The Synergistic Anticancer Effect of Bromodomain Inhibitor OTX015 and Histone Deacetylase 6 Inhibitor WT-161 in Osteosarcoma

Bo Yu
  The Third Affiliated Hospital of nanchang university

Lang Liu
  The Third Affiliated Hospital of nanchang university

Feng Cai
  The Third Affiliated Hospital of nanchang university

Yuanxiang Peng
  The Third Affiliated Hospital of nanchang university

Xiaofeng Tang
  The Third Affiliated Hospital of nanchang university

Duo Zeng
  The Third Affiliated Hospital of nanchang univercity

Teng Li
  The Third Affiliated Hospital of nanchang university

Feifei Zhang
  The Third Affiliated Hospital of nanchang university

Yiping Liang
  The Third Affiliated Hospital of nanchang university

Xuhui Yuan
  The Third Affiliated Hospital of nanchang university

Jiayu Li
  The Third Affiliated Hospital of nanchang university

Zhengzai Dai
  The Third Affiliated Hospital of nanchang university

Qi Liao
  The Third Affiliated Hospital of nanchang university

Xiao-Bin Lv（✉ nclvxiaobin@ncu.edu.cn ）
  Nanchang University   https://orcid.org/0000-0002-6493-4852

Primary research

Abstract

Background: Osteosarcoma (OS) is a tumor with a high malignancy level and a poor prognosis. First-line chemotherapy for OS has not been improved for many decades. Bromodomain and extra terminal domain (BET) and histone deacetylase (HDACs) regulate histone acetylation in tandem, and BET and HDACs have emerged as potential cancer therapeutic targets.

Methods: Cell proliferation, migration, invasion, colony formation, and sphere-forming assay were performed with the two inhibitors alone or in combination to evaluate their suppressing effect on malignant properties of OS cells. The apoptosis and cells-cycle profile were measured by flow cytometry. The synergistic inhibitory effect of OTX015/WT-161 on tumor was also examined in a nude mouse xenograft model.

Results: The combined therapy of OTX015/WT-161 synergistically inhibited the growth, migration, and invasion and induced apoptosis, resulted in the G1/S arrest of OS cells. Additionally, OTX015/WT-161 inhibits the self-renewal ability of OS stem cells (OSCs) in a synergistic manner. Further mechanistic exploration uncovered that the synergistic downregulation of β-catenin by OTX015-mediated suppression of FZD2 and WT-161-mediated upregulation of PTEN may be responsible for the synergistic effect. Finally, the result of an in vivo assay showed that tumor xenografts were significantly decreased after treatment with OTX015/WT-161 combination compared with OTX015 or WT-161 alone.

Conclusion: Our findings in this study demonstrated that OTX015 and WT-161 had a synergistic anti-cancer efficacy against OS, and their combination might be a promising therapeutic strategy for OS.

1. Introduction

OS is the most common primary malignant bone tumor. It mainly affects children and young adults, with an incidence peak at around 18 years old, and is mostly localized in long bones [1]. Due to the characteristics of early metastasis and poor prognosis of OS, this aggressive malignant tumor has become a major cause of death-threatening adolescents and young adults. The standard treatment of OS is surgery, radiotherapy, and adjuvant chemotherapy [2]. Unfortunately, 5 years survival rate drops from 75–25% for bad responders [3], hence further work is urgently required to discover novel therapeutic strategies for OS treatment.

Bromodomain and extra-terminal family proteins (BRDT, BRD2, BRD3, and BRD4) bind to acetylated lysine residues on histones as chromatin readers [4]. BRD4 is the most thoroughly researched BET family protein. The N-terminal bromodomain of BRD4 recognizes acetylated lysine on nucleosome histones and facilitates the expression of several oncogenes, such as c-myc, which contribute to the proliferation of cancer cells [5, 6].

OTX015 (inhibiting BRD2/3/4), a clinical-stage bromodomain inhibitor, can competitively replace bromodomain protein from chromatin, preventing oncogene expression and inducing cancer cell

apoptosis. OTX015 has been shown to inhibit the proliferation of leukemia, glioblastoma, and lung cancer cells [7–9]. Combination of OTX015 with other chemical has been attempted to enhance the therapeutic efficacy against cancers due to the limited efficacy of bromodomain inhibitors alone and the inevitable drug resistance [10].

HDACs regulates the de-acetylation of histone and non-histone proteins, thereby regulating gene expression or protein stability and activity [11, 12]. HDACs remove the acetyl group from the lysine residue on the histone tails, leading to chromatin compaction and transcriptional repression, mainly of tumor suppressor genes. The HDACs family consists of 18 members,which are divided into four types, including class I (HDAC1, HDAC2, HDAC3, HDAC8), class IIa (HDAC4, HDAC5, HDAC7, HDAC9), class IIb (HDAC6, HDAC10), class III Sir2-like enzymes (consisting of seven sirtuins), and class IV (HDAC11). HDACs inhibitors induce cancer cell growth inhibition and apoptosis in vitro and suppress tumor progression. Among all the HDACs, HDAC6 was reported to be implicated in multiple intracellular processes, including protein degradation, cell-cell interactions and cell mobility, and HDAC6 expression is raised in cancer to promote cancer development [13, 14]. Therefore, HDAC6 inhibitors have emerged as promising anticancer agents [15].

Because histone acetylation is co-regulated by BET and HDACs, the two have a close biological relationship. It has reported that the combination of bromodomain and HDACs inhibitors showed synergistic effects in killing several cancers including gallbladder cancer, glioblastoma and acute myelogenous leukemia [16–18]. In this study, we demonstrated the synergistic effect of bromodomain inhibitor OTX015 and HDAC6 inhibitor WT-161 in killing OS.

2. Methods
2.1 Cell culture and treatment

MG63, U2OS, MNNG and 143B human osteosarcoma cell lines were obtained from Stem Cell Bank of Chinese Academy of Sciences. The cells were tested for Mycoplasma before experiments. Cells were cultured in DMEM (Gibco, Grand Island, NY) medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and were incubated in a CO2 incubator with 5% CO_2 at 37°C. Alternatively, Cells were treated with inhibitors OTX015 (MCE, MedChem Express, HY-15743) and WT-161 (MCE, MedChem Express, HY-100871) for 48 hours before analyzing by qRT-PCR, western blotting, proliferation assay or transwell assay.

2.2 Cell viability assay

OS cells were seeded at an acceptable density (3×10^3 cells/well) in a 96-well culture plate for 18–24 hours, and then each cell line was treated independently or cooperatively for 24, 48, or 72 hours with OTX015 and WT-161 at different concentrations based on their IC_{50}. Meanwhile, an equal volume of DMSO was added as a negative control. After that, 10 μL CCK8 (Sigma-Aldrich) solution was added to each well and incubated for 1 hour away from light in a cell incubator. A microplate reader was used to

calculate the absorbance value (OD) of each well at 450 nm. The cellular viability was calculated using the formula: (OD of control – OD of treatment) / (OD of control – OD of blank) × 100%. Each sample was tested in triplicate. The Chou-Talalay method and CompuSyn software were used to analyze the synergistic effect of two drugs combination.

