliferation, CCK8 was used to analyze cell viability in vitro. Firstly, we detected cell viability treated U2OS cells with telmisartan at 0, 1, 10, 20, 30, 40, 50 µM and observed that the cell viability was dose-dependent with telmisartan treatment, but 30 µM and above concentration, the cell viability was less than 50% when compared with 0 µM (Fig. 1A), which was not conducive to the following experiment. Therefore, we selected 20 µM as the candidate concentration. Next, we found that antiproliferative abilities in U2OS cells induced by telmisartan presented time-dependent, and especially at 72 h timepoint, this proliferation-inhibitory effect was more evident than other timepoint (Fig. 1B, **p < 0.01).

Figure 1. The effect of telmisartan on the proliferation of OS cell lines in vitro by CCK8 assay. A. Dose-dependent effect of telmisartan on the viability of OS cells. **p < 0.01 versus 0 µM groups. B. Time-dependent effect of telmisartan on the viability of OS cells. Values are presented as mean ± SD. **p < 0.01 versus NC groups.

3.2 Effect of telmisartan on migration and invasion of OS cells

Following, transwell invasion and migration assay were performed to determine cell invasive and migratory capabilities. As shown in Fig. 2A, after treatment with telmisartan, the numbers of invaded cells were markedly decreased to $25 \pm 2$ compared with the control ($39 \pm 4$) (**p < 0.01). Likewise, result of transwell migration assay showed that telmisartan treatment dramatically reduced migrated cells numbers ($63 \pm 5$), comparable to negative control (NC) groups ($82 \pm 8$) (Fig. 2B, **p < 0.01).
Effect of telmisartan on OS cells

3.3 Apoptotic changes in the OS cells treated with telmisartan

To ascertain whether OS cell death induced by telmisartan was presented through apoptosis, cells were treated with telmisartan and then stained with Annexin-V-FITC/PI. We found that telmisartan administration of U2OS cells results in a markedly increase in apoptotic rate of treated groups (24.37% ± 0.92%) comparable to NC groups (6.13% ± 0.43%) (Fig. 3A). To study the mechanism mediating telmisartan-induced apoptosis, Bax, Bcl-2, Cleaved Caspase-3 activities were examined in telmisartan-administrated cells using western blotting and results discovered suggest that the expression of the anti-apoptotic protein, Bcl-2, was reduced while the pro-apoptotic protein Bax was increased significantly (Fig. 3B and C, **p < 0.01). Meanwhile, the expression level of Cleaved Caspase-3 was also upregulated in telmisartan-treated groups (**p < 0.01).

Figure 2. The change of invasive and migratory capabilities in OS cell lines after telmisartan treatment were assessed by Transwell membrane migration and invasion assays. A. Telmisartan obviously reduced the number of cells invaded to the chamber. B. Telmisartan also markedly reduced the number of cells migrated to the chamber. **p < 0.01 versus NC groups.
3.4 The effects of treatment with telmisartan on mTOR signaling activity

It has been demonstrated that mTOR signaling plays a crucial cancer-inhibitory role in a variety of tumors. Thus, to determine the role of telmisartan in the activation of mTOR signaling, we detected series of correlative molecules with this vital pathway. As shown in Fig. 4A and B, telmisartan treatment leads to significant reduction in the phosphorylation levels of AKT and mTOR, while the total AKT, and mTOR protein level were observed without substantial change. Besides, telmisartan administration attenuated activity of mTOR downstream molecules such as p-P70S6K and Cyclin D1 related to cell growth. Together, these results showed that telmisartan inhibited the activation of mTOR signaling pathway.

