# Original Article The STAT3 inhibitor pimozide impedes cell proliferation and induces ROS generation in human osteosarcoma by suppressing catalase expression

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Abstract: Currently, there is a considerable need to develop new treatments for osteosarcoma (OS), a very aggressive bone cancer. The activation of STAT3 signaling is positively associated with poor prognosis and aggressive progression in OS patients. Our previous study reported that the FDA-approved antipsychotic drug pimozide had anti-tumor activity against hepatocellular carcinoma and prostate cancer cells by suppressing STAT3 activity. Therefore, the aim of this study was to investigate the specific effect of pimozide on OS cells and the underlying molecular mechanism. Pimozide inhibited cell proliferation, colony formation, and sphere formation capacities of the OS cells in a dose-dependent manner, inducing GO/G1 phase cell cycle arrest. Pimozide reduced the percentage of side population cells representing cancer stem-like cells and enhanced the sensitivity of OS cells to 5-FU induced proliferative inhibition. In addition, pimozide induced apoptosis of U2OS cells, which showed increased expression of cleaved-PARP, a marker of programmed cell death. Moreover, pimozide suppressed Erk signaling in OS cells. Importantly, pimozide induced ROS generation by downregulating the expression of the antioxidant enzyme catalase (CAT). NAC treatment partially reversed the ROS generation and cytotoxic effects induced by pimozide. CAT treatment attenuated the pimozide-induced proliferation inhibition. The decrease of CAT expression induced by pimozide was potentially mediated through the suppression of cellular STAT3 activity in OS cells. Thus, pimozide may be a novel STAT3 inhibitor that suppresses cellular STAT3 activity to inhibit OS cells or stem-like cells and is a novel potential anti-cancer agent in OS treatment.

Keywords: Pimozide, osteosarcoma, STAT3 inhibitor, ROS, translational medicine

#### Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, predominantly affecting children and young adults [1, 2]. It is a very aggressive cancer and is universally fatal if left untreated. The 5-year survival rate of OS patients is 60%-70%, with no significant improvements in prognosis since the advent of multi-agent chemotherapy [3]. Many patients develop metastases that are difficult to treat and confer a poor prognosis [4]. The most frequent metastases sites are lung and bone [5]. The current available adjuvant chemotherapy provides no survival advantage for patients with pulmonary metastases [6]. Therefore, the development of novel strategies is critical to overcoming the poor prognosis of OS.

Importantly, the Signal Transducer and Activator of Transcription 3 (STAT3) signaling is constitutively active in various human cancers, including osteosarcoma [7, 8]. The activation of STAT3 signaling is positively associated with poor prognosis and aggressive progression in OS patients [9]. STAT3 signaling is involved in tumor initiation, proliferation, and metastasis of cancer cells [10]. Several studies have suggested that inhibiting STAT3 signaling by an antagonist or small interfering RNA induced apoptosis and prevented from metastasis of OS cells [11]. STAT3 signaling activity was blocked by an IL-6neutralizing antibody, leading to reduced proliferation, migration, and invasion of osteosarcoma cells through the downregulation of Survivin and Bcl-xL and the upregulation of pro-apoptotic genes [12]. Thus, it is indicated that targeting STAT3 activation appears to be an effective therapy for the treatment of OS.

Increasing numbers of FDA-approved clinical drugs have been re-purposed to treat various cancers, leading to increased choices and improved effectiveness of anti-tumor therapies [13]. Pimozide, an FDA-approved compound used to clinically treat psychotic diseases, has anti-cancer effects on various tumors and leukemia, such as breast cancer [14], melanoma [15] and myelogenous leukemia [16]. Pimozide mechanically inhibited the activation of STAT5 signaling to decrease the cell viability of leukemia cells [16]. Moreover, our previous study showed that the antipsychotic drug pimozide inhibited the proliferation and self-renewal abilities of cancer cells, including hepatocellular carcinoma and prostate cancer cells, by suppressing STAT3 activity [17, 18]. Furthermore, pimozide was reported to inhibit Wnt/β-catenin signaling and reduce the expression of the stem cell marker EpCAM in hepatocellular carcinoma cells [19]. However, neither the antitumor effect of pimozide nor the inhibition of STAT3 activity by pimozide treatment in OS cells has been fully demonstrated.

