Inhibition of polo-like kinase 1 leads to the suppression of osteosarcoma cell growth *in vitro* and *in vivo*

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Osteosarcoma is the most common type of primary bone cancer in children and adolescents. Treatment options for osteosarcoma may include surgery, chemotherapy, and radiotherapy. Unfortunately, many patients eventually relapse, resulting in an unsatisfactory outcome. The serine/threonine-specific polo-like kinase 1 (PLK1) is a kinase that plays an important role in mitosis and the maintenance of genomic stability. PLK1 has been found to be highly expressed in the malignant cells of osteosarcoma. Here, we describe the in-vitro and in-vivo effects of BI 2536, a small-molecule inhibitor of PLK1, which through inhibiting PLK1 enzymatic activity, causes mitotic arrest and eventually induces cancer cell apoptosis. In this study, we show that the PLK1 inhibitor, BI 2536, inhibits proliferation and induces apoptosis in two-dimensional and three-dimensional cultures of osteosarcoma cell lines, KHOS and U-2OS. A proliferation assay performed both in two-dimensional and three-dimensional culture showed that the growth of both cell lines was inhibited by BI 2536. Cell cycle analysis showed that the cells treated with BI 2536 were mainly arrested in the G_2/M phase. Immunofluorescence and western blotting analysis confirmed that the administration of BI 2536 led to significant decrease of PLK1 and Mcl-1 protein expression levels in dose-dependent and time-dependent manners. Furthermore, BI 2536-induced apoptosis in the osteosarcoma cell lines was shown by poly (ADP-ribose) polymerase cleavage and caspase assay. Finally, in mouse osteosarcoma xenografts, BI 2536-treated mice had significantly smaller tumors compared with the control mice. These findings offer evidence of the potential role for targeting PLK1 in osteosarcoma therapy. *Anti-Cancer Drugs* **22:444–453**

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Introduction

Osteosarcoma is the most common type of primary malignant bone tumor affecting children and young adults. Standard treatment includes surgical resection with pre-operative and postoperative chemotherapy [1]. Compared with surgery alone, when long-term survival rates were 10–20%, chemotherapy has increased the disease-free survival rate to more than 60% [2–5]. Doxorubicin, cisplatin, high-dose methotrexate, and ifosfamide are the most active chemotherapeutic reagents used in osteosarcoma patients [6]. However, if these agents are unable to obtain a favorable tumor response, further chemotherapeutic options are limited. Despite aggressive chemotherapy, one-third of patients with localized osteosarcoma experience recurrent or progressive metastatic disease and the average survival period after developing metastasis is less than 1 year. Therefore, to improve the survival rate of osteosarcoma patients, novel therapeutic strategies are needed.

The serine/threonine-specific polo-like kinase 1 (PLK1), found highly expressed in malignant tumors including sarcomas [7–9], is an essential kinase that plays an important role in mitosis and the maintenance of genomic stability. Earlier, we screened a human kinase short hairpin RNA (shRNA) library and identified several genes facilitating osteosarcoma cell growth and proliferation. Specifically, we observed that blocking the expression of PLK1 by shRNA or small interfering RNA (siRNA) could inhibit growth and induce apoptosis in osteosarcoma cells. Extensive evidence has shown that PLK1 is also overexpressed in human tumors and the depletion of PLK1 by siRNA leads to apoptosis in various cancer cell lines. PLK1 is also highly expressed in sarcoma cell lines [10,11] and osteosarcoma tissues compared with the very low expression levels in human osteoblast cells. Patients with osteosarcoma tissue expressing high levels of PLK1 had a significantly shorter survival period than those with lower levels [10]. These results seem to show that PLK1 is a logical point for pharmacological intervention and the inhibitor that specifically and potently targets PLK1 can potentially be used for the treatment of osteosarcoma.

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Recently, BI 2536, a specific inhibitor of PLK1, showed low nanomolar antiproliferative activity toward human cancer cells (EC_{50} values ranged between 2 and 25 nmol/l in a panel of 32 human cancer cell lines) [12]. BI 2536 binds with PLK1, resulting in mitotic arrest and apoptosis in a variety of tumor cells [12,13]. This compound showed more than 1000-fold selectivity relative to a panel of 63 other kinases [12,13]. The first phase I clinical trial of this inhibitor in patients with advanced solid tumor has since then been reported, which showed that BI 2536 was well tolerated and showed significant antitumor activities [14,15]. Up to now, however, the effect of BI 2536 on PLK1 in osteosarcoma is unknown. In this study, we show that the inhibition of PLK1 by BI 2536 blocks cell proliferation and tumor growth, induces mitotic arrest, and finally leads to apoptosis of osteosarcoma cell lines.

Materials and methods

Compound and antibodies

BI 2536 was purchased from ChemieTek (Indianapolis, Indiana, USA). Stock solutions were prepared in dimethyl sulfoxide at a concentration of 1 mmol/l and stored at –20°C. The rabbit monoclonal antibody PLK1, the rabbit polyclonal antibodies to human Mcl-1, and poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technologies (Danvers, Massachusetts, USA). The mouse and rabbit monoclonal antibodies to actin and the thiazolyl blue formazan [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). The Alexa Fluor 488 goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, California, USA). The goat anti-mouse horseradish peroxide (HRP) and goat anti-rabbit HRP were purchased from Bio-Rad (Hercules, California, USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce (Rockford, Illinois, USA).

Cell lines and cell culture

The osteosarcoma, KHOS, cell line was provided by Dr Efstathios Gonos (Institute of Biological Research & Biotechnology, Athens, Greece) [16]. The human osteosarcoma cell line, U-2OS was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Both the osteosarcoma cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 μg/ml; Invitrogen). The cells were incubated at 37°C in 5% CO_2–95% air atmosphere. Light microscopy images were documented using a Zeiss microscope from Carl Zeiss, Inc., (Oberkochen, Germany) with an attached Nikon D40 digital camera from Nikon Corp. (New York, USA).

