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Effect of AKT3 expression on MYC- and caspase-8-dependent apoptosis caused by polo-like kinase inhibitors in HCT 116 cells

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Polo-like kinase (PLK) is a cell-cycle regulator that is overexpressed in several cancer cell types. Polo-like kinase is considered a novel target for cancer therapies, and several PLK inhibitors (PLKis), including BI 2536, BI 6727, and GSK461364, have been developed. In this study, we established five BI 2536-resistant cell lines from human colorectal cancer HCT 116 cells, to explore the resistance mechanism and identify predictable biomarkers of PLKis. We showed that PLKi-induced caspase-8 activation was attenuated in the BI 2536-resistant cell lines. We also showed that the expression of P-glycoprotein (P-GP) and AKT3 was upregulated, whereas that of MYC was downregulated in some BI 2536-resistant cell lines. Expression of P-GP conferred resistance to PLKis, and PLKi-induced apoptosis was dependent on MYC and caspase-8 in HCT 116 cells. We also showed for the first time that AKT3 suppressed BI 6727-induced caspase-8 activation and conferred resistance to PLKis. Collectively, these results indicate that MYC, caspase-8, P-GP, and AKT3 play critical roles in PLKi-induced apoptosis. Therefore, they are candidate biomarkers of the pharmacological efficacy of PLKis.

Polo-like kinase (PLK) is a serine/threonine kinase with five isoforms, PLK1–5.[1] Polo-like kinases 1–4 have an N-terminal kinase domain and a C-terminal polo-box domain, whereas PLK5 lacks the N-terminal kinase domain.[1] Expression of PLK1 is low in G_0, G_1, and S phases of the cell cycle, begins to increase in G_2 phase, and is high in M phase.[2,3] Overexpression of PLK1 is associated with cancer progression and a poor prognosis.[4,5] Polo-like kinase 1 localizes to the centrosomes and spindle poles during prophase and metaphase, and then relocalizes to the spindle midzone during late anaphase.[6] Polo-like kinases phosphorylate their substrates during several steps of the cell cycle, and they play essential roles in promoting the entry into mitosis, as well as centrosome maturation and duplication.[1] Depletion of PLK1 induces G_2/M arrest and apoptosis with caspase-3 activation,[7,8] so it is considered an attractive target of cancer therapies.

Several PLK inhibitors (PLKis) have been developed, including BI 2536, BI 6727 (volasertib), and GSK461364.[9–11] BI 2536 and BI 6727 potently inhibit PLK1, PLK2, and PLK3. GSK461364 has >100-fold selectivity for PLK1 over PLK2 and PLK3.[12] BI 6727 has reported anticancer efficacy in various malignancies *in vitro* and *in vivo*.[13] It has a manageable safety profile and has been tested in a phase II clinical trial as a combination therapy with low-dose cytarabine for acute myeloid leukemia (AML).[14] A phase III clinical trial (NCT01721876; POLO-AML-2) is underway. Therefore, biomarker molecules involved in the efficacy or toxicity of BI 6727 and other PLKis are of interest, to improve the clinical benefits of these PLKis. The resistance mechanisms of PLKis are also an important issue. Various mechanisms, such as the increased expression of drug efflux transporters, the alteration of target molecules, the upregulation of survival signals, and the downregulation of cell death mechanisms, have been investigated.[15] The overexpression of P-glycoprotein (P-GP) is one of the commonest resistance mechanisms to BI 2536, BI 6727, and GSK461364, which are investigated in this study.[16–18] A PLK1 gene mutation has also been reported to confer resistance to BI 2536.[19]

In this study, we established five BI 2536-resistant cell lines (BI 10-1-5, BI 10-1-10, BI 20-1, BI 40-1, and BI 40-2) from human colorectal cancer HCT 116 cells, to investigate the mechanisms responsible for the sensitivity to PLKis. We found that the expression of P-GP and AKT3 was increased, whereas that of MYC was repressed in the BI 2536-resistant cell lines. We showed that PLKi-induced apoptosis was mediated by caspase-8 and modulated by AKT3 and MYC. These results suggest that P-GP and AKT3 are putative biomarkers of resistance to PLKis and, conversely, that MYC and caspase-8 reflect sensitivity to PLKis.

Materials and Methods

Cells and inhibitors. HCT 116 and HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 7% FBS and 50 μg/mL kanamycin at 37°C in a humidified atmosphere with 5% CO_2. The PLKis GSK461364, BI 2536, and BI 6727 were purchased from Selleck Chemicals

