BI 2536-mediated PLK1 inhibition suppresses HOS and MG-63 osteosarcoma cell line growth and clonogenicity

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Osteosarcoma is the most common primary malignant tumor of bone, which frequently occurs in the second decade of life. Despite the improvements in neoadjuvant chemotherapy, the outcome of patients with chemoresistant or metastatic tumors is still poor. Therefore, there is a need for the development of more efficient therapeutic agents. BI 2536, an innovative selective inhibitor of Polo-like kinase 1, has shown anticancer potential promoting mitotic arrest and apoptosis in a variety of tumor cells, including osteosarcoma. Here, we present more evidence of the antiproliferative effects of BI 2536 on HOS and MG-63 osteosarcoma cell lines. Our results showed that nanomolar concentrations (10, 50, and 100 nmol/l) of the drug significantly decreased cell proliferation and clonogenic capacity, inducing mitotic arrest and aneuploidy. Interestingly, although BI 2536 mediated a moderate increase of apoptosis after 48 h in HOS cells, no increased caspase-3 activity was detected for MG-63 cells. In contrast to previous studies, we show that perturbation of normal mitotic progression by BI 2536 in these osteosarcoma cell lines results in caspase-independent mitotic catastrophe followed by necrosis. Our findings reinforce the likelihood of directing against Polo-like kinase 1 as a therapeutic option in the treatment of osteosarcoma. ***Anti-Cancer Drugs*** **22:995–1001 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Osteosarcoma (OS) is a highly malignant tumor of the bone with a peak incidence during the adolescent growth period [1]. Current treatment consists of combined chemotherapy (mainly cisplatin, doxorubicin, and methotrexate) and surgical ablation. Nevertheless, despite the advances attained over the last 30 years, the improvement of the cure rate was modest, with a 5-year disease-free survival of 60% [2]. Still, a substantial proportion of patients relapse or fail conventional therapy, making the search for new therapeutic agents imperative. Different approaches are now being used to identify new molecular markers for therapeutic purposes. Among these, a lentiviral small hairpin RNA screening of human kinases recently identified Polo-like kinase 1 (PLK1) as a potential treatment target for OS, demonstrating that when knocked down its inactivation results in growth arrest and apoptosis [3].

The last volume of *Anti-cancer Drugs* published an interesting study by Liu *et al*. [4] in which the investigators described their findings on PLK1 inhibition by BI 2536 in KHOS and U-2OS OS cell lines as well as in xenograft mouse models. Here, we present more evidence on the antiproliferative effects of this specific inhibitor on two different OS cell lines (HOS and MG-63), reinforcing the likelihood of directing against PLK1 as a therapeutic option in the treatment of OS.

Materials and methods

Cell culture

The human OS cell lines, HOS and MG-63, were kindly provided by Dr Jeremy A. Squire (Department of Pathology and Molecular Medicine, Kingston General Hospital, Queen's University, Kingston, Ontario, Canada). Cells were cultured in Ham's F10 (Gibco BRL, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO_2 incubator.

Drug and treatment

BI 2536 was purchased from Boehringer Ingelheim Pharmaceuticals and diluted in dimethyl sulfoxide according to the manufacturer's instructions. For all experiments, cells were treated with nanomolar concentrations (10, 50, and 100 nmol/l) in accordance with the literature [5]. Corresponding control cultures received an equal volume of solvent.

Measurement of cell growth by the XTT cell proliferation assay

In brief, cells were seeded in 96-well flat-bottom plates (2500 cells/well) and were allowed to attach to the incubator overnight. After this period, BI 2536 was added in varying concentrations to each well and incubated for

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24, 48, and 72 h. After treatment, the culture medium was removed and replaced with a medium containing 10 µl of XTT dye (3 mg/ml) (XTT II; Roche Molecular Biochemicals, Indianapolis, Indiana, USA) in each well. The plates were incubated for 2 h at 37°C and the formazan product was measured at 455 and 650 nm by using an iMark microplate reader (Bio-Rad Laboratories Inc., Benicia, California, USA). Each experiment was performed in triplicate wells and repeated in three sets of tests.

