HDAC6 Inhibitor WT161 Performs Antitumor Effect on Ostersarcoma and Synergistically Interacts with 5-FU

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Abstract

Background: WT161 as a new selective HDAC6 inhibitor has been shown to play anti-tumor effects on multiple myeloma and breast cancer. However, the role of WT161 in osteosarcoma remains unclear. The aim of this study is to explore the role of WT161 in osteosarcoma and its underlying mechanisms.

Methods: The anti-proliferative effect of WT161 on osteosarcoma cells was examined using MTT assay and colony formation assay. Cell apoptosis was analyzed using flow cytometer. The synergistic effect was evaluated by isobologram analysis using CompuSyn software. The osteosarcoma xenograft models were esatablished to evaluate the anti-proliferative effect of WT161 in vivo.

Results: WT161 suppressed the cell growth and induced apoptosis of osteosarcoma cells in a dose- and time-dependent manner. Mechanistically, we found that WT161 treatment obviously increased the protein expression level of PTEN and decreased the phosphorylation level of AKT. Notably, WT161 shows synergistically inhibitory effects on osteosarcoma cell combined with 5-FU. Animal experiment results show WT161 inhibits the growth of osteosarcoma tumor and further illustrates that WT161 and 5-FU have a synergistic efficiency in osteosarcoma.

Conclusions: These results indicate that WT161 inhibiting the growth of osteosarcoma through PTEN and has a synergistic efficiency with 5-FU.

Background

Osteosarcoma is the most common primary solid malignant tumor in bones, which is defined as the presence of malignant mesenchymal cells that produce bone-like and/or immature bones[1].The incidence of osteosarcoma in the general population is 2–3 million per year. However the incidence of puberty is higher, with the highest incidence between 15 and 19 years old, reaching 8–11 million per year. In addition, osteosarcoma is usually accompanied by early metastasis, and the vast majority are lung metastases. 90% of osteosarcoma patients die of lung metastasis before using multiple chemotherapy[2, 3]. Therefore, it is particularly important to develop new strategies for treating osteosarcoma.

In recent years, HDAC inhibitors have become a new strategy for the treatment of tumors[4]. More and more HDAC inhibitors are being developed for the treatment of tumors. With regard to osteosarcoma, many studies have demonstrated that HDAC inhibitors can inhibit osteosarcoma cell growth, metastasis, and induce osteosarcoma cell apoptosis[5–7]. In addition, many studies have found that HDAC inhibitors and some commonly used clinical chemotherapeutic drugs have a synergistic effect on inhibiting osteosarcoma cells, thereby making osteosarcoma cells more sensitive to clinical chemotherapeutic drugs and improve drug resistance[8–10]. These studies have proved that epigenetic processes play an important role in the occurrence and development of osteosarcoma and HDAC inhibitors are a potential drug for the treatment of osteosarcoma. In addition to histone deacetylase activity on histones, HDAC6 also has deacetylase activity on non-histone proteins such as β-tubulin, HSP90, cortactin and

peroxiredoxin[11]. These unique functions of HDAC6 have aroused great interest in the medicinal chemistry community. Therefore, a lot of efforts have been made to develop selective HDAC6 inhibitors for treatment, hoping to minimize the side effects caused by pan-inhibition[12]. Recently, WT161 as a selective HDAC6 inhibitor has been shown to play anti-tumor effects on multiple myeloma, retinoblastoma and breast cancer[12, 13]. However, the role of selective HDAC6 inhibitor in osteosarcoma is rarely reported.

.In this study, we found that WT161 suppressed the proliferation of this study is to explore the role of WT161 in osteosarcoma and its potential mechanisms.

Methods

Cell culture and chemicals

The human osteosarcoma cancer cell lines U20S and MG63 were kindly provided by professor Kang[14]. The U20S and MG63 cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium supplied with 10% fetal bovine serum (FBS) (Gibco-Life Technologies) at 37℃ in 5% CO2 atmosphere. WT161 was purchased from MedChemExpress (NJ 08852, USA) and prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA).

MTT assay

Using complete medium to configure osteosarcoma cells into a single cell suspension, and inoculating cell suspension into 96-well plates at a density of 5000 cells/well. After the drug administration, the 96-well plate was placed in a cell incubator to continue culturing for 24h, 48h, and 96h. The MTT solution were added to each well. After 4 hours, the formation of purple crystals was observed. Then adding DMSO solution to fully dissolve the purple crystals and place them on the microplate reader for detection. The detection wavelength was 490nm. The relative OD value = OD value of experimental well/OD value of control well.

