

Cytotoxic mechanism of PLK1 inhibitor GSK461364 against osteosarcoma: Mitotic arrest, apoptosis, cellular senescence, and synergistic effect with paclitaxel

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Abstract. Polo-like kinase 1 (PLK1), a serine/threonine kinase and an oncogene, is crucial in regulating cell cycle progression. PLK1 also has been demonstrated as a potential target of osteosarcoma (OS) by using short hairpin RNA libraries in lentiviral vectors for screening of protein kinase. In preclinical studies, GSK461364, a potent and selective ATP-competitive PLK1 inhibitor, showed antiproliferative activity against multiple tumor cell lines. In the present study, we evaluated the expression level of PLK1 in OS and explored the cytotoxic mechanism of GSK461364 against OS. PLK1 was significantly overexpressed in OS compared with normal osteoblasts and other types of sarcoma. GSK461364 inhibited PLK1 and caused mitotic arrest by inducing G2/M arrest in OS cells. Moreover, GSK461364 exerted a cytotoxic effect by inducing apoptosis in OS, and induced cellular senescence in OS cell lines, as indicated by an increased senescence-associated β -galactosidase activity and enhanced DcR2 and interleukin-1 α expression. In addition, we demonstrated a synergistic cytotoxic effect of GSK461364 and paclitaxel, possibly resulting from combined mitotic arrest. In conclusion, the present study revealed that PLK1 was overexpressed in OS and that GSK461364 exerted its cytotoxic effect on OS by inducing mitotic arrest and subsequent apoptosis and induced cellular senescence; therefore, senescence-associated markers

can be used as treatment biomarkers, and a combination of GSK461364 and paclitaxel can potentially treat OS.

Introduction

Osteosarcoma (OS) is the major subtype of malignant bone tumors (1,2). In pediatric patients with localized disease, ~80-90% of 5-year overall survival is achieved when treated at experienced centers (3-5); however, in adult patients (6,7) and patients with recurrent or metastatic disease (3,8,9), response to chemotherapy is less satisfactory, and survival of such patients is generally poor.

Genetically, sarcomas can be classified as translocation-related and non-translocation-related. Non-translocation-related sarcomas can be further grouped as sarcomas with a simple genetic profile based on limited amplifications, such as dedifferentiated liposarcomas and parosteal OS (10,11), and major sarcomas with extremely complex genomic imbalances, such as leiomyosarcomas, undifferentiated pleomorphic sarcomas, pleomorphic liposarcomas and conventional OS (12). In conventional OS, these genomic aberrations cause oncogenic changes in such diverse processes as cell cycle regulation, cell death/cytokine pathways, proliferative signaling pathways, telomere dysfunction, metastasis and tumor suppression (13-20). Recently, several studies have revealed that the PI3K-mTOR pathway is crucial in conventional OS (15,16). However, the result of a clinical study of the mTOR inhibitor in OS treatment was unsatisfactory (21). Therefore, additional investigations exploring potential OS targets are necessary.

Polo-like kinase 1 (PLK1), a serine/threonine kinase and a known oncogene, is crucial in regulating cell cycle progression (22). Moreover, PLK1 has been demonstrated to be a potential target of OS when short hairpin RNA libraries were used in lentiviral vectors for protein kinase screening (23,24). However, few studies have evaluated PLK1 expression in

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OS (25). In our previous study, we revealed that 15-deoxy- Δ 12, 14-prostaglandin J2 (15d-PGJ2), a prostaglandin derivative, exerts cytotoxic effects against OS cells by downregulating the p-AKT and PKA-PLK1 pathways through reactive oxygen species-mediated c-Jun-N-terminal kinase activation (26). Therefore, inhibiting PLK1 may effectively treat OS.

GSK461364, a potent and selective ATP-competitive PLK1 inhibitor, has exhibited antiproliferative activity against multiple tumor cell lines in preclinical studies (27). Elevation of phosphorylated histone H3 (pHisH3) and suppression of PLK1 were indicators observed in tumor xenografts (27). In the present study, we evaluated the expression level of PLK1 in OS and explored the cytotoxic mechanism of GSK461364 against OS.

