

## ORIGINAL ARTICLE

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# Rapamycin inhibits tumor growth of human osteosarcomas

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## Summary

**Purpose:** Treatment options for osteosarcoma are limited due to its resistance to chemotherapy and radiotherapy. Signaling through the mammalian target of rapamycin (mTOR) pathway contributes to cell proliferation and chemoresistance of many cancers. Rapamycin, as an inhibitor of mTOR, has been developed as potentially valuable therapeutic agent. In this report, we evaluated the effects of rapamycin on human osteosarcoma cells' growth in vitro and in vivo.

**Methods:** Proliferation of osteosarcoma cells treated with rapamycin at different time periods was detected and changes in the cell cycle were measured by MTS and flow cytometry, respectively. Autophagy induced by rapamycin in osteosarcoma cells and the expression of cell cycle regulating proteins were detected by Western blotting. The effect of rapamycin on tumor growth in vivo was detected using

mice xenograph models.

**Results:** The proliferation of osteosarcoma cells was significantly inhibited by rapamycin treatment in a concentration-dependent manner and the cell cycle progression was impaired with G1 arrest. Rapamycin induced autophagy, increased the expression of p27 and decreased the expression of Cyclin D1. In addition, rapamycin suppressed the tumor growth in mice xenograph models.

**Conclusions:** The potent antiproliferative activities of mTOR inhibitor rapamycin has been proven. These results strongly indicate that rapamycin may be a promising agent against osteosarcomas.

**Key words:** autophagy, cell cycle, mTOR, osteosarcoma, proliferation, rapamycin

## Introduction

Osteosarcoma is the most commonly diagnosed malignant primary bone tumor affecting children and adolescents, usually occurring in the long bones [1-3]. It is a highly aggressive tumor and the most common metastatic site is the lung [1,4]. Due to its poor response to currently used chemotherapy and the high recurrence rate, current therapeutic and diagnostic strategies show poor prognosis [5,6]. Meanwhile, little is known about the signaling pathways that drive the progression of human osteosarcoma [7]. Therefore, investigation of novel therapeutic approaches that target specific cell survival pathways is urgently needed.

As an evolutionarily conserved serine/threonine kinase, mTOR plays a critical role in the regulation of protein translation, proliferation, differentiation and metabolic processes [1,2,8-10]. Perturbations in the mTOR signaling transduction pathway are common and likely to be involved in various cancers including osteosarcoma [1]. As a specific inhibitor of mTOR, rapamycin inhibits the growth of a number of cancers, such as small cell lung cancer, renal cancer, breast and pancreatic cancer [8,11,12]. Previous researches have shown that osteoblastic differentiation is enhanced by rapamycin in rat osteoblast-like osteosarcoma cells and rapamycin efficiently blocks the prolif-

eration of Ewing's sarcoma cells [13,14]. Recent studies demonstrated that rapamycin suppresses the growth of osteosarcoma cell lines HOS and KHOS/NP with cell cycle arrest in the G1 phase [15], but so far very few researches have been reported concerning the effects and the underlying associated mechanisms of rapamycin on human osteosarcoma cells.

The purpose of the present study was to investigate the effects of the mTOR inhibitor, rapamycin, on the activation of autophagy and the antiproliferative activities on osteo-sarcoma cells *in vitro* and *in vivo*.

## Methods

### Cell lines and culture

Human osteosarcoma cell lines SaOS2 and U2OS cells were purchased from Chinese Academy of Science (Shanghai, China) and maintained in DMEM (Gibco, USA) containing FBS (10%), penicillin (100 U/ml), streptomycin (10 U/ml) and glutamine (2 mmol/L) in 5% CO<sub>2</sub> saturated humidity, at 37°C.

### Reagents and antibodies

Rapamycin (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA), and the final concentration of DMSO was 0.1%. The anti-LC3 antibody (#3868), anti-p62 antibody (#8025), anti-p27 antibody (#3688) and anti-cyclin D1 antibody (#2978) were all purchased from Cell Signaling (MA, USA) and anti-β-actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology (TX, USA).