2.3 Apoptosis and cell cycle assay

Cell apoptosis were evaluated using a apoptosis assay kit (Tiangen, china) according to the manusfactory's instruction. In breaf, cells exposed to appropriate concentrations OTX015 or/and WT-161 or control group of DMSO for 48 hours were were resuspended in a 500 ml binding buffer before being washed with PBS for three times. Then the Cells were added with 5 μL of annexin V-FITC and propidium iodide (PI), respectively and incubated for 30 minutes at room temperature in the dark before measuring by flow cytometry assay (Becton-Dickinson, USA). For cell cycle analysis, cells treated with OTX015 or/and WT-161 and DMSO for 48 hours were washed with PBS for three times, fixed with 75% ethanol at 4°C overnight. Then the cells were then centrifuged, washed for three times, resuspended in pre-cooled PBS, and incubated in the dark for 30 minutes with PI and RNase. Finally, Flow cytometry was used to evaluate the cell cycle profile.

2.4 Transwell Assay

Transwell analysis was performed to assess the effects of OTX015 and /or WT-161 on the migration and invasion ability of osteosarcoma cells. Transwell chambers with polycarbonate membranes were used to detect the experiments. In the invasion experiment, 2×10^4 cells in 200 μL of serum-free medium were inoculated into the Matrigel (BD Biosciences, Franklin Lakes, NJ) coated upper chamber after being treated with a particular concentration of OTX015, WT-161, or their mixture for 48 hours. In the lower chamber, 750 μL medium supplemented with 10% FBS was added. The steps were the same for the migration experiment, except there was no Matrigel coating in the transwell chambers. The cells stained with 0.1% crystal violet in the upper compartment were washed clean with cotton swabs after 24 hours, and the number of invading cells in the lower chamber was observed under the microscope.

2.5 Colony forming Assay

Cells treated with OTX-015, WT-161, or their combination were seeded into 6-well plates at a condensation of 5000 cells/well for 14 days. Then the cells were fixed with 4% paraformaldehyde, stained 0.5% crystal violet and photoed by a microscope.

2.6 plasmid construction and Cell transfection

Full-length β-catenin was amplified from cDNAs reversely transcribed from mRNA extracted from 293T cells and and cloned into pcDNA3.1 vector. The primers for cloning β-catenin is froward: 5'-GCTACTCAAGCTGATTTGATGGA-3' and reverse: 5'-TTACAGGTCAGTATCAAACCAGGC-3'. The transfection of plasmids was performed using Lipofectamine ® 2000 (Invitrogen) according to the manufactory's instructions.

2.7 Quantitative real-time

Total RNA was extracted by TRIZOL reagent (Invitrogen), and cDNA was synthesized by PrimeScript™ RT reagent kit (TaKaRa). The PCR reaction was carried out with SYBR Premix Ex Taq II kit (Takara) according to the manufactory's instructions. GAPDH, a housekeeping gene, was used as an internal control. The result was analyzed via the $2^{-\Delta\Delta Ct}$ method. The primers sequences were as following:

CTNNB1-F: CGTGGACAATGGCTACTCAAGC

CTNNB1-R: TCTGAGCTCGAGTCATTGCATAC

FZD2-F: TCCTCAAGGTGCCATCCTATCTC

FZD2-R: TGGTGACAGTGAAGAAGGTGGAAG

FZD4-F: GTGTCACTCTGTGGGAACCAA

FZD4-R: GGCTGTATAAGCCAGCATCAT

FZD6-F: AGAGGTGAAAGCGGACGGA

FZD6-R: AGAGAGTCTGGAGATGGATGCT

LRP6-F: ACGATTGTAGTTGGAGGCTTG

LRP6-R: ATGGCTTCTTCGCTGACATCA

AXIN1-F: CAAGCAGAGGTATGTGCAGGA

AXIN1-R: CACAACGATGCTGTCACACG

GSK3β-F: GTGGTTACCTTGCTGCCATC

GSK3β-R: GACCGAGAACCACCTCCTTT

2.8 Sphere-forming assay

MG63 and U2OS cells (1000 cells/well) suspended DMEM/F12 (Invitrogen, Carlsbad, CA) medium supplemented with B27 (Invitrogen), human EGF (10 ng/ml, PeproTech) and human BFGF (10 ng/ml, PeproTech) were inoculated in six-well ultra-low attachment plates (Corning Inc., NY) and incubated in a CO_2 incubator with 5% CO_2 at 37°C. The spheres were photoed and countered under a microscope.

2.9 Western blot analysis

Cells were washed three times with PBS, lysed with RIPA buffer on ice for half an hour and centrifuged at 12000g/s for 20 minutes to collect the supernatant. Then the proteins were separated by SDS-PAGE and transfromed to PVDF membranes. The membranes were blocked with 5% nonfat milk, probed with primary antibodies and stained with with horseradish peroxidase (HRP)-linked secondary antibodies (Promega). Then the blots were visualized using ECL kit (Tiangen). The antibodies used in this study

were as follows: anti-PTEN (proteintech, 22034-1-AP), anti-β-actin (proteintech, 20536-1-AP), anti-β-Catenin (proteintech, 66379-1-lg); The anti-p-mTOR (5536), anti-mTOR (2983), anti-P65 (8242), anti-IKB (4814), anti-p-ERK1/2 (4370), anti-ERK1/2 (5013), anti-p-STAT3 (9131), anti-STAT3 (9132), anti-CDK2 (18048), anti-CyclinB1 (12231), anti-P21 (2947), anti-cleaved caspase-3 (9664), anti-cleaved caspase-9 (20750), anti-p-GSK-3β (5558) and anti-GSK-3β (12456) were purchased from Cell Signaling Technology (CST, Danvers, MA, United States). And the anti-p-JAK2 (ab32101), anti-JAK2 (ab108596), anti-Vimentin (ab92547), anti-N-cadherin (ab18203) and anti-E-cadherin (ab40772) were purchased from Abcam (MA, United States).

2.10 xenograft model

All nude mice (age of 4–6 weeks) were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). 1×10^6 U2OS cells in 200 uL of PBS was injected subcutaneously into left armpits of each mouse. After 5 to 7 days of inoculation when the xenograft grow to 200 cm^3, the mice were randomly divided into four groups (6 mice each group) and each group was injected intraperitoneally with drugs every 3 days: vehicle as a negative control; OTX015 (50 mg/kg); WT-161 (50 mg/kg); OTX015 combining with WT-161 (same concentrations as for single agent). About 2 weeks later, the nude mice were killed, the tumors were removed and weighed. All animal experiments have been approved by the institutional ethical review boards of the Third Affiliated Hospital of Nanchang University.

2.11 Statistical analyses

All of the experimental data were repeated at least three times and expressed as mean ± SD. Student's t-test was used to compare differences between two groups. P values < 0.05 was considered to be statistically significant.

3. Results

3.1 Co-treatment with OTX015 and WT-161 inhibitors inhibits OS cells growth in a synergistic manner

To begin, OS cell lines (MG63, U2OS) were exposed to increasing concentrations of OTX015 or WT-161 inhibitors for 24, 48, or 72 hours, respectively, and then OS cell proliferation was measured by the CCK-8 assay. Both OTX015 and WT-161 inhibited the growth of OS cells in a time-dependent and dose-dependent manner (Figure.1A and 1B). To determine the synergistic effect of the two chemicals, we evaluated the combination index (CI) in four OS cell lines using Chou-Talalay methods. Compared to the two inhibitors alone, the combined therapy of OTX015 and WT-161 showed significantly synergistic effect in killing the OS cells, and the CI values were significantly less than 1 in all the four OS cell lines (Figure.1C), suggesting that OTX015/WT-161 had a substantial synergistic inhibitory effect on OS cells. The colony formation experiment results also showed that OTX015/WT-161 combination has a greater

inhibitory effect on cancer cell proliferation than either of the inhibitors alone at the same dosage in MG63 and U2OS (Figure.1D).

