ORIGINAL RESEARCH

Azurin Regulates P21 and Enhances the Sensitivity of Osteosarcoma Cells to Cisplatin

Yu Chen, MS; Hui Zeng, BS; Mingming Hou, Mmed; Yuzhi Qiu, MS

ABSTRACT

Background • Osteosarcoma (OS) is the most common bone malignancy, with a high mortality rate in adolescents. Despite advancements in therapeutic interventions, OS prognosis remains poor due to drug resistance. P21, a cyclin-dependent kinase inhibitor, plays a critical role in cell cycle regulation and has been implicated in OS pathogenesis. Cisplatin (DDP) is a conventional chemotherapeutic agent for OS, but its efficacy is often limited due to drug resistance. Azurin, a bacterial redox protein, has been reported to exhibit antitumor activity. However, its interaction with P21 in OS remains unexplored. In this study, we sought to investigate the impact of azurin on the cytotoxic effect of DDP against OS cells in relation to P21 expression.

Methods • Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to determine the level of p21 and apoptosis-related factors in U2OS cells. A Cell Counting Kit-8 (CCK-8) was used to examine the effects of azurin-p21 on the U2OS cell proliferation rate. Flow cytometry (FCM)was used to analyze the impact of azurin-P21 on the apoptosis/cell cycle. Enzyme-linked immunosorbent assay (ELISA) was used to analyze the effects of azurin-P21 on the secretion of oxygen free radicals, glutathione and glutathione peroxidase.

Results • Azurin exhibited significant cytotoxic activity against U2OS cells expressing wild-type (WT) P21, with minimal impact on SAOS-2 and MG63 cells lacking endogenous P21. Azurin treatment resulted in increased expression of procaspase-3 and Bax, decreased expression of B-cell lymphoma-2 (Bcl-2) and a consequential increase in apoptosis. The depletion of P21 attenuated these effects, suggesting the crucial role of P21 in azurin-mediated cytotoxicity. Furthermore, azurin synergistically enhanced the cytotoxic effect of DDP against U2OS cells, which was mitigated by P21 depletion.

Conclusions • Our findings demonstrated that azurin selectively induces apoptosis and cell cycle arrest in U2OS cells, which is mediated via P21. This study highlights the potential of azurin as a sensitizer for DDP in the treatment of OS. Future studies on DDP-resistant OS cells may further elucidate the clinical relevance of our findings. (*Altern Ther Health Med.* 2023;29(7):119-125).

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INTRODUCTION

Osteosarcoma (OS) is a common malignant tumor of the bone marrow that affects young people.[1,2] Although chemotherapy such as cisplatin (DDP) has been successful in increasing patient survival rates and treating the primary tumor,[3] a percentage of patients with metastatic disease and patients with relapsed tumors still have a poor prognosis.[4] Drug resistance and damage to the body caused by frequent use of DDP remain important drawbacks in the treatment of OS.[5] Therefore, there is an urgent need for novel potent drugs in OS treatment, especially drugs that can improve DDP sensitivity to reduce chemotherapy-induced adverse events.

Azurin, a species of pathogenic bacterium *Pseudomonas aeruginosa*,[6] was found to selectively induce and cause apoptosis in OS cells[7,8] such as breast[9] and lung cancer.[10] In addition, atomic force microscopy revealed widespread effects of azurin at the cellular level; cell membrane stiffness was reduced upon exposure to azurin, indicating that membrane alterations may underlie the broad anticancer activity of the protein.[11] Of note, studies have pointed out that the physiological indicators of animals treated with azurin are within the normal range, so it can be assumed that this bacterial protein has no toxic adverse events.[12,13] Azurin, intriguingly, has been reported to interact

with the tumor suppressor protein p53, forming a complex that leads to the increased production of reactive oxygen species (ROS)[14] and leading to oxidative stress factor (hydrogen peroxide [H_2O_2], nitric oxide [NO]) overproduction,[15] resulting in glutathione (GSH),[16] causing cell toxicity. However, the molecular mechanism by which azurin prevents cell growth in osteosarcoma cells is not known.

