Research Article

Bavachin Induces Ferroptosis through the STAT3/P53/SLC7A11 Axis in Osteosarcoma Cells

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Ferroptosis is a new form of regulated cell death, which is mediated by intracellular iron and is quite different from other forms of cell death, such as apoptosis, autophagy, or necrosis [5]. Intracellular iron reacts with H2O2 through Fenton reaction (Fe2+ + H2O2 → Fe3+ + HO· + OH-) to generate many reactive oxygen species (ROS) and trigger lipid peroxidation (LP) to induce ferroptosis [6]. Aberrant iron metabolism, ROS generation, and abnormal LP are the hallmarks of ferroptosis [5, 7]. Morphologically, ferroptotic cells demonstrate shrunken mitochondria, higher density of the mitochondrial membrane, and reduced mitochondrial cristae [8]. Additionally, ferroptosis can be inhibited by iron chelators or antioxidants and activated by some small compounds (erastin), or by inhibiting glutathione peroxidase-4 (GPX4) [9]. The cystine/glutamate antiporter system Xc−, which is composed of two subunits, SLC7A11 (light chain) and SLC3A2 (heavy chain), is closely linked to ferroptosis [10]. SLC7A11, which transports cystine into the cells, enhances glutathione (GSH) synthesis, further promoting the inhibition of ferroptosis by GPX4. P53, as an upstream mediator of SLC7A11, mediates the repression of

1. Introduction

Osteosarcoma (OS) is the most frequent bone tumour in children and adolescents, in addition to being the most common aggressive malignancy originating from mesenchymal cells [1, 2]. Although neoadjuvant chemotherapy combined with surgery and postoperative chemotherapy has been used to treat OS, the five-year survival rate of OS is not satisfactory on account of development of early metastases and chemotherapy resistance [3, 4]. Thus, it is imperative to find new targets and pharmaceuticals to improve the prognosis.

Ferroptosis is a new form of regulated cell death (RCD) that is mediated by intracellular iron and is quite different from other forms of cell death, such as apoptosis, autophagy, or necrosis [5]. Intracellular iron reacts with H2O2 through Fenton reaction (Fe2+ + H2O2 → Fe3+ + HO· + OH-) to generate many reactive oxygen species (ROS) and trigger lipid peroxidation (LP) to induce ferroptosis [6]. Aberrant iron metabolism, ROS generation, and abnormal LP are the hallmarks of ferroptosis [5, 7]. Morphologically, ferroptotic cells demonstrate shrunken mitochondria, higher density of the mitochondrial membrane, and reduced or diminished mitochondrial cristae [8]. Additionally, ferroptosis can be inhibited by iron chelators or antioxidants and activated by some small compounds (erastin), or by inhibiting glutathione peroxidase-4 (GPX4) [9]. The cystine/glutamate antiporter system Xc−, which is composed of two subunits, SLC7A11 (light chain) and SLC3A2 (heavy chain), is closely linked to ferroptosis [10]. SLC7A11, which transports cystine into the cells, enhances glutathione (GSH) synthesis, further promoting the inhibition of ferroptosis by GPX4. P53, as an upstream mediator of SLC7A11, mediates the repression of
SLC7A11 to initiate ferroptosis in tumour cells [11]. In addition to breast cancer, colorectal cancer, and anaplastic thyroid cancer [12–14], ferroptosis also exerts anticancer effects in OS [15, 16]. Some clinical drugs including sulfasalazine and sorafenib have proven to induce ferroptosis to trigger anticancer effects [17], via inhibiting the system Xc [18, 19]. Therefore, activating P53 to downregulate SLC7A11/GPX4 to trigger ferroptosis could be a potential method to inhibit progression of OS cells.

Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family and is a vital transcription factor involved in inflammation and tumour progression [20]. Classical STAT3 activation involves the phosphorylation of STAT3 at Tyr705 (p-STAT3 (705)), which interacts with and inhibits P53 [21]. Wang et al. discovered that high expression of STAT3 is relevant to increased malignancy and poor prognosis [22]. As described above, it is critical to inactivate STAT3 to halt tumour progression.

Flavonoids have multiple biological functions, especially anticancer effects induced by the prooxidant activity [23]. Amentoflavone inhibits glioma cells via inducing ROS accumulation and ferroptosis [24]. Robustaflavone also induces ferroptosis by increasing ROS and lipid peroxidation in MCF-7 cells [25]. Bavachin belongs to flavonoids and is a bioactive compound extracted from the fruit of Psoralea corylifolia and displays various functions including anti-inflammatory, lipid-lowering, and cholesterol-reducing effects [26]. A previous study showed that bavachin stimulates osteoblast differentiation by activating the Wnt pathway and is considered an estrogen mimetic with 10% foetal bovine serum (MRC, China).