Materials and methods

Bioinformatics analysis. In total, 109 microarray and clinicopathological data sets of sarcoma from Gene Expression Omnibus (GEO) dataset GSE14827 (OS), GSE13433 (alveolar soft part sarcoma, ASPS), GSE8167 (gastrointestinal stromal tumor, GIST), GSE20196 (synovial sarcoma, SYN), and GSE20559 (liposarcoma, LPS) and from E-MEXP-1922 (leiomyosarcoma, LMS) were obtained from the NCBI and ArrayExpress websites, respectively. Nine microarray data sets of normal osteoblasts (GSE9451 and GSE10311) were retrieved from the NCBI website. All these datasets were in Affymetrix U133 Plus 2.0 platform. To decrease intrasubtype heterogeneity, not all retrieved samples were included in the analysis. For OS, only tumors without subsequent metastasis were selected; for GIST, only those with exon 11 mutation were included; for SYN, only tumor with SYT-SXX type 1 fusion gene and non-poorly differentiated histology were put into analysis; and for LPS, only well-differentiated (WD) and dedifferentiated (DD) tumors were chosen due to their similar genetic background. Gene expression data were normalized using dChip (28,29). The expression levels of individual genes were obtained through Z-score transformation, and the differences between different subtypes were subsequently compared using the Student's t-test.

Cell lines and reagent. Three OS cell lines, U2OS, MG63 and SJSA were selected for *in vitro* study and maintained in either Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium, or DMEM/F12 base media with 10% fetal bovine serum. GSK461364 was purchased from Calbiochem (San Diego, CA, USA). The following antibodies were used for immunoblotting: PLK1 (#4513; 1:1,000), phospho-cdc25C (Ser216) (pCDC25C) (#4901; 1:1,000), phospho-Histone H3 (Ser10) (pHisH3) (#9701; 1:1,000), and phospho-Histone H2A.X (Ser139) (pH2AX) (#2577; 1:1,000), all from Cell Signaling Technology, and anti-actin (ABS 24-100; 1:10,000).

Cell viability assay. Two methods were used in the cell viability assay, the first being the TACS™ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Trevigen, Gaithersburg, MD, USA). Approximately 2,000-20,000 cells/100 μ l/well were seeded in 96-well plates overnight. Subsequently, reagents at different concentrations

were added in triplicates. The plates were incubated for the desired time at 37°C, pulsed with 10 μ l MTT reagent, and incubated for an additional 4 h at 37°C. Furthermore, detergent reagent of 100 μ l/well was added and mixed thoroughly to dissolve the blue crystals. Absorbance of the converted dye was measured using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm (test) and 650 nm wavelengths. Cell survival was calculated using the following equation: % survival = (mean experimental absorbance/mean control absorbance) x 100 (30).

The possible synergistic effect of GSK461364 and chemotherapy was measured using the combination index (CI) calculated using CalcuSyn software (Biosoft, Ferguson, MO, USA) (31). CI >1 was defined as antagonism, CI = 1 as additivity, and CI <1 as synergy; the experiment was performed in triplicate.

Cell viability was also measured through a trypan blue exclusion assay (32). OS cells were plated at 5x10⁴ cells/well in 24-well plates; a variable concentration of GSK461364 was added to each well the following day, and the plates were incubated at 37°C for 72 h. Subsequently, the cells were trypsinized and mixed with trypan blue. Viable cells with intact cell membranes and not absorbing trypan blue were counted using a light microscope. All experiments were performed in triplicate.

Apoptosis assessment by Annexin V staining. Annexin V staining was used to detect apoptosis in cell lines treated with GSK461364. The cells were washed once with 1X phosphate-buffered saline (PBS) following drug treatment and resuspended in 100 μ l staining solution [containing Annexin V fluorescein and propidium iodide in an Annexin V-binding buffer, Annexin V-fluorescein isothiocyanate (FITC); BD Biosciences, San Diego, CA, USA]. Subsequently, the cells were incubated at room temperature for 15 min and diluted in 1X Annexin V-binding buffer. The percentages of apoptotic cells were measured through flow cytometry.

Cell cycle analysis. Cell cycle analysis was performed through flow cytometry, as previously described (33). Briefly, cells were trypsinized, washed twice with PBS, and fixed with 70% ethanol at -20°C overnight, following which the fixed cells were washed twice with cold PBS, suspended in 420 μ l PBS, added with 50 μ l 10 mg/ml RNase A (Sigma), and shaken at 37°C for 15 min. Subsequently, 20 μ l of 0.2 mg/ml propidium iodide (PI) was added and cells were retained at room temperature for 1 h. Flow cytometry was performed using FACSCalibur (Becton-Dickinson and Co., Oxford, CA, USA) to measure relative DNA content based on red fluorescence levels. The percentages of the cells in the different phases of the mitotic cell cycle were calculated using CellQuest software (Becton-Dickinson).