Colony formation assay

Clonogenic assays were performed according to Franken *et al.* [6]. After trypsinization, single cell suspensions of 200 cells were seeded in six-well plates and treated with BI 2536 at 10, 50, and 100 nmol/l concentrations for 48 h. After this period, the culture medium was removed and replaced with drug-free medium. The cell cultures were incubated for 7 days and then the colonies were rinsed with PBS, fixed with methanol, and stained with Giemsa stain. The colonies with more than 50 cells were counted. Assays were performed in triplicate.

Detection of apoptotic cells

For apoptosis 3×10^4 cells were seeded on six-well plates containing 3 ml of culture medium. After 24 h, the medium was replaced and cells were treated with different concentrations of BI 2536 and cultured for an additional 48 h. Caspase activity was measured through the NucView 488 Caspase-3 Detection in Living Cells kit (Biotium Inc. Hayward, California, USA) according to the manufacturer's instructions. Concisely, treated cells were trypsinized and incubated for 40 min at room temperature with the capase-3 substrate. After this period, cells were fixed in formaldehyde and counterstained with 4′,6-diamidino-2-phenylindole. Then, samples were mounted, coverslipped, and analyzed by fluorescence microscopy with a triple filter. Five hundred nuclei were analyzed per treatment and cells were scored and categorized according to differential staining.

Detection of necrotic cells by differential staining

Dead cells were recognized by differential staining according to Lee and Shacter [7]. Treated cells were centrifuged and incubated for 5 min at 37°C with bisbenzimide (Hoechst 33342), propidium iodide, and fluorescein diacetate (Sigma Chemical Co., St. Louis, USA). Then, samples were mounted, coverslipped, and analyzed by fluorescence microscopy with a triple filter. Cells were scored and categorized as follows: (a) normal = blue nucleus and green cytoplasm, (b) apoptotic = fragmented blue nucleus and green cytoplasm, and (c) necrotic = red nucleus. Five hundred nuclei were analyzed per treatment.

Cytogenetic analysis and chromosome preparations

A total of 2×10^5 cells were seeded in 25 cm^2 tissue culture flasks containing 5 ml of culture medium. After 24 h, the medium was replaced and cells were treated with BI 2536 in varying concentrations and then incubated for 24, 48, and 72 h until harvesting. After each period, cells were trypsinized, hypotonically treated (0.075 M KCl) for 20 min at 37°C, and then fixed three times with methanol : glacial acetic acid (3 : 1). The fixed cells were then dropped onto glass slides, allowed to air dry, and stained with Giemsa stain. The mitotic index was calculated as the ratio between the number of cells in mitosis and the total number of cells (1000 cells were scored). For the study of aneuploidy, colcemid (0.1 µg/ml) was added 90 min before harvesting time. Fifty metaphases were analyzed per treatment.

Cell cycle analysis

After drug treatment, cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed on a Guava Personal Cell Analysis system (Guava Technologies, Hayward, California, USA) according to the standard protocol provided by the manufacturer. Percentages of cells in G0/G1, S, or G2/M phase were collected and processed using the Guava CytoSoft 4.2.1 version software.

Statistical analysis

Statistical analyses were carried out by using the SigmaStat software (Jandel Scientific Company, San Rafael, California, USA). One-way repeated measures analysis of variance followed by the Holm–Sidak pairwise multiple comparison was used to establish whether significant differences existed between groups. All tests were carried out for $\alpha = 0.05$.