2. Materials and Methods

2.1. Reagents. Bavachin was purchased from MCE (China), and pifithrin-α (PFT-α), deferoxamine (DFO), ferrostatin-1 (Fer-1), and liproxstatin-1 (Lip-1) were purchased from Topscience (China). Vitamin E was purchased from Beyotime (Shanghai, China). Rabbit polyclonal anti-transferrin receptor antibody (TFRC, AF5343, 1:1000), rabbit polyclonal anti-divalent metal transporter-1 antibody (DMT1, DF12740, 1:1000), rabbit polyclonal anti-ferritin light chain antibody (FTL, DF6604, 1:1000), rabbit polyclonal anti-ferritin heavy chain antibody (FTH, DF6278, 1:1000), rabbit polyclonal anti-SLC7A11 antibody (DF12509, 1:1000), rabbit polyclonal anti-P53 antibody (AF0879, 1:1000), rabbit polyclonal anti-STAT3 antibody (AF6294, 1:1000), rabbit polyclonal anti-p-STAT3 (705) antibody (AF3293, 1:1000), and rabbit polyclonal anti-glutathione peroxidase-4 antibody (GPX4, DF6701, 1:1000) were purchased from Affinity Bioscience (China). HRP goat anti-rabbit IgG was purchased from Earthox (USA).

2.2. Cell Culture. The human OS cell lines MG63 and HOS were purchased from Procell (China). The cells were cultured in a humidified CO2 incubator at 37°C and 5% CO2 and grown in DMEM/high-glucose (Gibco, USA) supplemented with 10% foetal bovine serum (MR, China).

2.3. Cell Viability. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (APEXBio, China). According to the protocol, the absorbance values of the samples were read at 450 nm using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). Subsequently, the cell death ratio was calculated using the formula: cell death ratio (%) = (A_sample − A_blank)/(A_control − A_blank) × 100.

2.4. Transmission Electron Microscope (TEM). MG63 and HOS cells were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at room temperature for 24 h. Then, the cells were fixed in 2% osmium tetroxide in PBS at room temperature for 1 h. Next, the samples were dehydrated with a graded series of ethanol solutions (50% to 100%). Subsequently, the samples were embedded in epoxy resin and sectioned into ultrathin sections (60 nm). Finally, the ultrathin sections were deembedded with uranyl acetate (1%) and lead citrate (0.1%) and observed and photographed using an H-7500 TEM (Hitachi, Japan).

2.5. Mitochondrial Membrane Potential (MMP) Detection. MMP was measured using JC-1 assay kit (Beyotime, China). According to the protocol, the cells were stained with the JC-1 probe and observed under a fluorescence microscope (DMIL4000, Leica, China). Green fluorescence indicates a low MMP, whereas red fluorescence indicates a high MMP.

2.6. Ferrous Iron Assay. Cellular ferrous iron levels were detected using FerroOrange (Dojindo, China). According to the protocol, the cells were incubated with FerroOrange for 0.5 h. Fluorescence intensity (Ex: 543 nm, Em: 580 nm) was measured using a fluorescence microplate reader (Varioskan &LUX, Thermo Fisher, China). The ferrous iron levels were finally expressed as a ratio to the fluorescence intensity value of the control.

2.7. ROS Assay. Cellular ROS levels were measured using a commercial ROS assay kit (Beyotime, China). According to the protocol, the cells were loaded with a 2,7-dichlorodihydrofluorescein diacetate probe (10 μM). The cells were then incubated at 37°C for 20 min. Finally, the fluorescence intensity (Ex: 488 nm, Em: 525 nm) was measured using a fluorescence microplate reader (Varioskan &LUX, Thermo Fisher, China).
2.8. GSH Assay. GSH levels were assayed using the GSH assay kit (Nanjing Jiancheng, Nanjing, China). The harvested cells were crushed by sonication to obtain the supernatant used for measuring the GSH levels in a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). The levels of GSH were expressed as a ratio to the absorbance value of the control cells assessed at 405 nm.

2.9. Malondialdehyde (MDA) Assay. MDA levels were assayed using the MDA Assay Kit (Beyotime, China). First, the cells were lysed using the RIPA Lysis Buffer (Servicebio, China) with a protease inhibitor and centrifuged at 100,000 g for 10 min at 4°C to obtain the supernatant. The protein concentration of the samples was measured using the BCA Protein Assay Kit (Beyotime, China). Subsequently, the collected supernatant (0.1 ml) was added to thiobarbituric acid (0.2 ml) and incubated at 100°C for 15 min. Finally, the samples were read at 532 nm using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). The MDA levels were expressed as a ratio to the absorbance value of the control.