Western blotting. Monolayers of cultured cells were rinsed in PBS and scraped into lysis buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo Fisher Scientific)] containing protease and phosphatase inhibitor cocktail (1:100 dilution; Thermo Fisher Scientific). Lysates were incubated for 30 min at 4°C and subsequently clarified through centrifugation for 30 min at

13,200 rpm at 4°C. Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Fisher Scientific). Protein extracts (20-50 µg/lane) were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to polyvinyl difluoride membranes (Millipore), and blotted with specific antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (Millipore) and exposed to X-ray film.

Senescence-associated β-galactosidase assay. Senescence-associated β-galactosidase (SA-β-gal) activity was detected using the cellular senescence assay kit (Millipore) according to the manufacturer's instructions. U2OS cells were treated with GSK461364 for 72 h; the adherent cells were fixed and stained with X-gal in a staining solution at pH 6.0 and washed twice with 1X PBS. The percentage of SA-β-gal-positive cells (the number of positive cells relative to the total number of cells) was quantified by counting 100 cells in 3 randomly chosen fields per dish by using an Olympus IX51.

DcR2 expression assayed by flow cytometry. DcR2 expression was detected through flow cytometry. OS cells treated with GSK461364 for 72 h were washed twice with 1X PBS and incubated with Alexa Fluor 488-labeled anti-DcR2 (R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature in the dark. After washing twice with 1X PBS and resuspending in 1X PBS, the mean of fluorescence intensity on the cell surface was measured through flow cytometry using FACSCalibur (Becton-Dickinson).

Analysis of interleukin-1α expression. RNAs were extracted from the cell lines using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed with 1 µg RNA by using SuperScript® III First-Strand Synthesis system (Invitrogen) for reverse transcription polymerase chain reaction (RT-PCR). The copy number for both interleukin (IL)-1α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was measured through qRT-PCR by using Maxima SYBR-Green/ROX qPCR Master Mix (Thermo Fisher Scientific) and a LightCycler® 480 system (Roche). The primer sequences used in the reaction were as follows: IL-1α forward, 5'-CCGTGAGTTTCCCAGAAGAA-3' and IL-1α reverse, 5'-ACTGCCCAAGATGAAGACCA-3'; GAPDH forward, 5'-GCCAAGGTCATCCATGACAAC-3' and GAPDH reverse, 5'-GAGGGGCCATCCACAGTCTT-3' (34). The PCR cycling conditions were as follows: 95°C for 5 min followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec followed by 72°C for 40 sec. The gene expression levels were calculated as previously described (35).

Results

Overexpression of PLK1 in OS. We explored the expression level of PLK1 among normal osteoblasts and OS as well as among other types of sarcoma. The expression level of PLK1 was compared between 27 OS and 9 normal osteoblasts. As depicted in Fig. 1A, PLK1 was significantly overexpressed in OS compared with normal osteoblasts. We further compared the expression of PLK1 in OS with other types of sarcoma. As depicted in Fig. 1B, the transcript level of PLK1 was significantly

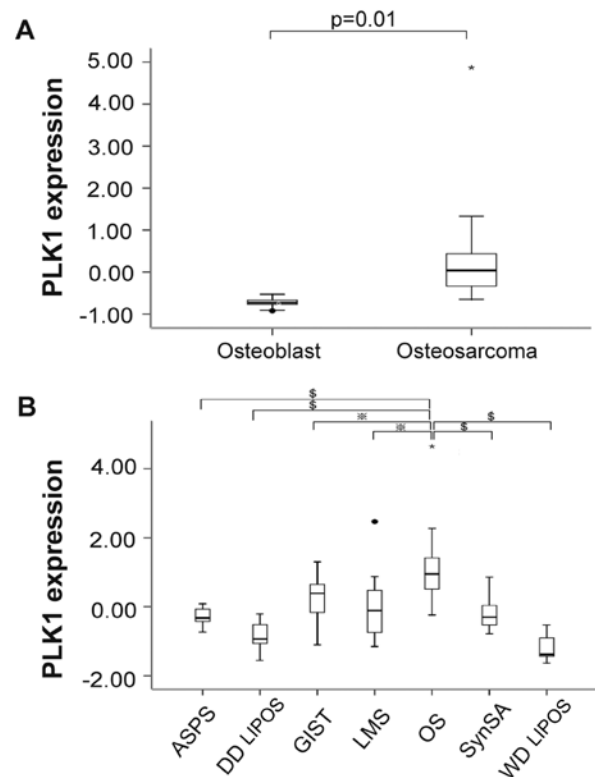


Figure 1. Comparing the expression level of PLK1 (A) between osteoblast and osteosarcoma, (B) between different types of sarcoma. Osteosarcoma showed significant higher expression level than either osteoblast or other types of sarcoma. ASPS, alveolar soft part sarcoma; DD LipoS, dedifferentiated liposarcoma; GIST, gastrointestinal stromal tumor; LMS, leiomyosarcoma; OS, osteosarcoma; SYN, synovial sarcoma; WD LipoS, well-differentiated liposarcoma. *P<0.01; §P<0.001.

cantly higher in OS compared with other types of sarcoma. These results indicated that PLK1 was overexpressed in OS.