Results

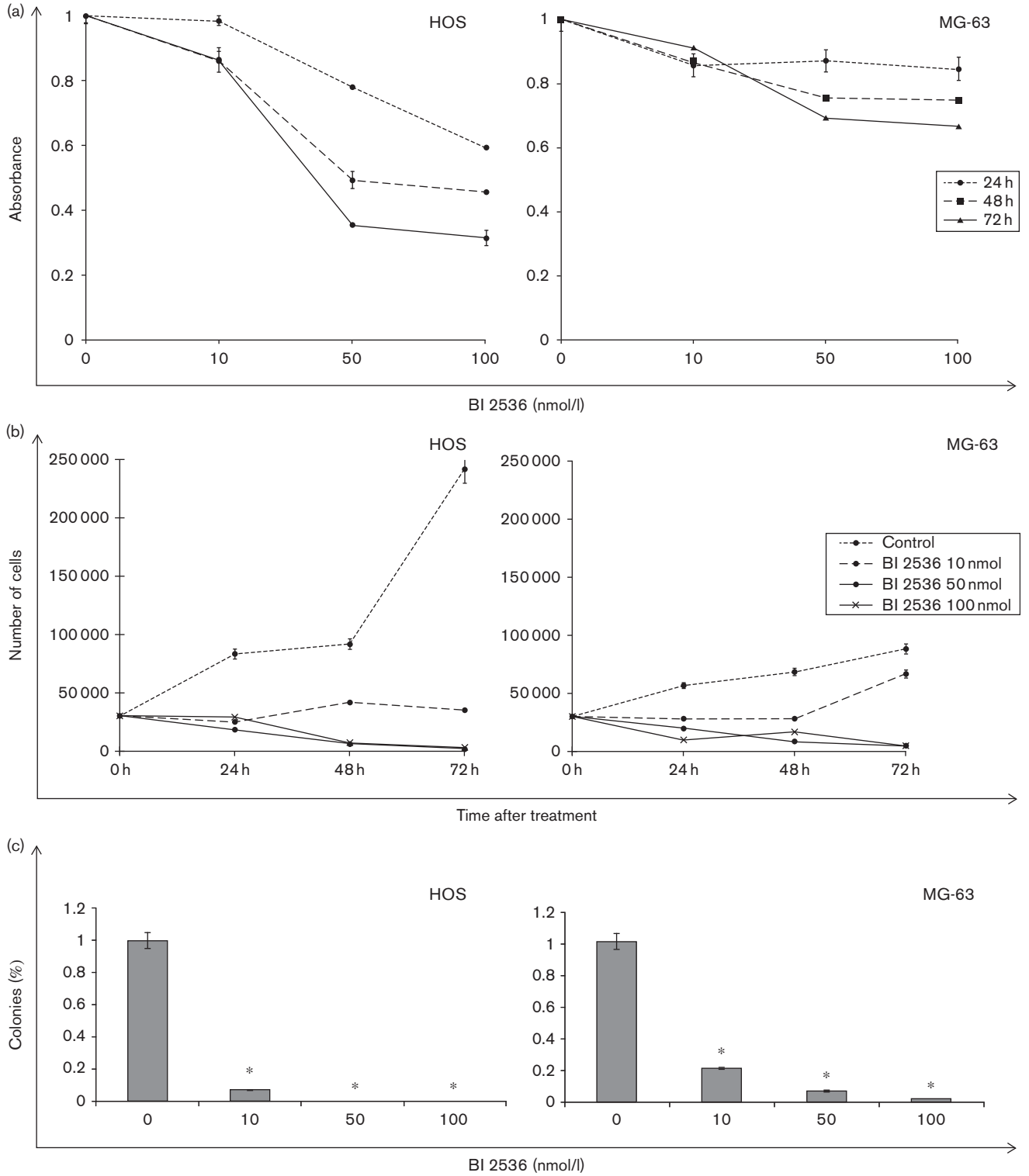
BI 2536 inhibits cell proliferation *in vitro*

BI 2536 at 50 and 100 nmol/l significantly inhibited growth of both OS cell lines when compared with the control (dimethyl sulfoxide: 0.1%) at all times tested ($P < 0.05$) (Fig. 1a). BI 2536 (10 nmol/l) concentration also induced a significant reduction in cell proliferation in MG-63 cells at all times tested ($P < 0.05$), although this effect in HOS cells was only observed at 48 h. The drug presented a maximum effect at 72 h, reducing proliferation in 31% and 65% for MG-63 and HOS cells, respectively (Fig. 1a). Cell growth monitored at selected intervals through Trypan blue exclusion also demonstrated a decrease in the counts of viable cells along with increasing BI 2536 concentrations (Fig. 1B).

BI 2536 potently abrogates the clonogenic capacity of osteosarcoma cell lines

PLK1 inhibition by BI 2536 significantly reduced the colony formation capacity for both cell lines when compared with the control ($P < 0.05$) (Fig. 1b). The clonogenic capacity of HOS cell line was reduced to 94% at the 10 nmol/l treatment, whereas no colonies were observed at higher concentrations. In the case of MG-63 cells, BI 2536 reduced the clonogenic capacity in 79%, 94%, and 99% for 10 nmol/l, 50 nmol/l, and 100 nmol/l, respectively.