MTT cell proliferation assay

In brief, 2×10^3 cells were planted into each well of the 96-well plates in a culture medium [RPMI 1640 supplemented with 10% FBS, penicillin (100 μg/ml), and streptomycin (100 μg/ml)]. After 24 h of incubation, BI 2536 was added at an increasing concentration to each well and incubated for 5 days. For the first 72 h, 10 μl of MTT was added to each well every 24 h of the culture. Then the plates were incubated for another 3 h. The formazan products of MTT were dissolved in acid–isopropanol and the absorbance was read at a wavelength of 490 nm on a SPECTRAmax Microplate Spectrophtometer from Molecular Devices (Sunnyvale, California, USA). All procedures were repeated on the fifth day. The relative absorbance values were obtained by assigning the absorbance value of cells without the administration of BI 2536 to 1. The experiments were performed in triplicate. All MTT data were processed with the use of GraphPad Prism 4 Software from GraphPad Software, Inc (San Diego, California, USA).

Three-dimensional culture

Three-dimensional (3D) cultures were prepared on a reconstituted basement membrane to mimic the growth pattern of tumors in the human body and evaluated whether the proliferation of osteosarcoma cells could be inhibited by BI 2536 [17]. Each well of eight-well glass chamber slides was covered evenly by 2-mm growth factor-reduced, Matrigel-formed basement membrane. A single cell suspension was seeded on the layer after the basement membrane was solidified in a cell culture incubator. The cells were grown in an Assay Medium [RPMI 1640 containing 2% horse serum, hydrocortisone (0.5 μg/ml), cholera toxin (100 ng/ml), insulin (10 μg/ml), penicillin G (100 U/ml), and streptomycin (100 mg/ml)] and epidermal growth factor (5 ng/ml), and 2% Matrigel. Twenty-four hours after being seeded, U-2OS and KHOS were treated with BI 2536 at concentrations of 10, 20, 40, 60, and 80 nmol/l.

Flow cytometry

The cells were cultured in a normal growth medium with FBS for 24 h and BI 2536 was added to each flask. After 72 h, the cells were collected from each flask and pelleted by spinning at 1300 rpm at 4°C for 5 min. The cell pellets were re-suspended in 1 ml of cold phosphate buffer solution (PBS) and fixed by adding 4 ml of –20°C absolute ethanol. Those samples were sent to the Flow Cytometry Core Facility Center for Regenerative Medicine, Harvard Stem Cell Institute, Massachusetts General Hospital for analysis.

Western blotting

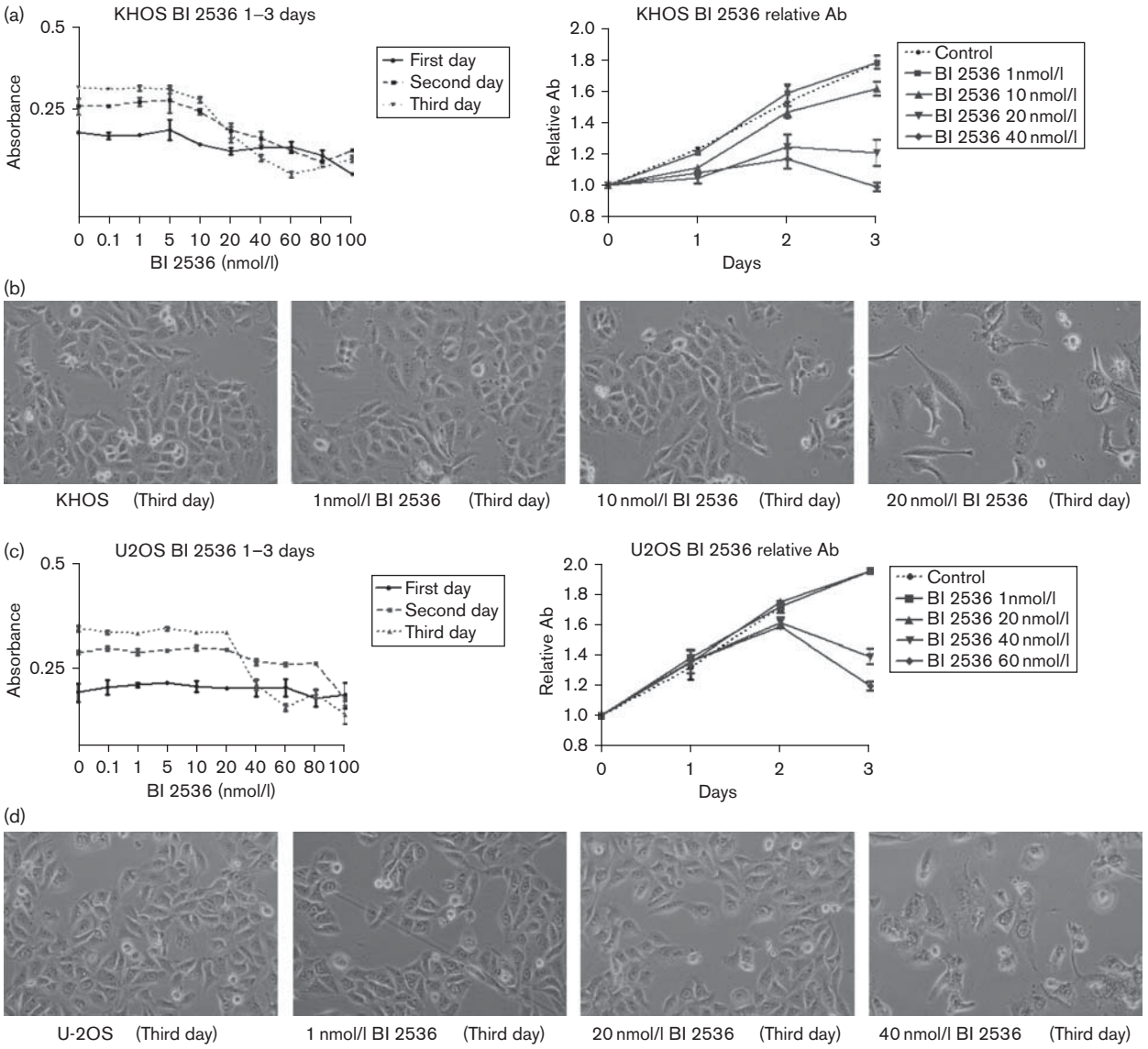
Protein lysates were harvested from the osteosarcoma cells and xenograft tumor tissues by lysing with 1 × RIPA Lysis Buffer from Upstate Biotechnology (Charlottesville, Virginia, USA). The protein concentrations were determined using Protein Assay Reagents (Bio-Rad) and spectrophotometer quantification from Beckman DU-640, Beckman Instruments, Inc., (Columbia, Maryland, USA). Twenty-five micrograms of total protein was processed on Nu-Page 4–12% Bis–Tris Gel (Invitrogen) and transferred

to a pure nitrocellulose membrane from Whatman International Limited (Banbury, UK). The rabbit monoclonal antibody PLK1, the rabbit polyclonal antibodies to human Mcl-1 and PARP were purchased from Cell Signaling Technologies. The mouse and rabbit monoclonal antibodies to actin were purchased from Sigma-Aldrich. Primary antibodies were diluted to 1 : 1000 and incubated in Tris-buffered saline (pH 7.4) with 0.1% Tween-20 for overnight at 4°C. Signals were generated through the incubation with HRP-conjugated secondary antibodies (Bio-Rad) in Tris-buffered saline (pH 7.4) with 5% nonfat milk and 0.1% Tween-20 at 1 : 2000 dilution for 1 h at room temperature. Immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence

