Cdc20 inhibitor apcin inhibits the growth and invasion of osteosarcoma cells

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Abstract. Osteosarcoma (OS) is the most common primary malignant bone tumor worldwide, primarily affecting children and adolescents (1). OS typically presents between the ages of 10 and 20 years or in elderly patients (2,3) and generally develops from primitive mesenchymal bone-forming cells in the long bones (4). At present, the therapeutic options for OS are surgery and intensive multi-agent chemotherapy, including cisplatin, doxorubicin, ifosfamide and methotrexate (5). The 5-year survival rate of patients with OS without metastases is 60-70%, depending on the use of chemotherapy (6). However, the 5-year survival rate for OS patients with metastases, especially pulmonary metastases, is only 20% (7). The current treatment strategies for metastatic OS typically result in poor prognoses and relapse (8). It would, therefore, be beneficial to develop novel therapeutic agents and innovative treatment approaches to improve survival rates in patients with OS.

The ubiquitin-proteasome system (UPS) is highly regulated and plays major roles in several biological processes. UPS controls different protein functions and important processes associated with the cell cycle, proliferation, metabolism, apoptosis and survival by targeting substrates for ubiquitination and degradation (9). The anaphase promoting complex/cyclosome (APC/C) is an unusually large multimeric cullin-RING ubiquitin ligase that plays a role in the cell cycle. RING finger E3 ubiquitin ligase cell division cycle 20 homolog (Cdc20) serves as an activator of APC/C during the metaphase-anaphase transition (10-12). High levels of Cdc20 have been reported in a number of malignancies and are associated with tumorigenesis and tumor progression (13,14). It has previously been reported that Cdc20 overexpression is associated with the occurrence of glioblastomas, while Cdc20 downregulation occurs in patients with low-grade tumors (15). Recently, Mao et al (16) demonstrated that Cdc20 overexpression may serve as an independent predictor of biochemical recurrence in patients with clinically localized prostate cancer undergoing laparoscopic radical prostatectomy without neoadjuvant therapy. Furthermore, Cdc20 upregulation was reported to be associated with poor prognosis in uterine cervix (18), colorectal cancer (19), pancreatic ductal adenocarcinoma (20), oral squamous cell carcinoma (21), gastric (22) and lung cancer (23). These findings revealed that Cdc20 may be a promising novel therapeutic target for cancer treatment (13). One study revealed that small interfering (si)RNA-mediated Cdc20 knockdown suppressed metastatic castration-resistant prostate cancer growth and enhanced chemosensitivity to docetaxel (24). Recently, Zhang et al (25) revealed that polyphenol curcumin inhibited pancreatic cancer cells via downregulation of the expression of Cdc20.

The APC/C inhibitor apcin is a small novel cell-permeable molecule that blocks the interaction between APC/C and Cdc20 or Cdh1 (26). It is accepted that apcin prevents substrate recognition by binding to Cdc20. Furthermore, apcin has been reported to induce metaphase arrest and apoptotic cell death in multiple myeloma (27). de Lange et al (28) demonstrated...
that several cancer cell lines with cohesion fatigues exhibited an increased response to apcin, indicating that APC/C-Cdc20 inhibitors may be effective therapeutic agents targeting cohesion defective cancers. However, the antitumor properties of apcin in OS have not been previously investigated. The aim of the present study was to determine whether apcin exhibited its antitumor properties in a human OS cell line. The possible molecular target that regulated cell death was also investigated.

Materials and methods

Cell culture. Human osteosarcoma cell lines MG63 and U2OS were purchased from the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (HyClone™; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C with 5% CO2.