GSK461364-mediated PLK1 inhibition and mitotic arrest in OS cell lines. An *in vitro* model was used to explore the potential of PLK1 as a therapeutic target in OS. GSK461364, a potent PLK1 inhibitor, has demonstrated favorable activity in various types of cancers (27). OS cells were treated with GSK461364 and the levels of PLK1 and pCDC25C (a downstream effector of PLK1) were measured through western blotting. Except for U2OS in time-dependent studies, decreased level of PLK1 and pCDC25C were noted in all three GSK461364-treated OS cell lines (Fig. 2). Moreover, we demonstrated that all OS cell lines treated with GSK461364 exhibited a dose- and time-dependent increase in pHisH3, an indicator of mitotic arrest (Fig. 2).

Effects of GSK461364 on cell cycle progression in OS cell lines. Furthermore, we explored the effects of GSK461364 on cell cycle progression in OS cell lines. As depicted in Fig. 3, flow cytometric analysis of DNA content in cells treated with GSK461364 for 24 h demonstrated marked accumulation of cells at G2/M DNA content in all 3 OS cell lines.

Cell viability assays were used to detect the possible cytotoxic activity of GSK461364 against OS cell lines. As depicted in Fig. 4, GSK461364 displayed cytotoxicity against all the three OS cell lines when assessed using either an MTT assay (Fig. 4A), or a trypan blue exclusion assay (Fig. 4B). Moreover,

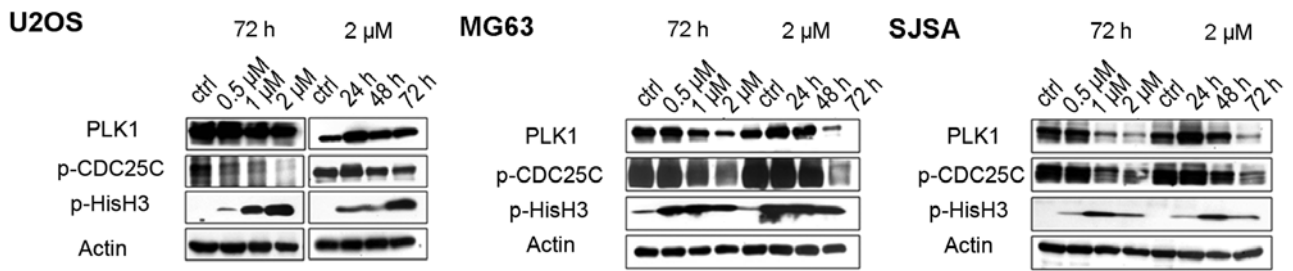


Figure 2. Immunoblotting with antibodies against PLK1, phosphorylated CDC25C (pCDC25C), phosphorylated histone H3 (pHisH3), and actin in three osteosarcoma cell lines after being treated with GSK461364. Except for U2OS in time-dependent studies, decreased level of PLK1 and pCDC25C were noted in the three GSK461364-treated OS cells. Also a dose-dependent and time-dependent increase in pHisH3 was observed the GSK461364-treated OS cells.

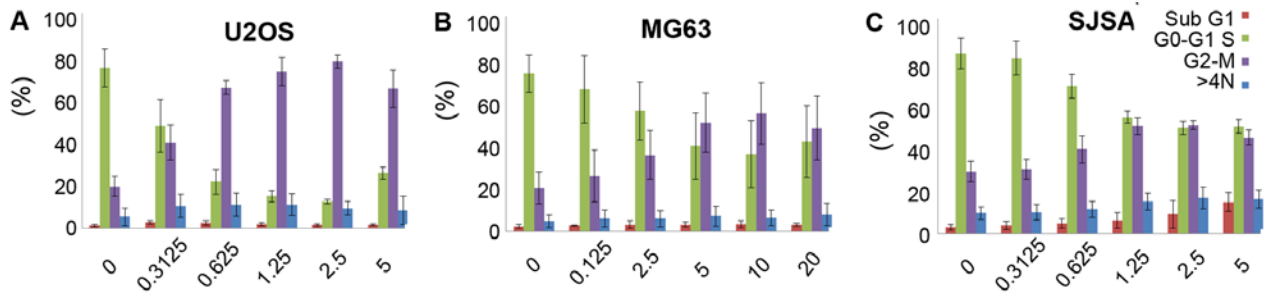


Figure 3. GSK461364 induces G2/M arrest in osteosarcoma cell lines. The DNA profiles of (A) U2OS, (B) MG63 and (C) SJSA treated with dimethyl sulfoxide (DMSO) or GSK461364 for 24 h were evaluated by flow cytometry. The percentages of cells in the sub G1, G0/G1, S and G2/M phases, as well as the percentage of cells with a DNA content >4N are shown in bars with different colors.