### Cell proliferation assay

Cell proliferation assay was performed by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS). 2×10<sup>5</sup> SaOS2 or 4×10<sup>5</sup> U2OS cells were seeded in triplicate in 96-well plates with growth medium and allowed for attachment for 8 hrs. Various concentrations of rapamycin and vehicle (0.1% DMSO) were added to each well. After 48 and 72 hrs, 20 μl MTS (Sigma, USA) were added for an additional 3 hrs at 37°C. The absorbance of the solution was measured at 490 nm using a microplate reader (BioRad, USA). Each experiment was performed in triplicate.

### Cell cycle analysis

Human osteosarcoma cells were grown to 65% confluency and then treated with rapamycin (1 μMol) for 72 hrs. Cells were then suspended in ice-cold phosphate-buffered saline (PBS) and fixed in 70% cold eth-

anol at 4°C overnight. Then, cells were stained with propidium iodide and RNase for 30 min and were analyzed using flow cytometry (BD Biosciences, USA). The number of nuclei in each phase of the cell cycle was determined using CellQuest™ software (BD Biosciences, USA) and the number of gated cells in G1, G2/M or S phase was presented as %.

### Western blotting

Equal amounts of proteins (50 μg) were resolved on 10% or 15% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membrane (Millipore, USA). The membranes were blocked with 5% nonfat milk at room temperature and then incubated with various specific antibodies recognizing the target protein at 4°C overnight. Subsequently, the membranes were incubated with appropriate horseradish peroxidase-labelled secondary antibodies for 1 h at room temperature and then visualized by an enhanced chemiluminescence detection system. The bands of proteins were analyzed by using the Image-Pro Plus 5.0 software.

### Animal studies

Eight-week-old nude mice were used to establish *in vivo* mice xenograph model. All animal operations were approved by the Animal Ethics Committee of Shanghai Jiaotong University School of Medicine. U2OS cells (2×10<sup>6</sup>) and SaOS2 cells (5×10<sup>6</sup>) were injected subcutaneously into the flanks of the animals. Once the tumor volume reached 50 mm<sup>3</sup>, mice were randomized into different groups and treated intraperitoneally with vehicle (0.1% DMSO) or rapamycin (5 mg/kg or 50 mg/kg) once daily for 3 weeks. Body weight and tumor volume were examined every 3 days and tumor volume was determined using the formula: (length\*width<sup>2</sup>)/2.

### Statistics

Statistical analyses were performed using Student's t-test. Differences with p values < 0.05 were considered statistically significant.

## Results

Concentration-dependent effects of rapamycin on the proliferation of human osteosarcoma cells

To assess the activity of rapamycin on osteosarcoma cell proliferation, SaOS2 and U2OS cells were exposed to rapamycin various concentrations (0, 0.04, 0.02, 0.1, 0.5, 2.5, 12.5, 62.5 μMol) for 48 and 72 hrs and analyzed by the MTS assay.

As shown in Figure 1a, treatment with rapamycin both for 48 and 72 hrs resulted in a concentration-dependent antiproliferative activity in SaOS2 cells compared with vehicle-treated cells. Meanwhile, U2OS cells treated with rapamycin both for 48 and 72 hrs also showed concentration-dependent inhibition of cell growth (Figure 1b). Our results indicated that rapamycin elicited a concentration-dependent decrease in cell viability.