The aim of this study was to investigate whether the neuroleptic drug pimozide has anti-cancer effects on osteosarcoma cells through the suppression of STAT3 signaling activity. The results showed that pimozide inhibited U2OS cancer cell proliferation, colony formation, and sphere formation and induced GO/G1 phase cell cycle arrest. In addition, pimozide reduced the percentage of side population and induced the apoptosis of cancer cells. Importantly, pimozide induced the reactive oxygen species (ROS) generation by suppressing catalase expression. Therefore, pimozide may be a novel potential anti-tumor agent in treating OS cells or stem-like cells.

## Materials and methods

## Cell line and cell culture

Human osteosarcoma U2OS cell lines (provided by Medical Centre of Shenzhen University, Shenzhen, China) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) in incubator with 5%  $CO_2$  at 37°C.

## Cell proliferation assay using WST-8

Cell proliferation was assessed by WST-8 colorimetric assay (Dojindo Laboratories, Gaithersburg, MD). Human osteosarcoma cells were plated in 96-well plates with 2,500 cells per well and exposed to the treatment of different concentrations of pimozide for various time intervals (24 h, 48 h, and 72 h). The WST-8 solution was added to each well after indicated time. After incubated at 37°C for another 4 hours, the absorbance was measured at 450 nm using a multi-well plate reader (BioTek, Vermont, USA).

## Cell cycle assay

Cell cycle was determined by propidium iodide (PI, BD Biosciences Clontech, USA) staining. Briefly, equal amounts of cells were seeded in 6-well plates and treated with pimozide at different concentration for 48 h. The cells were harvested, washed with PBS containing 0.1% BSA, and then, cold 70% ethanol was added while mixing the cells. PI buffer (40  $\mu$ g/mL, containing 100  $\mu$ g/mL RNase) was added, and the cells were analyzed by flow cytometry.

## Colony formation assay

Cells with different concentrations of pimozide were plated in 10% FBS medium for 7 days. Cells were stained with 0.5% crystal violet and photographed. The morphology and the number of colonies were observed using the microscope.

# Sphere formation assay

Sphere formation assay was performed as previously described [20]. To establish sphere cultures, single cells were cultured in 200  $\mu$ l of serum-free DMEM/F12 medium (Gibco) supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF, PeproTech), 20 ng/ ml human recombinant basic fibroblast growth factor (bFGF, PeproTech), and B27 (1:50; Gibco). Cells were cultured in ultra-low attachment plates with or without pimozide treatment. After

Name	Genbank accession	Sense sequence (5'-3')	Antisense sequence (5'-3')
HPRT1	NM_000194	GCGTCGTGATTAGTGATGATGA	GCACACAGAGGGCTACAATG
MCL1	NM_021960	GGCAGGATTGTGACTCTC	CTCCTACTCCAGCAACAC
BCL-xL	NM_138578	GCTGGTGGTTGACTTTCTCTC	GGTCTCCATCTCCGATTCAGT
MYC	NM_002467	AGGAACAAGAAGATGAGGAAGA	CTGCGTAGTTGTGCTGATG
STAT3	NM_003150	GGTCTGGCTGGACAATATCATT	GAGGCTTAGTGCTCAAGATGG
SOD1	NM_000454	GGTCCTCACTTTAATCCTCTAT	CATCTTTGTCAGCAGTCACATT
SOD2	NM_000636	TGACAAGTTTAAGGAGAAGC	GAATAAGGCCTGTTGTTCC
CAT	NM_001752	TTAATCCATTCGATCTCACC	GGCGGTGAGTGTCAGGATAG
GPX1	NM_000581	CGCCACCGCGCTTATGACCG	GCAGCACTGCAACTGCCAAGCAG
CHIP FOS	/	ATTGAACCAGGTGCGAATGT	GGAGGGATTGACGGGAACT
CHIP CAT-1	/	GGGTGCTAAAGATCAATTTGTG	CCTCAGGTGCTAGGATTTATCT
CHIP CAT-2	/	ACCAGTCTTGTTTCTCCATTTC	TCATCTCCAAAGTCCACAGTTT

 Table 1. The primers for qPCR

Abbreviations: *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *MCL1*, myeloid cell leukemia 1; *STAT3*, signal transducer and activator of transcription 3; *SOD1*, cytosolic superoxide dismutase; *SOD2*, mitochondrial superoxide dismutase; *CAT*, catalase; *GPX1*, glutathione peroxidase 1.

plating for 14 days, all spheres in each well were photographed.