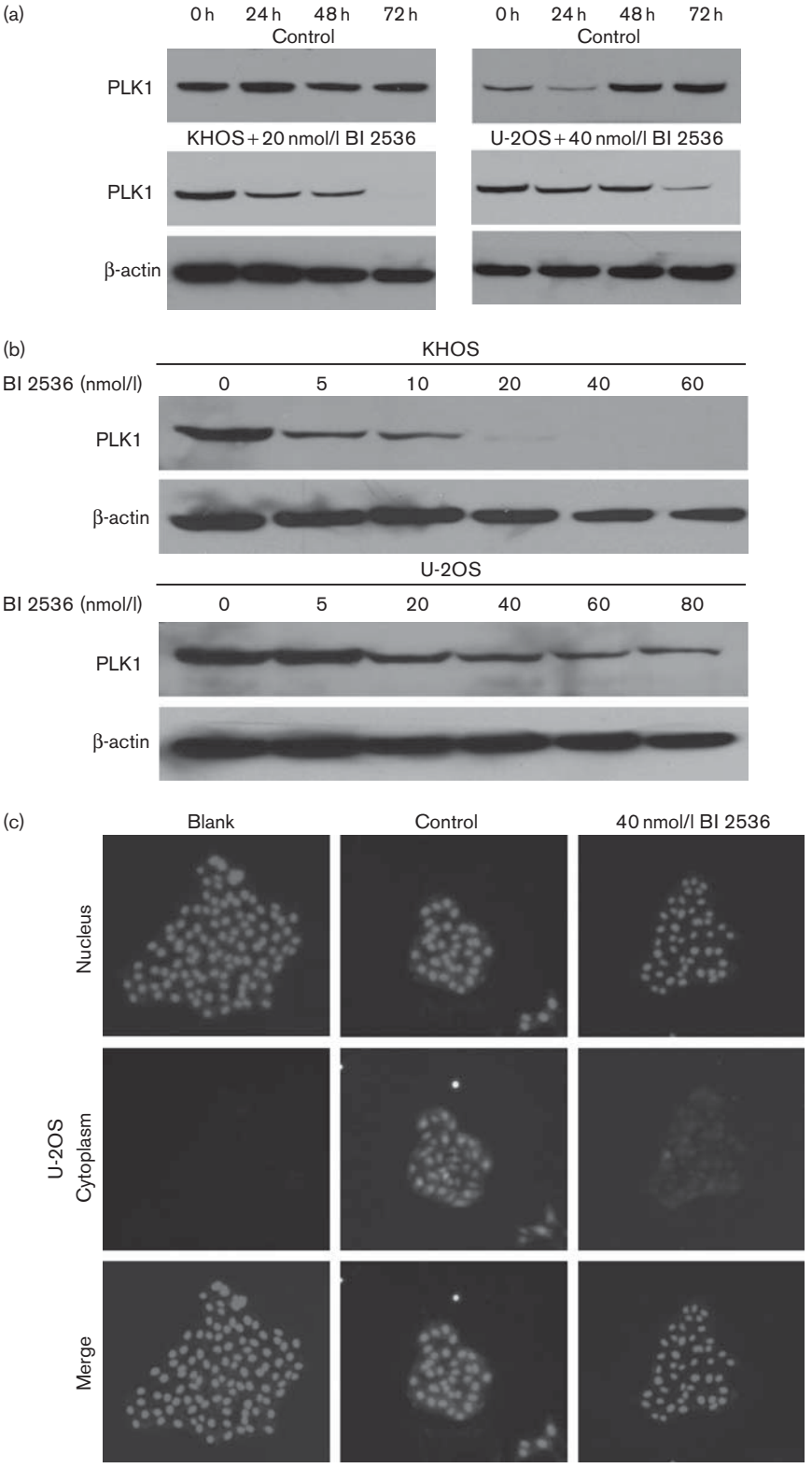
For the purposes of immunofluorescence, 5×10^3 osteosarcoma cells were seeded in each well of eight-well glass chamber slides. After 24 h of culturing, BI 2536 was added with different concentrations to each well and incubated for 72 h. Osteosarcoma cells were briefly rinsed with PBS and, after aspirating PBS, fixed in 2% paraformaldehyde for 15 min at room temperature, followed by permeabilization with ice-cold absolute methanol for 10 min at −20°C. The permeabilized cells were blocked with a blocking buffer for

Fig. 1



Reduction of growth (a and c) and viability of osteosarcoma cells (b and d) induced by BI 2536-mediated PLK1 inhibition. (a and c) PLK1 inhibitor, BI 2536, inhibits osteosarcoma cell proliferation. Cells were treated with BI 2536 at the indicated concentrations and for indicated times. (b and d) Phase-contrast photomicrographs of cell lines treated with different concentrations of BI 2536 for 72 h.

Fig. 2



BI 2536-mediated inhibition of PLK1 expression in dose-dependent and time-dependent manner (a and b). Immunofluorescence analysis of PLK1 expression inhibited by BI 2536 in two-dimensional culture of U-2OS (c). (a) BI 2536 reduces the expression of PLK1 in time-dependent manner. (b) BI 2536 reduces the expression of PLK1 in dose-dependent manner. (c) U-2OS was treated with minimal effective concentrations of BI 2536 for 72 h. The nuclei were counterstained with Hoechst 33 342 (1 μg/ml) for 1 min. Cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope equipped with a SPOT RT digital camera. Blank: normal osteosarcoma cells were stained without PLK1 primary antibody and staining of cytoplasm was absent. Control: no BI 2536 was administrated to cell lines.