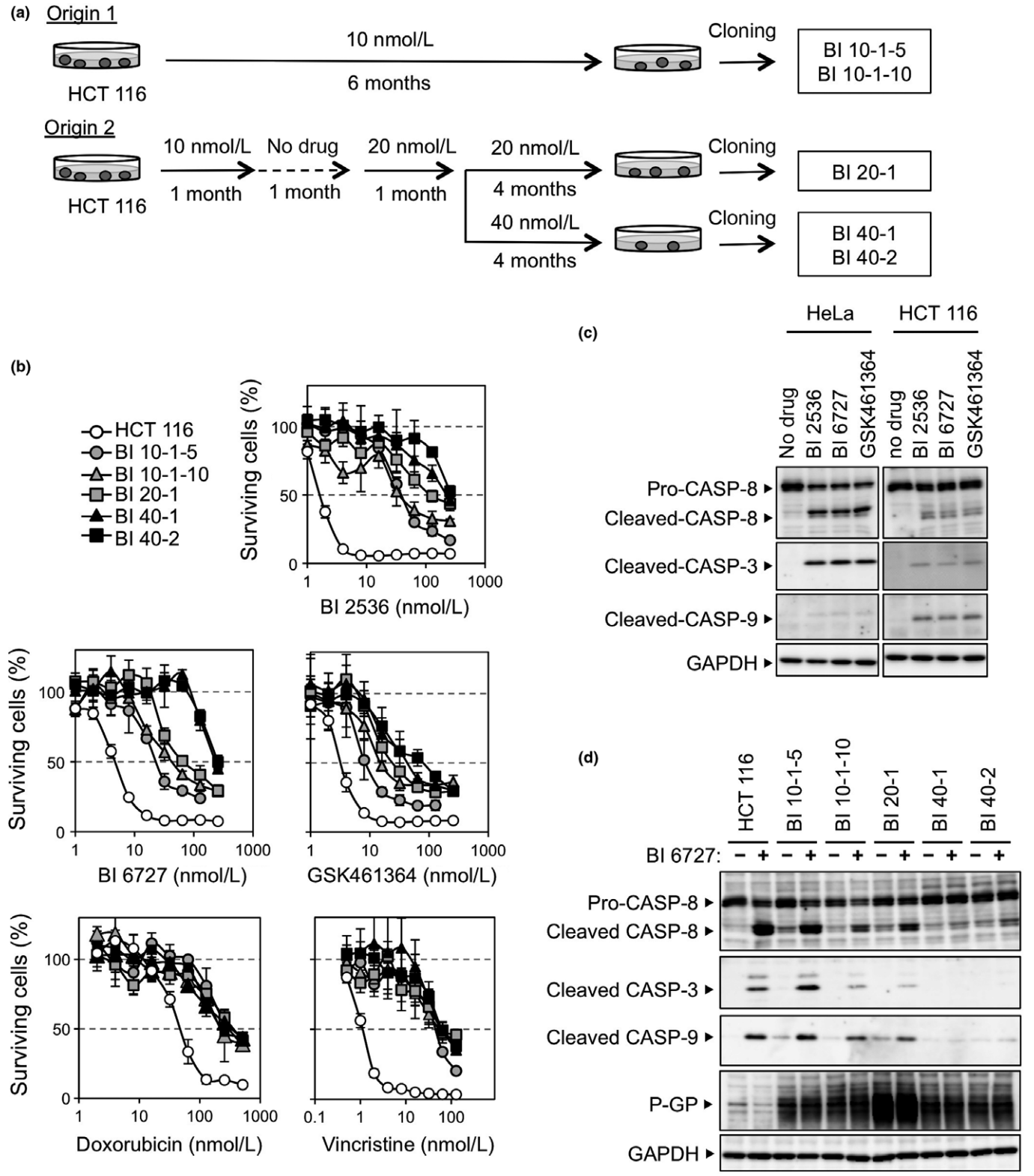


Fig. 1. Drug resistance of BI 2536-resistant cell lines. (a) Schematic protocol for the isolation of BI 2536-resistant cell lines from HCT 116 cells. The BI 10-1-5 and BI 10-1-10 cells were cloned in the presence of 10 nmol/L BI 2536, BI 20-1 cells in the presence of 20 nmol/L BI 2536, and BI 40-1 and BI 40-2 cells in the presence of 40 nmol/L BI 2536. (b) Sensitivity to polo-like kinase inhibitors (PLKis), doxorubicin, and vincristine in BI 2536-resistant cells. The cells were treated with BI 2536, BI 6727, GSK4561364, doxorubicin, or vincristine for 96 h and subjected to WST-8 assay. (c) PLKi-induced caspase activation. HeLa cells were treated with BI 2536 (10 nmol/L), BI 6727 (68 nmol/L), or GSK461364 (11 nmol/L), and HCT 116 cells were treated with BI 2536 (20 nmol/L), BI 6727 (70 nmol/L), or GSK461364 (12 nmol/L) for 48 h. The levels of cleaved caspase (CASP)-3, -8, and -9 were examined by Western blot analysis. (d) PLKi-induced caspase activation in BI 2536-resistant cell lines. Cells were treated with BI 6727 (100 nmol/L) for 48 h, and the levels of P-glycoprotein (P-GP) and cleaved caspase-3, -8, and -9 were examined by Western blot analysis.

(Houston, TX, USA). To establish BI 2536-resistant cell lines, HCT 116 cells were treated with increasing concentrations of BI 2536, starting from 10 nmol/L and gradually increasing to 40 nmol/L. Resistant cells were cloned from two independent origins (Fig. 1a).

Plasmid and transfectants. Wild-type (WT) and myristoylated (Myr) *AKT3* cDNAs (GenBank accession no. AF 135794) were isolated with a standard PCR method. A myristoylation sequence was added to the N-terminus, and the cDNA subcloned into the pD3HA plasmid vector.[20] To establish stable WT-*AKT3* transfectants, HCT 116 cells were transfected with the plasmid using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) and then selected with 800 μg/mL G418 (Thermo Fisher Scientific, Waltham, MA, USA). Stable Myr-*AKT3* transfectants were established similarly (Noguchi *et al.*, unpublished data). The expression of AKT3–hemagglutinin (HA) was confirmed by Western blot analysis.