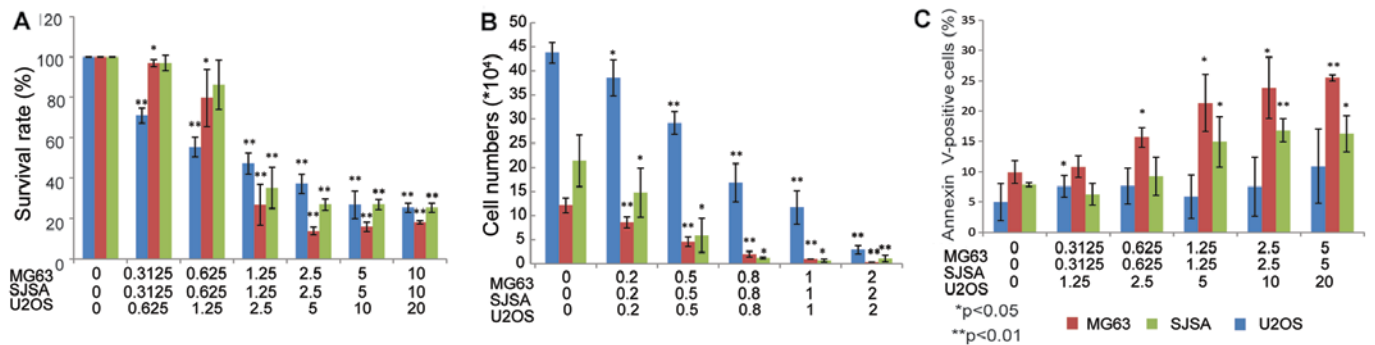


Figure 4. Treatment with GSK461364 inhibits PLK1 and results in growth inhibition and apoptosis in osteosarcoma cell lines. The viability of U2OS, MG63 and SJSA cells after treatment with various concentration of GSK461364 for 72 h was measured with (A) the TACS™ MTT cell proliferation assay (expressed as a percentage of viability under controlled culture conditions) and (B) a trypan blue exclusion assay (expressed as viable cell number). (C) Apoptosis assay. Three osteosarcoma cell lines were treated with various concentration of GSK461364 for 72 h. The percentage of apoptotic cells was determined using Annexin V-FITC/PI staining. All data represent the mean \pm SD of three independent experiments. *P<0.05; **P<0.01.

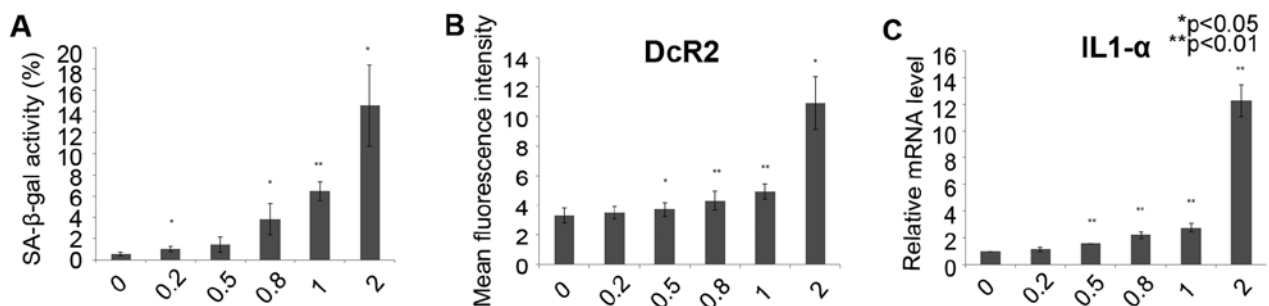


Figure 5. GSK461364 treatment induces cellular senescence in U2OS cell lines. (A) Treating U2OS cells with GSK461364 for 72 h dose-dependently increased senescence-associated β -galactosidase (SA- β -gal) staining. (B) Flow cytometric analysis of the expression of DcR2 in U2OS cells treated with GSK461364 for 72 h. (C) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of interleukin-1 alpha (IL-1 α) expression relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in U2OS cells treated with GSK461364. *P<0.05; **P<0.01.