60 min at room temperature (to prepare 25 ml, add 2.5 ml of 10X PBS, 1.25 ml of goat serum, and 21.25 ml of dH_2O and mix well). While stirring, add 75 µl of Triton X-100. The cells were then incubated with the rabbit monoclonal antibody PLK1 from Cell Signaling Technologies at 1 : 200 dilutions in an antibody dilution buffer overnight at 4°C. (To prepare 40 ml, add 4 ml of 10X PBS to 36 ml of dH_2O. Add 0.4 g of BSA and mix well.) While stirring, add 120 µl of Triton X-100. The cells were washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG at room temperature overnight. For counterstaining nuclei, the osteosarcoma cells were incubated in PBS containing Hoechst 33 342 (1 µg/ml; Invitrogen) for 1 min. Osteosarcoma cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope equipped with a SPOT RT digital camera from Diagnostic Instruments, Inc. (Sterling Heights, Michigan, USA).

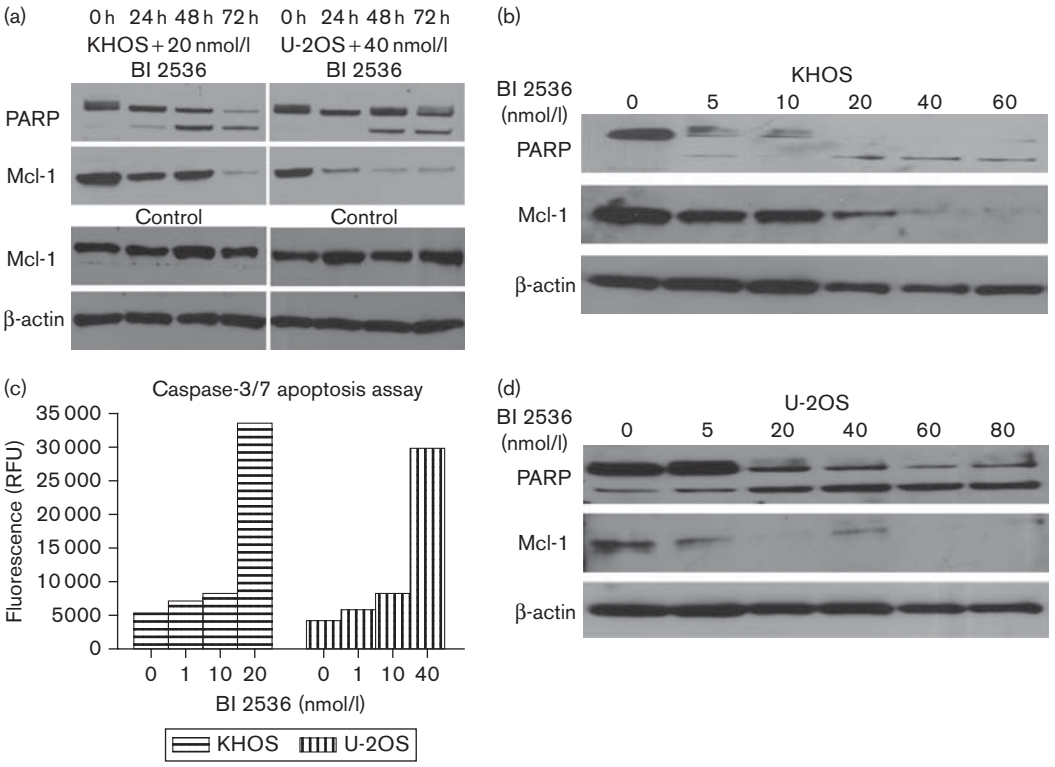
Apoptosis assay

The quantification of apoptosis was evaluated by using the Apo-ONE Homogeneous Caspase-3/7 Assay kit from Promega according to manufacturer's instructions (Madison, Wisconsin, USA). U-2OS (3×10^3) and KHOS cells were seeded per well in a white 96-well plate for 24 h before the addition of BI 2536. The U-2OS cells were treated with 1 and 40 nmol/l of BI 2536 and the concentrations of BI 2536 for KHOS cells were 1 and 20 nmol/l. Both osteosarcoma cell lines were treated for 72 h and the manufacturer's instructions for the apoptosis assay were then followed. The fluorescence of each well was measured at an emission wavelength of 521 nm and an excitation wavelength of 499 nm on a SPECTRAmax GeminiXS Microplate Spectrophtometer from Molecular Devices.

Animal studies

The protocol for animal use in this project has been approved by the Massachusetts General Hospital Subcommittee on Research Animal Care under the protocol number 2009N000229. KHOS cells (1×10^6) were inoculated subcutaneously with matrigel from BD Biosciences (San Jose, California, USA) into the right flank of 4-week-old Crl:SHO-*Prkdc*^{SCID}*Hr*^{hr} nude female mice purchased from the Charles River Laboratories (Ann Arbor, Michigan, USA). Seven days after the injection, the mice were randomized

Fig. 3



BI 2536-induced apoptosis in human osteosarcoma cell lines in dose-dependent and time-dependent manner (a–d). (a) The osteosarcoma cell lines were incubated with minimal effective concentrations of BI 2536 for 0, 24, 48, and 72 h, respectively. BI 2536 induces apoptosis in human osteosarcoma cell lines in time-dependent manner. (b and d) Both KHOS and U-2OS cells were released in different concentrations of BI 2536 for 72 h. BI 2536 induces apoptosis of human osteosarcoma cell lines in dose-dependent manner. (c) After osteosarcoma cells being treated with four different concentrations of BI 2536 for 72 h, apoptosis was evaluated by using the Apo-ONE Homogeneous Caspase-3/7 Assay kit as described in the Materials and methods section.

into a control group and a treatment group. BI 2536 was dissolved in dimethylsulfoxide, diluted with 0.9% NaCl and 0.1N HCl, and injected into the tail vein. The mice were treated once with BI 2536 (50 mg/kg) or the vehicle control. The health of the mice and evidence of tumor growth were examined daily and tumor volume was measured once every 3 days with a digital caliper. Tumor volume (mm^3) was calculated as $(W^2 \times L)/2$ (W as width and L as length).