Cell growth inhibition assay. WST-8 assays were carried out with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). In brief, cells were seeded into a 96-well plate at a density of 1×10^3 cells/well and incubated for 24 h. After incubation, the cells were treated with PLKis or anticancer drugs for 96 h. Cell counting kit solution (10 μL) was added to each well. After incubation for 3 h, the absorbance at 450 nm was measured with an Infinite M1000 microplate reader (Tecan Japan, Kanagawa, Japan). The data are presented as the means ± SD of triplicate determinations. The degree of resistance was calculated by dividing the IC_{50} of the cells of interest by those of the parent cells, as described previously.[21]

Western blot analysis. Cells were lysed in whole cell extract buffer (20 mmol/L Tris–HCl [pH 8.0], 50 mmol/L NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 5 mmol/L EDTA, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1% aprotinin, 10 μmol/L pepstatin, 10 μmol/L leupeptin, 10 μmol/L E-64d, and 0.1 μmol/L calyculin A) by sonicating them on ice. The protein in the cell lysates was estimated with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The protein samples were solubilized in SDS sample buffer (2% SDS, 50 mmol/L Tris-HCl [pH 8.0], 0.2% bromophenol blue, and 5% 2-mercaptoethanol) with boiling for 5 min at 70°C. Whole-cell lysates containing 10 μg protein were separated with SDS-PAGE, and then transferred onto PVDF membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). Western blotting signals were detected with the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and recorded with an ImageQuant LAS 4000 imager (GE Healthcare Japan, Tokyo, Japan). GAPDH was used as the loading control. The antibodies used were directed against: p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); MDR1 + 3 (C219) (Abcam, Cambridge, MA, USA); GAPDH (6C5) (Millipore); cleaved caspase-3 (Asp175; 5A1E), caspase-8 (1C12), cleaved caspase-9 (Asp330; #9501), AKT1 (C73H10), AKT2 (D6G4), AKT3 (L47B1), phospho-AKT (Ser473; D9E), glycogen synthase kinase 3β (GSK-3β) (D5C5Z), phospho-GSK-3β (Ser-9; D85E12), PLK1 (208G4) (Cell Signaling Technology, Danvers, MA, USA); and HA (3F10) and MYC (9E10) (Roche Diagnostics, Indianapolis, IN, USA).

Knockdown assay. HCT 116 cells were transfected with *CASP8*-targeting siRNA (Hs_CASP8_7 and Hs_CASP8_11; Qiagen, Valencia, CA, USA), *MYC*-targeting siRNA (siGENOME Human MYC [4609] siRNA-SMARTpool; Dharmacon, GE Healthcare, Amersham, UK) or a control scrambled siRNA (AllStars Negative Control siRNA; Qiagen) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). During the knockdown experiments, at 72 h after transfection, Western blot analysis and annexin V assay were carried out, and WST-8 assays were undertake at 96 h after transfection.

Annexin V assay and sub-G_1 population assay. The annexin V assay was carried out to calculate the proportions of apoptotic cells, using the Annexin-V–FLUOS Staining Kit (Roche Diagnostics). In brief, cells were seeded into 60-mm dishes (2×10^5 cells/dish) and incubated for 24 h. After incubation, the cells were treated with 100 nmol/L BI 6727 alone or in combination with 5 μmol/L MS-209,[22] a P-GP inhibitor, for 48 h. The cells were then trypsinized, washed twice with cold PBS, and centrifuged (1500 *g*, 5 min, 4°C). The cell pellets were suspended in HEPES buffer, and then stained with annexin V–FITC and propidium iodide (PI), and then incubated for 30 min at room temperature in the dark. The annexin-V-positive cells were analyzed with a BD LSR II flow cytometer (BD Biosciences, Tokyo, Japan).

For the sub-G_1 population assay, cells were seeded into 60-mm dishes (5×10^5 cells/dish) and on the next day, transfected with FuGENE HD Transfection Reagent. At 24 h after transfection, the cells were treated with BI 6727 (20 nmol/L) for 48 h. The cells were trypsinized and washed with cold PBS. The cells were suspended in 70% ethanol for 30 min for fixation, collected, stained with PI (40 μg/mL), and treated with RNase A (200 μg/mL). The cells were analyzed with a BD LSR II flow cytometer (BD Biosciences).

Colony formation assay. Cells were seeded into 60-mm dishes (5×10^5 cells/dish), and treated on the following day with BI 6727 (0, 3, 10, or 30 nmol/L). After 24 h, the cells were replated into a six-well plate at a density of 500 cells/well. After 14 days, the colonies were stained with 0.5%

Table 1. Drug sensitivity of BI 2536-resistant cell lines

Cell line	IC_{50} (nmol/L)/[fold of parent HCT 116]									
	BI 2536		BI 6727		GSK461364		Doxorubicin		Vincristine	
HCT 116	1.6	[1.0]	4.7	[1.0]	3.2	[1.0]	47	[1.0]	1.1	[1.0]
BI 10-1-5	39	[24]	23	[5.0]	8.5	[3.0]	310	[7.0]	45	[41]
BI 10-1-10	37	[23]	43	[9.0]	15	[5.0]	230	[5.0]	56	[51]
BI 20-1	120	[76]	67	[14]	25	[8.0]	360	[8.0]	61	[56]
BI 40-1	220	[140]	230	[49]	43	[13]	330	[7.0]	62	[57]
BI 40-2	>260	[>160]	260	[54]	77	[24]	240	[6.0]	62	[56]