3.2 OTX015 and WT-161 synergistically induce apoptosis and inhibit cell cycle progression in OS cells

To further investigate the inhibitory effect of OTX015 and WT-161 on the proliferation of OS cells, MG63 and U2OS cells were treated with DMSO, OTX015, WT-161 or OTX015/WT-161 at the optimum concentration for 48h, then the cell apoptosis and cell-cycle profiles were analyzed by flow cytometry. OTX015 and WT-161 combination induces a dramatically higher apoptosis rate than OTX015 or WT-161 alone (Figure.2A). In addition, cell-cycle profile analysis showed that OTX015 and WT-161 combination treatment resulted in much more cells arresting in G1/S phase than OTX015 or WT-161 treatment alone (Figure.2C). Furthermore, the expression of cleaved caspase 3, cleaved caspase 9 and p21 proteins were substantially higher during OTX015/WT-161 combination treatment compared to single treatment (Figure.2B and 2D). These results were consistent with the higher apoptosis rates and more pronounced G1/S arrest by OTX015 and WT-161 treatment. Taken together, these results indicate that OTX015/WT-161 suppresses OS cell proliferation by inducing apoptosis and blocking the cell cycle.

3.3 OTX015 and WT-161 synergistically suppress the metastasis and self-renewal of OS cells

The transwell assay was used to evaluate the effects of OTX015 and WT-161 on the migration and invasion of OS cells. As shown in Figure.3A and 3B, the invasion and migration of OS cells were dramatically weakened after 48 hours of treatment with OTX015/WT-161. In addition, EMT (epithelial-mesenchymal transition), a critical step metastasis progression of cancer cells. the combination treatment synergistically decreased vimentin and N-cadherin expression and increased E-cadherin expression, indicating that OTX015/WT-161 combination synergistically suppresses the EMT of OS cells (Figure.3C). To further confirm the role of OTX015/WT-161 combination on the tumorigenesity of OS cells, we investigated whether OTX015 and WT-161 can inhibit the self-renewal ability of OSCs by Sphere-forming assay. As shown in Fig. 3D and 3E, the combined treatment of OTX015 and WT-161 resulted in the smallest spheroid development and the greatest inhibitory effect.

3.4 Co-treatment of OTX015 and WT-161 represses OS cells growth via WNT and PTEN/AKT signaling pathways

To explore the underline mechanisms by which OTX015 and WT-161 synergistically suppress the tumorignesity of OS cells, we examined the changes of several classical survival signaling pathways including WNT, mTOR, NF-kB, JAK-STAT, and MAPK in OS cells upon OTX015, WT-161 alone or combination treatment. Among them, β-catenin protein in the WNT pathway was significantly reduced upon the OTX015/WT-161 combination treatment compared with single chemical treatment alone in both MG63 and U2OS cells (Figure.4A), indicating that β-catenin may be a crucial protein responsible for the

synergistic repression of OS cells by OTX015 and WT-161 combination. To address the detailed mechanisms by which OTX015/WT-161 synergistically reduced β-catenin protein level, we examine the effect of OTX015/WT-161 on classical upstream signal pathway. We found that OTX015 treatment significantly reduces the mRNA level of FZD2 in both cells (Figure.4B). PTEN, an upstream protein of GSK3β which phosphonates and facilitates β-catenin protein degradation, was reported to be upregulated upon HDAC6 inhibition. We thus examined whether OTX015 and WT-161 play roles on PTEN expression. As shown in Fig. 4C, WT-161 but not OTX015 treatment increased PTEN expression. Altogether, these results indicate that OTX015 mediated FZD2 downregulation and WT-161 mediated PTEN upregulation may result in the synergistic repression of β-catenin expression.

3.5 Reintrodction of β-catenin "rescure" in part the anti-cancer effets in OS cells upon OTX015/WT-161 combination treatment .

To further confirm the role of β-catenin in mediating the anti-cancer effects of OTX015/WT-161, we reintroduced β-catenin into OTX015/WT-161-treated OS cells and examine whether β-catenin transfection could reverse OTX015/WT-161-exerted killing of OS cells. A CCK-8 assay showed that transfection of β-catenin increased the viability of MG63 and U2OS upon OTX015/WT-161 treatment (Figure.5A). Besides, reintroduction of β-catenin increased the migratory and invasive abilitiy and reduced the apoptosis rates of OS cells upon OTX015/WT-161 compared to vector control (Figure.5B and 5C). Taken together, these results indicate that β-catenin mediated in part the anti-cancer roles of OTX015/WT-161 in OS cells.

3.6 OTX015/WT-161 synergistically inhibit the growth of OS xenografts in vivo

We next evaluated the antitumor activity of OTX-015 and/or WT-161 against OS tumor xenografts in immunocompromised mice. Both OTX-015 and WT-161 reduced the growth and weight of OS in NOD-SCID mice. Notably, the combination of OTX015 and WT-161 inhibited tumor xenografts growth better than either OTX-015 or WT-161 alone (Figure.6A and 6B).These results indicate that OTX-015 and WT-161 synergistically inhibit the growth of OS in vivo.

4. Discussion

Chemotherapy is especially important for cancer treatment, and it is the preferred systemic treatment for almost all cancers [19]. For OS, high-dose chemotherapeutic treatments work well, but strong reverse effect are usually accompanied, limiting their efficacy on the treatment of OS patients [20]. Therefore, the more effective and economical approaches are combination therapy, which increase the sensitivity and reduce the revers effect of single drug alone[21].

BET inhibitors including OTX015, have anticancer effects through the epigenetic repression of multiple pro-oncogene. Although BET inhibitors have been used as tumor-targeted tents, early clinical trial findings have been mixed, and drug susceptibility has restricted the therapeutic efficacy of BET inhibitors. However, several preclinical data indicated that the combination of BET inhibitors and HDAC inhibitors

has a broader prospect[22, 23]. It was reported in several studies that combining BET inhibitors with specific HDAC inhibitor improved their efficacy. BRD4 inhibitors and histone deacetylase inhibitors can prevent the proliferation and metastasis of gallbladder cancer cells through the PI3K/Akt and MAPK/ERK pathways, and induce cell apoptosis, according to Liu et al [16]. Shahbazi et al. found that JQ1 and panobinostat synergistically inhibited the growth of neuroblastoma cells and induced apoptosis by reducing the expression of LIN28B and N-Myc [24]. Fiskus et al. reported that JQ1 and the HDAC inhibitor panobinostat could induce apoptosis in acute myeloid leukemia cells and have a synergistic anti-tumor effect [18].

Our results indicated that the combination of OTX015 and WT-161 synergistically inhibited cell proliferation and induced apoptosis, and resulted in a cell cycle arrest in OS cells. By the sphere-forming assay, we discovered that OTX015/WT-161 could worked together to inhibit OSCs' self-renewal ability. Mechanistical exploration showed that WNT and PTEN/PI3K/Akt signaling pathways were linked in the synergistic effect of OTX015/WT-161. Besides, overexpression of β-catenin recovered the killing effect of OTXWT-161, indicating β-catenin is responsible for the synergistic efficacy of OTX/WT-161 on OS cells. Finally, in vivo experiments revealed that OTX015/WT-161 had a strong inhibitory effect on the tumor xenografts.