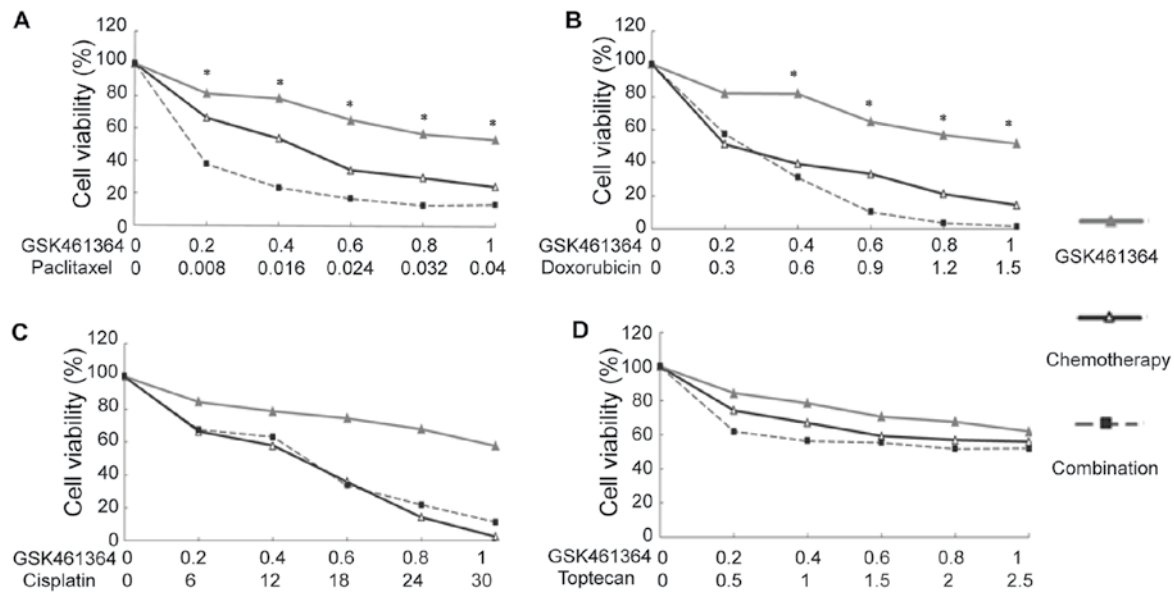


Figure 6. The viability of U2OS cells treated with GSK461364 in combination with different chemotherapeutic agents for 72 h was measured with the TACS™ MTT cell proliferation assay (expressed as a percentage of viability under controlled culture conditions). All data represent the mean \pm SD of three independent experiments. *A combination index (CI) <1.0 indicates a synergistic effect.