Colony formation

Using complete medium to configure osteosarcoma cells into a single cell suspension, and inoculating cell suspension into 6-well plates at a density of 500 cells/well. After the drug administration, the 6-well plate was placed in a cell incubator to continue culturing for 10 days. After 10 days, the cells were carefully rinsed twice with PBS and immobilized with methanol for 30 minutes. After immobilized, adding 2ml 0.1% crystal violet to each well and stain overnight. Photograph and count the colonies containing > 50 cells

Apoptosis assay

Using complete medium to configure osteosarcoma cells into a single cell suspension, and inoculating cell suspension into 6-well plates at a density of 2×10^5 cells/well. After the drug administration, the 6-well plate was placed in a cell incubator to continue culturing for 48h. After 48 hours, following the annexin V-FITC/PI apoptosis detection kit (BD Biosciences, USA) to prepare samples for testing. Flow cytometer was used to detect the apoptosis rate of the cells in each sample.

Western blot analysis

The cells were washed 1–2 times using PBS and then added radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors incubating on ice for 30 min. The lysates were collected and centrifuged at 1,000 rpm for 20 min at 4 ℃. We used the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA) to measure protein concentration. The proteins of each group were fractionated by SDS-PAGE, and then transferred to PVDF membrane. After that, the membranes were soaked in PBST with 5% nonfat dry milk for 1.5 hours at room temperature and then probed with primary antibodies overnight at 4 ℃. After wash extensively, the membranes were probed with second antibody and then wash with PBST for 3 times. Then the proteins were visualized by chemiluminescence (Beyotime Biotechnology, Shanghai, China).

xenograft murine model

24 female BALB/c mice aged 4-6 weeks were prepared for tumor implantation.. Every nude mice were inoculated with 3×10^6 osteosarcoma cells and to ensure that the number of inoculated cells per nude mouse was consistent. After subcutaneous injection of cells, the xenograft tumor formation were monitored every day. When the tumor size reached about 2cm, nude mice were randomly divided into 4 groups, 2 cages in each group, and 3 nude mice in each cage. The mice were injected intraperitoneally with PBS, WT161 (80 mg/kg), 5-FU (5 mg/kg) or WT161&5-FU combination, respectively once a day. The tumors volumes were measured using a vernier caliper, and the formula for calculating the tumor volume = 0.5×tumor length×(tumor width)^2. After about two weeks, the nude mice were euthanized. The tumors were removed, weighed, and the tumors volume were calculated.

Evaluation of drug interaction

Evaluation of drug interaction was conducted as described previously[15]. Briefly, using drug concentrations based on the half maximal inhibitory concentration(IC50) value of each drug as a single drug to produce growth inhibition of about 10% to 90%. When the drugs are used in combination, the drug concentration of the two drugs should be kept at a certain ratio. The combination index(CI) was calculated by the CompuSyn software(Biosoft).

Statistical analysis

We used statistical software SPSS 16 to analyze the experimental data. Results are shown as the mean ± standard deviation(SD). LSD test was used to detect the statistical difference between experimental groups. P values less than 0.05 were considered statistically significant.

Results

WT161 suppresses the cell growth of osteosarcoma cells

To investigate whether WT161 exerts an anti-tumor effect on osteosarcoma, U2OS and MG63 cells were treated with a series concentration of WT161. The enhancement of acetyl-α-tubulin level by WT161 treatment in U2OS cells indicated the efficiency of WT161 in osteosarcoma cells (Fig. 1A). We then examined the anti-proliferative effect of WT161 using MTT assay. As shown in Fig. 1B and 1C, WT161 suppressed the cell growth of U2OS and MG63 cells in a dose- and time-dependent manner. To further investigate the effect of WT161 on osteosarcoma cell, we performed the colony forming assay. As shown in Fig. 1D, the results revealed that WT161 inhibits the colony formation of MG63 cells in a dose-dependent manner.

WT161 treatment induces the apoptosis of osteosarcoma cells

In order to determine whether WT161 affect the apoptosis of osteosarcoma cells, we tested apoptotic cell number of osteosarcoma cell through flow cytometry. The osteosarcoma cell line was cultured with a series concentration of WT161 for 48 hours. As shown in Fig. 2A and 2B,The results indicate that WT161 increases the apoptosis of U2OS and MG63 cells osteosarcoma cell in a dose-dependent manner.

WT161 increases the apoptosis of osteosarcoma cells mainly through regulating PTEN/AKT signaling pathway

Previous studies had demonstrated that PTEN/AKT signaling pathway was usually overactivated in osteosarcoma and promotes tumorigenesis and development[16, 17]. In addition, HDAC6 expression level was related with PTEN translocation and expression. Therefore, we sought to examine whether WT161 induces osteosarcoma cell apoptosis by regulating the PTEN/AKT signaling pathway. As expected, WT161 treatment obviously increased the protein expression level of PTEN and decreased the phosphorylation level of AKT(Fig. 3A and 3B). These results indicate that WT161 increases the apoptosis of osteosarcoma cells mainly through the regulation of PTEN/AKT signaling pathway.