Metastasis is a critical factor that affects cancer treatment [25]. OS is a kind of highly aggressive cancer usually with distant metastases, especially lung metastases, at the time of diagnosis. To date, the 5-year survival rate for OS with pulmonary metastasis is as low as 20–30%, making metastasis as a significant challenge in OS therapy [26, 27]. EMT refers to a biological process in which epithelial cells lose their polarity, weaken cell-to-cell connections, and become more aggressive [28]. As EMT makes cancer cells more aggressive, resulting in distant metastasis, inhibiting EMT can increase cancer patients' survival. We found that both OTX015 and WT-161 inhibited OS cells migration and invasion, reduced the mesenchymal marker proteins vimentin and N-cadherin and increased the epithelial marker E-cadherin. Notably, the efficacy were more pronounced when combination of OTX015 with WT-161.

Multiple genes balance control the dead and alive of cells during apoptosis, a physiological process. Caspase family proteins are essential determinants of apoptosis signaling and are required for the initiation and progression of apoptosis. Caspase-3 and caspase-6, which carry out the apoptotic process, and caspase-9, which is activated by signaling factors, are members of the caspase family of proteins [29]. In our research, we found that treating OS cells with OTX015 and WT-161 together promoted apoptosis while also increasing cleaved caspase-3 and cleaved caspase-9 proteins. The expression of apoptosis-related proteins corroborated the flow cytometry findings. As for the phenomenon that OTX015/WT-161 can arrest the cell cycle in the G1/S phase, it has been previously reported that BET inhibitors can arrest the cell cycle in the G1 phase of a variety of malignant tumors [22, 30]; HDAC inhibitors induced apoptosis and blocked cells in the G1 phase [31], which was consistent with the effect of OTX015/WT-161 on the cell cycle of OS in this study. Furthermore, treatment with OTX015/WT-161 resulted in a decrease in CDK2 protein and an increase in p21 protein, confirming the role of OTX015/WT-161 on the cell cycle arrest.

The synergistic mechanism of OTX015/WT-161 on the repression of OS cells was explored in this study. We found that OTX015 mediated the degradation of β-catenin through inhibiting the expression of FZD2, a WNT receptor, whose activation increases the activity of the degradation complex against β-catenin. In addition, HDAC6 inhibitors have been demonstrated to cause the activation and membrane translocation the phosphatase and tensin homologue (PTEN), a well-known tumor suppressor gene inhibiting PI3K/ Akt signaling [32]. Both the WNT and PI3K/Akt pathways can promote the development of cancer, and they are often interacted [33]. Therefore, we suspected that WT-161 could also inhibit the growth of OS cells through the PTEN/PI3K/ Akt signaling pathway to participate in the synergistic therapeutic effect [34]. Indeed, we found WT-161 treatment increased the expression of PTEN, an upstream regulator of GSK-3β/ β-catenin. In a word, the WNT and PI3K/Akt pathways complemented each other to degrade β-catenin and thus play an anti-cancer role in the treatment of OTX015/WT-161 (Fig. 6C).

It's worth noting that OTX015/WT-161 has a synergistic inhibitory effect on OSCs. At the present, CSCs are considered to be a subtype of tumor cells that can facilitate tumor proliferation, metastasis, and self-renewal [35]. The molecular mechanism of the synergistic effect of OTX015/WT-161 on OSCs needs to be analyzed further, as it will be promise the development of novel OS therapeutic strategies.

5. Conclusion

In conclusion, this research indicates that OTX015/WT-161 synergistically inhibits the malignant properties of OS cells by blocking the WNT and PI3K/ Akt pathways, triggering apoptosis, G1/S cycle arrest and repressing metastasis. This study provided a promising therapy strategy for clinical drug treatment of OS.

Declarations

Ethics approval and consent to participate

This study was approved by the institutional ethical review boards of the Third Affiliated Hospital of Nanchang University.

Consent for publication

All authors declared no financial conflict of interest.

Competing interests

The authors declared no .financial conflict of interest.

Competing interests

The authors declare that they have no competing interests

Funding

Authors' contributions

XBL and QL designed this research; BY, LL, SF and FC performed experiments; XFT and YXP performed western blotting and the drug treatment experiments; DZ, TL and FFZ performed the in vivo assay; YPL, XHY, JYL and ZZD cultured of osteosarcoma cell lines and assist with data analysis; XBL and QL wrote the manuscript.

Acknowledgments

Footnotes

Publisher's Note

Conflict of Interest

The authors declare no potential conflicts of interest.

Availability of data and materials

All have been shown in the manuscript.

References

1. Lamoureux F, et al. Recent advances in the management of osteosarcoma and forthcoming therapeutic strategies. Expert Rev Anticancer Ther. 2007;7(2):169–81.
2. Chen D, et al. Super enhancer inhibitors suppress MYC driven transcriptional amplification and tumor progression in osteosarcoma. Bone Res. 2018;6:11.
3. He JP, et al. Review of the molecular pathogenesis of osteosarcoma. Asian Pac J Cancer Prev. 2014;15(15):5967–76.

4. Wu SY, Chiang CM. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. J Biol Chem. 2007;282(18):13141–5.

5. Qin ZY, et al. BRD4 Promotes Gastric Cancer Progression and Metastasis through Acetylation-Dependent Stabilization of Snail. Cancer Res. 2019;79(19):4869–81.

6. Borbely G, et al. Induction of USP17 by combining BET and HDAC inhibitors in breast cancer cells. Oncotarget. 2015;6(32):33623–35.

7. Berthon C, et al. Bromodomain inhibitor OTX015 in patients with acute leukaemia: a dose-escalation, phase 1 study. Lancet Haematol. 2016;3(4):e186-95.

8. Berenguer-Daize C, et al. OTX015 (MK-8628), a novel BET inhibitor, displays in vitro and in vivo antitumor effects alone and in combination with conventional therapies in glioblastoma models. Int J Cancer. 2016;139(9):2047–55.

9. Riveiro ME, et al. OTX015 (MK-8628), a novel BET inhibitor, exhibits antitumor activity in non-small cell and small cell lung cancer models harboring different oncogenic mutations. Oncotarget. 2016;7(51):84675–87.

10. Zhang W, et al. Combinational therapeutic targeting of BRD4 and CDK7 synergistically induces anticancer effects in head and neck squamous cell carcinoma. Cancer Lett. 2020;469:510–23.

11. Petta V, et al. Histones and lung cancer: Are the histone deacetylases a promising therapeutic target? Cancer Chemother Pharmacol. 2013;72(5):935–52.

12. Tang J, Yan H, Zhuang S. Histone deacetylases as targets for treatment of multiple diseases. Clin Sci (Lond). 2013;124(11):651–62.

13. Barneda-Zahonero B, Parra M. Histone deacetylases and cancer. Mol Oncol. 2012;6(6):579–89.

14. Valenzuela-Fernandez A, et al. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. Trends Cell Biol. 2008;18(6):291–7.

15. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov. 2014;13(9):673–91.

16. Liu S, et al. BRD4 inhibitor and histone deacetylase inhibitor synergistically inhibit the proliferation of gallbladder cancer in vitro and in vivo. Cancer Sci. 2019;110(8):2493–506.

17. Meng W, et al. Enhanced efficacy of histone deacetylase inhibitor combined with bromodomain inhibitor in glioblastoma. J Exp Clin Cancer Res. 2018;37(1):241.

18. Fiskus W, et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. Mol Cancer Ther. 2014;13(5):1142–54.

19. Mansoori B, et al. The Different Mechanisms of Cancer Drug Resistance: A Brief Review. Adv Pharm Bull. 2017;7(3):339–48.