P21 is a member of the cyclin-dependent kinase inhibitor family[17] and plays a pivotal role in cancer development and progression.[18,19] It exerts its effects primarily by interfering with the function of cyclin-dependent kinase complexes,[20] which modulates both the cell cycle[21] and DNA repair mechanism[22] by interfering with the function of cyclin-dependent kinase complexes. This interaction is particularly important in the context of OS as it directly links the inhibitory effects of p21 in OS with cell cycle control processes. Enhanced p21 expression causes cell cycle S-phase arrest and restricts cell growth, helping cells prevent DDP-induced apoptosis.[23] The B-cell lymphoma-2 (Bcl-2) protein family is an established apoptosis regulator.[24] The Bcl-2 family can act as pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) regulators.[25,26] The percentage of anti-apoptotic/pro-apoptotic proteins partly dictates the cellular response to apoptotic or survival signals.

Although azurin has been extensively studied for its anti-tumor activity, its capacity to modulate the growth of osteosarcoma is not well characterized. The aim of our study was to determine the effect of azurin on OS cells and its molecular mechanism, inducing cell apoptosis via regulation of p21.

METHODS

Cell Culture

Human OS cells SAOS-2, U2OS and MG63 (Procell, Wuhan, China) were cultured (37°C; 5%) in McCoy's 5A containing 10% FBS (Gibco™, Grand Island, New York, USA), MG63 cultured in minimum essential medium (MEM) (Gibco) containing 10% FBS. U2OS is p21 wild type (WT), SAOS-2 and MG63 lack endogenous p21. Small interfering (si) RNA p21 (GenePharma Biotechnology Co., Ltd, Shanghai, China) were transfected into U2OS using Lipofectamine® 3000 (Invitrogen, Carlsbad, California, USA), and the sequences were: p21 si-1: 5′-GATGGAACTTCGACTTTGT–3′; p21 si-2: 5′-CAGTTTGTGTGTCTTAATTAT–3′; p21 si-3: 5′-: CTGGCATTAGAATTATTTAAA–3′; si-NC: 5′-: UUCUCCGAACGUGUCACGUTT–3′.

Azurin (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in MEM, and exposed to different concentrations of azurin (0 to 400 μg/ml) plus DDP (0 to 40 μg/ml).

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR Assay)

Total RNA was extracted with TRIzol reagent (Invitrogen). After centrifugation at 12000g (4°C; 10 min) in a high speed refrigerated centrifuge (JIDI-17RS, Guangzhou JiDi Instrument Co., Ltd., China), RNA were reverse-transcribed to cDNA and analysis using One Step SYBR Green RT-qPCR Kit (Biomarker, Beijing, China), and performed under the following conditions: 95°C for 60 sec followed by 40 cycles of 95°C for 6 sec and 60°C for 32 sec. Sequences of the primer pairs used for amplification were as follows: β-actin, F: 5'-CATTGCCGACAGGATGCAG-3', R: 5'-CTCGTCATACTCCTGCTTGCTG-3'; p21, F: 5'-CATGTGGACCTGTCACTGTCTTGTA-3', R: 5'-GAAGATCAGCCGGCGTTG-3'. P21 expression was calculated using the 2-delta-delta Ct $2^{-\Delta\Delta Ct}$ method,[27] with β-actin as internal reference for normalization.

Proliferation Assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) following the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at a density of 5 x 10^3 cells per well and allowed to adhere overnight. The next day, cells were treated with the indicated concentrations of azurin. After 48 hours, 10 μl of CCK-8 solution was added to each well and incubated for 2 h at 37°C. The absorbance was then measured at 450 nm using an enzyme-labeled instrument (Fluoroskan™; Fisher Scientific, Waltham, Massachusetts USA).

Flow Cytometry (FCM) Analysis of Apoptosis/Cycle

For apoptosis analysis, cells were harvested, washed with PBS, and resuspended in binding buffer. They were then stained with Annexin V-FITC and propidium iodide (PI) using an Apoptosis Detection Kit (Beyotime) according to the manufacturer's instructions. Briefly, cells were incubated with Annexin V-FITC and PI for 15 minutes at room temperature in the dark and then analyzed using a FCM (BD Biosciences). For cell cycle analysis, cells were fixed with 70% ethanol at -20°C overnight. Fixed cells were then washed with PBS and stained with PI using the Cycle Detection Kit (Beyotime). Briefly, cells were treated with RNase A and PI for 30 min at 37°C in the dark. The DNA content was then analyzed by FCM.