#### Effects of rapamycin on cell cycle of human osteosarcoma cells

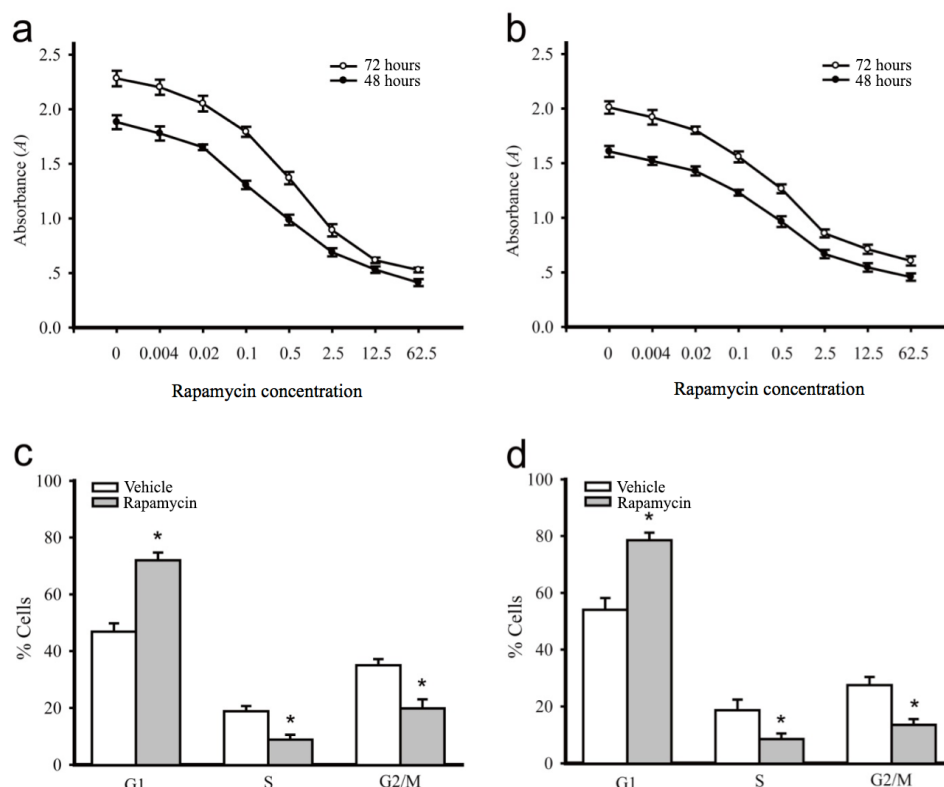
To investigate whether the antiproliferative effects of rapamycin on osteosarcoma cells might be due to the induction of cell cycle arrest, cells treated with rapamycin were analyzed by flow cytometry. Consistent with the MTS assay, our results showed that rapamycin induced a G1 phase cell cycle arrest in SaOS2 and the percentage of cells in the G2/M and S phase significantly decreased (Figure 1c). In accordance with the observation of the cell cycle arrest effect of rapamycin on SaOS2 cells, we found that the G1 phase of U2OS cells treated with rapamycin were also significantly increased with a decrease in the percentage of G2/M and S phase in cells (Figure 1d).

These results indicated that the growth inhibitory effect of rapamycin was via cell cycle arrest.

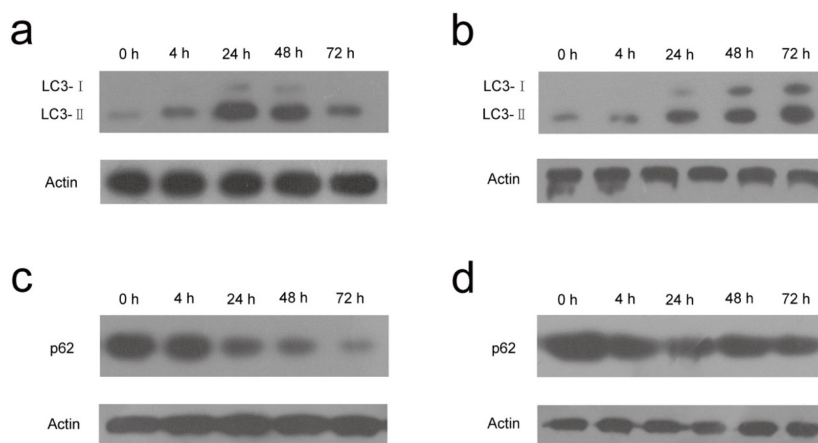
#### Rapamycin induces autophagy in human osteosarcoma cells