20. Harrison DJ, et al. Current and future therapeutic approaches for osteosarcoma. Expert Rev Anticancer Ther. 2018;18(1):39–50.

21. Bayat Mokhtari R, et al. Combination therapy in combating cancer. Oncotarget. 2017;8(23):38022–43.

22. Doroshow DB, Eder JP, LoRusso PM. BET inhibitors: a novel epigenetic approach. Ann Oncol. 2017;28(8):1776–87.

23. Janouskova H, et al. Opposing effects of cancer-type-specific SPOP mutants on BET protein degradation and sensitivity to BET inhibitors. Nat Med. 2017;23(9):1046–54.

24. Shahbazi J, et al. The Bromodomain Inhibitor JQ1 and the Histone Deacetylase Inhibitor Panobinostat Synergistically Reduce N-Myc Expression and Induce Anticancer Effects. Clin Cancer Res. 2016;22(10):2534–44.

25. Bielack SS, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol. 2002;20(3):776–90.

26. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531–43.

27. Yang C, et al., *Bone Microenvironment and Osteosarcoma Metastasis*. Int J Mol Sci, 2020. 21(19).

28. Kong D, et al. Cancer Stem Cells and Epithelial-to-Mesenchymal Transition (EMT)-Phenotypic Cells: Are They Cousins or Twins? Cancers (Basel). 2011;3(1):716–29.

29. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013;5(4):a008656.

30. Filippakopoulos P, et al. Selective inhibition of BET bromodomains. Nature. 2010;468(7327):1067–73.

31. Li Y, Seto E. *HDACs and HDAC Inhibitors in Cancer Development and Therapy*. Cold Spring Harb Perspect Med, 2016. 6(10).

32. Haddadi N, et al. PTEN/PTENP1: 'Regulating the regulator of RTK-dependent PI3K/Akt signalling', new targets for cancer therapy. Mol Cancer. 2018;17(1):37.

33. Shorning BY, et al., *The PI3K-AKT-mTOR Pathway and Prostate Cancer: At the Crossroads of AR, MAPK, and WNT Signaling*. Int J Mol Sci, 2020. 21(12).

34. Meng Z, Jia LF, Gan YH. PTEN activation through K163 acetylation by inhibiting HDAC6 contributes to tumour inhibition. Oncogene. 2016;35(18):2333–44.

35. Adorno-Cruz V, et al. Cancer stem cells: targeting the roots of cancer, seeds of metastasis, and sources of therapy resistance. Cancer Res. 2015;75(6):924–9.
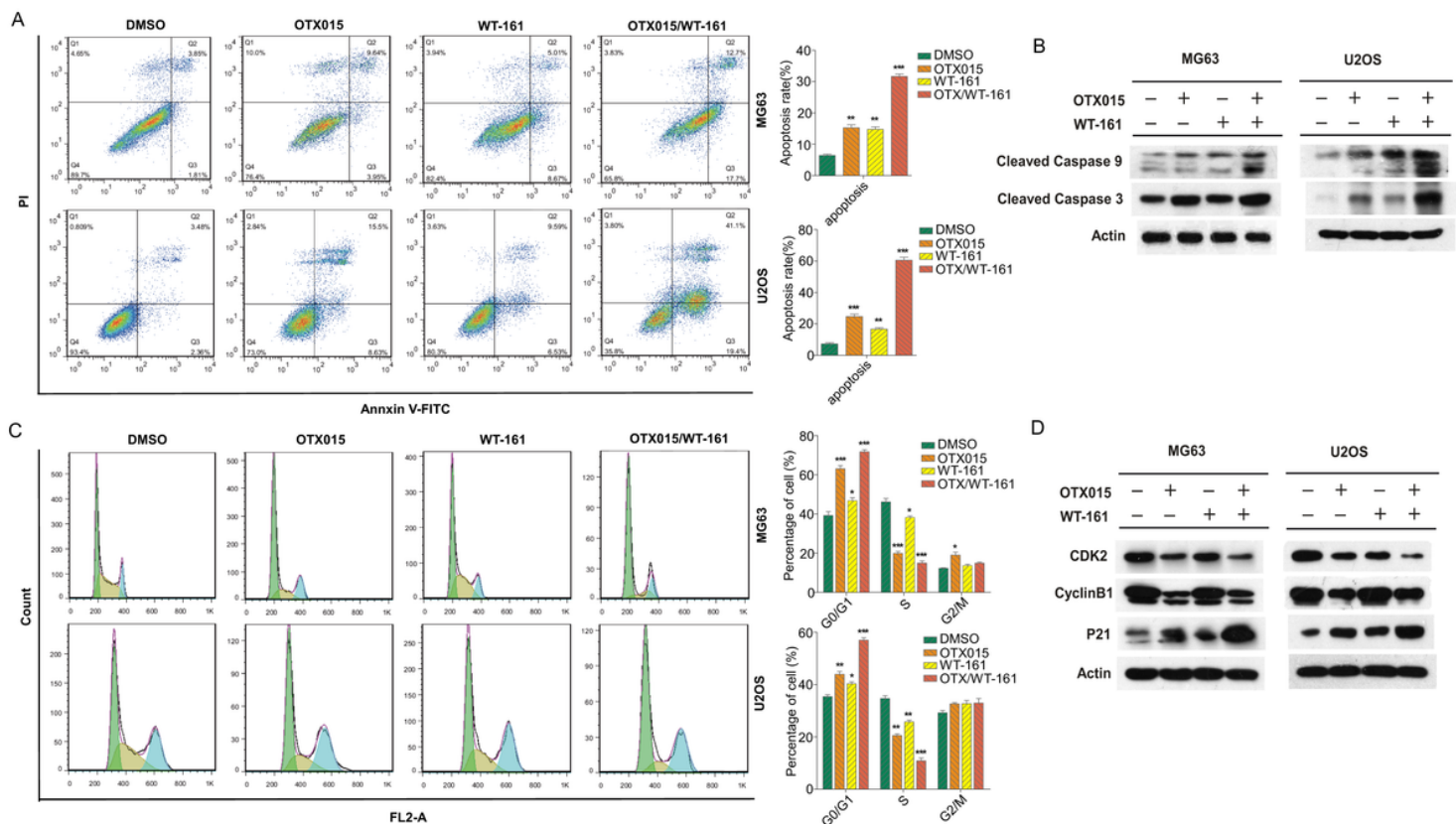
Figures
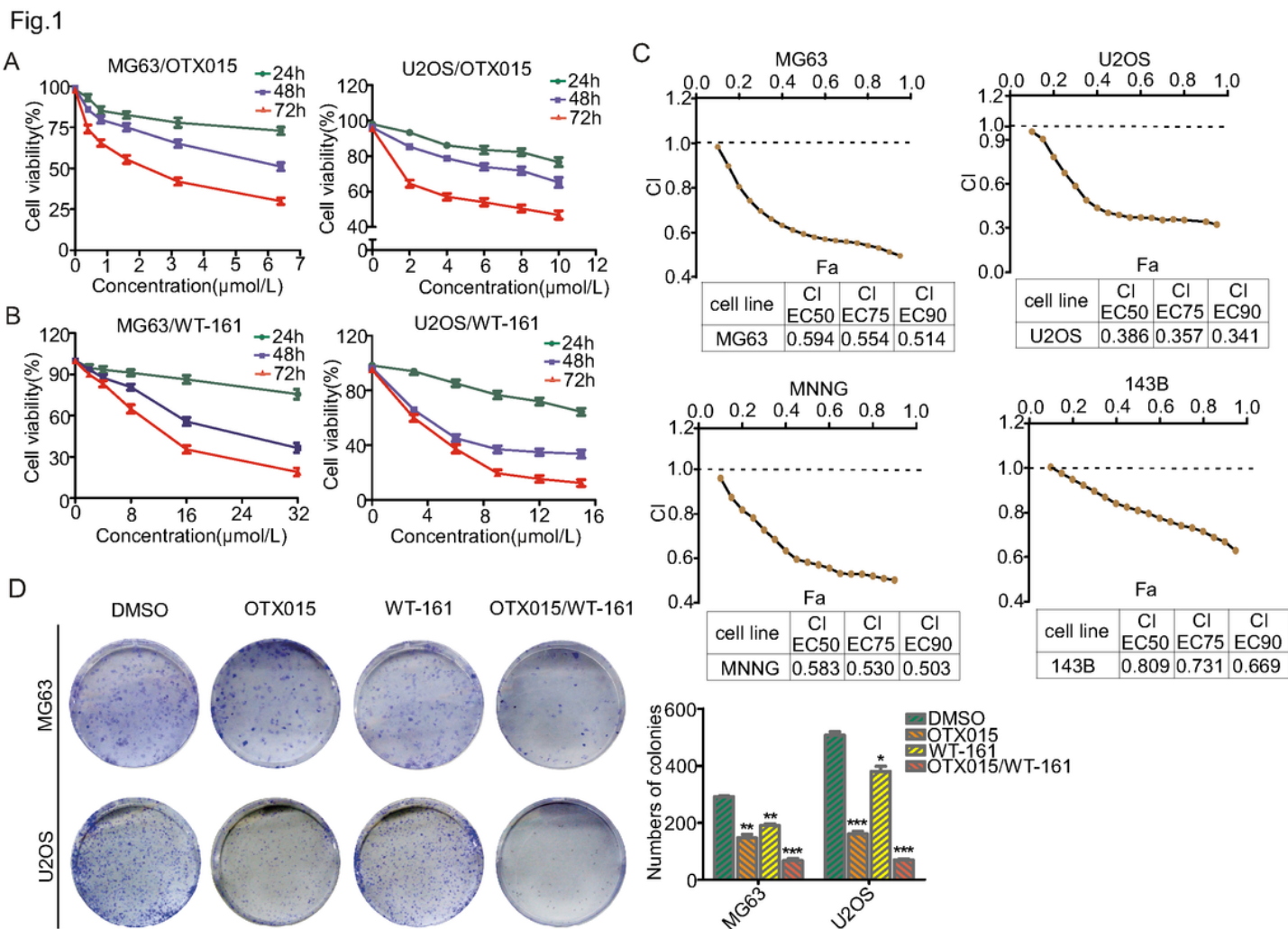
Figure 1