WT161 shows synergistic effects on osteosarcoma cell combined with 5-FU

Several studies have found that PTEN/PI3K/AKT signaling pathway was related to the sensitivity of cancer cells to chemotherapy especially 5-FU[18–22]. Thus, we explored whether WT161 could show synergistic inhibitory effects on osteosarcoma cells combined with 5-FU. Indeed, the results showed the CI values are lower than 0.7, indicating that WT161 enhanced the inhibitory effect of 5-FU on osteosarcoma cells (Fig. 4A and 4B).

WT161 shows synergistic effects on osteosarcoma tumor growth combined with 5-FU in a mouse xenograft model

To further confirm the effect of WT161 on osteosarcoma, we evaluated the anti-proliferative effect and synergistic effects combing with 5-FU in xenograft murine model. As shown in Fig. 5A and 5B, WT161 and 5-FU significantly inhibit the growth and weight of osteosarcoma tumor. In addition, compared with the WT161 administration group and the 5-FU administration group, the WT161&5-FU combination administration group inhibited tumor growth more significantly (Fig. 5C). This result further illustrates that the histone deacetylase inhibitor WT161 and the clinical chemotherapeutic drug 5-FU have a synergistic efficiency in osteosarcoma.

Discussion

Osteosarcoma is the most common primary malignant bone tumor. And it is usually accompanied by early metastasis, most of which are lung metastases. 90% of osteosarcoma patients died of lung metastasis before using a variety of chemotherapy[23]. At present, chemotherapy is still the most important method for the treatment of osteosarcoma, but as the application of chemotherapy is more and more widely used, the problem of drug resistance is becoming more and more serious. Therefore, it is very important to develop a method with good curative effect and little side effect[2, 3].

PTEN is an effective tumor suppressor. Its loss of function is often observed in hereditary and sporadic tumors, and it is also one of the most common mutated tumor suppressor genes in human cancers. PTEN has phosphatase activity and non-enzymatic functions(scaffolding) in cells. It relies on these functions to control various biological processes, including genome stability, cell survival, migration, proliferation, and metabolism[24–26]. Even a small decrease in the expression level of PTEN or enzyme activity can lead to cancer susceptibility and contribute to tumor development. In osteosarcoma, some studies have found that the absence of PTEN can enhance the growth of osteosarcoma and lung metastasis[27]. In addition, it has been found that many drugs and genes regulate osteosarcoma proliferation, apoptosis, migration, invasion and increase the sensitivity of osteosarcoma cells to chemotherapy by affecting the expression level or activity of PTEN[28–32]. Therefore, PTEN plays a vital

role in osteosarcoma. In our study, it was also demonstrated that WT161 affects the proliferation and apoptosis of osteosarcoma cells mainly by increasing the protein expression level of PTEN.

PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase-B) signaling pathway is one of the most important intracellular pathways, which regulates cell survival, growth, differentiation, metabolism and cytoskeletal reorganization. The main proteins involved in this signaling pathway are PI3K and Akt[33–35]. When most oncoproteins and tumor suppressors are involved in cell metabolism and signal regulation, these regulation converge in the PI3K signal transduction pathway, forming a balance[34, 36]. However, in some tumors, through activation or inactivation mechanisms, this balance is broken to target interrelated proteins. At present, more and more studies have shown that PI3K/AKT signaling pathway is often over-activated in osteosarcoma and promotes the occurrence and development of tumors, including cell proliferation, invasion, cell cycle progression, apoptosis inhibition, angiogenesis, metastasis and chemical resistance. Inhibiting this pathway through small molecule compounds has also become an attractive potential treatment for osteosarcoma[17]. PTEN acts as a major negative regulator of the PI3K/Akt signaling pathway, and loss of PTEN activity is often found in osteosarcoma[16]. Therefore, PTEN activators may be an alternative method of inhibiting this pathway in osteosarcoma, and some studies have found that it is indeed feasible. Some researchers have found that treatment of osteosarcoma with 5-azacytidine can significantly increase the expression of PTEN, and the downstream AKT signal is also inactivated, causing apoptosis of osteosarcoma cells[37]. In our study, it is also found that WT161 can also increase PTEN expression and inhibit the downstream PI3K/Akt signal pathway, thus inducing apoptosis of osteosarcoma cells.