Western Blotting

Denatured proteins from U2OS cells were resolved by 10% SDS-PAGE (Beyotime). The separated protein bands were subsequently transferred to polyvinylidene difluoride (PVDF) membranes and incubated for 12h at 4°C) with p21 (1:1000, ab109520; Abcam, Cambridge, UK), Bax (1:1200, ab32503; Abcam), cleaved caspase-3 (1:800, ab32042; Abcam), Bcl-2 (1:5000, ab133504; Abcam), and GAPDH (1:3000, ab8245; Abcam) primary antibodies. They were then incubated for 2h at 25°C with goat anti-rabbit antibody (1:20,000: ab205718; Abcam).

Reactive Oxygen Species ROS) Activation Assay

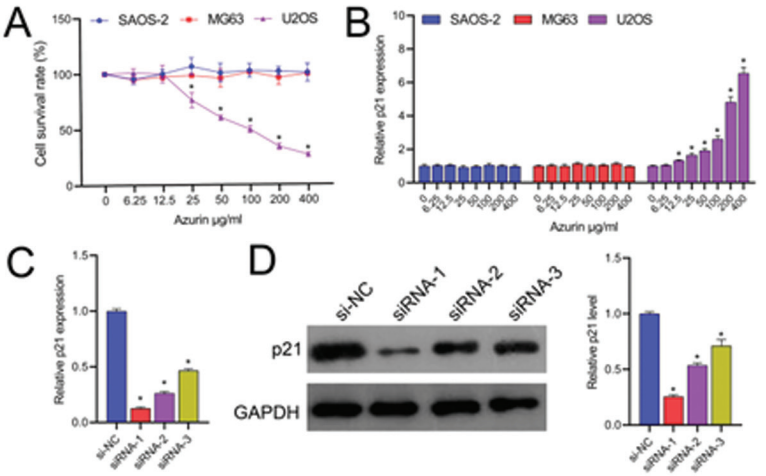
U2OS cells (5×10^3) were cultured (12h at 37°C) in a 96-well plate. Then, we added 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) and incubated the

compound for 30 min. This non-fluorescent compound is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) when oxidized within the cell, thus providing a measure of ROS activity. The cells were then incubated at 37°C for 30 min, allowing the H2DCFDA to passively diffuse into the cells, where it reacts with ROS to form DCF. U2OS cells were then harvested and the cell ROS activity was analyzed with Fluoroskan (Fisher Scientific) enzyme-labeled instrument.

ELISA Assay

ELISA kits (Beyotime) were used to test for items including H_2O_2, reduced glutathione (GSH) and oxidized glutathione (GSSG). In brief, 100 μg of total protein lysate was loaded onto the pre-coated 96-well plates and incubated for 2h at room temperature. After washing to remove unbound proteins, a biotinylated detection antibody specific for the target protein was added and incubated for 1h at 25°C and streptavidin was added to each well and incubated for 45 min. The absorbance was measured at 450 nm using a Fluoroskan enzyme-labeled instrument. The concentration of each protein was calculated based on the standard curve obtained from the respective protein standards.

Figure 1. Azurin exerts differential effects on OS cells through the regulation of p21. (**1A**) CCK-8 analysis of toxicity of different concentrations of azurin to SAOS-2, MG63 and U2OS cells. (**1B**) RT-qPCR analysis of the effect of different concentrations of azurin on the transcriptional activity of p21 in SAOS-2, MG63 and U2OS cells. (**1C**) RT-qPCR analysis of p21 gene expression in U2OS cells by 3 siRNAs against p21. (**1D**) Western blot analysis of p21 protein expression in U2OS cells by 3 siRNAs against p21.



*$P < .05$.

Abbreviations: CCK-8, Cell Counting Kit-8; OS, osteosarcoma; P21, a cyclin-dependent kinase inhibitor; siRNA, small interfering RNA.

Statistical Analysis

Data are presented as mean±standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9 software (Dotmatics, Boston, Massachusetts USA). Differences between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test. A $P < .05$ was considered statistically significant.