2.10. Western Blotting Analysis. The cells were lysed and homogenized using RIPA Lysis Buffer (Servicebio, China) with a protease inhibitor. The protein concentration of the samples was measured using the BCA Protein Assay Kit (Beyotime, China). The proteins were added to the loading buffer (Beyotime, China) and denatured in boiling water.
for 5 min. Each protein sample (40 μg) was loaded onto an SDS-PAGE (10%–12%) gel and transferred to PVDF membranes. The membranes were blocked with NcmBlot Blocking Buffer (NCM Biotech, China) and incubated with the primary antibodies at 4°C overnight. The membranes were subsequently incubated with the secondary antibody at room temperature for 1.5 h. Finally, the bands were developed using a HyperSignal ECL kit (4A Biotech, China).

2.11. Cell Transfection. For evaluating the overexpression of STAT3 and SLC7A11, an overexpressing plasmid along with a negative control (NC) plasmid was purchased from Vigene Biosciences (China). Briefly, cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen, China). After 48h, the cells were subjected to subsequent experiments.

2.12. Statistical Analysis. Data are presented as means ± SD (n = 3). One-way ANOVA and Tukey’s multiple comparisons test were used to analyze the data. All statistical analyses were performed using GraphPad Prism 7 (https://www.graphpad.com/). Statistical significance was set at p < 0.05.

3. Results

3.1. Bavachin Inhibits OS Cell Viability. To evaluate the effects of bavachin on OS cells, MG63 and HOS cells were treated with bavachin (5 μM, 10 μM, 20 μM, 40 μM, and...
80 μM) for 24 h, 48 h, and 72 h, and then cell viability was measured using the CCK8 assay. As shown in Figure 1(b), bavachin inhibited the viability of both MG63 and HOS cells in a concentration- and time-dependent manner. According to CCK8 assay, the half-maximal inhibitory concentration (IC$_{50}$) of bavachin at 24 h was determined. The IC$_{50}$ values for MG63 and HOS cells were 42.32 μM and 34.2 μM, respectively. Therefore, 40 μM of bavachin concentration was selected for the following experiments.

3.2. Bavachin Induces Ferrous Iron Accumulation to Initiate OS Cell Death. To evaluate whether bavachin affects ferrous iron accumulation, we assessed the expression of ferrous iron in OS cells treated with bavachin (10 μM, 20 μM, and 40 μM) for 24 h. Compared to the control group, bavachin increased the ferrous iron concentration in a concentration-dependent manner (Figure 2(a)). As illustrated in Supplementary Figures 1(a) and 1(b), ferrous iron levels reached maximum at 24 h of 40 μM bavachin stimulation. Furthermore, OS cells pretreated with 100 μM of DFO (iron chelator) for 1 h recovered from bavachin-induced (40 μM, 24 h) cell death (Figure 2(b)). Finally, western blotting was used to explore the mechanism of ferrous iron accumulation induced by bavachin. As shown in Figures 3(c) and (d), bavachin treatment increased TFRC and DMT1 expressions and decreased FTH and FTL expression in a concentration-dependent manner (10 μM, 20 μM, and 40 μM).

3.3. Bavachin Induces Ferroptosis in OS Cells. To demonstrate bavachin-induced ferroptosis in OS cells, we looked into the morphology of the treated cells. TEM showed that...
Figure 4: Bavachin induces GSH depletion and lipid peroxidation accumulation in osteosarcoma (OS) cells. (a, b) Bavachin induces GSH depletion in a dose-related manner over 24 h. (c, d) Bavachin greatly induces ROS generation in OS cells over 24 h. (e, f) Bavachin increases MDA accumulation in a dose-related manner. (g, h) Western blotting analysis shows the expression of p-STAT3, STAT3, P53, SLC7A11, and GPX4 in OS cells treated with bavachin (10 μM, 20 μM, and 40 μM) for 24 h. *p < 0.05 vs. the control group, **p < 0.01 vs. the control group, ***p < 0.001 vs. the control group.
Figure 5: Continued.
was observed in the bavachin (40 μM) gradually as bavachin concentration (10 μM) stress in OS cells. It was found that the level of GSH declined to determine whether bavachin induces oxidative stress in OS cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells.