At present, although the adjuvant chemotherapy for osteosarcoma has made progress and the survival rate has been significantly improved, the overall prognosis is still poor[17]. Therefore, the treatment of osteosarcoma requires novel treatment methods. Recently, combined chemotherapy has received more and more attention. This method can increase the anti-tumor effect of clinical chemotherapeutics and reduce the dosage of clinical chemotherapeutics, thereby reducing toxic and side effects[38]. In recent years, some researchers have found that the use of combination chemotherapy in osteosarcoma can increase the cytotoxicity of clinical chemotherapy drugs and is a potentially valuable method for the treatment of osteosarcoma[39]. More importantly, people have found that by regulating PTEN and downstream PI3K/AKT pathway, it can increase the efficacy of 5-FU chemotherapy and improve drug resistance[18–22]. And, we found that WT161 inducing apoptosis of osteosarcoma cells by regulating PTEN/AKT pathway in our study. Therefore, we suspect that WT161 and 5-FU may have a synergistic effect. As we expected, WT161 can enhance the cytotoxicity of the clinical chemotherapy drug 5-FU, showed significant synergy when used in combination with 5-FU. In addition, in the osteosarcoma xenograft model, the tumor size and weight of the WT161&5-FU combination administration group are smaller than either of the single administration groups. These results indicate that the combined use of WT161 and 5-FU may be an effective strategy for the treatment of osteosarcoma in the future.

Conclusions

Overall, we demonstrated that WT161 inhibits the growth of osteosarcoma cells in a dose- and time-dependent manner and increases its apoptosis mainly by increasing the protein expression of pro-apoptotic protein PTEN, thereby inhibiting the activation of PI3K/Akt pathway. In addition, It showed significant synergy when used in combination with 5-FU on osteosarcoma cells. The results of in vitro experiments also proved that WT161 can inhibit the growth of osteosarcoma transplanted tumors and combined with the clinical chemotherapy drug 5-FU can synergistically inhibit the growth of osteosarcoma.WT161 might be a promising agent against osteosarcoma.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JS and XFT carry out most experiments of the study and drafted the manuscript. FFZ, CJ, RFL, YRZ, BY, BL, YHG and DZ finished cell culture, MTT assay, apoptosis assay, western blot analysis and animal experiment. MW and ZPZ carried out the statistical analysis and organized the figures. CHZ and XBL designed the study and associated with all the steps of the study. All authors read and approved the final manuscript.

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Not applicable.