Reagents and antibodies. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) and Calcein-AM were purchased from Sigma-Aldrich; Merck KGaA (St. Louis, MO, USA). MTT and Calcein-AM were diluted in dimethyl sulfoxide (DMSO) and stored at -20°C, respectively. Primary antibodies for Bim [B-cell lymphoma 2 (Bcl-2) interacting mediator of cell death] (dilution 1:1,000; cat. no. 2933) and p21 (dilution 1:1,500; cat. no. 2947) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Monoclonal anti-tubulin (dilution 1:3,000; cat. no. T9028) was purchased from Sigma-Aldrich. Secondary antibodies (dilution 1:2,500; cat. no. A-10031; dilution 1:3,000; cat. no. A-10343) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

MTT assay. MG63 and U2OS (2.9x10⁴ cells/well) were seeded in 96-well plates and cultured overnight. Different concentrations of apcin were added to the medium and cultured for 24, 48 and 72 h, respectively. An MTT assay was then carried out to assess the cell viability according to the manufacturer’s protocols. Then, 10 µl of MTT solution (0.5 mg/ml) was added to each well followed by incubation for 4 h. The liquid supernatant was then drained off and 100 µl of DMSO was added to each well to dissolve the crystals. The absorbance of each well at 490 nm was determined using a Multimode Reader of SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Cell apoptosis assay. MG63 and U2OS cells (2x10⁵ cells/well) were seeded in 6-well plates and treated with different concentrations of apcin for 48 h. Then, the cells were harvested and washed with phosphate-buffered saline (PBS). Subsequently, the cells were resuspended in 500 µl binding buffer with 5 µl propidium iodide (PI) and 5 µl FITC-conjugated anti-Annexin V antibody. The apoptosis was analyzed by a flow cytometer (FACScalibur; BD Biosciences, San Jose, CA, USA).

Transwell assays. MG63 and U2OS cells were suspended at a density of 3x10⁴ cells/well in 200 µl of DMEM. The cell suspension was placed on the upper layer of a cell permeable membrane in Matrigel-precoated Transwell inserts (24-well insert; Corning Inc., Corning, NY, USA) and were incubated for 24, 48 and 72 h, respectively. An MTT assay was then performed for MG63 and U2OS cells following treatment with the designated concentrations of apcin for 24, 48 and 72 h, respectively. The results revealed that OS cell proliferation was significantly suppressed by apcin in a time- and dose-dependent manner (Fig. 1).
Treatment with 25 µM apcin caused slight OS cell growth inhibition at 24 and 48 h, but 25% inhibition at 72 h. However, treatment with 50 or 75 µM apcin led to ~50 and ~75% cell growth inhibition, respectively, at 72 h in both MG63 and U2OS cells. These results demonstrated that apcin exhibited its antitumor characteristics in human OS cells.

Apcin induces apoptosis. It was further investigated whether apcin affected apoptosis in human OS cells. MG63 and U2OS cells were treated with the designated concentrations of apcin for 48 h and cell apoptosis was assessed using the PI/FITC-Annexin V assay. We revealed that apcin treatment induced significant cell apoptosis in a dose-dependent manner (Fig. 2). These results indicated that apcin inhibited human OS cell proliferation by inducing apoptosis.

Apcin inhibits Transwell cell migration and invasion. Thereafter, we determined whether apcin reduced OS cell migration and invasion. Cells were stained with crystal violet or Calcein-AM and Transwell migration and invasion assays were performed. The results revealed that apcin treatment suppressed the migration in MG63 cells (Fig. 3). In addition, it was revealed that apcin treatment suppressed the invasion of both MG63 and U2OS cells through the pores of the Matrigel-coated membrane (Fig. 4). Furthermore, the inhibition of migration and invasion was demonstrated to be inhibited in a dose-dependent manner.

Apcin inhibits cell migration in a wound healing assay. We also examined whether apcin suppressed OS cell migration. Both MG63 and U2OS cells were treated with apcin and a
wound healing assay was performed. It was observed that apcin treatment markedly suppressed OS cell migration in a dose-dependent manner (Fig. 5).