## Apoptosis assay using Annexin V/PI staining

The percentage of apoptotic cells was measured using an Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions (Keygen, Nanjing, China). Briefly, cells were treated with the indicated concentrations of pimozide. The cells were then collected and used for Annexin V/PI staining. The stained cells were analyzed by flow cytometry (Calibur, BD Biosciences, San Diego, CA, USA).

## Reactive oxygen species assay

ROS generation was detected using the fluorescent dyes 2,7-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) (Beyotime, Jiangsu, China). Briefly, after pimozide treatment for 48 h, cells were washed with medium and were incubated with DCFH-DA (10  $\mu$ M) or DHE (10  $\mu$ M) in DMEM for 30 min at 37°C in the dark. Then, cells were washed twice with DMEM and analyzed using a flow cytometer (FACSCalibur, BD Biosciences, San Diego, CA, USA) at an excitation/emission wavelength of 488/525 nm and 488/610 nm, respectively [21].

## Total RNA extraction and real-time PCR assay

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol and

reversely transcribed into cDNA using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA). The primers are provided in **Table 1**. *HPRT*1 expression was used as an internal control [22].

#### Western blotting assay

Equal amounts of protein from cells sample harvested with RIPA Lysis Buffer were subjected to electrophoresis in SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Merck Millipore, Billerica MA, USA). Blots were detected using primary antibodies against GAPDH (Ambion, Austin, TX, USA), p21, p27, Nanog, SOX2, phospho-STAT3 (Tyr705) (p-STAT3), STAT3, cleaved poly ADP-ribose polymerase (Asp214) (cleave-PARP), Erk, phosphorylated Erk (p-Erk) (Cell Signaling Technology, Beverly, MA, USA), CAT (Beyotime, Jiangsu, China), Cyclin D1, c-Myc and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody binding was detected with an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Rockford, USA) [23].

## STAT3 luciferase reporter assay

Transient transfection was conducted using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. For the luciferase reporter assay, U2OS cells were seeded in 24-well plates and transfected with the STAT3 luciferase reporter plasmid STAT3-



**Figure 1.** The antipsychotic agent pimozide inhibits STAT3 signaling activation and reduced cell proliferation of osteosarcoma cells. U2OS cells were incubated with the indicated doses of pimozide for 48 h before being subjected to Western blot or qPCR assays to analyze cellular STAT3 signaling activity. A. Western blot analysis of the protein expression of p-STAT3 and STAT3. B. U2OS cells were transfected with STAT3 reporter and pRL-TK renilla luciferase reporter plasmids. After 24 h of transfection, the cells were treated with different doses of pimozide for another 48 h. A dual luciferase assay was performed to detect the relative luciferase activity. C. An RT-qPCR assay was performed to analyze the expression of the STAT3 downstream genes *MYC*, *BCL-xL*, *MCL*1, and *STAT3*. D. U2OS cells were treated with various concentrations of pimozide for various times, and cell viability was determined by WST-8 colorimetric assay. The results are shown as the mean values  $\pm$  SD of 3 independent experiments. \*P < 0.05, \*\*P < 0.01, compared with the control.

Luc (pGL3.0, Promega, USA). The cells were collected 48 h after transfection, and the luciferase activities in the cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega, WI, USA). Each transfection was performed in triplicate and repeated at least three times.

#### Chromatin immunoprecipitation assay

Chromatin Immunoprecipitation (ChIP) assay was performed by the Chromatin Immuno-

precipitation Kits (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Fold enrichment method as described was used to analyze the ChIP-qPCR data. The primers are provided in **Table 1**.

## Statistical analysis

The data were presented as the mean ± SD and analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The Student's two-tailed t-test was used to assess statistically significant differences between experimental and control groups. Analysis of variance (ANOVA) was used when more than two data sets were analyzed. followed by an appropriate post hoc test. The level of significance was set at P < 0.05. The statistical results were shown, \*P < 0.05, \*\*P < 0.01.