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Figures

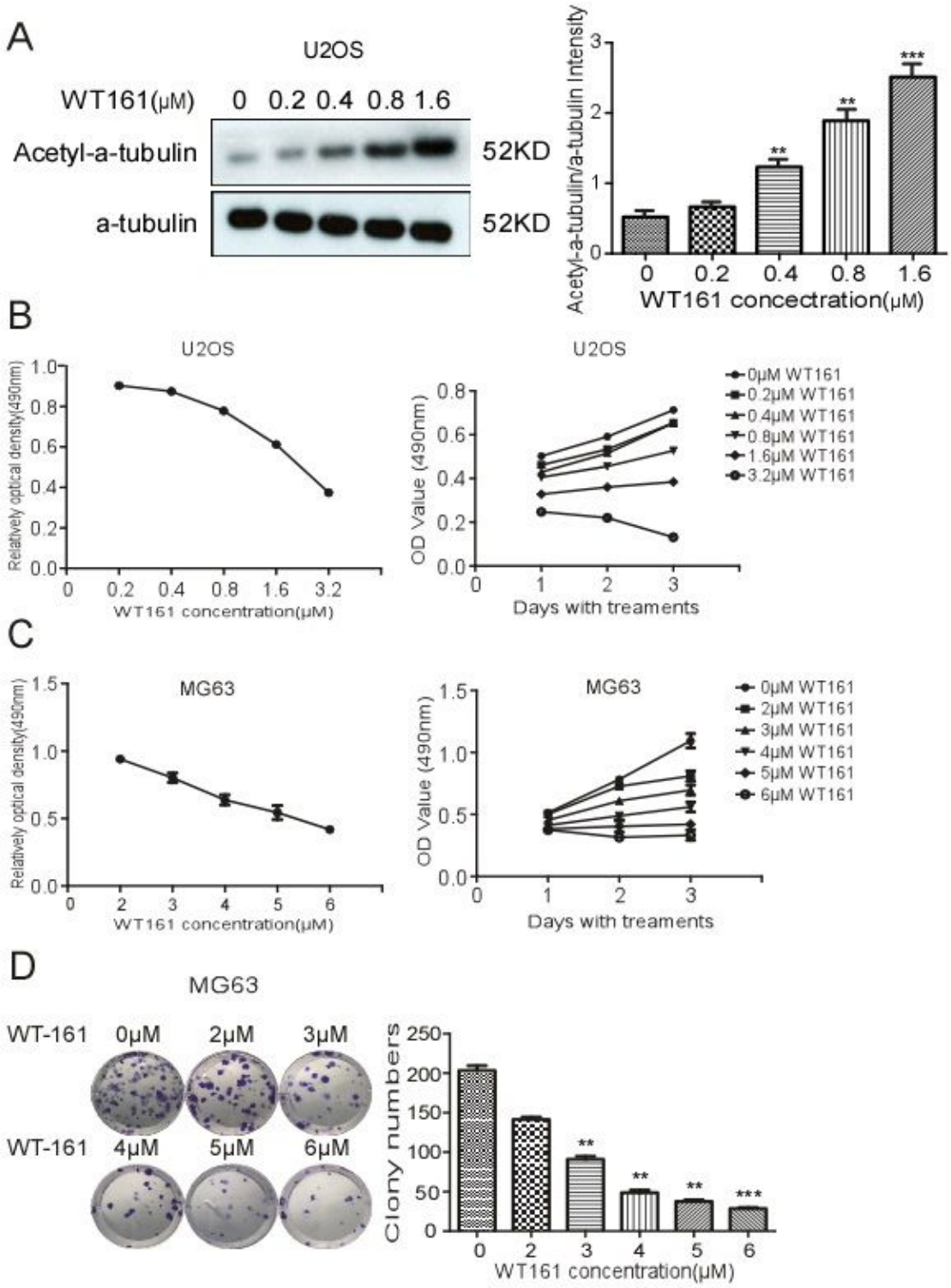


Figure 1

WT161 suppresses the cell growth of osteosarcoma cells. A)U2OS cells were subjected to various concentrations of (0-1.6μM) WT161 for 48h. Means from three independent experiments are presented as data. B)Various concentrations of WT161 was administered to U2OS cells for 72h with quantitative absorbance per 24h. C)Various concentrations of WT161 was administered to MG63 cells for 72h with quantitative absorbance per 24h. D)MG63 cells were inoculated into 6-well plates(1,000 cells/well). Each

well was treated with different concentrations of (0-6μM) WT161 for two weeks. After two weeks, the cells were stained 0.1% crystal violet dye overnight. n=3, **P<0.01, ***P<0.001.