Results

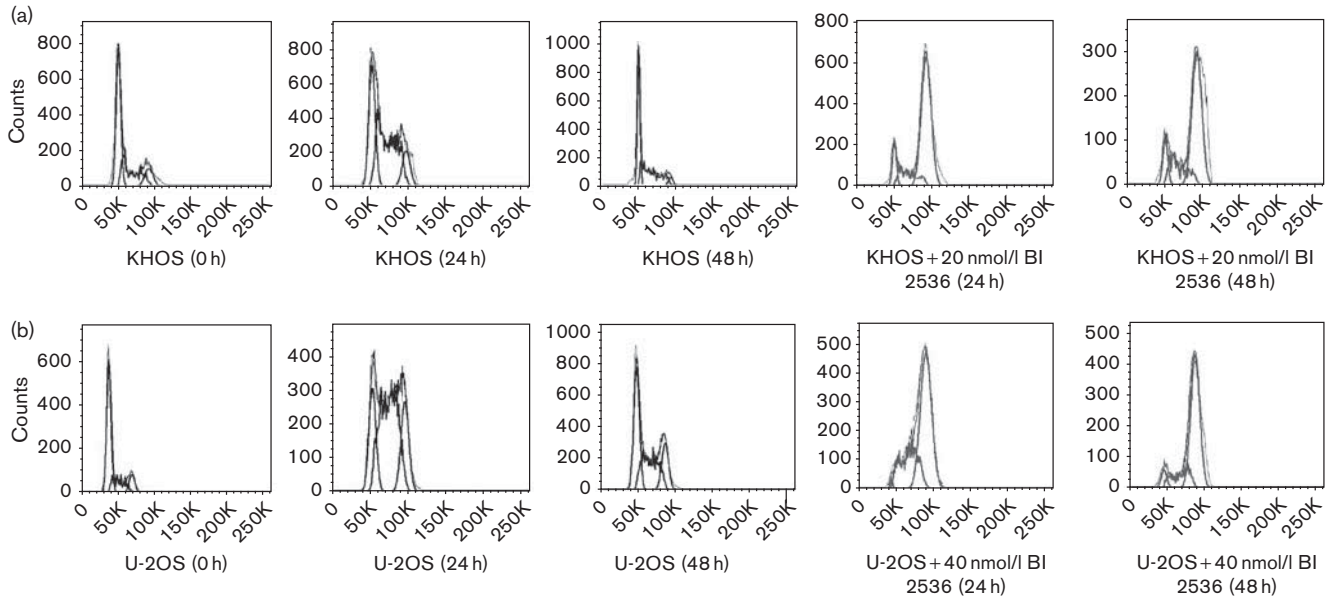
Administration of BI 2536 reduces growth and viability of osteosarcoma cell lines

We used osteosarcoma cell lines, KHOS and U-2OS, to test the effects of BI 2536 on cell growth and viability [10]. Cells were treated with BI 2536 in different concentration ranges (starting from 0.1 to 100 nmol/l) and the absorbance of each well was read every 24 h. Compared with the control, BI 2536 had maximum effect at 72 h of treatment and the minimally effective concentrations of BI 2536 on KHOS and U-2OS were established as 20 and 40 nmol/l, respectively (Fig. 1a and c and Fig. 5a). Next, KHOS and U-2OS were treated with a range of concentrations of BI 2536 for 72 h in six-well plates and the images of cells were then taken. The treatment of cells with BI 2536 decreased cell proliferation in both osteosarcoma cell lines, especially at or greater than the minimally effective concentrations (Fig. 1b and d).

BI 2536 leads to a reduction of PLK1 expression in time-dependent and dose-dependent manners

To evaluate whether the PLK1 inhibitor, BI 2536, can lead to a reduction of PLK1 expression in the osteosarcoma cell lines, KHOS and U-2OS were incubated either with a range of concentrations or the minimal effective concentration of BI 2536 for 24, 48, and 72 h. The effects of BI 2536 on the expression of PLK1 were analyzed by western blot and immunofluorescence. After both osteosarcoma cell lines were treated with BI 2536, the expression of PLK1 was downregulated in time-dependent and dose-dependent manners (Fig. 2a and b).