Fig. 1



(a) Growth inhibition in HOS and MG-63 cell lines treated with BI 2536 at the indicated concentrations for 24, 48, and 72 h (XTT assay); (b) Cell growth curve after treatment with the drug; (c) BI 2536 potently abrogated the clonogenic capacity of both osteosarcoma cell lines ($*P < 0.05$).

Table 1 Cell cycle analysis of HOS and MG-63 osteosarcoma cell lines treated with BI 2536

	HOS			MG-63		
	24 h	48 h	72 h	24 h	48 h	72 h
G1 (%)						
Control	48.7 ± 2.6	54.7 ± 1.2	67.1 ± 1.1	42.9 ± 0.5	55.1 ± 1.2	67.6 ± 1.5
10 nmol/l	15.5 ± 1.2	27.6 ± 2.9	31.7 ± 3.1	20.6 ± 6.5	22.2 ± 0.2	39.9 ± 9.0
50 nmol/l	4.8 ± 0.4	42.3 ± 25.7	22.7 ± 7.4	3.6 ± 0.1	19.4 ± 6.6	27.7 ± 11.7
100 nmol/l	5.0 ± 1.0	48.7 ± 18.4	18.3 ± 4.9	3.0 ± 0.4	17.5 ± 4.1	18.9 ± 5.6
S (%)						
Control	16.5 ± 3.5	12. ± 0.4	10.3 ± 1.5	19.0 ± 3.2	12.9 ± 0.1	9.7 ± 1.7
10 nmol/l	12.4 ± 1.7	11.4 ± 4.1	11.5 ± 0.4	16.6 ± 4.3	11.1 ± 1.2	20.5 ± 2.0
50 nmol/l	6.0 ± 0.9	7.0 ± 1.6	7.2 ± 6.2	5.5 ± 0.8	10.9 ± 0.3	16.4 ± 2.1
100 nmol/l	5.6 ± 0.8	9.8 ± 3.7	7.8 ± 0.9	5.0 ± 1.6	8.4 ± 0.3	17.8 ± 1.3
G2/M (%)						
Control	34.6 ± 1.1	32.4 ± 1.63	25.5 ± 2.13	37.9 ± 2.8	31.6 ± 1.1	22.7 ± 3.3
10 nmol/l	71.9 ± 2.2	60.8 ± 5.2	60.0 ± 2.16	65.6 ± 4.3	66.3 ± 1.1	39.4 ± 11.3
50 nmol/l	89.0 ± 0.5	49.8 ± 23.6	66.9 ± 12.0	90.7 ± 0.8	66.1 ± 7.0	56.4 ± 9.3
100 nmol/l	89.3 ± 1.4	41.1 ± 14.9	71.4 ± 7.6	91.8 ± 2.0	70.5 ± 4.5	63.8 ± 6.8

Percentages of cells in G1, S, and G2/M phases are expressed as mean ± standard deviation

BI 2536 induces necrosis-like death in osteosarcoma cells

To determine the induction of apoptosis in OS cells treated with BI 2536, caspase-3 activity was measured. Compared with the control, BI 2536 treatment of the HOS cell line mediated a moderate increase of apoptosis after 48 h ($P < 0.05$). However, no increased caspase-3 activity was observed after the treatment of MG-63 at either of the concentrations tested (Fig. 2a). Instead, the microscopic analysis of treated cells by differential staining with propidium iodide demonstrated a higher frequency of necrotic-like cells for both cell lines in a dose-dependent manner and time-dependent manner, especially for treatments with 100 nmol/l that showed more than 60% of propidium iodide-stained cells after 72 h (Fig. 2b).

BI2536 induces mitotic arrest of osteosarcoma cell lines

BI 2536 treatment induced a strong G2/M arrest in HOS and MG-63 cells when compared with untreated cells at all times tested in a dose-dependent manner (Table 1). This effect was also demonstrated by the significant increase of mitotic cells in a dose-dependent manner and time-dependent manner ($P < 0.05$) (Fig. 3a). Of note, a higher number of mitotic cells were observed after 24 h of treatment, especially in the case of HOS with 60% of mitotic cells after treatment with 100 nmol/l BI 2536.

BI 2536 increases chromosome instability in osteosarcoma cells

Treatment with different concentrations of BI 2536 induced an increase in chromosome instability in HOS and MG-63 cells that escaped mitotic arrest. As shown in Fig. 2b, even though both cell lines present some inherent chromosome instability, cells without treatment showed constant numbers of chromosomes along treatment times. However, in treated cells the chromosome number distribution showed a displacement, being more evident for the 100 nmol/l treatment (Fig. 3b).