RESULTS

Azurin Exerts Differential Effects on OS Cells via P21 regulation

Different concentrations of azurin (0, 6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml) were administered to the SAOS-2, MG63 and U2OS cell lines. CCK-8 analysis showed that the inhibition of U2OS cells was dose-dependent with azurin, and azurin left SAOS-2 and MG63 cells almost undamaged. The inhibition of U2OS cells was dose-dependent (Figure 1A). Half maximal inhibitory concentration (IC_{50}) of azurin on U2OS cells for 48h was 102.4 μg/ml. Meanwhile, we observed that the transcriptional activity of p21 was dose-dependent with azurin in U2OS cells, while the transcriptional activity of p21 in SAOS-2 and MG63 cells was not affected by azurin (Figure 1B).

P21 siRNA Screening

To confirm that azurin mediates the activation of p21, we constructed 3 p21 siRNAs and transfected them into U2OS cells. P21 siRNA inhibited the expression and protein level of p21 in U2OS cells (**Figure 1C**, **1D**). Among thexsm, the inhibition efficiency of p21 siRNA-1 was the best, and we used this for the follow-up molecular mechanism research.

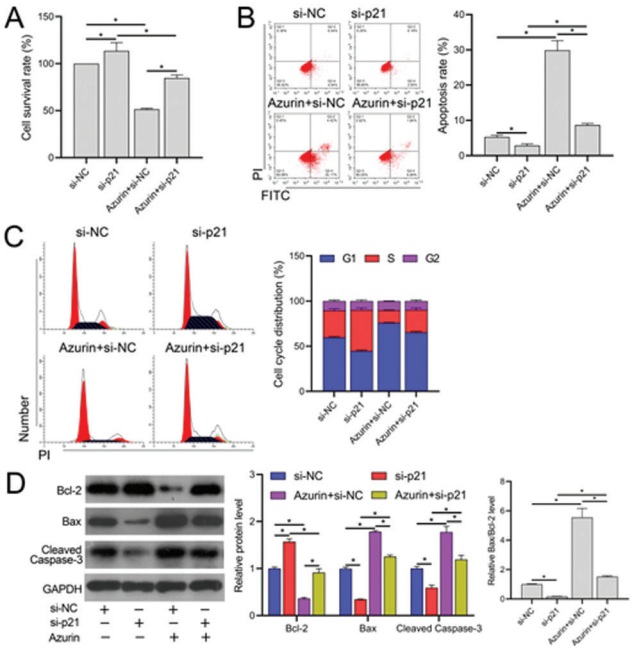
Azurin-Mediated Proliferation of U2OS Cells is Achieved Through P21

We observed that compared with si-NC, p21 siRNA promoted cell proliferation, and the survival rate of U2OS cells decreased under the action of azurin at IC50 concentration. In the presence of p21 siRNA, azurin significantly alleviated the reduction in U2OS cell viability (Figure 2A).

Azurin Mediates Apoptosis and Cycle Arrest of U2OS Cells via P21

Compared with si-NC, p21 siRNA inhibited the apoptosis of U2OS cells, and the apoptosis level was significantly upregulated with azurin at IC50 concentration. In the presence of p21 siRNA, the promotion of apoptosis in U2OS cells by azurin was significantly suppressed (Figure 2B). In addition, we observed that compared with si-NC, p21 siRNA promoted the cell cycle entry of U2OS cells into the S phase, and arrested in the G1 phase by azurin at IC_{50} concentration. In the presence of p21 siRNA, the effect of azurin was inhibited (Figure 2C).

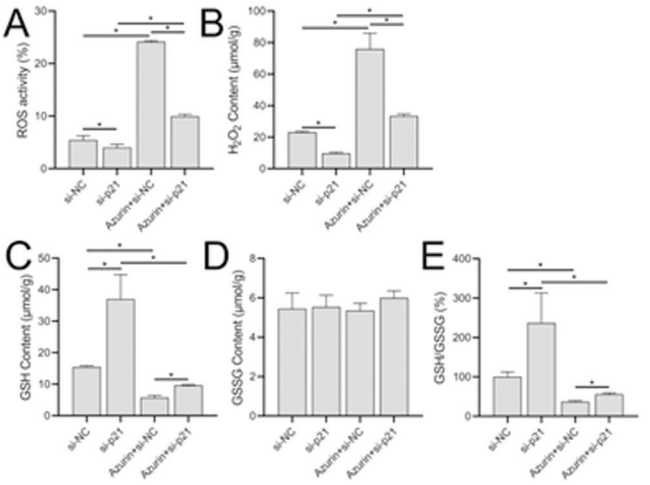
Figure 2. Azurin activates apoptotic proteins via p21, resulting in decreased U2OS cell viability, upregulation of apoptosis, and cycle arrest. (**2A**) CCK-8 analysis of the effect of azurin-p21 on the viability of U2OS cells. (**2B**, **2C**) FCM analysis of the effects of azurin-p21 on apoptosis (2B) and cycle (2C) of U2OS cells. (**2D**) Western blot analysis of the effect of azurin-p21 on the protein levels of Bcl-2, Bax and cleaved-caspase-3.