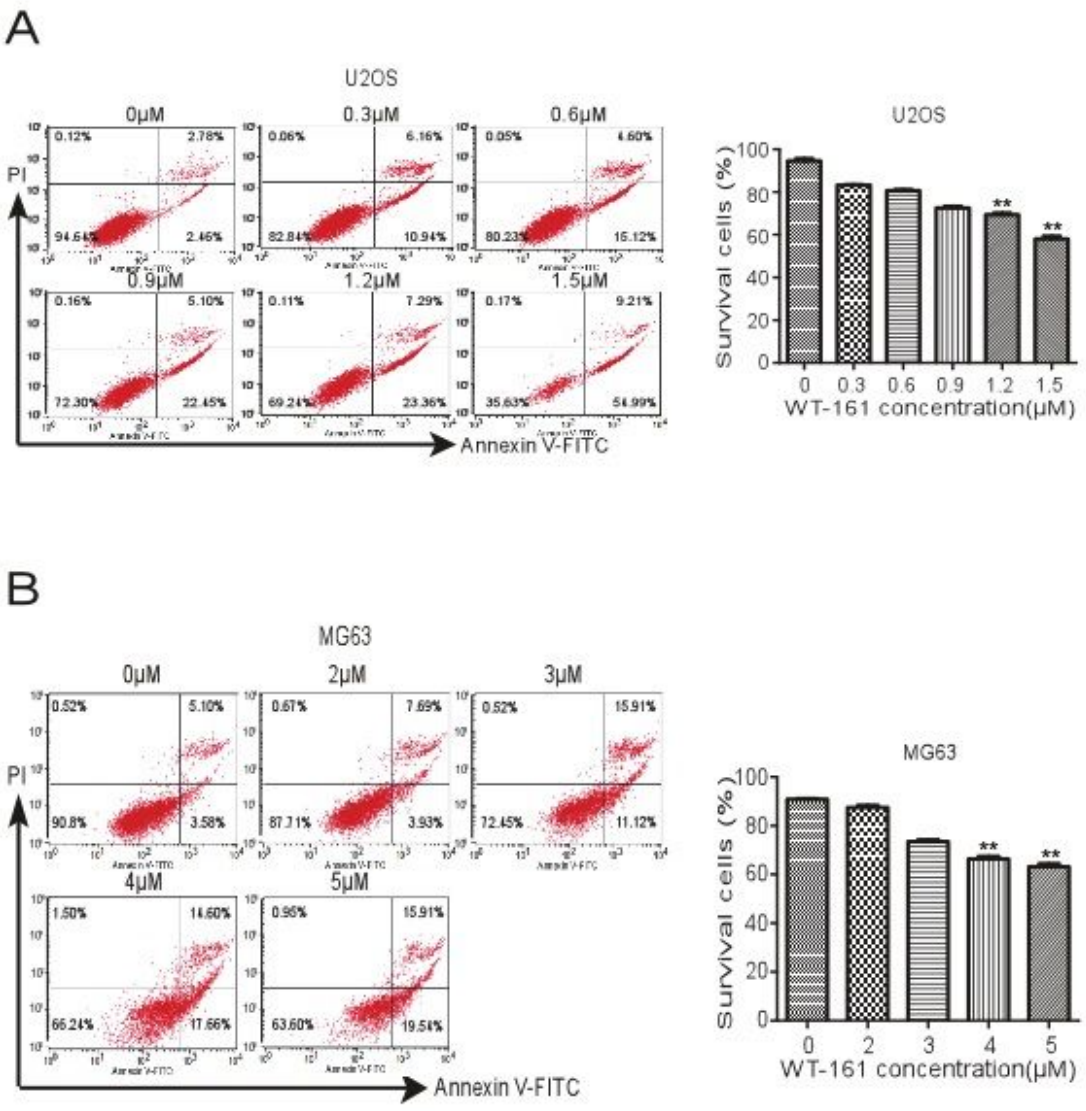


Figure 2

WT161 treatment induces the apoptosis of osteosarcoma cells. A)U2OS cells were subjected to various concentrations of WT161 for 48h. The cell suivival levels were assessed by flow cytometry. n=3,**P<0.01.

B)MG63 cells were subjected to various concentrations of WT161 for 48h. The cell suivival levels were assessed by flow cytometry. n=3,**P<0.01.

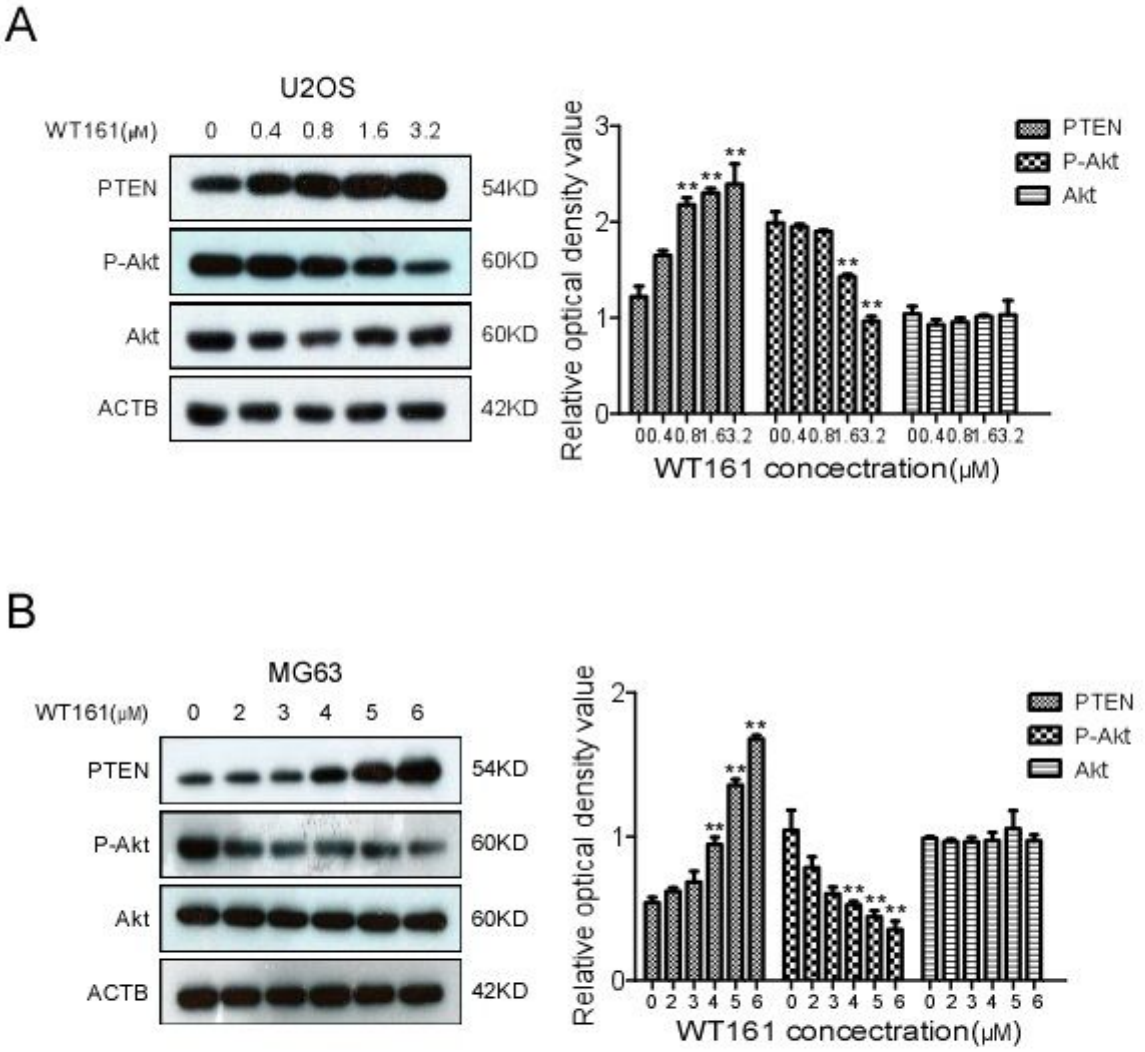
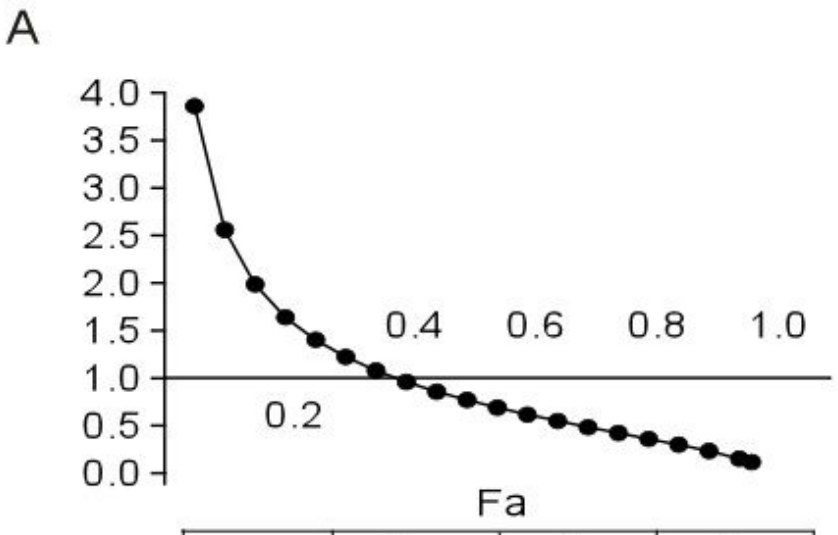