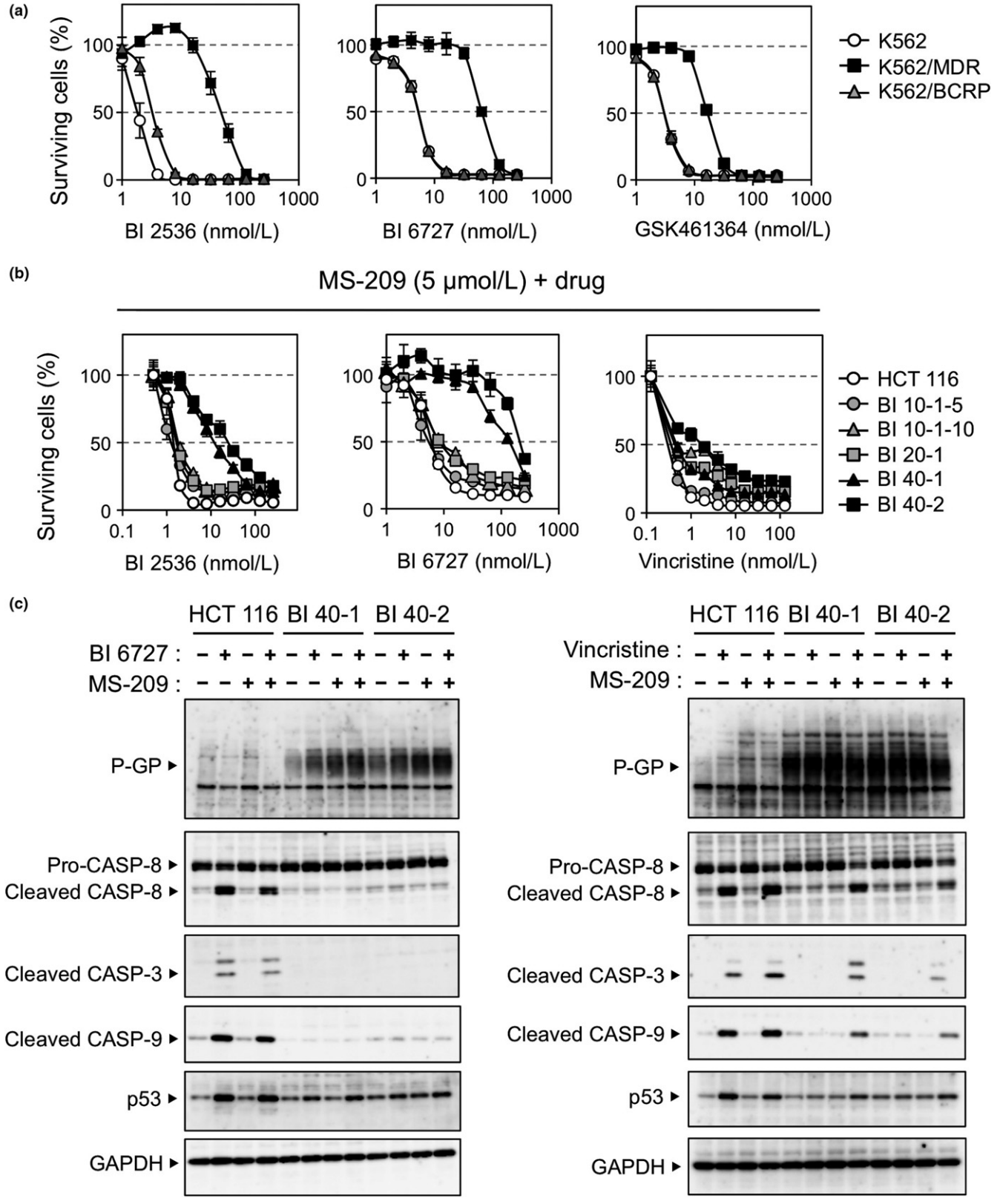


Fig. 2. Resistance to polo-like kinase inhibitors (PLKis) is not abolished by MS-209 in BI 40-1 and BI 40-2 cells. (a) Sensitivity to PLKis in P-glycoprotein (P-GP)- or breast cancer resistance protein (BCRP)-overexpressing K562, K562/MDR, and K562/BCRP cells. Cells were treated with PLKis for 96 h and subjected to WST-8 assay. (b) Effects of MS-209 on BI 2536-resistant cell lines. Cells were treated with BI 2536, BI 6727, or vincristine in the absence or presence of 5 μmol/L MS-209 for 96 h and subjected to WST-8 assay. (c) BI 6727- and vincristine-induced caspase activation in BI 40-1 and BI 40-2 cells. The cells were treated with either BI 6727 (100 nmol/L) or MS-209 (5 μmol/L) alone or in combination for 48 h (left panels). Similarly, the cells were treated with either vincristine (10 nmol/L) or MS-209 (5 μmol/L) alone or in combination for 48 h (right panels). The levels of P-GP, p53, and cleaved caspase (CASP)-3, -8, and -9 were examined by Western blot analysis.