Discussion

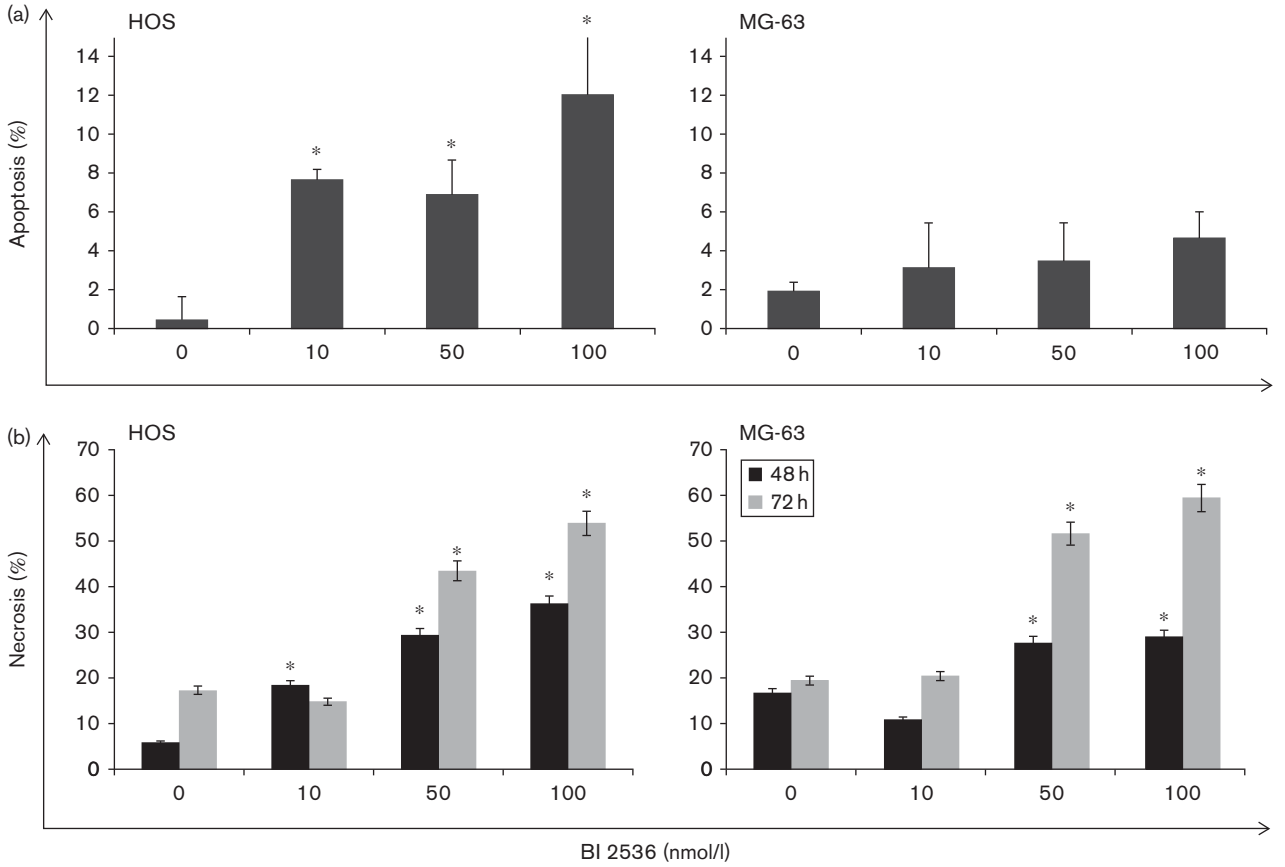
PLK1 is a serine/threonine kinase that functions in regulating many stages of mitosis progression [8]. This protein is highly expressed in various human malignant neoplasms, such as gastric cancer, breast cancer, lung cancer, hepatomas, and among others, OS [9–10]. Particularly, it has been demonstrated that patients with this aggressive bone tumor and high PLK1 expression levels show significantly shorter survival [3].