#### Results

The antipsychotic agent pimozide inhibits STAT3 signaling activation and reduces cell proliferation of osteosarcoma cells

Initially, we examined whether the antipsychotic agent pimozide inhibited STAT3 signaling activity. A Western blot analysis was performed to determine the phosphorylation of STAT3 at tyrosine 705 (pY-STAT3), which refl-

ects STAT3 signaling activation. Figure 1A shows that pimozide reduced the basal expression of pY-STAT3 in U2OS cells in a dose-dependent manner. Moreover, pimozide inhibited STAT3-dependent luciferase activity (Figure 1B). RT-qPCR results demonstrated that pimozide treatment inhibited the transcriptional levels of the STAT3 signaling downstream genes *MYC*, *BCL-xL* and *MCL1* (Figure 1C). Thus, it indicated that U2OS cells showed decreased STAT3 activity after pimozide treatment.

# Anti-cancer activity of pimozide in osteosarcoma cells



# Anti-cancer activity of pimozide in osteosarcoma cells

Figure 2. Pimozide inhibits the colony- and sphere-forming abilities of osteosarcoma cells.U2OS cells were treated with indicated concentrations of pimozide for the indicated times. A. Colony formation assay of U2OS cells treated with pimozide. The numbers of colonies were counted after staining with crystal violet. The graph indicates the number of colonies (a) and the statistical results are displayed (b). B. Sphere formation assay of U2OS cells treated with pimozide. The spheres were imaged under a light microscope (magnification,  $100^{\times}$ ) (a), and the statistical results are shown (b). C. Western blot analysis of the expression of stemness factors in U2OS cells. Cells were treated with the indicated concentrations of pimozide for 48 h. The cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against Nanog, Sox2, and c-Myc.  $\beta$ -actin was used as a loading control. D. Side population analysis of U2OS with indicated treatment. The population rate was shown. Relative representative figures are shown from 1 of 3 independent experiments. E. The cell viability assay was performed to show whether pimozide enhanced the sensitivity of U2OS cells to 5-FU induced proliferative inhibition. The results are from 3 independent experiments. The statistical results are shown, \*P < 0.05, \*\*P < 0.01.

Next, the anti-proliferative effect of pimozide in U2OS cells was detected using a WST-8 colorimetric assay. U2OS cells were exposed to a series of concentrations of pimozide for 24, 48, and 72 h. As shown in **Figure 1D**, pimozide inhibited the proliferation of U2OS cells in both a dose- and time-dependent manner. The IC50 value at 24, 48, and 72 h was 22.16  $\pm$ 2.54, 17.49  $\pm$  1.14 and 13.78  $\pm$  0.34 µM, respectively. Similar results were observed in two other OS cell lines, MG-63 and SW1353 (<u>Supplementary Figure 1</u>). Pimozide, might, therefore have therapeutic potential in treating OS cells.

## Pimozide inhibits the colony- and sphere-forming abilities of osteosarcoma cells

We next examined whether pimozide inhibited the colony- and sphere-forming abilities of U2OS cells. Colony and sphere formation assays showed that pimozide treatment suppressed the numbers of colonies or spheres in a dose-dependent manner (Figure 2A and 2B). After treatment with 10 µM pimozide for 7days, U20S cells showed a 95.34% ± 0.577% decrease in the number of colonies. A sphere formation assay showed that the inhibition rate was 56.24% ± 5.08%, 87.12% ± 2.22% and 98.44% ± 0.22% in U2OS cells treated with 5, 10, and 15 µM pimozide, respectively. Similar results were observed in the two other OS cell lines, MG-63 and SW1353 (Supplementary Figure 2). In addition, a Western blot assay showed that U2OS cells had significantly reduced expression of the stemness genes Nanog, Sox2, and c-Myc after treatment with pimozide for 48 h (Figure 2C).

We then examined the percentage of side population cells in U2OS cells treated with pimozide using flow cytometry. Pimozide treatment decreased the percentage of side population cells from 0.269% to 0.012% (Figure 2D). Moreover, pimozide treatment enhanced the sensitivity of U2OS cells to 5-Fluorouracil (5-FU) induced proliferative inhibition (Figure 2E). Therefore, these results suggested that pimozide decreased the stemness of OS cells, leading to inhibit OS cells or stem-like cells.