*$P < .05$.

Abbreviations: CCK-8, Cell Counting Kit-8; FCM, flow cytometry; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Figure 3. Azurin-p21 siRNA mediates the production of oxygen free radicals in U2OS cells. (**3A**) Fluoroskan™ enzyme-labeled instrument was used to measure the effect of azurin-p21 on ROS level in U2OS cells. (**3B**-**3E**) ELISA was used to analyze the effect of azurin-p21 on the content of H_2O_2 (3B), GSH (3C), and GSSG (3D) in U2OS cells. (3E) The ratio of GSH/GSSG was calculated.



*$P < .05$.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; siRNA, small interfering RNA.

Azurin-P21 Kills U2OS Cells by Activating Apoptotic Proteins

We observed that azurin can activate Bax (upregulated)/Bcl-2 (downregulated) and caspase-3, whereas p21 siRNA reverses its effects and p21 siRNA could also interfere with azurin efficacy when coexisting with azurin (Figure 2D).

Azurin-p21 siRNA Mediates the Production of Oxygen Free Radicals in U2OS Cells

Our study found that azurin mediates the production of ROS and H_2O_2 and inhibits GSH levels. p21 siRNA alone showed the opposite effect to azurin. With azurin and p21 siRNA treatment, the effects of either azurin or p21 siRNA were partially reversed. Neither azurin nor p21 siRNA had an effect on GSSG levels, so the GSH/GSSG ratio was similar to GSH levels (Figure 3A-3E).

The Role of Azurin-p21 on U2OS Resistance Susceptibility

Azurin promotes apoptosis and inhibits the growth of U2OS cells by activating p21, causing cell damage and resulting in reduced DNA synthesis and replication, thus being unable to complete the cell cycle and blocked in the G1 phase. It is not known whether azurin-p21 has a synergistic effect on DDP killing OS cells, so we treated U2OS cells with p21 siRNA or azurin combined with DDP (IC_{50}). Azurin was used at a concentration of 6.25 μg/m that was nontoxic to U2OS. The results showed that different concentrations of DDP (0, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μg/ml) treated U2OS cells, and the IC_{50} of DDP on U2OS cells was measured at 2.44 μg/ml (Figure 4A). We then observed that azurin had a synergistic effect on the effect of DDP (IC_{50}) on the reduction of viability. With p21 siRNA, both DDP and the combined effect of DDP and azurin were partially inhibited (Figure 4B). Meanwhile, DDP promoted the apoptosis of U2OS cells and arrested the cells in the G1 phase, and the effect of DDP was enhanced by azurin. However, the synergistic effect of DDP or azurin plus DDP was partially alleviated by p21 siRNA (Figure 4C, 4D).

Azurin Regulates DDP to Influence the Regulation of Apoptosis-Related Proteins

DDP promoted the levels of Bax (upregulated)/Bcl-2 (downregulated) and caspase-3. Azurin alone further promoted the effect of DDP, and p21 siRNA alone attenuated part of the effect of DDP. The synergistic effect of azurin on DDP was reversed when azurin was administered in combination with p21 siRNA (Figure 4E).