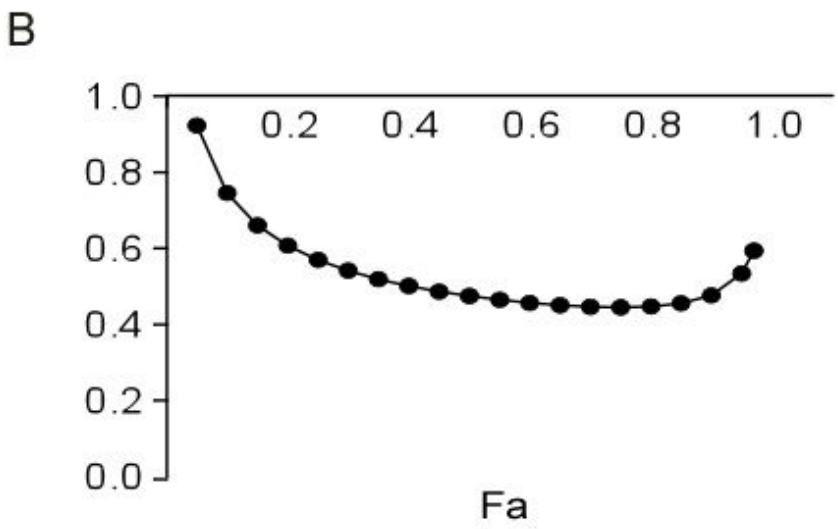
Figure 3

WT161 increases the apoptosis of osteosarcoma cells mainly through regulating PTEN/AKT signaling pathway. A)The protein expression levels of PTEN, P-Akt and Akt were tested in U2OS cells treated with WT161 for 48h using the Western blot analysis. n=3, **P<0.01. B)The protein expression levels of PTEN,

P-Akt and Akt were tested in MG63 cells treated with WT161 for 48h using the Western blot analysis. n=3, **P<0.01.