## Pimozide induces GO/G1 phase cell cycle arrest in osteosarcoma cells

To determine whether pimozide could induce cell cycle arrest to inhibit cell growth, we analyzed the effect of pimozide on cell cycle distribution using PI staining. After U2OS cells were treated with different concentrations of pimozide for 24 h, the percentage of cells in GO/G1 phase increased significantly in a dose-dependent manner (Figure 3A and 3B). Following treatment with 15 µM pimozide, U2OS cells exhibited a significant increase in the percentage of GO/G1 phase cells, from  $48.23 \pm 2.34\%$ to 64.95 ± 3.18%. In addition, we assessed whether pimozide inhibited the expression of molecular markers associated with GO/G1 phase arrest. A Western blot assay indicated that the p21 and p27 levels were remarkably increased and the Cyclin D1 level was decreased in U2OS cells treated with pimozide compared with control cells (Figure 3C). Besides, the expression of Ki67, a proliferation marker, was decreased from 83.2% to 45.2% in U2OS cells treated with 15 µM pimozide for 48 h (Figure 3D). Thus, these data indicated that pimozide decreased the viability of OS cells in positive association with G0/G1 phase cell cycle arrest.

## Pimozide promotes apoptosis in U2OS osteosarcoma cells

An Annexin-V-FITC/PI double staining assay showed that pimozide promoted the apoptosis



of U2OS cells in a dose-dependent manner (Figure 4A and 4B). Flow cytometry showed

that the total apoptosis rate was 20.22%  $\pm$  2.04%, 25.63%  $\pm$  2.16% and 38.09%  $\pm$  2.18%,



respectively, in U2OS cells treated for 24 h with 5, 10 and 15  $\mu$ M pimozide, while the apoptosis rate of the control cells was 8.12% ± 1.08%. Moreover, the expression levels of apoptosis-related proteins were measured by a Western blot assay (**Figure 4C**). Cells treated with pimozide for 24 h exhibited significantly increased expression of cleaved PARP, which is produced by caspase-3 cleavage during programmed cell death. These results indicated that pimozide promoted apoptosis in U2OS cells.

## Pimozide suppresses the extracellular signalregulated kinase (Erk) signaling to inhibit cell viability of osteosarcoma cells

Previous studies have found cross talk between the STAT3 and Erk pathways [24]. To examine whether pimozide inhibited Erk signaling, a

Western blot analysis was performed to evaluate the expression of Erk phosphorylation (p-Erk). The results showed that pimozide reduced the expression of p-Erk of U2OS cells in a dose-dependent manner (Figure 5A). In addition, a proliferation curve showed that the rate of cell viability was 39.63% ± 1.44% in U2OS cells treated with 15 µM pimozide alone and 38.23% ± 1.39% in cells treated with 15 µM pimozide plus 10 µM U0126, an indirect Erk signaling inhibitor (Figure 5B). There was no statistically significant difference in the antiproliferative effect of pimozide between the groups treated with and without U0126. Similar results were found after treatment with 20 µM pimozide and in U2OS cells treated with pimozide alone or with pimozide plus the direct Erk signaling inhibitor FR180204 (Figure 5D).

#### Anti-cancer activity of pimozide in osteosarcoma cells



**Figure 5.** *Pimozide suppresses the extracellular signal-regulated kinase (Erk) signaling to inhibit cell viability of osteosarcoma cells.* U2OS cells were incubated with the indicated doses of pimozide for 48 h before being subjected to a Western blot assay to analyze cellular Erk signaling. A. Western blot analysis of the level of p-Erk and Erk expression. U2OS cells were treated with the indicated doses of pimozide alone or with the Erk signaling inhibitor U0126 or FR180204 for 72 h before being subjected to a cell viability assay or Western blot assay. B, Da. Cell proliferative analysis of U2OS cells after specific treatments. The results are shown as the mean values  $\pm$  SD of 3 independent experiments. \*P < 0.05, \*\*P < 0.01, compared with the control. C, Db. Western blot analysis of the level of p-Erk and Erk expression.  $\beta$ -actin was used as a loading control.