OTX015 and WT-161 synergically inhibit the proliferation of OS cells. (A-B) The effects of either OTX015 or WT-161 on OS cells are dose-dependent and time-dependent. CCK-8 was used to detect cell proliferation in MG63 and U2OS after they were treated separately with various doses of OTX015 or WT-161 for 24, 48, or 72 hours. (C) CCK-8 assayed the viability of OS cells at a certain concentration of OTX015/WT-161 and calculated CI values according to the Chou-Talalay method to determine the synergistic effect of OTX015 and WT-161. CI<1 indicates that OTX015 and WT-161 have a synergistic effect. (D) OTX015/WT-161 synergically inhibited the colony formation of MG63 and U2OS. Data are means ± SD (n=3). *P < 0.05，**P < 0.01, ***P < 0.001，compared to DMSO.

Fig.2

Figure 2

Effect of OTX015/WT-161 on apoptosis and cell cycle of OS cells in vitro. (A) Flow cytometry was used to assess the apoptosis of MG63/U2OS cells. VFITC and PI reagents were used to treat OS cells that had been infected with OTX015 or/and WT-161 for 48 hours. The percentage of apoptosis in various drug treatment groups was measured. (B) The expression of cleaved caspase 3 and cleaved caspase 9 protein in DMSO, OTX and/or WT-161. (C) The cell cycle distribution was analyzed by flow cytometry. The percentage of G0/G1, S, and G2/M phases of OS cells in the bar chart indicates that OTX015/WT-161 cooperatively arrests the cell cycle in the G1/S phase. (D) Western blot analysis of cyclin-related proteins was performed in MG63 and U2OS. Actin was used as a loading control. Data are means ± SD (n=3). * P <0.05, ** P <0.01 and *** P <0.001, compared to DMSO.