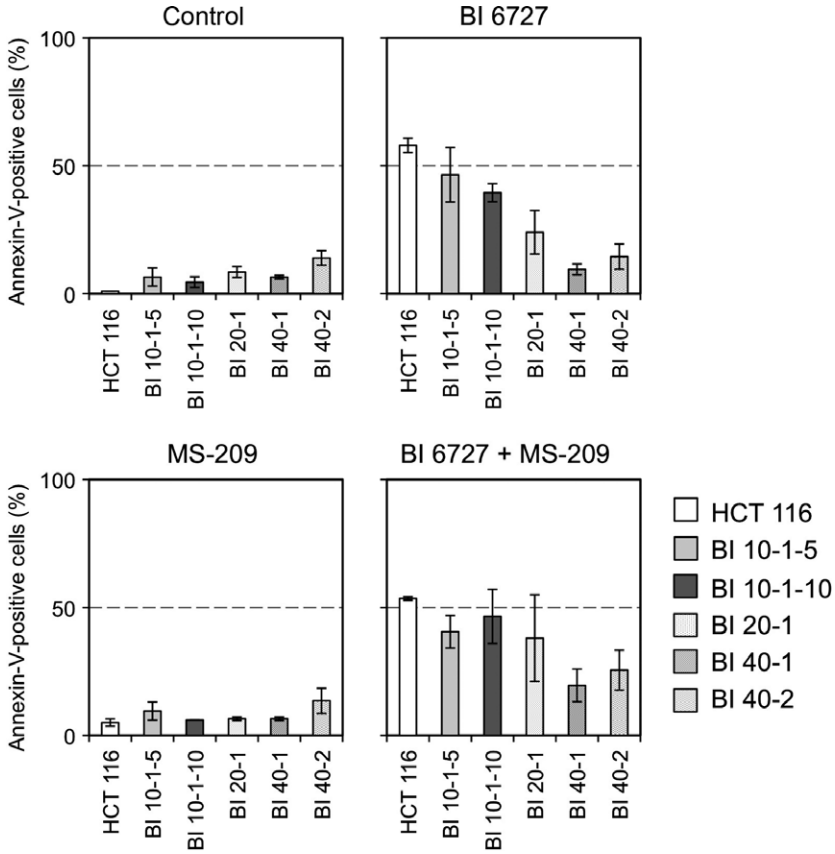


Fig. 3. Effect of MS-209 on BI 6727-induced apoptosis in HCT 116 and BI 2536-resistant cells. The proportions of BI 6727-induced apoptosis were examined in the presence or absence of MS-209. Cells were treated with either BI 6727 (100 nmol/L) alone, MS-209 (5 μmol/L) alone, or their combination for 48 h. The cells were then harvested, stained with annexin-V–FITC and propidium iodide, and analyzed by flow cytometer. Flow cytometric analysis defined apoptotic cells as annexin-V-positive cells, and the proportions of apoptotic cells are indicated by bar graphs. Data are expressed as mean ± SE for two independent experiments.

crystal violet. The number of colonies containing more than 50 cells was counted under a microscope, and the results are presented as means ± SD (n = 3).

Statistical analysis. The quantitative results are presented as means ± SD (n = 3). The two-tailed Student's t-test was used to evaluate the significance of differences. A value of $P < 0.05$ was considered statistically significant.

Results

Drug resistance of BI 2536-resistant cell lines. We established five BI 2536-resistant cell lines (BI 10-1-5, BI 10-1-10, BI 20-1, BI 40-1, and BI 40-2) from HCT 116 cells with two independent protocols (Fig. 1a). The BI 40-1 and BI 40-2 cells showed 140-fold greater resistance to BI 2536 than the parental HCT 116 cells, and the other three lines showed 23–76-fold greater resistance to BI 2536 than the parental cells (Table 1). The BI 2536-resistant cell lines showed cross-resistance to the other PLKis, BI 6727 and GSK461364 (Fig. 1b). The BI 40-1 and BI 40-2 cells showed higher cross-resistance to these two PLKis than the other three lines. These five BI 2536-resistant cell lines showed similar levels of resistance to doxorubicin and vincristine (Fig. 1b).

A Western blot analysis confirmed that the three PLKis induced the cleavage of caspase-3, -8, and -9 in HeLa and HCT 116 cells (Fig. 1c). The cleaved forms of caspase-3, -8, and -9 were still detected in the BI 10-1-5, BI 10-1-10, and BI 20-1 cells but not in the BI 40-1 and BI 40-2 cells after treatment with BI 6727 (100 nmol/L), supporting our findings that the BI 40-1 and BI 40-2 cells were highly resistant to BI 6727 (Fig. 1d). P-glycoprotein expression was upregulated in all five resistant cell lines. Among them, the BI 20-1 cells showed the highest level of P-GP expression, so the level of P-GP expression did not correlate with the level of BI 6727 resistance.

Polo-like kinase inhibitor resistance not abolished by P-GP inhibitor MS-209. To determine the effect of P-GP on the cellular resistance to PLKis, we first examined the sensitivity of P-GP- or breast cancer resistance protein-overexpressing K562 cells to PLKis. A cell growth inhibition assay showed that P-GP expression conferred strong resistance to the three PLKis (Fig. 2a). The P-GP inhibitor MS-209 completely reversed the resistance to vincristine in all five cell lines and to the PLKis in BI 10-1-5, BI 10-1-10, and BI 20-1 cells. The BI 40-1 and BI 40-2 cells still retained significant levels of resistance to PLKis in the presence of MS-209 (Fig. 2b). Consistent with this, MS-209 did not restore BI 6727-induced caspase activation or p53 accumulation in these cells (Fig. 2c, left panels). The proportions of annexin-V-positive cells in the BI 40-1 and BI 40-2 cells were lower than in the other cells when the cells were co-treated with BI 6727 and MS-209 (Fig. 3). Because MS-209 apparently restored vincristine-induced caspase activation and p53 accumulation in BI 40-1 and BI 40-2 cells (Fig. 2c, right panels), MS-209 cancelled P-GP-mediated drug resistance under these conditions. These results suggest that mechanisms other than P-GP are involved in the resistance of BI 40-1 and BI 40-2 cells to PLKis.