Erk and p-Erk expression were further examined using a Western blot assay to detect Erk signaling activation. As shown in **Figure 5C**, U2OS cells did not exhibit significantly decreased p-Erk expression after treatment with pimozide alone or with pimozide plus U0126. Similar data were observed in the FR180204 group (**Figure 5D**). According to these results, pimozide could suppress Erk signaling to inhibit the viability of OS cells.

Pimozide produces ROS generation in osteosarcoma cells through inhibiting antioxidant enzyme gene catalase expression

Reports have indicated that ROS production could partially contribute to cell apoptosis and



**Figure 6.** *Pimozide produces ROS generation in osteosarcoma cells through inhibiting antioxidant enzyme gene catalase expression.* U2OS cells were treated with 15  $\mu$ M pimozide alone, 2 mM NAC alone or a combination of the two for 48 h, and the cells were then collected, stained with DCFH-DA or DHE and subjected to flow cytometry. A. ROS analysis of U2OS cells treated as indicated. The intensity or the population rate is shown. Relative representative figures are shown from 1 of 3 independent experiments. B. The expression of the ROS production-related genes SOD1, SOD2, CAT, and GPX1 were analyzed by RT-qPCR. C. Cell viability was determined by a WST-8 colorimetric assay. D. Western blot analysis of the expression of the CAT protein in U2OS cells treated as indicated. E. Western blot analysis of the expression of the CAT protein in U2OS cells treated with a CAT expression plasmid. F. Cell viability was determined by a WST-8 colorimetric assay. G. The conserved STAT3 binding sequence "TTCN<sub>3-4</sub> GAA" was searched in the promoter of the CAT gene, and two putative STAT3-binding sites were found. A ChIP assay was performed with an antibody against STAT3 in U2OS cells. Real-time PCR was then used to measure the enrichment of the putative STAT3-binding sites in the *CAT* gene. The results are shown as the mean values ± SD of 3 independent experiments. \*P < 0.05, \*\*P < 0.01, compared with the control.

affect cell proliferation. We next evaluated the effect of pimozide on the ROS levels in U2OS cells using the fluorescent dyes DCFH-DA and DHE. U20S cells were incubated with 15 µM pimozide for 48 h, and the cells were then collected, stained with DCFH-DA or DHE and subjected to flow cytometry. As expected, the ROS levels increased in U2OS cells after treatment with pimozide (Figure 6A). Moreover, the ROS production-related genes SOD1, SOD2, CAT and GPX1 were analyzed by gPCR to demonstrate the decreased ROS levels induced by pimozide. The results showed that pimozide treatment inhibited the transcription levels of the CAT gene but had little effect on the SOD1. SOD2 and GPX1 genes (Figure 6B). Presumably, the pimozide-induced ROS generation was associated with the expression of the antioxidant enzyme gene CAT in OS cells.

To determine whether the pimozide-induced ROS generation was affected by the presence of antioxidant compounds, we analyzed the pimozide-induced effects in the presence of NAC. NAC treatment partially reversed the level of ROS generation induced by pimozide in U2OS cells (Figure 6A). The cytotoxic effects observed in U2OS cells treated with pimozide were decreased in the presence of NAC (Figure 6C). In addition, pimozide reduced the expression levels of the CAT protein (Figure 6D). Moreover, we examined whether increased CAT expression reversed the pimozide-induced inhibitory effect on OS cells. A Western blot analysis revealed increased expression of the CAT protein in U2OS cells transfected with CAT overexpression plasmid (Figure 6E). CAT treatment attenuated the pimozide-induced proliferation inhibition (Figure 6F). These results suggested that pimozide induced ROS generation in OS cells by inhibiting the expression of the antioxidant enzyme gene CAT.

To determine whether CAT is a direct target of STAT3 signaling, we searched for STAT3-binding sites in the regulatory regions of the *CAT* gene and found two putative STAT3-binding sites (**Figure 6G**). We then performed a ChIP analysis of STAT3 binding to the promoter of the *CAT* gene in OS cells and found that STAT3 was able to bind the *CAT* promoter. These data indicated that the decrease in CAT expression induced by pimozide was potentially mediated through the suppression of cellular STAT3 activity in OS cells.