Fig. 4

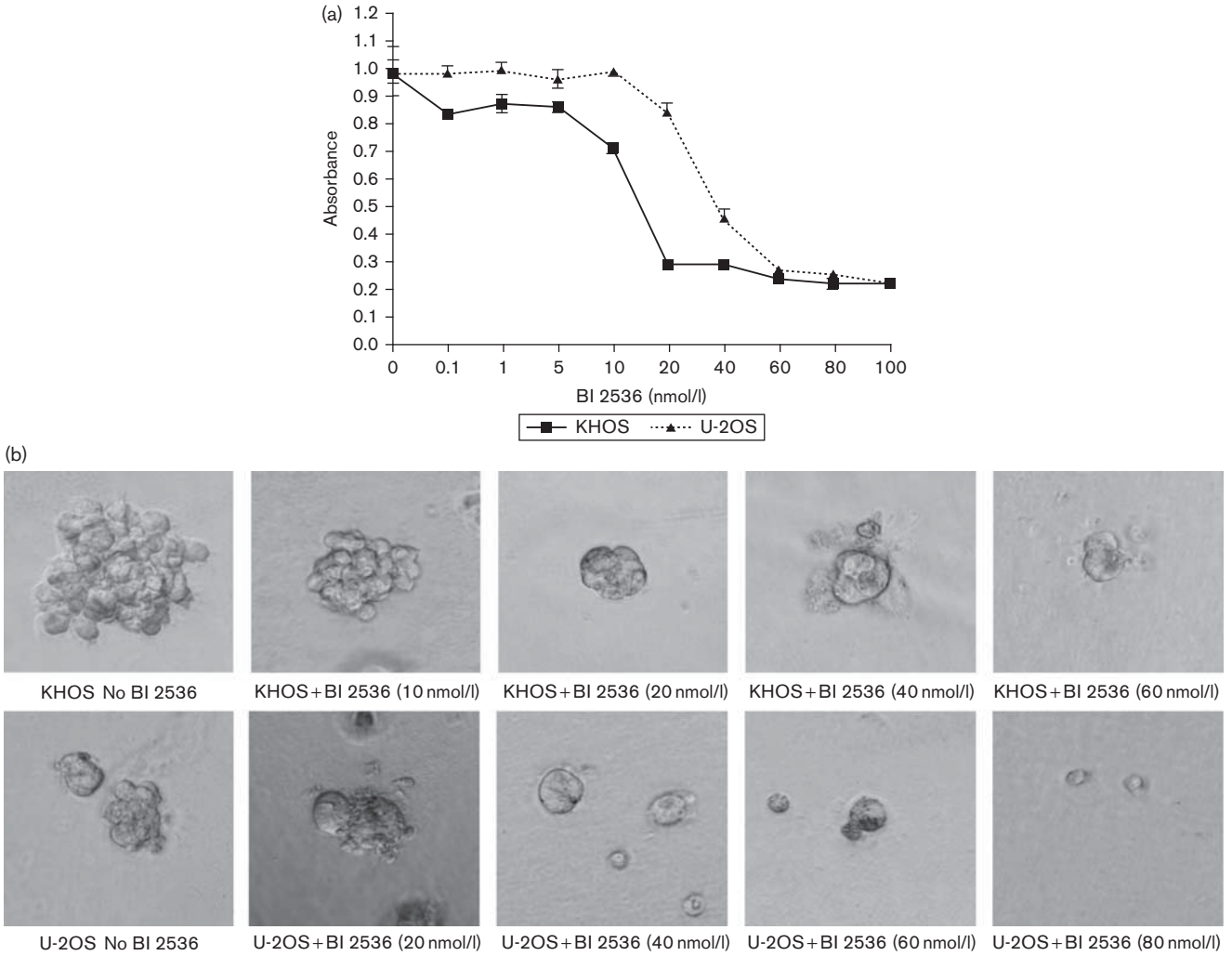


(c)

	KHOS					U-2OS				
	(0 h)	(24 h)	(48 h)	20 nmol/l BI 2536 (24 h)	20 nmol/l BI 2536 (48 h)	(0 h)	(24 h)	(48 h)	40 nmol/l BI 2536 (24 h)	40 nmol/l BI 2536 (48 h)
G_1 (%)	52.1	33.7	47.0	9.87	9.99	73.3	21.3	43.7	1.13	5.58
S (%)	30.1	51.3	43.5	16.0	20.0	21.8	56.9	38.5	32.2	17.1
G_2/M (%)	12.9	12.6	5.93	69.6	66.3	3.2	21.2	20.9	61.6	64.6

Cell cycle analysis of osteosacroma cell lines treated with or without BI 2536 (a–c). (a and b) KHOS and U-2OS were treated with minimal effective concentration of BI 2536 for 24 and 48 h, respectively. Cell cycle was analyzed by flow cytometry. (c) Percentages of cells in G_1, S, and G_2/M phases for each group. 24 and 48 h after treatment with BI 2536, G_2/M mitotic arrest is evident at concentrations of 20 and 40 nmol/l in both KHOS and U-2OS.

Fig. 5



Effects of BI 2536 on the proliferation of osteosarcoma cells and inhibition of cell growth by BI 2536 in three-dimensional culture (a and b). (a) Cells were treated with BI 2536 in complete growth medium at the indicated concentrations. The relative sensitivity of each cell to BI 2536 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay. (b) Osteosarcoma cells were seeded into eight-well slides and 24 h later treated with BI 2536 at minimal effective concentrations for 3 days. Cell images were documented using a Zeiss microscope with an attached Nikon D40 digital camera.

BI 2536-mediated PLK1 inhibition could be observed after 72 h and at concentrations of 20 and 40 nmol/l for U-2OS and KHOS (Fig. 2c and Supplementary Fig. 1). These results are similar to earlier studies of BI 2536 in leukemia and anaplastic thyroid carcinoma cells [18–20].

BI 2536 induces apoptosis and mitotic arrest in human osteosarcoma cell lines

We examined the proapoptotic effects of BI 2536 by apoptosis assay and immunoblotting for the proteins, Mcl-1 and PARP cleavage. Significant decrease in the expression level of Mcl-1 and an increase in PARP cleavage were observed in time-dependent and dose-dependent manners (Fig. 3a, b and d). Compared with groups administered 1 and 10 nmol/l of BI 2536, the addition of BI 2536 in minimally effective concentrations resulted in greater levels of apoptosis in both osteosarcoma cell lines KHOS and U-2OS (Fig. 3c). In a next step, KHOS and U-2OS cells were released in the presence of BI 2536 with minimally effective concentrations for 24 and 48 h. They were then analyzed by flow cytometry. Compared with the control group, the majority of the cells treated with BI 2536 for 24 and 48 h were arrested in the G_2/M phase (Fig. 4a–c).

BI 2536 inhibits osteosarcoma cell proliferation in a three-dimensional culture and a mouse model

We asked whether the growth of osteosarcoma cells could be inhibited by BI 2536 in a 3D culture. It is possible that the drug efficacy might be significantly lower in

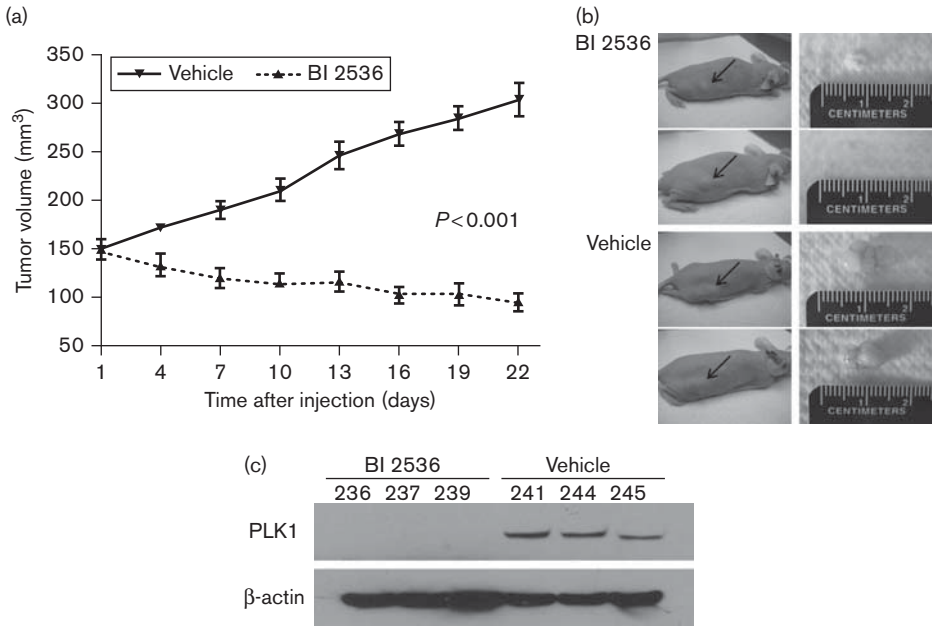
3D culture models than in two-dimensional (2D) models as cellular architecture might play an important role in drug uptake and distribution [21]. KHOS and U-2OS cells were seeded to a 3D culture system (as described in methods). After 24 h, BI 2536 was added to each well with a range of concentrations and was set to incubate for 72 h. The growth of osteosarcoma cells was inhibited in the 3D culture by approximately the same concentrations of BI 2536 as the 2D culture (Fig. 5b). As the concentrations of BI 2536 increased, the growth of cells became more and more impaired. For animal studies, BI 2536 or the vehicle control was administrated once intravenously into the tail vein of Crl:SHO-*Prkdc*^{SCID}*Hr*^{hr} nude female mice with 150 mm^3 of KHOS xenograft tumors. After the mice were treated with BI 2536 once, there was no major toxic (> 20%) body weight loss. BI 2536-treated mice had significantly smaller tumors compared with control mice ($P < 0.001$, Fig. 6a and b). The expression of PLK1 at a protein level was much lower in tumor tissues of the mice treated with BI 2536 than with vehicle control (Fig. 6c).