Fig.3
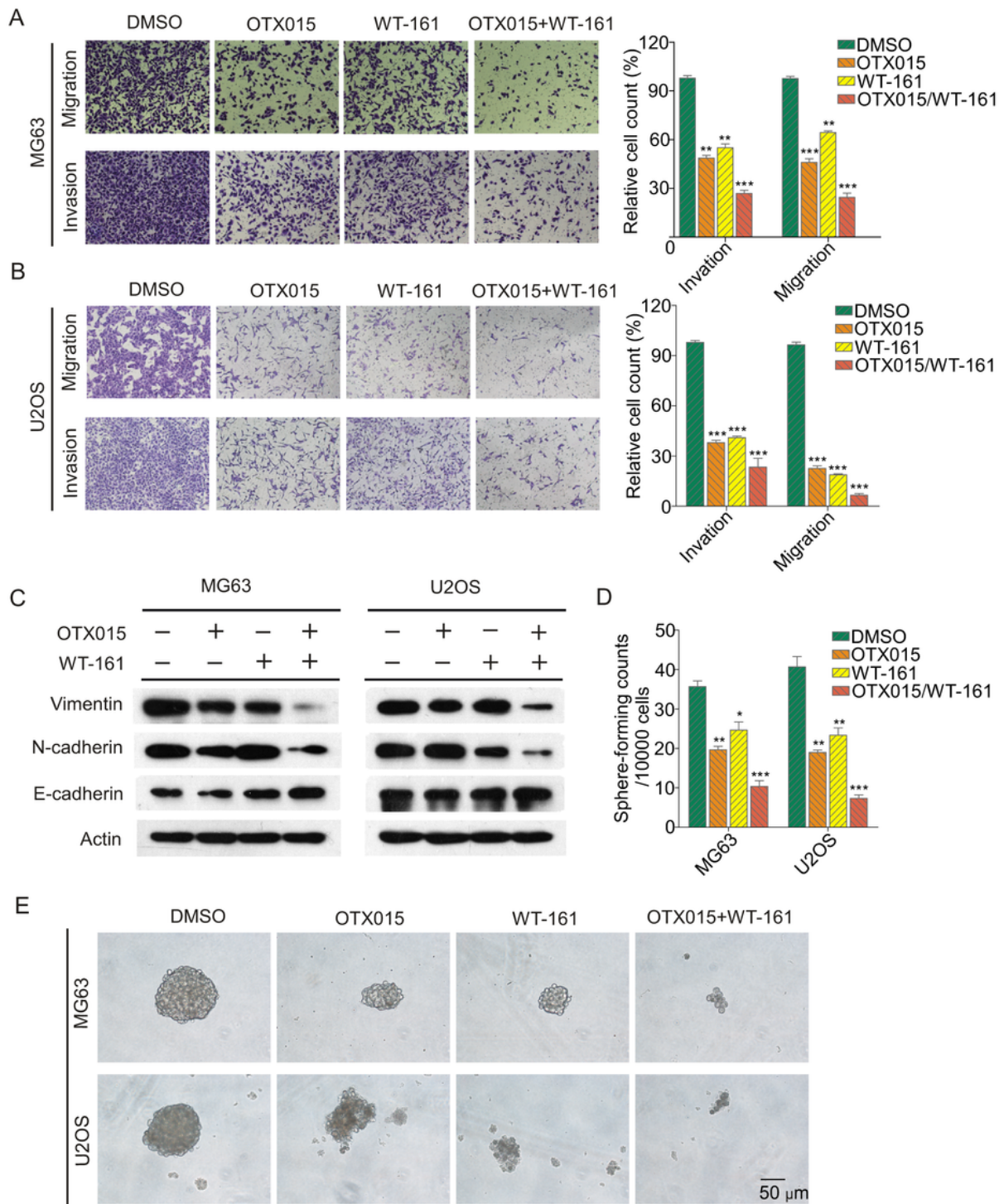
Figure 3

OTX015/WT-161 synergically inhibits metastasis and stem cell like properties of OS cells. (A and B) In transwell tests, the combination of OTX015 and WT-161 inhibited the invasion and migration of MG63/U2OS cells synergistically. Histograms was used to measure the number of OS cells. (C) In the presence of OTX015 or/and WT-161, Western blot analysis of EMT-associated proteins in OS cells was performed. (D and E) MG63/U2OS cells were treated with 4μmol/L OTX015 or/and 4μmol/L WT-161 to

evaluated their sphere - forming capacity. Scale bars represent 50µm. Actin was used as a loading control. Data are means ± SD (n=3). * P <0.05, ** P <0.01 and *** P <0.001, compared to DMSO.
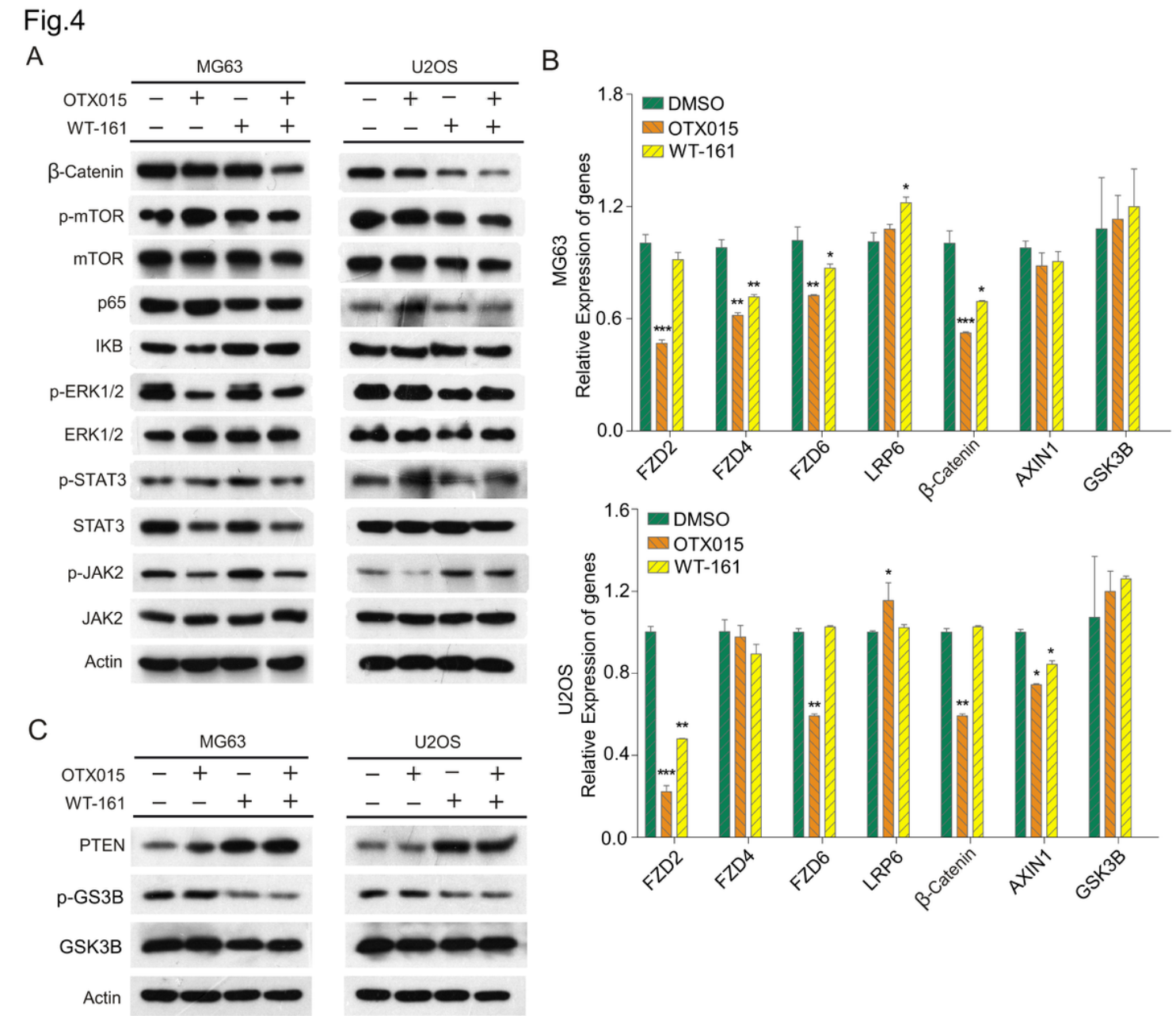
Figure 4