# Discussion

Drug discovery and development for the clinical treatment of OS has been taken seriously. Drug repurposing, new applications for existing or abandoned pharmacotherapies, is one of the most important strategies used to treat cancer cells [25]. For example, metformin, an anti-diabetic drug, can inhibit cancer cell growth in vitro and in vivo, functioning as an anti-cancer drug [26]. Clinically approved drugs used to treat a new disease typically have two important advantages. One is that the drug often has low toxicity to cells in vivo. The other is that the drug has clear molecular targets or treatment for specific diseases [27]. Drug repurposing brings new advances in drug discovery and development, making pharmaceutical research more predictable and reliable and less costly. In addition, since the common chemotherapeutics cannot obviously improve the disease conditions of OS patients, it is meaningful that an FDA approved drug is repurposed for the treatment of OS. In this study, we found that pimozide, a clinical drug approved by the FDA to treat neuroleptic disorders, has anti-cancer effects in OS cells, reducing the proliferation ability and increasing the apoptosis of the osteosarcoma cells in vitro. The antipsychotic agent pimozide may, therefore, be a potential and novel curative for patients with osteosarcoma.

Although pimozide can induce cardiac toxicity. it has not been reported to have adverse effects on other normal functional cells, such as hepatic or hematopoietic cells. A previous study showed that pimozide has almost no effect on hematopoietic progenitors derived from healthy donors [16]. Pimozide treatment was well tolerated in a mouse model with no significant effects on body weight [16]. Similar results were observed in our previous study [16]. The dose (25 mg/kg) of pimozide used to treat cancer in-vivo is relatively low compared to the commonly used dose for treating CNS diseases. Additionally, according to the previous study, the precise lethal dose of pimozide in humans is unknown. The oral LD50 is 228 mg/kg in mice, 5120 mg/kg in rats, 188 mg/ kg in guinea pigs, and 40 mg/kg in dogs (DrugBank: pimozide (DB01100)). Therefore, pimozide may also be a safe drug for treating OS cells or stem-like cells.

In our previous study, we reported that the neuroleptic drug pimozide had anti-tumor activity against hepatocellular carcinoma and prostate cancer cells through the suppression of STAT3 activity [17, 18]. Numerous studies have demonstrated constitutive activation of STAT3 in a wide variety of human malignancies, including osteosarcoma [9, 12]. Aberrantly STAT3 activation contributes to oncogenesis by preventing apoptosis, inducing cell proliferation, angiogenesis, invasion, and metastasis as well as suppressing anti-tumor immune responses [28, 29]. Since STAT3 signaling is important for OS cell proliferation, we hypothesized that pimozide may inhibit the proliferation of OS cells and reduce STAT3 activity. Similarly, pimozide also reduced the basal expression of pY-STAT3 in U2OS cells in a dose-dependent manner, inhibited the transcription levels of the STAT3 signaling downstream genes MYC, BCL-xL and MCL1 and weakened cellular STAT3 reporter luciferase activity. Pimozide also inhibited U2OS cell proliferation, colony formation, and sphere formation and induced GO/G1 phase cell cycle arrest. In addition, pimozide reduced the percentage of side population cells. Our present study showed that pimozide, as a STAT3 inhibitor, inhibited the proliferation of OS cells or stem-like cells. Thus, these results further suggest that pimozide may be a novel STAT3 inhibitor that can be used to suppress cellular STAT3 activation for anti-cancer treatment.

In addition, our results showed that pimozide could suppress Erk signaling to inhibit the viability of osteosarcoma cells, reducing the expression of p-Erk in a dose-dependent manner, while no statistically significant difference in the anti-proliferative effect of pimozide was observed between cells treated with and without U0126. Because there is cross talk between the STAT3 and Erk pathways, it is likely that pimozide is a potential STAT3 activity inhibitor, leading to its anti-cancer effects in cancer cells. The mechanism will further demonstrate why pimozide has an anti-cancer effect on cancer cells, paving the way for the translational application of pimozide in cancer therapy.

Previous studies show that pimozide is a wellknown antagonist of serotonin 5HT7 receptors ( $K_i = 0.5$  nM) for anti-depressant effect and of dopamine receptor D2 (D2R) ( $K_i = 0.33$  nM) for treating schizophrenia [30]. Pharmacologic antagonists pimozide reduced proliferation of pancreatic cancer cells by inhibiting DRD2 expression [30]. No reports have been demonstrated how 5HT7 receptor or DRD2 to activate STAT3 signaling in OS cells. In future, it needs to determine whether 5HT7 or DRD2 expression is associated with STAT3 activation of OS cells.