The inveterate anticancer potential of PLK1 inhibition in OS has been corroborated through different functional studies [11–13]. More recently, Liu *et al.* [4] demonstrated that BI 2536, an ATP-competitive PLK1 inhibitor, efficiently inhibits proliferation and induces mitotic arrest followed by apoptosis in KHOS and U-2OS cell lines. Here, we present more evidence on the antiproliferative effects of this synthetic compound on HOS and MG-63 OS cell lines. Our results also show that nanomolar concentrations (10, 50, and 100 nmol/l) of the drug significantly decrease cell proliferation, inducing G2/M arrest and aneuploidy.

Commonly, OS tumors show intrinsic resistance to preoperative chemotherapy based on methotrexate, cisplatin, and doxorubicin. Previous studies have shown that these fast-proliferating cell lines are resistant to such drugs [14]. In contrast, the results presented by Liu *et al.* [4] and our group show that these cell lines are remarkably vulnerable to BI 2536.

Binding of this drug with PLK1 results in mitotic arrest and apoptosis in a variety of tumor cell types with different oncogene signatures [5]. The in-vitro effect of this drug on the HOS and MG-63 cell-cycle progression by flow cytometry showed an accumulation of cells with a doubled DNA content (G2/M). Further cytogenetic analysis demonstrated a blockage in mitosis, with higher accumulation of dividing cells after 24 h of treatment. Remarkably, at 100 nmol/l BI 2536 concentration, HOS cell line showed

Fig. 2



(a) After 48 h of treatment, BI 2536 moderately increased apoptosis in HOS cell lines; however, no caspase-3 activity was detected for MG-63 cells, suggesting that perturbation of normal mitotic progression might be followed by a caspase-3-independent mitotic catastrophe. (b) Differential staining with propidium iodide demonstrated a significant increase of necrotic-like cells in a dose-dependent manner and time-dependent manner. Statistically different $*P < 0.05$.

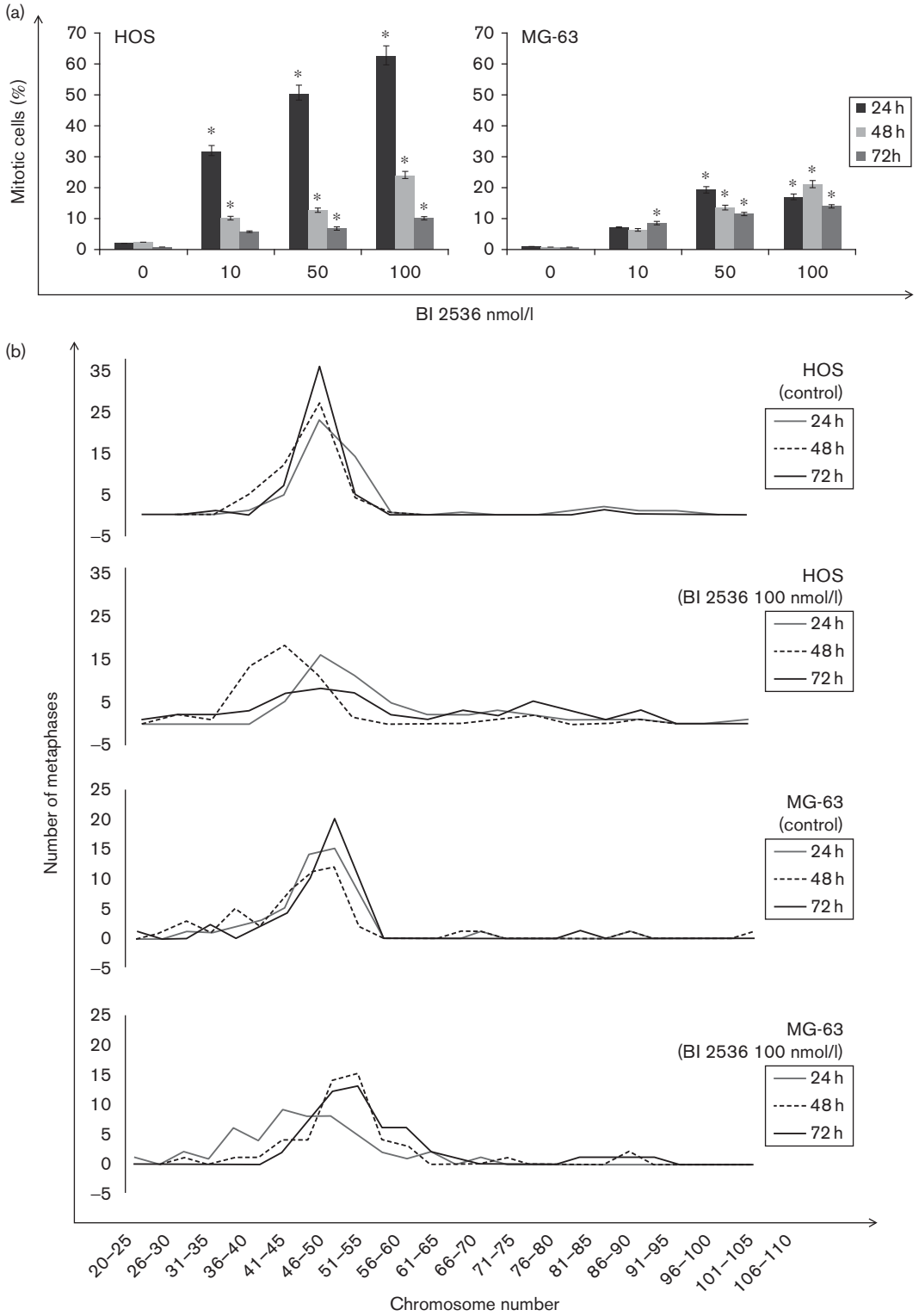
60% of cells in mitosis at 24 h, although the number of mitotic cells was reduced to 25% and 10% at 48 h and 72 h, respectively, coinciding with the decrease in proliferation observed by the XTT assay. Furthermore, along with the decrease of cells in mitosis, those that escape death tend to divide asymmetrically in the subsequent cycles, with the consequent generation of aneuploid cells.