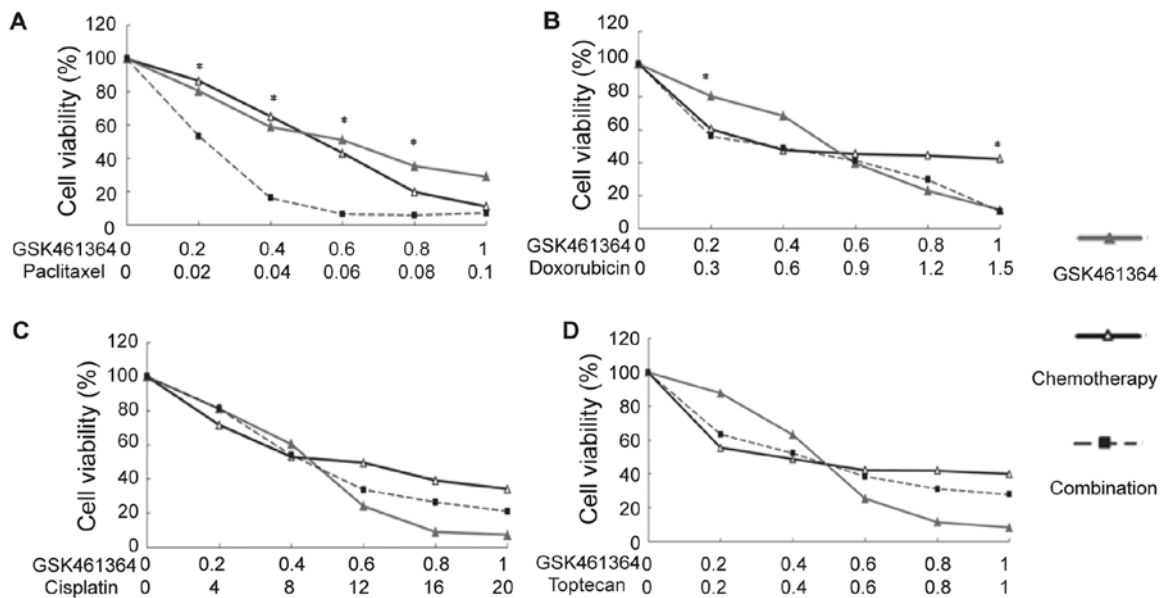


Figure 7. The viability of MG63 cells treated with GSK461364 in combination with different chemotherapeutic agents for 72 h was measured with the TACS™ MTT cell proliferation assay (expressed as a percentage of viability under controlled culture conditions). All data represent the mean \pm SD of three independent experiments. *A combination index (CI) <1.0 indicates a synergistic effect.

a significant induction of apoptosis in OS cell lines was revealed on co-staining with PI and FITC-labeled Annexin V (Annexin V-FITC; Fig. 4C).

GSK461364 induces cellular senescence in U2OS cell lines.

Cellular senescence can be induced by cell cycle inhibition. To explore whether GSK461364 induces senescence in OS cells, a SA- β -gal assay was used in the U2OS cells following treatment with GSK461364 for 72 h. A significant increase in SA- β -gal activity in the U2OS cells was revealed following GSK461364 treatment (Fig. 5A). In addition, the expression of DcR2, a well-known senescence biomarker (34,36), was

dose-dependently enhanced following GSK461364 administration (Fig. 5B). Furthermore, the expression of IL-1 α , a cytokine associated with the senescence-associated secretory phenotype (SASP) (34,36), was upregulated in the U2OS cells treated with GSK461364, as measured through qRT-PCR (Fig. 5C). These results indicate that GSK461364 treatment induces cellular senescence in OS cells.

Synergistic effect of GSK461364 with paclitaxel. We evaluated the possible synergistic effect of GSK461364 with other chemotherapies. As depicted in Figs. 6 and 7, except for the U2OS cells treated with doxorubicin, GSK461364 showed no

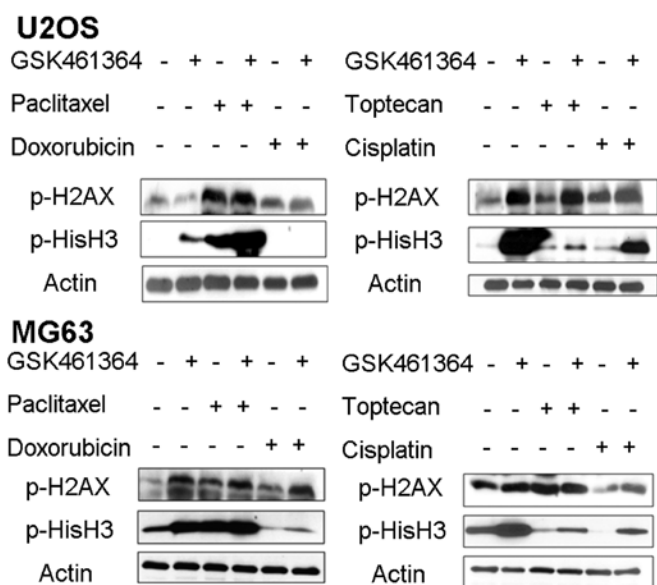


Figure 8. Immunoblotting with antibodies against phospho-Histone H2A.X (pH2AX), phosphorylated histone H3 (pHisH3), and actin in U2OS (upper panel) and MG63 (lower panel) after being treated with variable combination of GSK461364 and chemotherapy. Only GSK461364 plus paclitaxel showed increased level of pHisH3 in comparison with other combinations. No significant changes were observed in the pH2AX level.

significant synergistic effect with DNA damaging agents, such as doxorubicin or cisplatin, or with the topoisomerase inhibitor topotecan. However, it showed a significant synergistic effect with paclitaxel in both U2OS and MG63 cells.

Furthermore, we explored the underlying mechanism of the synergistic effect of GSK461364 and paclitaxel. As depicted in Fig. 8, a combination of GSK461364 and paclitaxel produced significantly increased mitotic arrest, as indicated by the increased pHisH3 level. By contrast, a combination of GSK461364 with other chemotherapeutic agents reduced mitotic arrest. No significant changes were observed in the pH2AX level, an indicator of DNA damage.