Discussion

Recently, PLK1 has been identified as a crucial kinase for osteosarcoma cell growth and survival. Targeting PLK1 with shRNA or siRNA resulted in tumor cell growth inhibition and induced apoptosis [10,22]. Similar results have been reported in rhabdomyosarcoma [11]. In this study, we further analyzed the effect of PLK1 down-regulation mediated by its inhibitor, BI 2536. We observed the antitumor activities of BI 2536 both *in vitro* and in animal tumor models of osteosarcoma. First, we observed that BI 2536 could obviously inhibit cell growth and viability in 2D and 3D culture models of KHOS and U-2OS cells. The expression of PLK1 is decreased after treatment with BI 2536 in dose-dependent and time-dependent manners. Second, the administration of BI 2536 induced mitotic arrest and cell apoptosis in KHOS and U-2OS cells. Finally, in mouse xenograft models, BI 2536 showed the ability to block the growth of human osteosarcoma.

PLK1 is an evolutionarily conserved serine/threonine kinase, which functions as a regulator of mitosis and genomic stability [23]. It has also been shown that PLK1 and survivin interact during mitosis, and PLK1 phosphorylates survivin at serine 20. Interestingly, both PLK1 and survivin are highly expressed in cancers [24–27]. More recently, BI 2536 was found to synergize with imatinib and nilotinib in chronic myelogenous leukemia cells and inhibit proliferation of both imatinib-sensitive and imatinib-resistant chronic myelogenous leukemia cells [18]. However, in this study, we did not find a synergistic

Fig. 6



Inhibition of tumor growth with BI 2536 in subcutaneous xenograft mouse model. Four-week-old Crl:SHO-*Prkdc*^{SCID}*Hr*^{hr} nude female mice with KHOS xenograft tumors were treated intravenously into the tail vein with vehicle or BI 2536 at the dose of 50 mg/kg once. (a) Effects of BI 2536 on KHOS cell xenograft tumor growth. (b) Representative tumor photographs of the mice treated with vehicle control or BI 2536. (c) Expression levels of PLK1 in tumor tissues of the mice treated with BI 2536 or vehicle (236, 237, and 239 were mice treated with BI 2536; 241, 244, and 245 were mice treated with vehicle control). Black arrows point to the position of the subcutaneously implanted tumor on each mouse.

effect of BI 2536 with doxorubicin in osteosarcoma cells (data not shown). The potential role of PLK1 during tumorgenesis has also been investigated. For example, normal fibroblast NIH3T3 cells with a constitutive expression of PLK1 by transfection could grow in soft agar and form tumors in nude mice [28]. PLK1 has been established as a novel target for cancer treatment and inhibition or depletion of PLK1 activity has been tested in cancer therapy [23,29–31].

The expression and activity of PLK1 begin to rise in G_2 and peak during the M phase. Exposure to BI 2536 results in spindle defects, chromatin separation failure, and metaphase arrest, which may inhibit cell proliferation, cause arrested cell death, and lead to a reduction of PLK1 expression [23]. We validated the inhibition effects of BI 2536 on PLK1 expression and established the minimally effective concentration in osteosarcoma cell lines. Concentrations as low as 20–40 nmol/l of BI 2536 were sufficient to cause mitotic arrest, growth pattern changes, and eventually proceed toward apoptosis and cell death. These effects, which were hallmarks of PLK1 inhibition, suggest that the inhibitor was exerting its effect in osteosarcoma cells [13,23]. Importantly, the expression of PLK1 was significantly higher in sarcoma cell lines and osteosarcoma tissues than in human osteoblast cells [10]. Osteoblast cells proved to be less sensitive to PLK1 inhibition by siRNA or shRNA, implying that PLK1 has a unique function in maintaining the integrity and promoting the proliferation of malignant osteoblast cells. According to xenograft mouse models, BI 2536 significantly decreased the tumor volume and its PLK1 expression level, which also suggests the possibility of directing against PLK1 as a therapeutic target in the treatment of osteosarcoma.

In conclusion, these findings suggest that PLK1 can be exploited as a potential target in the treatment of osteosarcoma, and its inhibitor BI 2536 could be examined in clinical trials of human cancer therapy in the future.

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Conflicts of interest statement: no potential conflicts of interest were disclosed.