3.4. Bavachin Induces GSH Depletion and LP Accumulation in OS Cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells. It was found that the level of GSH declined gradually as bavachin concentration (10 μM, 20 μM, and 40 μM) increased over 24 h (Figures 4(a) and 4(b)). However, ROS levels were remarkably upregulated by bavachin stimulation (Figures 4(c) and 4(d)). Moreover, ROS levels reached their highest values at 24 h of 40 μM bavachin stimulation (Supplementary Figure 1(c) and 1(d)). As illustrated in Figures 4(e) and 4(f), bavachin markedly elevates the MDA level, which is a marker of LP, in a concentration-dependent manner for 24 h. To further analyze the molecular mechanism of bavachin-induced LP in OS cells, western blotting was performed to detect the expression of related proteins. The results demonstrated that bavachin inhibited GPX4, SLC7A11, and p-STAT3 expressions and decreases P53 expressions in OS cells (Figures 4(g) and 4(h)).

3.5. SLC7A11 Overexpression Alleviates Bavachin-Induced Ferroptosis in OS Cells. OS cells were transfected with the SLC7A11 overexpressing plasmid to examine whether SLC7A11 overexpression could rescue bavachin-induced ferroptosis. As indicated in Figures 5(a)–5(c), SLC7A11 upregulation increases the decline in GPX4 expression and GSH content induced by bavachin (40 μM, 24 h). SLC7A11 overexpression attenuates the bavachin-induced accumulation of ROS and MDA (Figures 5(d) and 5(e)). Importantly, SLC7A11 overexpression rescues bavachin-induced OS cell death (Figure 5(f)). Overall, SLC7A11 overexpression inhibits bavachin-induced ferroptosis in OS cells.

3.6. P53 Inactivation Upregulates SLC7A11 and Alleviates Bavachin-Induced Ferroptosis in OS Cells. Our previous experiments (Figure 4) showed that bavachin upregulated P53 and downregulated SLC7A11-induced ferroptosis in OS cells. To investigate the regulatory relationship between P53 and SLC7A11, OS cells were pretreated with 5 μM PFT-α, a P53 inhibitor, for 0.5 h followed by treatment with 40 μM of bavachin for 24 h. As shown in Figures 6(a) and 6(b), PFT-α inactivates P53 to upregulate the expression of SLC7A11 and GPX4 induced by bavachin. PFT-α also restores bavachin-induced GSH consumption (Figure 6(c)). Meanwhile, PFT-α significantly reduced bavachin-induced ROS and MDA accumulation (Figures 6(d) and 6(e)). Subsequently, PFT-α rescued bavachin-induced ferroptosis in OS cells (Figure 6(f)). In summary, these results show that P53 negatively regulates SLC7A11 and plays a promoting role in bavachin-induced ferroptosis in OS cells.

3.7. P-STAT3 Activation Downregulates P53 Expression to Rescue Bavachin-Induced Ferroptosis in OS Cells. To evaluate whether STAT3 inhibits P53 expression to alleviate bavachin-induced ferroptosis in OS cells, OS cells were transfected with a STAT3 overexpressing plasmid, followed by treatment with 40 μM bavachin for 24 h. As shown in Figures 7(a) and 7(b), compared with that in the NC group, p-STAT3 was activated, although the p-STAT3/STAT3 ratio was unchanged. p-STAT3 activation inhibited the upregulation of P53 expression induced by bavachin, while increased GPX4 expression suppressed by bavachin. p-STAT3 activation recovered GSH depletion (Figure 7(c)) and decreased

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**Figure 5:** SLC7A11 overexpression alleviates bavachin-induced ferroptosis in osteosarcoma (OS) cells. OS cells were transfected with a SLC7A11 overexpressing plasmid and treated with bavachin (40 μM, 24 h). (a, b) Western blotting demonstrates the relative levels of SLC7A11 and GPX4 protein expression. (c) Overexpression of SLC7A11 restores bavachin-induced GSH depletion in OS cells. (d, e) Overexpression of SLC7A11 reduces bavachin-induced ROS and MDA accumulation. (f) Overexpression of SLC7A11 recovers bavachin-induced ferroptosis in OS cells. *p < 0.05, **p < 0.01, and ***p < 0.001.
Figure 6: Continued.
the accumulation of ROS and MDA, which is stimulated by bavachin (Figures 7(d) and 7(e)). Moreover, p-STAT3 upregulation rescued bavachin-induced ferroptosis in OS cells (Figure 7(f)). As described above, p-STAT3 activation inhibits P53 expression and rescues bavachin-induced ferroptosis in OS cells.