Discussion

In the present study, we revealed that PLK1 was significantly overexpressed in OS compared with normal osteoblasts or other types of sarcoma. GSK461364, a PLK1 inhibitor, inhibited PLK1 and induced mitotic arrest through G2/M arrest in OS cell lines, with subsequent apoptosis. In addition, GSK461364 induced cellular senescence in OS cell lines and showed a synergistic effect with paclitaxel.

Identifying treatment targets in sarcoma is difficult because of its complex genomic background. However, using genomic and expression profiling in 183 soft tissue sarcoma, Chibon *et al* (37) established a prognostic gene expression signature, namely, the Complexity index in sarcomas (CINSARC), comprising 67 genes related to mitosis and chromosome management. CINSARC had predicted metastasis outcome in an independent validation set of 127 sarcomas. Moreover, by reanalyzing a dataset of GIST, our group identified aurora kinase A (AURKA), along with other cell cycle and mitosis genes, as a prognostic factor for recurrence. In

addition, AURKA is a potential treatment target (38,39). Therefore, cell cycle regulation genes are a crucial group of potential biomarkers or targets in sarcoma.

PLK1, essential for regulation of mitosis and maintenance of genomic stability, is overexpressed in human tumors and has prognostic potential in cancers, indicating its involvement in carcinogenesis and its potential as a therapeutic target (22). Sero *et al* (25) demonstrated enhanced PLK1 expression in clinical OS samples and cell lines compared with normal human tissues. In addition, an enhanced PLK1 expression at diagnosis appeared to be associated with an unfavorable clinical outcome. In the present study, we demonstrated significant PLK1 overexpression in OS by comparing the transcription levels of different subtypes of sarcoma in a microarray database. However, PLK1 was not found to have a prognostic influence in OS (data not shown), and its role in oncogenesis of OS requires further investigation.

Several PLK1 inhibitors have been demonstrated to exert a cytotoxic effect on OS. BI 2536 suppresses cell line growth and clonogenicity (40), and decreases the xenograft tumor size of OS (41). Bogado *et al* (42) demonstrated that both BI 6727 and GSK461364 induce cell growth arrest, apoptosis and radiosensitization in OS cell lines. Sero *et al* (25) revealed that another PLK1 inhibitor, NMS-P937, was highly active in both drug-sensitive and drug-resistant OS cell lines.

We revealed that GSK461364 exerts significant cytotoxic activity on 3 OS cell lines. We discovered that GSK461364 targets and downregulates PLK1 and pCDC25C in the OS cell line and induces mitotic arrest, as indicated by an enhanced pHisH3 expression level and a marked G2/M arrest. Our results demonstrated that GSK461364 is cytotoxic to all 3 OS cells when assessed either through an MTT assay or a trypan blue exclusion assay. In addition, a significant induction of apoptosis in OS cell lines was detected by co-staining with PI and Annexin V-FITC, indicating an apoptosis-inducing effect. These data are consistent with previous reports.

Cell cycle inhibitor induces cellular senescence, wherein cells remain viable but typically arrested at the G1 or G2/M phases of the cell cycle, failing to proceed even after mitogen stimulation. Senescence cells are usually characterized by specific cellular phenotypes (such as increased SA- β -gal activity), secretory phenotype (SASP, usually a cytokine, such as IL-1 α), and apoptosis-regulatory protein (such as DcR2) (36). In the present study, GSK461364 treatment significantly increased SA- β -gal activity and enhanced the expression of DcR2 and IL-1 α in OS cell lines. In addition, similar findings were revealed in our previous study of AURKA inhibitor MLN8237 in the treatment of GIST cell line (39). Our studies indicated that cellular senescence is a crucial phenotype of PLK1, or in treatment of other cell cycle inhibitors and that senescence-associated markers may be valuable biomarkers for therapy with these compounds.

Furthermore, GSK461364 and paclitaxel were demonstrated to act synergistically in inducing a cytotoxic effect on OS, probably because of enhanced mitotic arrest. A previous OS study failed to demonstrate any synergistic effect of GSK461364 with chemotherapy (42). However, in a breast cancer study, PLK1-specific antisense oligonucleotides acted synergistically with paclitaxel in inducing cell cycle arrest, apoptosis, and reduction of tumor growth of xenograft (43).

Therefore, a combination of GSK461364 and paclitaxel deserves further investigation in a clinical setting.

In conclusion, the present study revealed that PLK1 is over-expressed in OS. GSK461364, a PLK1 inhibitor, exerted its cytotoxic effect on OS through the induction of mitotic arrest and subsequent apoptosis and induced cellular senescence; therefore, senescence-associated markers can be used as probable treatment biomarkers, and a combination of GSK461364 and paclitaxel may be effective in OS treatment.

Acknowledgements

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