Azurin-p21 Enhances the Generation of Oxygen Radicals by DDP

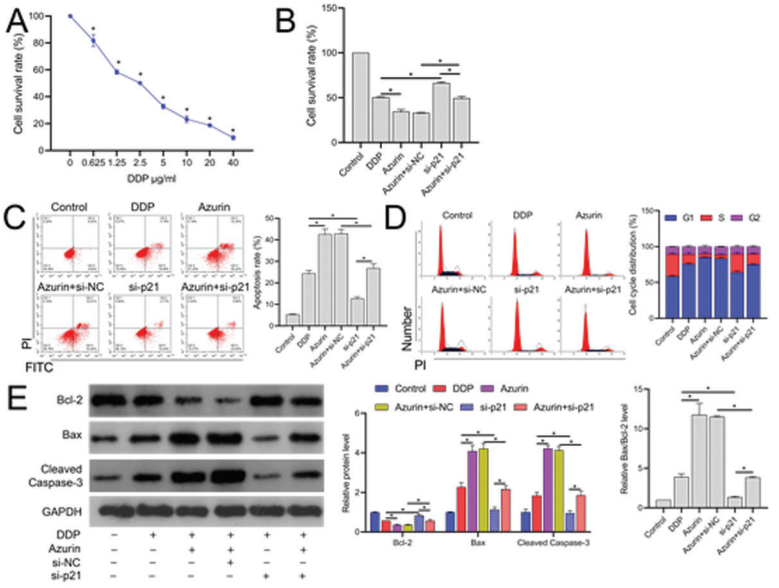
DDP mediates the formation of intracellular ROS and H_2O_2 and depletion of GSH, and azurin aggravates the toxic effect of DDP on U2OS. p21 siRNA alone can inhibit the effect of DDP. However, under the combined effect of azurin-p21 siRNA, the inhibition of DDP by p21 siRNA alone and the synergistic effect of azurin on DDP were inhibited. GSSG levels did not change significantly, so the GSH/GSSG ratio was similar to GSH levels (Figure 5A-5E).

DISCUSSION

Previous studies have shown that azurin has antitumor activity,[6,28] including on OS.[29] Our research found that the impact of azurin on the growth of OS cells and only had a significant killing effect on p21 wild-type (WT) U2OS cells, and there was no significant killing effect on SAOS-2 and MG63 cells lacking endogenous p21. The proliferation of SAOS-2 and MG63 cells had little effect. Thus, the apoptosis pathway of U2OS cells requires functional p21. How azurin interferes with the proliferation of U2OS through p21 is still unknown.

As p21 is closely related to the cell cycle, its inhibitory effect on the cell cycle is related to its nuclear localization.[30,31] Other important functions of p21 include regulating transcription and inhibiting apoptosis,[32,33] and are highly dependent on p21 protein interactions.[34] In addition, p21 can inhibit apoptosis through association with procaspase-3 in the cytoplasm.[35] Our study found that azurin can promote the apoptosis of U2OS cells and inhibit cell growth. Deletion of p21 expression promotes cell growth, inhibits apoptosis and partially reverses the effects of azurin. Forming procaspase-3/p21 complexes can accelerate cell death.[36] Here, we report that procaspase-3 protein levels are upregulated by azurin, but p21 siRNA inhibits this effect. Therefore, azurin-treated U2OS cells activate caspase-3, which leads to a cascade response that leads to apoptotic cell death.

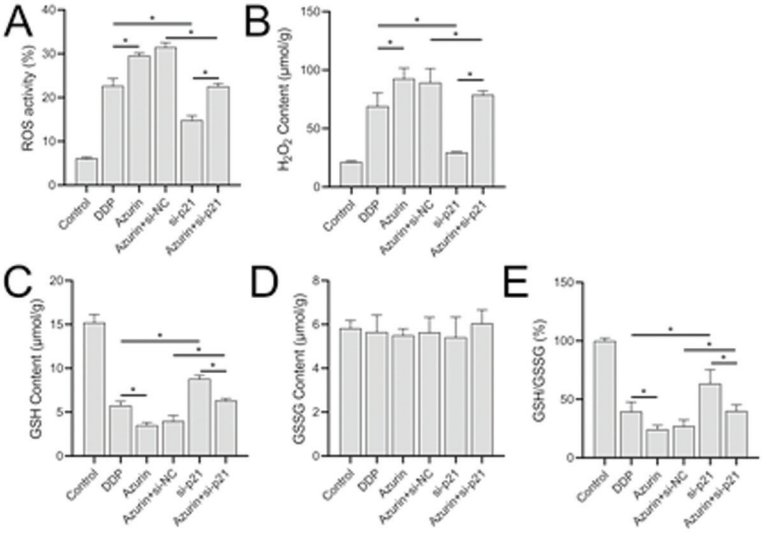
Figure 4. Azurin-p21 mediates proliferation of U2OS cells. (**4A**) CCK-8 analysis of the effect of DDP on the viability of U2OS cells. (**4B**) CCK-8 analysis of azurin-p21 for DDP survival in U2OS cells. (**4C**, **4D**) FCM analysis of the effect of azurin-p21 on the apoptosis level (4C) and cell cycle (4D) of DDP in U2OS cells. (**4E**) Western blot analysis of the effect of azurin-p21 on the protein levels of Bcl-2, Bax and cleaved caspase-3.