Apcin induces Bim expression. The pro-apoptotic molecule Bim has attracted increasing attention as a possible target for tumor therapy. In the present study, we determined whether
Bim expression was induced by apcin treatment and whether it exhibited its antitumor activity in human OS cells. The results of western blotting demonstrated that the expression of Bim was increased in MG63 and U2OS cells following apcin treatment (Fig. 6). It was also observed that apcin promoted Bim expression in a dose-dependent manner. Furthermore, p21, a downstream target of Bim, was also significantly upregulated in a dose-dependent manner following apcin treatment (Fig. 6). These findings revealed that the antitumor activity of apcin in OS cancer cells may be attributable, at least in part, to the increased expression of Bim and p21.

**Discussion**

Osteosarcoma (OS) is a malignant tumor of mesenchymal origin that primarily affects the long bones in children and...
young adults. Typically, it has a bimodal age distribution in the second decade of life and late adulthood (3,29). At present, patients with low-grade OS lesions are typically treated with surgical resection alone. However, for high-grade lesions, neoadjuvant therapy followed by wide surgical resection of the tumor and a course of adjuvant chemotherapy is the typical treatment regimen. The use of adjuvant chemotherapy improves the 5-year survival rate of patients with OS from <20 to ~70% (6,30). Neoadjuvant chemotherapy with high-dose methotrexate, doxorubicin, cisplatin and ifosfamide is commonly used to treat patients with OS. Other agents, including vincristine, bleomycin and cyclophosphamide, are sometimes used as well (31).

Previous studies have revealed that Cdc20 controls the substrate specificity of APC/C to bind and ubiquitinate its target proteins for subsequent degradation. Cdc20 is often upregulated in tumors and is associated with clinicopathological parameters (13). For instance, high expression of Cdc20 was associated with higher tumor grades in bladder, cervical, colonic, endometrial, gastric, liver, ovarian, prostatic and renal carcinomas (32). There was a significant correlation between Cdc20 upregulation and advanced tumor stage in breast, colon, endometrium and prostate cancers (32). Furthermore, Cdc20 overexpression predicted a poor prognosis in a wide range of human malignant tumors (17-22,23), and thus may be used as a biomarker of tumor prognosis. As such, inhibiting Cdc20 expression or binding may serve as an effective cancer treatment. Inhibition of Cdc20 by its siRNA reduced the cell growth and invasion of OS cells (33). A number of Cdc20 inhibitors have been identified and extracted from natural compounds, including withaferin A, which was demonstrated to modulate the spindle assembly checkpoint by degrading the Mad2-Cdc20 complex in colorectal cancer cells (34). Compound 331 was demonstrated to selectively induce glioma cell death by upregulating miR-494 and downregulating Cdc20 (35). Furthermore, rottlerin was demonstrated to inhibit cell growth and invasion by downregulating Cdc20 in glioma cells (36). It has also been reported that curcumin suppressed Cdc20 expression in pancreatic cancer cells (25). A number of natural compounds exhibit multiple non-specific targets and it would be beneficial to discover a specific Cdc20 inhibitor for cancer treatment. To this end, Zeng et al (37) demonstrated that pharmacologically disrupting the APC-Cdc20/Cdh1 interaction with tosyl-L-arginine methyl ester inhibited E3 ligase activity, inducing spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. In addition, they identified another Cdc20-specific APC inhibitor, apcin, that was able to bind with Cdc20, block substrate recognition and inhibit the ubiquitination of Cdc20 substrates (26).