As has been previously shown, mTOR is a kinase whose activity suppresses autophagy in cells from yeast to humans [16]. We subsequently investigated whether rapamycin, the specific inhibitor of mTOR, might induce autophagy in osteosarcoma cells. The conversion of microtubule-associated protein 1 light chain 3 (LC3) is a specific marker to monitor autophagy [17]. During autophagy activation, LC3-I is processed into LC3-II. LC3 proteins were detected by Western blot analysis of osteosarcoma cells treated with rapamycin for 0, 4, 24, 48 and 72 hrs. The LC3-II fragment appeared as early as 4 hrs in SaOS2 cells after rapamycin treatment and persisted after 48 hrs (Figure 2a), while the LC3-II fragment emerged 24 hrs after rapamycin treatment in U2OS cells (Figure 2b).

p62, a molecule accompanying the induction of autophagy was also investigated. As shown in Figure 2c and 2d, in accordance with the autophagy induction, rapamycin reduced the expres-



**Figure 1.** Inhibitory effects of rapamycin on human osteosarcoma cells. Rapamycin significantly suppressed the growth of SaOS2 (a) and U2OS (b) osteosarcoma cells in a concentration-dependent manner ( $p < 0.01$ ). Rapamycin treatment led to a significant increase in the proportion of cells in the G1 phase and decrease in the S and G2/M phase in SaOS2 (c) and U2OS cells (d) compared with that of the vehicle-treated cells. Data are presented as mean  $\pm$  standard error of the mean. \*  $p < 0.05$ , compared with vehicle-treated osteosarcoma cells.



**Figure 2.** Rapamycin induced autophagy in human osteosarcoma cells. Representative Western blotting shows the time course changes of the protein levels of LC3-II and p62 in SaOS2 (**a,c**) cells and U2OS cells (**b,d**) treated with rapamycin.

sion of p62 in SaOS2 and U2OS cells.

#### *Effects of rapamycin on the expression of cell cycle regulating proteins*

To further elucidate the molecular basis underlying the antiproliferative and cell cycle arrest activity of rapamycin, the expression of cell cycle regulating proteins was examined. Following exposure to rapamycin for 72 hrs, the cyclin D1 was found to be significantly downregulated in SaOS2 cells (Figure 3a), while the levels of p27 significantly increased compared to that with vehicle-treated cells (Figure 3b). Similar results were obtained in U2OS cells treated with rapamycin. Our results revealed that rapamycin regulated the expression of cell cycle regulating proteins.