WNT and PTEN/Akt signalling pathways were markedly inhibited by the OTX015/WT-161 combination therapy, resulting in the synergistic anticancer effects. (A) Under various drug conditions, the main proteins of five classical pathways, WNT, mTOR, NF-kB, JAK-STAT and MAPK, were examined by western blotting. (B) The effects of OTX015 or/and WT-161 on multiple genes in the WNT pathway were analyzed by qPCR. (C) The western blotting result of PTEN, p-GSK3β and GSK3β. Actin was used as a loading control. Data are means ± SD (n=3). * P <0.05, ** P <0.01 and *** P <0.001, compared to DMSO. ns = not significant.
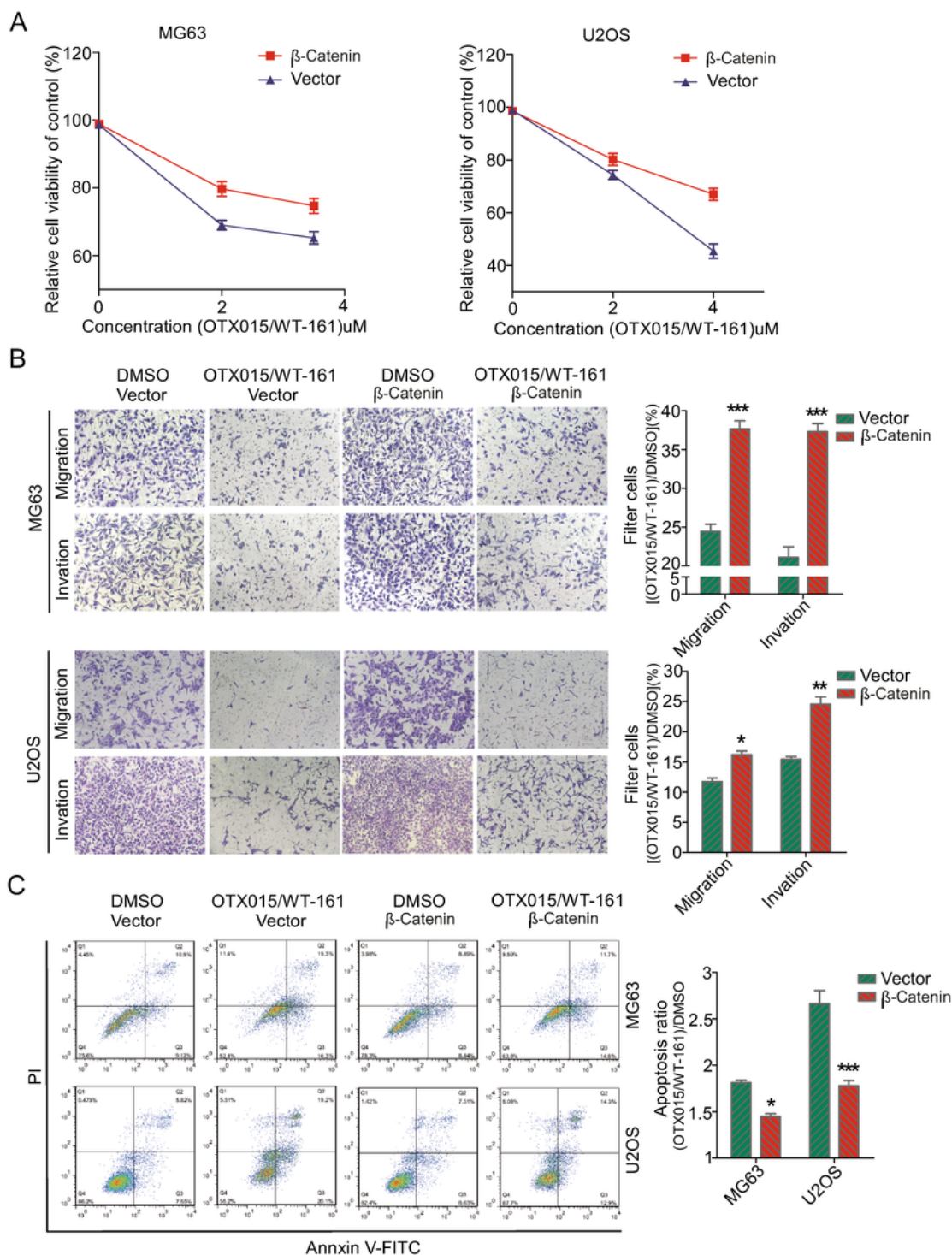
Figure 5

Resistance of overexpressed β-catenin to the tumor-suppressing effects of OTX015/WT-161. (A) CCK-8 was used to detect cell activity after OTX015/WT-161 treated MG63/U2OS with overexpression of the β-catenin gene. (B and C) Between the overexpressed β-catenin group and the control group, the inhibitory impact of OTX015/WT-161 on the migration, invasion, and apoptosis of OS cells was contrasted. The

ratio of cell penetration and apoptosis was visualized by a bar chart. Data are means ± SD (n=3). * P <0.05, ** P <0.01 and *** P <0.001, compared to DMSO. ns = not significant.
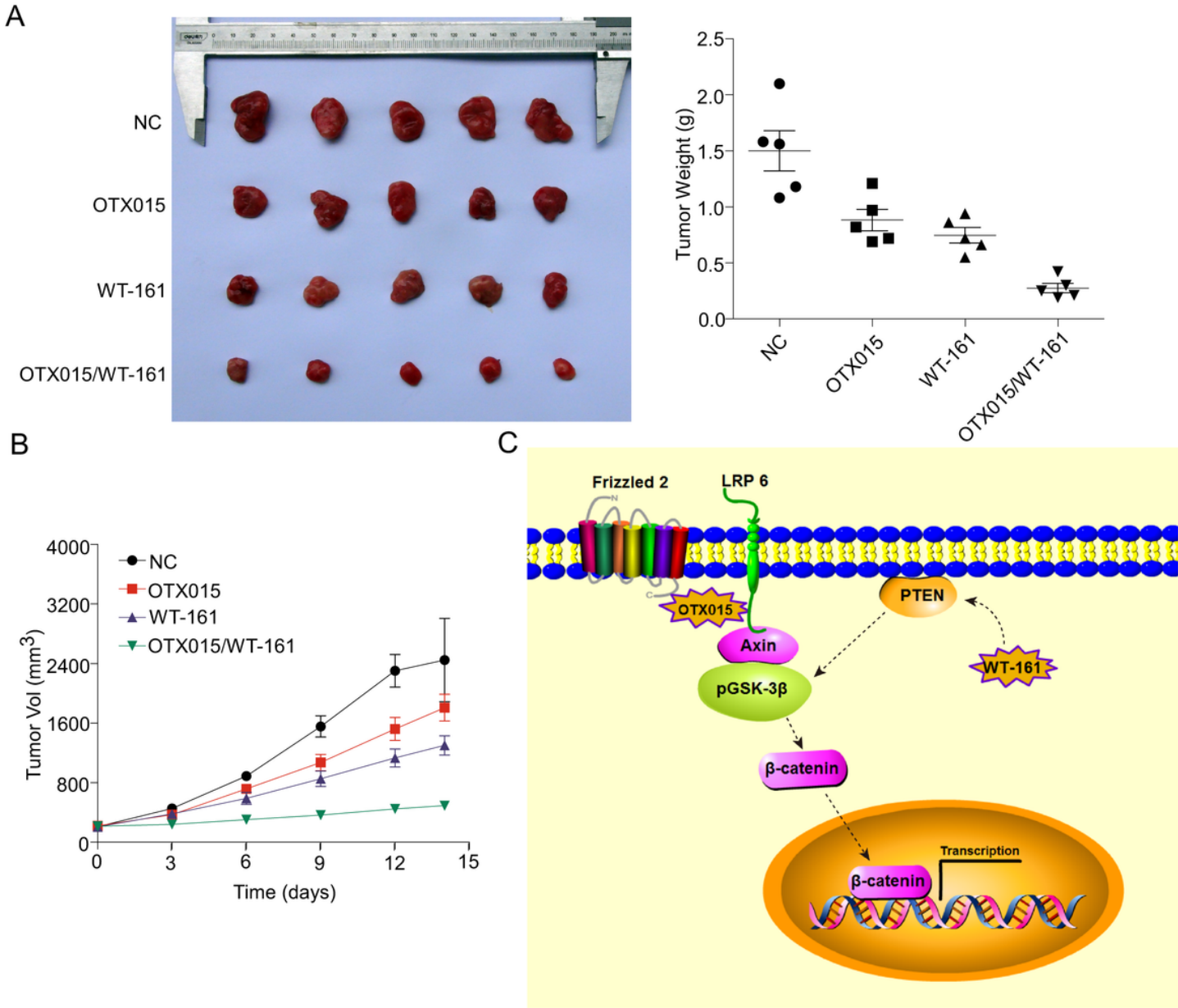
Fig.6

Figure 6

OTX015/WT-161 synergistically inhibit the growth of transplanted tumors in vivo. (A) OS cells were injected into the axilla of nude mice. After three days, four classes of nude mice (NC, OTX015, WT-161, and OTX015+WT-161) were fed with the previous material method. After two weeks of therapy, the morphology and size of xenografts excised from nude mice were seen in this picture. (B) Tumor volume changes every three days in the four treatment groups, as well as the final weight of the tumor. (C) Summary of working model.