4. Discussion

Our study revealed that bavachin could induce OS cell death, which was reversed by iron chelator (DFO) and ferroptosis inhibitors (Fer-1, Lip-1, and Vit E). Moreover, bavachin reduced the MMP and led to mitochondrial shrinkage, increased mitochondrial membrane density, and reduced mitochondrial cristae in OS cells. Furthermore, bavachin elevated intracellular ferrous iron levels by increasing TFRC and DMT1 expression and decreasing FTH and FTL expressions. Bavachin also reduced SLC7A11 and GPX4 expression and DMT1 expression and decreasing FTH and FTL expression of TFRC could increase iron levels to increase ferroptosis [34]. Although the expression of FTH and DMT1 is also a positive regulator of iron accumulation, leading to ferroptosis [36]. Consistent with previous studies, our findings show that TFRC and DMT1 expressions are positively related to ferroptosis induced by bavachin. Additionally, ferritin, composed of FTL and FTH, also serves as a critical mediator of iron storage and a regulator of iron homeostasis. Published studies have revealed that ferritin has a two-sided function, ferritinophagy-degrading ferritin to release Fe^{2+} that is involved in ferroptosis progression [9].

Triggering LP, which is described as a process by which ROS attacks polyunsaturated fatty acids to cause dehydrogenation, is an essential event that drives ferroptosis [39]. Both ROS, generated from iron in the Fenton reaction, and MDA, the final product of LP, are contributing factors for ferroptosis activation [40]. Bavachin has been demonstrated to promote ROS generation via suppressing the MMP to trigger HepG2 cell apoptosis [28]. However, whether bavachin induces MDA has not been reported in the literature. Similarly, our results suggest that bavachin elevates the level of ROS in a dose-dependent manner and suppresses the MMP. Moreover, we found that bavachin distinctly increased the MDA content to promote ferroptosis.

The SLC7A11/GPX4 axis efficiently protects cells from ferroptosis [41]. SLC7A11, a key subunit of the system Xc-, is responsible for transporting cysteine, which is reduced to cysteine for synthesis of GSH. As a vital nonenzymatic antioxidant, GSH not only scavenges free radicals but also acts as a
Figure 7: Continued.
cofactor of the GPX4-mediated reduction reaction of lipid hydroperoxides (LOOH). Inhibition of SLC7A11/GPX4, which is the most important antioxidant enzyme in the body, can trigger ferroptosis, while activation of SLC7A11/GPX4 facilitates the suppression of ferroptosis [42]. Shi et al. reported that tirapazamine induced ferroptosis in OS cells via downregulation of SLC7A11 and GPX4 expression, whereas the phenotype of cells induced by tirapazamine was reversed after overexpression of SLC7A11 [43]. Lin et al. also found that EF24, a synthetic drug, triggered ferroptosis in OS cells via inhibiting GPX4 [16]. Similarly, our results showed that bavachin led to ferroptosis in OS cells with accumulation of ROS and MDA, GSH depletion, and downregulation of GPX4 expression, while overexpression of SLC7A11 rescued OS cells from ferroptosis caused by bavachin. Therefore, we believe that bavachin triggers OS cell ferroptosis via the SLC7A11/GPX4 axis.

**P53 is known as a tumour suppressor protein and can inhibit tumour cells by triggering ferroptosis [44]. Jiang et al. discovered that P53 induced ferroptosis by negatively regulating SLC7A11 to inhibit cystine transport [11]. However, a few studies have reported a contrasting view about the function of P53 in ferroptosis. In colorectal cancer cells, P53 inhibited LP and ferroptosis by binding to DPP4, while the loss of P53 led to the recovery of DPP4 to increase cell ferroptosis [45]. Tarangelo et al. found that P21, a downstream effector of P53, elevates intracellular GSH levels and GPX4 expression, leading to P53-delayed ferroptosis in**

![Figure 7](image_url)

**Figure 7:** p-STAT3 activation downregulates P53 expression to rescue bavachin-induced ferroptosis in osteosarcoma (OS) cells. (a, b) Western blotting shows the relative levels of p-STAT3, STAT3, P53, and GPX4 protein expression. (c) p-STAT3 activation restores bavachin-induced GSH depletion. (d, e) p-STAT3 activation reduces bavachin-induced ROS and MDA accumulation. (f) p-STAT3 activation rescues bavachin-induced ferroptosis in OS cells. *p < 0.05, **p < 0.01, and ***p < 0.001.

![Figure 8](image_url)

**Figure 8:** Schematic diagram displays potential mechanism of bavachin-induced ferroptosis in osteosarcoma cells. LP: lipid peroxidation.
Supplementary Materials

Supplementary Figure 1: Bavachin induces ferrous iron accumulation and ROS generation in a time-dependent manner in osteosarcoma (OS) cells. (Supplementary Materials)

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