#### *Antitumor activity of rapamycin in human osteosarcoma xenografts*

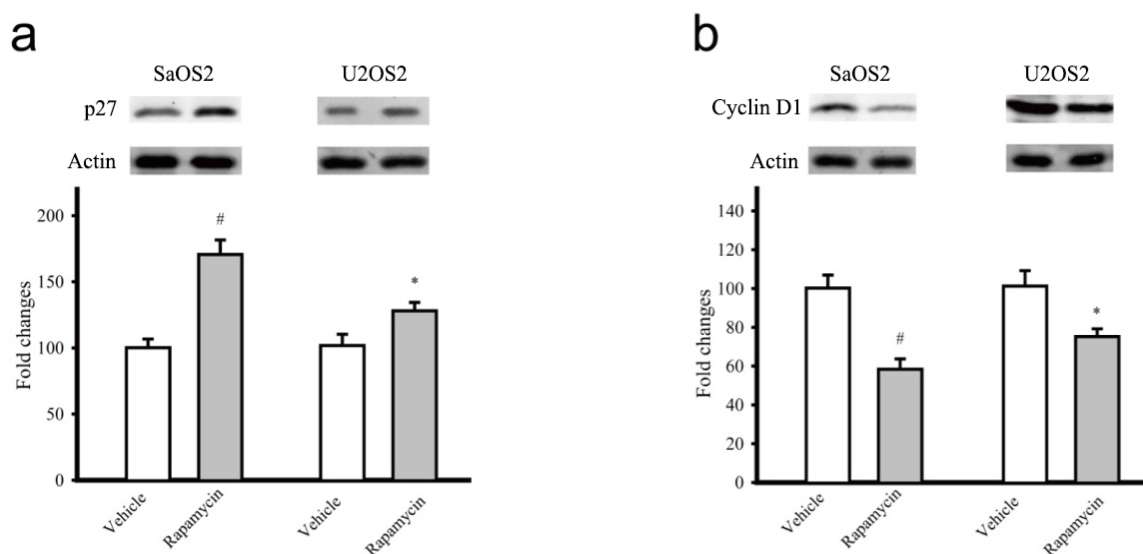
We next evaluated the *in vivo* efficacy of rapamycin on the growth of U2OS and SaOS2 xenografts. As described above, nude mice bearing U2OS and SaOS2 tumor xenografts were treated daily with vehicle or different concentrations of rapamycin for 21 days. No significant difference was found in the body weight of mice in different groups after 21 days of treatment (data not shown). As shown in Figure 4a, we found that the tumor volume of rapamycin (50 mg/kg)-treated group was significantly reduced compared with the vehicle-treated group, however rapamycin (5 mg/kg) did not significantly reduce the tumor

volume of SaOS2 tumor xenografts. Meanwhile, rapamycin (50 mg/kg) significantly suppressed the tumor growth of U2OS tumor xenografts (Figure 4b). Taken together, these results suggest that rapamycin inhibits the tumor growth of human osteosarcomas *in vivo*.