![Figure 3](image-url)

**Figure 3.** Inductive impact of apoptosis by telmisartan in OS cells. A. The percentage of apoptotic cells were observed after stained with Annexin-FITC/PI and detection of flow cytometry to be significantly increase by administration of telmisartan. B. Expression of the apoptosis-related proteins including Bax, Bcl-2 and Cleaved Caspase-3 treated with telmisartan in OS cells were determined by western blot analysis. C. Quantified results that analyzed by Image-Pro Plus6.0 software were presented with histogram types. All values are expressed as mean ± SD. **p < 0.01 versus NC groups.
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4 Discussion

In this study, our present finding indicated that the effect of telmisartan on the growth of OS cell lines and relevant mechanism of action. Above all, we found telmisartan markedly inhibited OS cells proliferation, migration and invasion. Further, we discovered that telmisartan induced apoptosis of OS cells. Moreover, we revealed that the above antitumorigenic potent effects of telmisartan might be regulated by inhibition of the mTOR pathway.

Accumulating evidence shows that telmisartan takes on a critical anticarcinogenic action in various types of cancers via in vitro and in vivo experiments. For example, a current study showed that telmisartan effectively suppressed abdominal aortic aneurysms pathogenesis [19]. Another research reported that telmisartan has antiproliferative and apoptotic effects on human colon cancer in vitro [20]. In concert with these findings, in present study, we found that telmisartan is able to down-regulate growth, invasion and migration in OS cell lines obviously. Additionally, administration of OS with telmisartan could induce apoptosis occurrence. Previous studies have shown that some of the cancerous cell proliferation inhibitory action was contributed by apoptosis [21, 22]. As is known that Bcl-2, Bax and Cleaved Caspase-3 play crucial role in apoptosis process [23]. Thus, in our study, OS cells apoptotic ratio was also incremented after telmisartan treatment, further, this induction was accompanied by a strong decrement in anti-apoptotic protein Bcl-2 expression level, while a high increase in pro-apoptosis proteins Bax and Cleaved Caspase-3. Above of these data indicate that telmisartan has a tumor suppressor potential in OS. In this case, series of anti-tumor potencies with telmisartan treatment in OS were presented, which prompt us to explore its underlying mechanism.

To investigate relevant molecular mechanism, we assess activity of mTOR signaling pathway. It has been well-established that mTOR pathway is involved in cell survival, growth and metastasis, and is one of the major signaling
mTOR pathway tightly correlated with cancer progression. mTOR pathway is reported to be activated in osteosarcoma cell lines [24, 25]. Once PI3K activated, its catalytic subunit activates AKT, successively mTOR complex 1 (mTORC1) is activated, and subsequently activation of mTORC1 upregulates P70S6K phosphorylation and then control protein synthesis like Cyclin D1, consequently modulating cellular growth and apoptosis process [26, 27]. Increasing reports demonstrated that mTOR pathway mediated tumorigenicity in OS [28-30]. What is more, there is some reports mentioned that telmisartan participated in regulation of mTOR pathway [16, 31]. In the current study, we evaluated mTOR signaling coregulating proteins and found that telmisartan led to reduction of p-mTOR, p-AKT expression levels, and also discovered that expression of Cyclin D1 and p-P70S6K were suppressed by telmisartan treated. Collectively, these results indicated that mTOR signaling pathway was mediated human OS cell potent anti-proliferative, anti-metastatic, and pro-apoptotic effects. However, the limitation of our study were that we merely apply single OS cell line U2OS, and confined to study in cellular level. Thereby, further studies with different cell lines or in vivo and more elaborate molecular mechanism related pathway or others might be needed to investigate.

In conclusion, the present results manifest that telmisartan has anticancer function in human OS cells through inhibiting the potency of cell proliferation, invasion, migration and enhancing apoptosis of human OS cells. In addition, telmisartan develops its potential action in resisting OS cells probably through mTOR inactivation. Based on our findings, telmisartan could be considered as a promising novel therapeutic candidate for OS.

Ethical approval: The conducted research is not related to either human or animals use.

Conflict of interest: Authors state no conflict of interest.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| OS           | osteosarcoma |
| mTOR         | mammalian target of rapamycin |
| ATIR         | angiotensin II type 1 receptor |
| ARB          | angiotensin II type 1 receptor blockers |
| AKT          | serine/threonine protein kinase B |
| Bcl-2        | B-cell lymphoma 2 |
| Bax          | Bcl-associated X |
| p-P70S6K     | phospho-p70 ribosomal S6 kinase |
| ECL          | enhanced chemiluminescence |

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