Polo-like kinase inhibitor-induced apoptosis requires caspase-8 and MYC. We next examined whether PLKi-induced apoptosis is attributable to caspase-8. Transfection with *CASP8* siRNA suppressed the expression of caspase-8 protein (Fig. 4a) and the BI 2536-induced cleavage of caspase-3 and -9 (Fig. 4b). The proportion of annexin-V-positive cells after treatment with BI 2536 also decreased after *CASP8* knockdown (Fig. 4c). Furthermore, cells transfected with *CASP8* siRNA (black

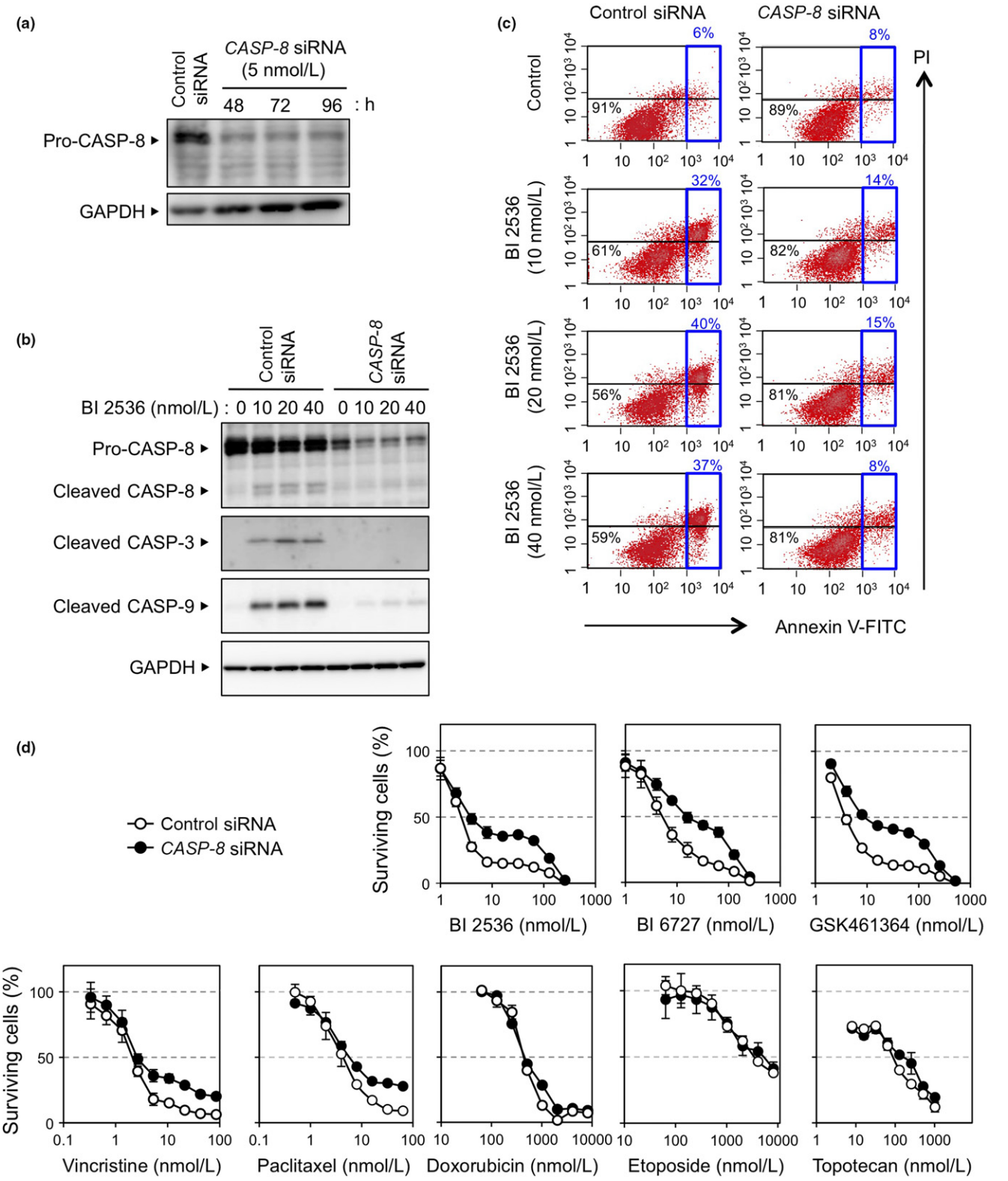


Fig. 4. Caspase-8 plays an essential role in polo-like kinase inhibitor (PLKi)-induced apoptosis. (a) Knockdown of caspase (CASP)-8. HCT 116 cells were transfected with *CASP8*-targeting siRNA, and caspase-8 protein expression was examined by Western blot analysis. (b) PLKi-induced caspase activation in *CASP8*-knockdown cells. Cells were then treated with BI 2536 (0, 10, 20, or 40 nmol/L) for an additional 48 h. Levels of cleaved caspase-3, -8, and -9 were examined by Western blot analysis. (c) PLKi-induced apoptosis in *CASP8*-knockdown cells. The cells were treated as in (b). The cells were then harvested and stained with annexin-V–FITC and propidium iodide (PI). Apoptotic cells were defined as annexin-V-positive cells, and the proportions of apoptotic cells are indicated in blue. (d) Sensitivity to PLKis, vincristine, paclitaxel, doxorubicin, etoposide, or topotecan in *CASP8*-knockdown cells. HCT 116 cells were transfected with *CASP8* siRNA or control siRNA. At 48 h after transfection, the cells were treated with BI 2536, BI 6727, GSK461364, vincristine, paclitaxel, doxorubicin, etoposide, or topotecan for an additional 48 h and subjected to WST-8 assay.