References

1 Messerschmitt PJ, Garcia RM, Abdul-Karim FW, Greenfield EM, Getty PJ. Osteosarcoma. *J Am Acad Orthop Surg* 2009; **17**:515–527.
2 Chou AJ, Geller DS, Gorlick R. Therapy for osteosarcoma: where do we go from here? *Paediatr Drugs* 2008; **10**:315–327.
3 Bacci G, Ferrari S, Mercuri M, Longhi A, Capanna R, Tienghi A, *et al.* Neoadjuvant chemotherapy for extremity osteosarcoma: preliminary results of the Rizzoli's 4th study. *Acta Oncol* 1998; **37**:41–48.
4 Fuchs N, Bielack SS, Epler D, Bieling P, Delling G, Korholz D, *et al.* Long-term results of the co-operative German–Austrian–Swiss osteosarcoma study group's protocol coss-86 of intensive multidrug chemotherapy and surgery for osteosarcoma of the limbs. *Ann Oncol* 1998; **9**:893–899.
5 Trent JC. Rapid evolution of the biology and treatment of sarcoma. *Curr Opin Oncol* 2008; **20**:393–394.
6 Tan ML, Choong PF, Dass CR. Osteosarcoma: conventional treatment vs. gene therapy. *Cancer Biol Ther* 2009; **8**:106–117.
7 Liu X, Lei M, Erikson RL. Normal cells, but not cancer cells, survive severe plk1 depletion. *Mol Cell Biol* 2006; **26**:2093–2108.
8 Martin BT, Strebhardt K. Polo-like kinase 1: target and regulator of transcriptional control. *Cell Cycle* 2006; **5**:2881–2885.
9 Van Vugt MA, Medema RH. Getting in and out of mitosis with polo-like kinase-1. *Oncogene* 2005; **24**:2844–2859.
10 Duan Z, Ji D, Weinstein EJ, Liu X, Susa M, Choy E, *et al.* Lentiviral shRNA screen of human kinases identifies plk1 as a potential therapeutic target for osteosarcoma. *Cancer Lett* 2010; **292**:220–229.
11 Hu K, Lee C, Qiu D, Fotovati A, Davies A, Abu-Ali S, *et al.* Small interfering RNA library screen of human kinases and phosphatases identifies polo-like kinase 1 as a promising new target for the treatment of pediatric rhabdomyosarcomas. *Mol Cancer Ther* 2009; **8**:3024–3035.
12 Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, Krssak M, *et al.* Bi 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth *in vivo*. *Curr Biol* 2007; **17**:316–322.
13 Lenart P, Petronczki M, Steegmaier M, Di Fiore B, Lipp JJ, Hoffmann M, *et al.* The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr Biol* 2007; **17**:304–315.
14 Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, Kaiser R, *et al.* Phase I dose escalation and pharmacokinetic study of BI 2536, a novel polo-like kinase 1 inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2008; **26**:5511–5517.
15 Hofheinz RD, Al-Batran SE, Hochhaus A, Jager E, Reichardt VL, Fritsch H, *et al.* An open-label, phase I study of the polo-like kinase-1 inhibitor, BI 2536, in patients with advanced solid tumors. *Clin Cancer Res* 2010; **16**:4666–4674.
16 Lourda M, Trougakos IP, Gonos ES. Development of resistance to chemotherapeutic drugs in human osteosarcoma cell lines largely depends on up-regulation of clusterin/apolipoprotein j. *Int J Cancer* 2007; **120**:611–622.
17 Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of mcf-10a mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 2003; **30**:256–268.
18 Gleixner KV, Ferenc V, Peter B, Gruze A, Meyer RA, Hadzijusufovic E, *et al.* Polo-like kinase 1 (plk1) as a novel drug target in chronic myeloid leukemia: overriding imatinib resistance with the plk1 inhibitor BI 2536. *Cancer Res* 2010; **70**:1513–1523.
19 Nappi TC, Salerno P, Zitzelsberger H, Carlomagno F, Salvatore G, Santoro M. Identification of polo-like kinase 1 as a potential therapeutic target in anaplastic thyroid carcinoma. *Cancer Res* 2009; **69**:1916–1923.
20 Renner AG, Dos Santos C, Recher C, Bailly C, Creancier L, Kruczynski A, *et al.* Polo-like kinase 1 is overexpressed in acute myeloid leukemia and its inhibition preferentially targets the proliferation of leukemic cells. *Blood* 2009; **114**:659–662.
21 Horning JL, Sahoo SK, Vijayaraghavalu S, Dimitrijevic S, Vasir JK, Jain TK, *et al.* 3-D tumor model for in-vitro evaluation of anticancer drugs. *Mol Pharm* 2008; **5**:849–862.
22 Yamaguchi U, Honda K, Satow R, Kobayashi E, Nakayama R, Ichikawa H, *et al.* Functional genome screen for therapeutic targets of osteosarcoma. *Cancer Sci* 2009; **100**:2268–2274.
23 Strebhardt K, Ullrich A. Targeting polo-like kinase 1 for cancer therapy. *Nat Rev Cancer* 2006; **6**:321–330.
24 Colnaghi R, Wheatley SP. Liaisons between survivin and plk1 during cell division and cell death. *J Biol Chem* 2010; **285**:22592–22604.
25 Glinsky GV. Genomic models of metastatic cancer: functional analysis of death-from-cancer signature genes reveals aneuploid, anoikis-resistant, metastasis-enabling phenotype with altered cell cycle control and activated polycomb group (pcg) protein chromatin silencing pathway. *Cell Cycle* 2006; **5**:1208–1216.
26 Noh EJ, Lim DS, Jeong G, Lee JS. An hdac inhibitor, trichostatin a, induces a delay at G_2/M transition, slippage of spindle checkpoint, and cell death in a transcription-dependent manner. *Biochem Biophys Res Commun* 2009; **378**:326–331.

27 Noh EJ, Lim DS, Lee JS. A novel role for methyl cpg-binding domain protein 3, a component of the histone deacetylase complex, in regulation of cell cycle progression and cell death. *Biochem Biophys Res Commun* 2009; **378**:332–337.
28 Smith MR, Wilson ML, Hamanaka R, Chase D, Kung H, Longo DL, *et al.* Malignant transformation of mammalian cells initiated by constitutive expression of the polo-like kinase. *Biochem Biophys Res Commun* 1997; **234**:397–405.
29 Cogswell JP, Brown CE, Bisi JE, Neill SD. Dominant-negative polo-like kinase 1 induces mitotic catastrophe independent of cdc25c function. *Cell Growth Differ* 2000; **11**:615–623.
30 Lane HA, Nigg EA. Antibody microinjection reveals an essential role for human polo-like kinase 1 (plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol* 1996; **135**:1701–1713.
31 Liu X, Erikson RL. Polo-like kinase (plk)1 depletion induces apoptosis in cancer cells. *Proc Natl Acad Sci USA* 2003; **100**:5789–5794.