*$P < .05$.

Abbreviations: CCK-8, Cell Counting Kit-8; DDP, cisplatin; FCM, flow cytometry

Figure 5. Azurin-p21 enhances the generation of oxygen radicals by DDP (**5A**) Fluoroskan™ enzyme-labeled instrument was used to evaluate the effect of azurin-p21 for DDP on ROS level of U2OS cells. (**5B**-**5E**) ELISA analysis of the effect of azurin-p21 for DDP on the content of H_2O_2 (5B), GSH (5C), and GSSG (5D) in U2OS cells. (5E) Calculation of GSH/GSSG ratio.



*$P < .05$.

Abbreviations: DDP, cisplatin; ELISA, enzyme-linked immunosorbent assay; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species.

The Bcl-2 family of proteins has been shown to exert anti-apoptotic/pro-apoptotic effects by homologous or heterologous dimer formation.[37,38] Lack of this pro-apoptotic protein is involved in drug resistance, mediating apoptosis induced by p53 and increasing sensitivity to apoptosis induced by chemotherapy;[39,40] when Bcl-2 is activated or ubiquitous, apoptosis is blocked.[41] Our study showed that Bax (upregulated)/Bcl-2 (downregulated) were activated in U2OS cells, suggesting that azurin-induced apoptosis was related to the Bcl-2 family. Therefore, functional regulation of Bax/Bcl-2 by P21 may be involved in the mechanism of azurin-induced apoptosis.

Downregulation of p21 reduces G1 arrest and apoptotic cells,[42] and that loss of p21 function may induce a resistance phenotype.[43,44] It has also been reported that p21 can enhance DDP-induced cytotoxicity against OS cells by activating the caspase-3 cascade and increasing the Bax/Bcl-2 ratio.[45] We observed a synergistic influence of azurin on the killing effect of DDP on U2OS cells, which was at least partially prevented by depletion of p21. Furthermore, the effects of azurin-p21 on procaspase-3 and Bax/Bcl-2 levels were consistent with those without DDP. This means that azurin promotes the sensitivity of DDP to U2OS through p21, which is caused by azurin-mediated activation of procaspase-3 and Bax and inhibition of Bcl-2.

P21 plays an essential role in regulating the level of oxygen free radicals.[46] Knockdown of p21 reduced the binding of p21 to procaspase-3 and the accumulation of ROS.[47,48] Excessive ROS accumulation leads to cell type damage, which has been correlated with reduced GSH levels.[49] GSH exists in cells in either the oxidized (GSSG) or reduced (GSH) state and is a water-soluble tripeptide consisting of the amino acid triplet glutamine, cysteine and glycine.[50] Therefore, the maintenance of an optimal ratio of GSH to GSSG in cells is critical for survival. Mechanistically, GSH is an important antioxidative enzyme involved in the quenching of ROS. We found that when p21 expression i inhibited, the accumulation of ROS was reduced and H_2O_2 levels were greatly inhibited. On the contrary, the level of GSH/GSSG ratio was significantly upregulated. However, the effect of p21 depletion was counteracted by DDP or azurin. This means that the effect of azurin in mediating U2OS apoptosis or synergizing with DDP to increase the sensitivity to U2OS is caused by the regulation of ROS, H_2O_2 and GSH/GSSG by p21.

CONCLUSION

Our study demonstrated that azurin selectively induces apoptosis/cycle arrest in U2OS cells. Cell apoptosis/cycle were closely related to p21-mediated Bax/Bcl-2 ratio and caspase-3. This is one of the mechanisms by which azurin induces apoptosis.

CONFLICT OF INTEREST

None.

FUNDING

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