Cell line	CI EC_{50}	CI EC_{75}	CI EC_{90}
U20S	0.77086	0.42429	0.23419



Cell line	CI EC_{50}	CI EC_{75}	CI EC_{90}
MG63	0.47504	0.44565	0.47707

Figure 4

WT161 shows synergistic inhibitory effects on osteosarcoma cell combined with 5-FU. A)U2OS cells were subjected to 5-FU only or in combined with WT161. The synergistic effect of various concentration range of WT161 and 5-FU were showed using CDI. B)MG63 cells were subjected to 5-FU only or in combined

with WT161. The synergistic effect of various concentration range of WT161 and 5-FU were showed using CDI.

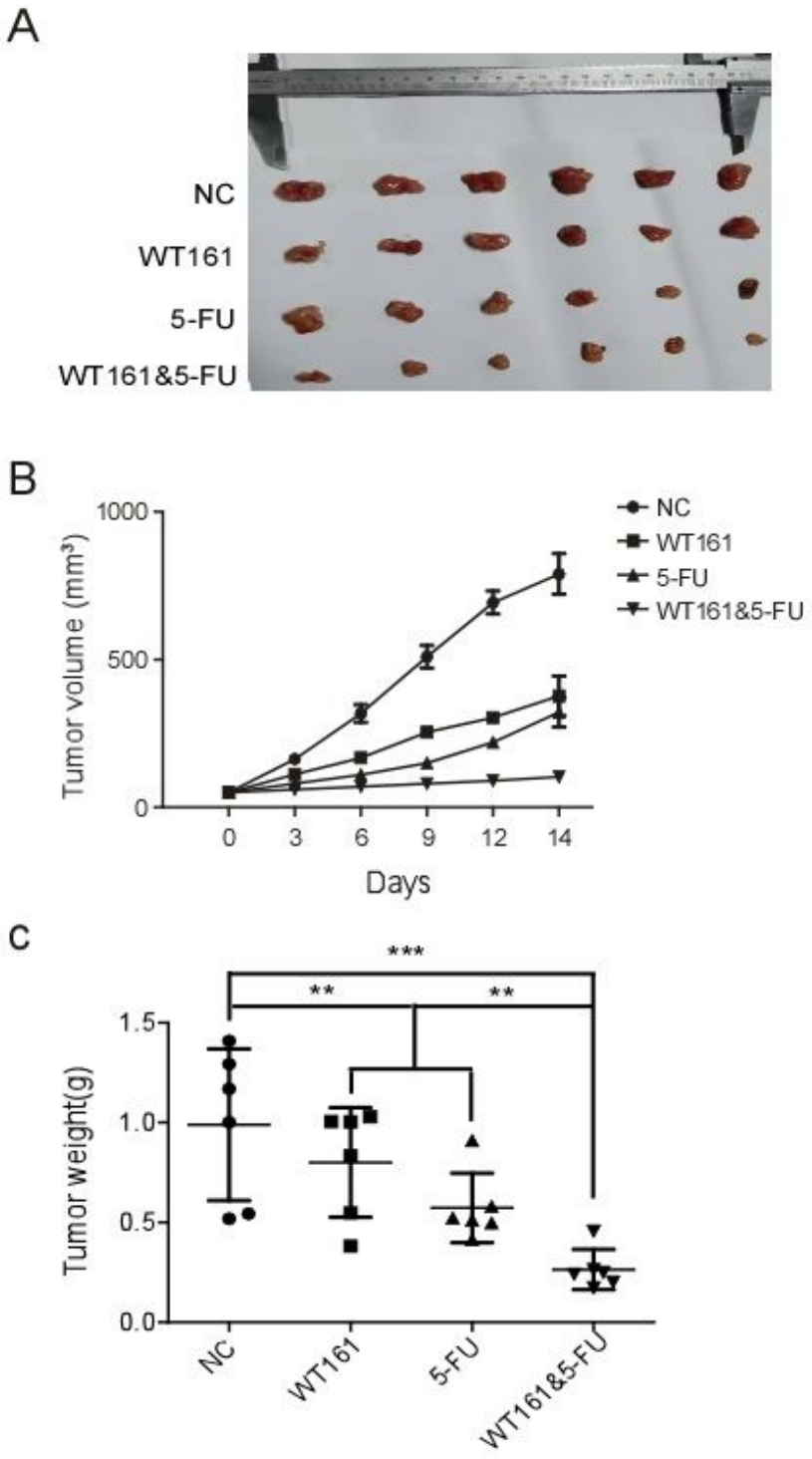


Figure 5

WT161 shows synergistic effects on osteosarcoma tumor growth combined with 5-FU in a mouse xenograft model. A)Tumor display map of each experimental group. B)Tumor volume statistics of each experimental group. C)Tumor statistics of each experimental group. **P<0.01, ***P<0.001.