Our current study is to provide novel insight that pimozide produced ROS generation in cancer cells through inhibiting antioxidant enzyme gene catalase expression. ROS are closely related to carcinogenesis and play an important role in cancer [31, 32]. Previous studies have shown that ROS may be involved in multiple tumorigenesis processes, including tumor initiation and transformation, tumor progression, tumor promotion, tumor angiogenesis, and tumor metastasis [33, 34]. ROS are generated by both mitochondria and NADPH oxidases and scavenged by antioxidant enzyme [35]. By screening the expression of antioxidant enzyme genes, we found that pimozide treatment inhibited CAT gene expression but had little effect on the expression of SOD1, SOD2, and GPX1. Moreover, CAT treatment attenuated the pimozide-induced proliferation inhibition. Presumably, the pimozide-induced ROS generation was associated with the expression of the antioxidant enzyme gene *CAT* in cancer cells. Mechanically, pimozide inhibited cellular STAT3 activity to reduce CAT expression in OS cells.

In conclusion, this study illustrated that the neuroleptic drug pimozide has anti-tumor activity against OS cells or stem-like cells. Moreover, pimozide as a STAT3 inhibitor can suppress Erk signaling and promote ROS production possibly through reducing the expression of the antioxidant enzyme gene *CAT*. Thus, the antipsychotic agent pimozide may be a potential and novel therapeutic for OS patients. Of note, the development of new pimozide derivatives with stronger anti-cancer effects but lower side effects is urgently needed.

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# Disclosure of conflict of interest

None.

## Authors' contribution

JJ Chen and G Chang made substantial contributions to conception and design of the study and to data interpretation. N Cai and W Zhou performed the real-time PCR analysis, western blot analysis and preformed statistical analysis. LL Ye and J Chen carried out the experiments of cell culture and cellular proliferation. QN Liang made AnnexinV/PI staining using FASC. N Cai, W Zhou, JJ Chen and G Chang wrote the manuscript. All authors reviewed and approved the manuscript.

## Abbreviations

OS, osteosarcoma; STAT3, the Signal Transducer and Activator of Transcription 3; ROS, reactive oxygen species; PI, propidium iodide; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DCFH-DA, 2,7-dichlorofluorescein diacetate; DHE, dihydroethidium; SP, side population; 5-FU, 5-fluorouracil; ChIP, chromatin immunoprecipitation; pY-STAT3, phosphorylation of STAT3 at tyrosine 705; Erk, extracellular signal regulated kinase; p-Erk, Erk phosphorylation; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *MCL1*, myeloid cell leukemia 1; *STAT3*, signal transducer and activator of transcription 3; *SOD1*, cytosolic superoxide dismutase; *SOD2*, mitochondrial superoxide dismutase; *CAT*, catalase; *GPX1*, glutathione peroxidase 1.

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**Supplementary Figure 1.** *Pimozide inhibits cell viability of osteosarcoma cell line SW1353 and MG-63.* OS cells SW1353 (A) and MG-63 (B) were treated with various concentrations of pimozide for different times, and cell viability was determined by WST-8 colorimetric assay. The results are shown as the mean values  $\pm$  SD of 3 independent experiments. \*P < 0.05, \*\*P < 0.01, compared with the control.



**Supplementary Figure 2.** *Pimozide inhibits the capacity of colony- and sphere-forming in osteosarcoma SW1353 and MG-63 cells.* SW1353 and MG-63 cells were treated with indicated concentrations of pimozide for the indicated times. Colony formation assay of SW1353 (A) and MG-63 (B) cells treated with pimozide. The numbers of colonies were counted after staining with crystal violet. The graph indicates the number of colonies (a). And the statistical results are displayed (b). Sphere formation assay of U2OS cells treated with pimozide. The spheres were imaged under a light microscope (magnification,  $100 \times$ ) (c), and the statistical results are shown (d).The results are from 3 independent experiments. The statistical results are shown, \*P < 0.05, \*\*P < 0.01.