It has been demonstrated that small interfering RNA-mediated PLK1 inhibition in U-2OS cells also interferes with the correct assembly of the anaphase-promoting complex, inhibiting the separation of sister chromatids and leading to failure of cytokinesis [15]. Eventually, after repeated attempts of division, cells proceed toward apoptosis and cell death. In this study, BI 2536 mediated an increase of apoptosis after 48 h in HOS cells. However, although statistically significant, apoptosis induction was not as evident as shown by Liu *et al.* [4] for KHOS and U-2OS. In addition, no increased caspase-3 activity was detected for MG-63 cells, suggesting that disruption of normal mitotic progression by BI 2536 in these cell lines might lead to other mechanisms of death rather than apoptosis. Noteworthy, cell cycle analysis showed a rise of 'sub-G1' peaks in cells treated with higher concentrations of BI 2536 (data not shown). Similarly, the microscopic analysis of differential staining with propidium iodide demonstrated the disruption of plasmatic membrane integrity, which is also an indicative of necrosis-like death.

Interestingly, cytological studies have demonstrated that BI2536-treated cells die mostly due to mitotic catastrophe [16], a form of cell death occurring from within mitosis that can also follow caspase-independent pathways [17]. Indeed, it has been demonstrated that apoptosis is not always required for mitotic catastrophe lethality, as cells can also undergo slow death in a necrosis-like manner with the characteristic loss of nuclear and plasma membrane integrities [18].

In contrast, OS treatment outcome is often hampered by the tumor's properties of metastasis and subsequent recurrence, making the study of the ability of cells to form colonies an essential requisite for testing potential therapeutic targets. Our results showed that BI 2536

Fig. 3



BI 2536 treatments induced blockage in mitosis, with higher accumulation of dividing cells after 24 h of treatment (a) and increasing chromosome instability in subsequent rounds of cell division (b). Statistically different $*P < 0.05$.

potently abrogated HOS and MG-63 clonogenic capacity. Similar findings have been reported in BI2536-treated KG1 cells and small interfering RNA-treated primary AML cells [19]. Interestingly, BI 2536 inhibited growth of OS tumor xenografts in nude mice [4]. In addition, it was shown to induce regression of large tumors with well-tolerated intravenous dose regimens [5], giving pharmacologic perspectives to avoid severe nephrotoxicity and neurotoxicity displayed by this treatment.

Taken together, our data, along with that previously reported, reinforce the likelihood of directing against PLK1 as a therapeutic option in the treatment of OS.

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Conflicts of Interest

There are no conflicts of interest.

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