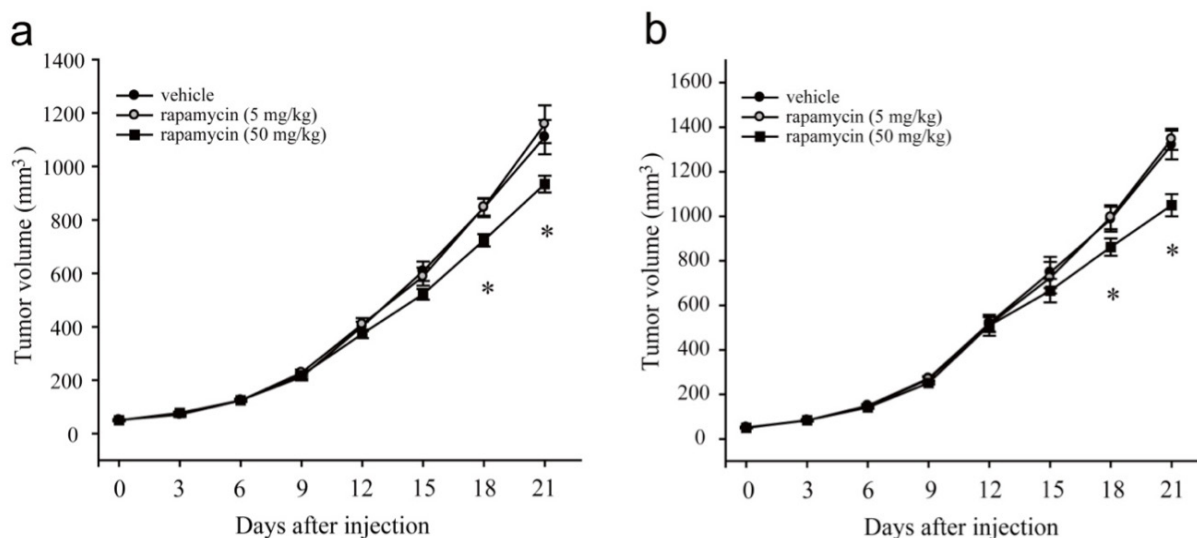
## Discussion

Many reports have indicated that signaling through the mTOR pathway contributes to protein synthesis, cell survival, growth, progression and chemoresistance of numerous cancers [18-20]. Therefore, mTOR inhibitors have been exploited as potentially useful agents in cancer therapeutics [11,21-23]. Rapamycin, an antimicrobial agent isolated from *Streptomyces hygroscopicus*, can inactivate mTOR signaling pathway and has been shown to inhibit the growth of a variety of tumor cell lines [11,24-27]. However, the effects of rapamycin on human osteosarcoma cell lines SaOS2 and U2OS cells have not been investigated. The purpose of this study was to assess the role of rapamycin as a possible therapeutic factor in human osteosarcomas.

The data presented herein demonstrated the ability of rapamycin to inhibit growth, induce cell cycle arrest and autophagy and enhance the expression of cell cycle regulating proteins in human osteosarcomas. We firstly found that rapamycin treatment led to a significant growth inhibition and a concentration-dependent decrease in the proliferation of SaOS2 and U2OS cells. According-



**Figure 3.** Rapamycin regulated the expression of cell cycle regulating proteins in osteosarcoma cells. Rapamycin increased the expression of p27 (**a**) and decreased the expression of Cyclin D1 (**b**) in SaOS2 and U2OS cells. Data are presented as mean  $\pm$  standard error of the mean. \*  $p < 0.05$ , #  $p < 0.01$ , compared with vehicle-treated osteosarcoma cells.



**Figure 4.** Intraperitoneal injection of rapamycin resulted in significant inhibition of tumor growth *in vivo*. A significant reduction of tumor growth in SaOS2 (**a**) and U2OS (**b**) was found in rapamycin-treated nude mice group compared with vehicle-treated group. \*  $p < 0.05$ , compared with vehicle-treated group.

ly, cell cycle analysis displayed cell cycle arrest in G1 phase. Thus, these results demonstrated that rapamycin showed potent growth-inhibitory effects on cell proliferation of SaOS2 and U2OS osteosarcoma cells. Finally, we found that the tumor growth of human osteosarcoma was significantly

suppressed by rapamycin in xenografts models.

Autophagy is an evolutionarily conserved process induced by inhibition of mTOR that involves bulk degradation of cytoplasmic organelles and proteins and LC3-II is a suitable marker for this process [28,29]. mTOR is commonly recognized



as the master autophagy regulator [30]. However, autophagy functions as a survival mechanism or contributor of apoptosis is still a matter of debate [30,31]. In our study, we found that inhibition of mTOR signaling pathway by rapamycin in osteosarcoma cells induced cell autophagy. Since several studies found that the deregulated mTOR signaling pathway induces not only autophagy but also protein translation and cell growth [32,33], in addition to investigating the role of rapamycin in cell growth and cell cycle progression of SaOS2 and U2OS osteosarcoma cells, we examined its effect on the expression of cell cycle regulating proteins cyclin D1 and p27. In addition to cell growth, cyclin D1 and p27 have critical regulatory roles in cell cycle control, apoptosis and metabolism [34-39]. Our results showed that rapamycin up-regulated p27 expression and downregulated D1 expression. The regulatory effects of rapamycin on the expression of cyclin D1 and p27 might con-

tribute to its antiproliferative and cell cycle arrest activities on osteosarcoma cells and the results suggest that the inhibitory ability of rapamycin on osteosarcoma cells could be attributed to the modulation of cell cycle regulating proteins expression.

Our research demonstrates that rapamycin effectively inhibited the proliferation of human osteosarcoma *in vitro* and *in vivo*. The antiproliferative efficacy of rapamycin in osteosarcoma cells is, at least in part, attained by targeted reduction of tumor promoting cytokines cyclin D1 and p27. Therapeutically targeting mTOR might yield significant benefits in patients with osteosarcoma.

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