Apcin was initially reported as an inhibitor of cyclin proteolysis in mitotic Xenopus egg extract (38). Recently, Sackton et al (26) reported that apcin directly disturbed the binding of Cdc20 to its substrates and subsequently blocked mitotic exit in human cancer cells. Apcin inhibited the oncogenic function of Cdc20 by reducing cell viability and inducing apoptosis in a dose-dependent manner in prostate cancer cells (39). Furthermore, it was reported that Speckle-type POZ protein (SPOP)-deficient prostate cancer cells were resistant to apcin, suggesting that apcin could be clinically used to treat patients with SPOP-WT prostate cancer, but not SPOP-deficient prostate cancer. It was reported that Warsaw breakage syndrome cell lines and several cancer cell lines with cohesion defects exhibited a significantly increased response to apcin (28). In the present study, it was demonstrated that apcin inhibited OS cell growth and induced significant apoptosis. The mobility of OS cells was also markedly inhibited by apcin treatment. Our findings revealed that apcin could have potential application in chemotherapy for OS patients.

A previous study reported that Cdc20 suppressed cell apoptosis largely via targeting Bim for polyubiquitination and degradation (40). Bim belongs to the Bcl-2-homology 3-only family (41). It has been reported that Bim interacts with Bcl-2, Bcl2L1 and other Bcl-2 proteins to act as an apoptotic activator under both physiological and pathological conditions to initiate the indigenous apoptotic pathway (41-44). Bim is widely expressed in a variety of tissues and regulates a number of normal and pathological systems (45). In the past few years, Bim has attracted more and more attention as a plausible target for cancer therapy (46). It has been reported that Bim plays a vital role in the anoikis of a number of cancer cells, including breast and lung cancer, fibrosarcoma, melanoma and OS (47-50). It was further demonstrated that aberrant levels of Bim affected chemotherapy response (51). For instance, Bim overexpression was revealed to enhance the efficacy of microtubule-targeting chemotherapy and may be a powerful predictor of tumor response to microtubule-targeting agents, including paclitaxel and vinorelbine (52). In contrast, Bim downregulation following siRNA transfection delayed paclitaxel-induced apoptosis, suggesting that low expression of Bim is responsible for paclitaxel-resistance in cancer cells (52). It has also been demonstrated that Bim deletion polymorphisms are associated with a poor clinical response to erlotinib and may be an independent prognostic factor for patients with non-small cell lung cancer with the EGFR mutation (53,54). Collectively, these previous studies suggest that many chemotherapeutic agents use Bim to trigger apoptosis in a variety of cancer cells. Bim-targeting therapies may offer more effective and individual options for cancer treatment in the future.

A number of chemotherapeutic agents, including molecular-targeting agents imatinib, gefitinib and bortezomib, use Bim as an executioner. These agents could be classified as ‘primitive’ Bim-targeting agents (46). Recently, Gambichler et al (55) demonstrated that Bim protein expression was significantly inversely correlated with melanoma features that are associated with poor prognosis. They further reported that Bim was an independent predictor of advanced disease, confirming that this pro-apoptotic BH3-only protein may be a potent biomarker and promising therapeutic target. High Bim expression is a potential prognostic marker as well as a chemotherapeutic target for cervical cancer (56). The Bim deletion polymorphism was found to be associated with primary resistance to crizotinib in patients with ALK fusion-positive non-small cell lung cancer (57). As such, targeting and manipulating Bim expression or activity may affect the outcomes of human cancers. Pharmacological inhibition of APC/C with proTAME induced apoptosis in multiple myeloma cells and was partially mediated by Bim, as well as the phosphorylation of Bcl-2 and Bcl-xL (27). It was reported that Bim expression was upregulated by proTAME treatment, while the apoptotic consequences of Cdc20 depletion have
been attributed to the accumulation of Bim. The results of the present study demonstrated that Bim was upregulated in OS cells following apcin treatment. Furthermore, apcin-induced Bim upregulation was dose-dependent. These results revealed that apcin exhibited its antitumor activity in OS cells. Bim overexpression may be due, at least in part, to disruption of the apcin-induced APC-Cdc20 interaction. As such, apcin may have potential as a treatment for human OS and Cdc20 may be a promising molecular target for chemotherapy.

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All data generated or analyzed during this study are included in this published article.

Authors' contributions

YG and GS were involved in project administration and supervised the study; YG and GS wrote and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