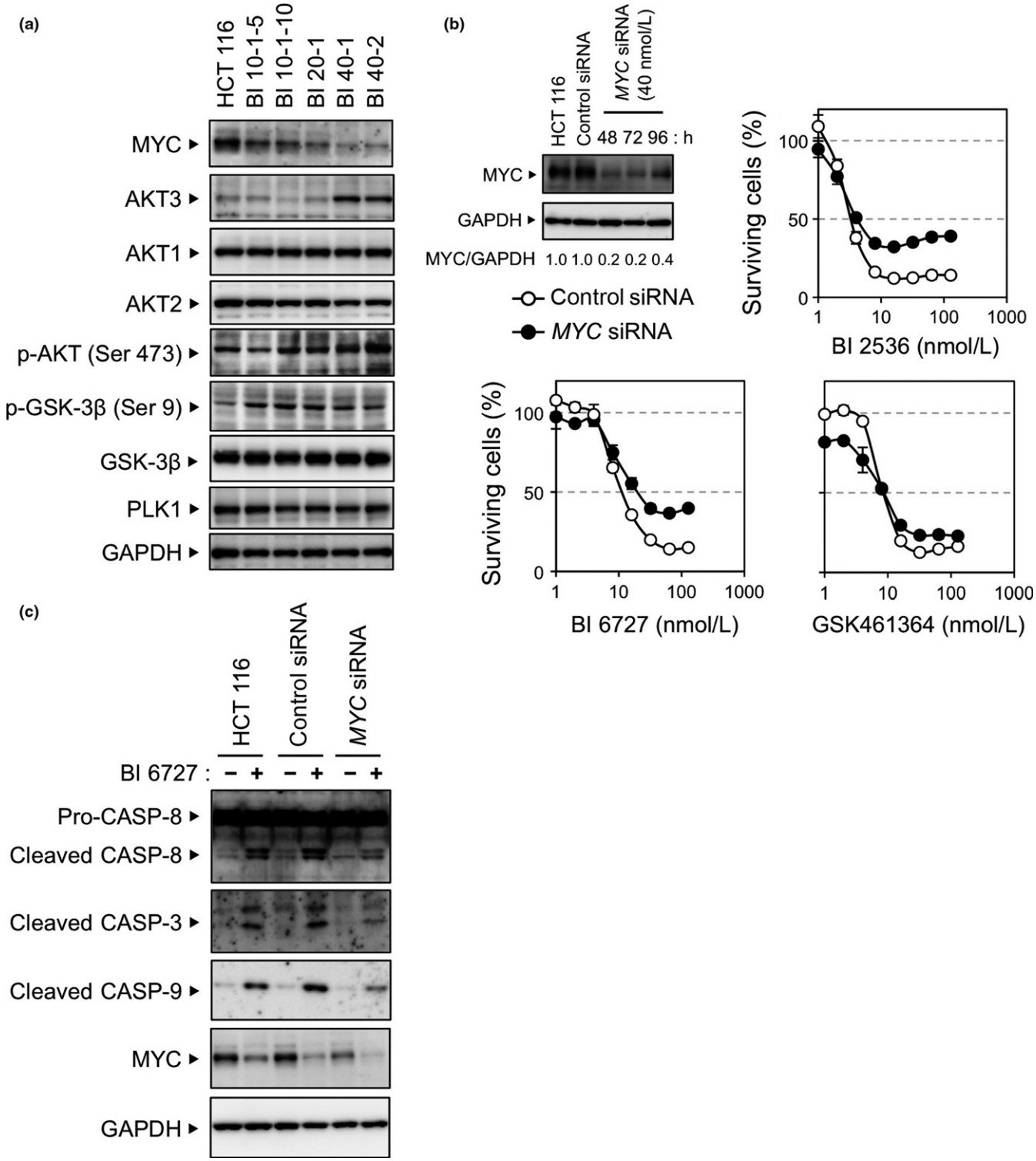
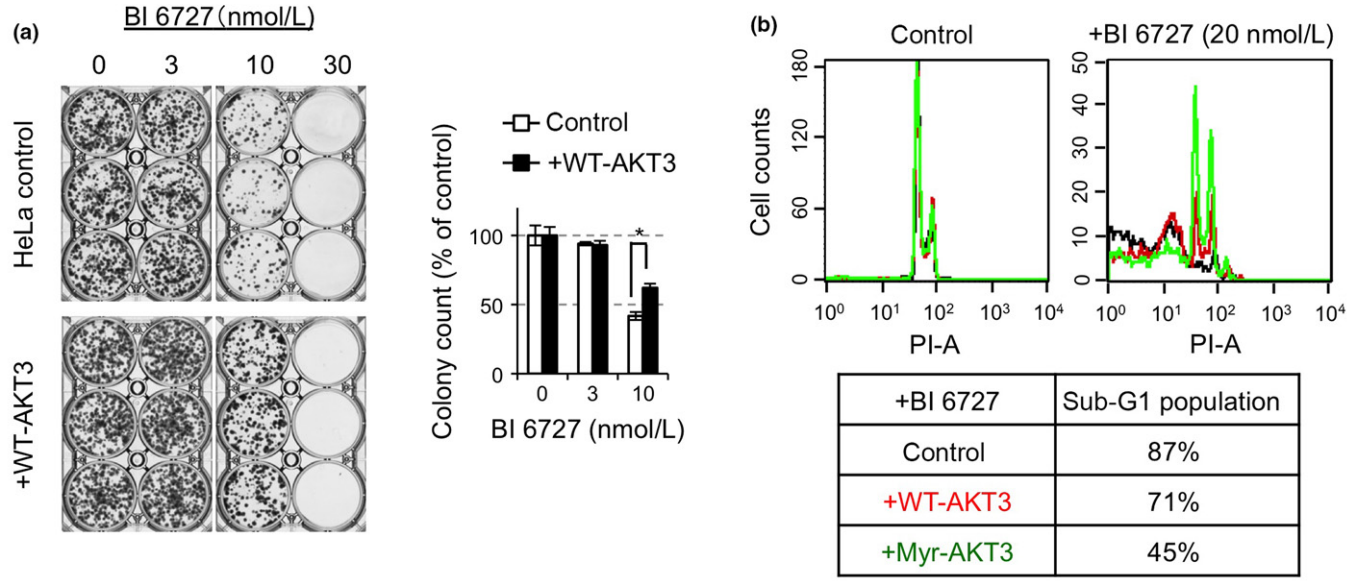
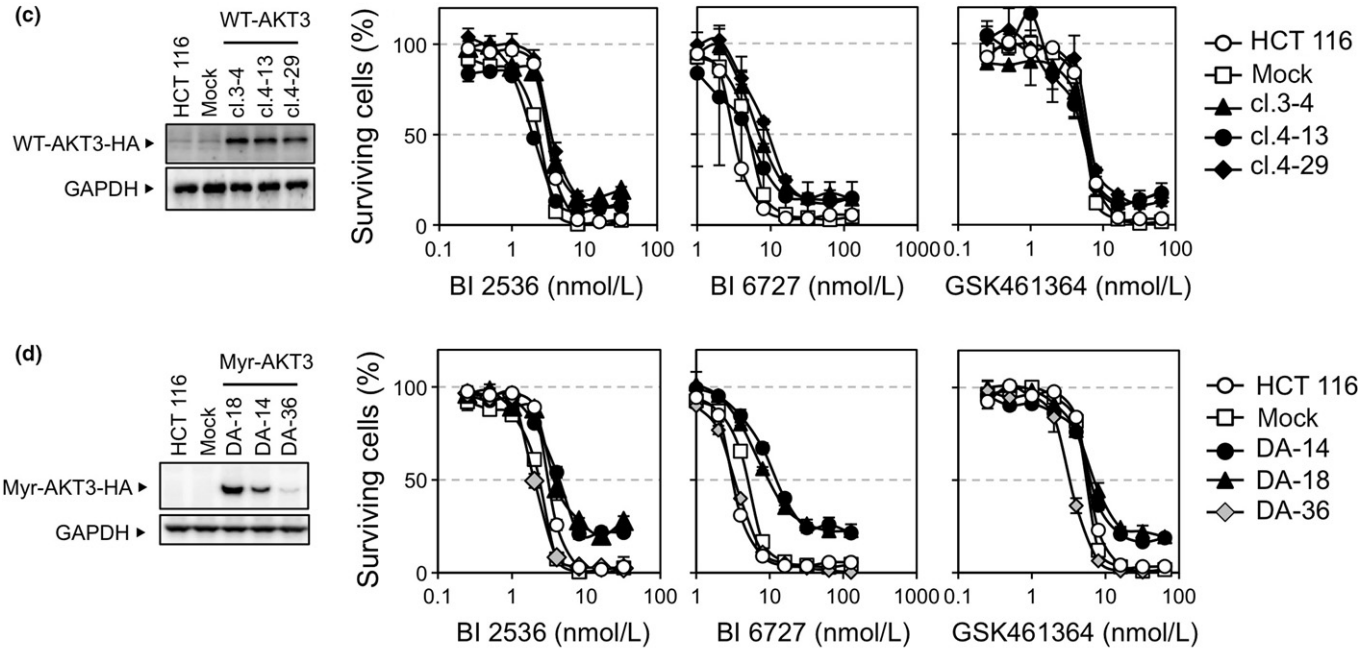


Fig. 5. Downregulation of MYC is involved in resistance to polo-like kinase inhibitors (PLKis). (a) Expression levels of MYC, AKTs, AKT downstream proteins, and polo-like kinase 1 (PLK1) in BI 2536-resistant cell lines. (b) Sensitivity to PLKis in *MYC*-knockdown cells. HCT 116 cells were transfected with *MYC*-targeting siRNA, and MYC protein expression was examined by Western blot analysis (upper left panels). The normalized intensity ratio (MYC/GAPDH) is indicated at the bottom of the blots. At 48 h after transfection, the cells were treated with PLKis for an additional 48 h and subjected to WST-8 assay. (c) PLKi-induced caspase activation in *MYC*-knockdown cells. HCT 116 cells were transfected with *MYC*-targeting siRNA. At 48 h after transfection, the cells were treated with BI 6727 (100 nmol/L) for an additional 24 h. The levels of cleaved caspase (CASP)-3, -8, and -9 and MYC were examined by Western blot analysis. GSK-3β, glycogen synthase kinase 3β; p-, phosphorylated.

symbols in Fig. 4d) showed 2.6-, 3.0-, and 2.4-fold higher resistance to BI 2536, BI 6727, and GSK461364, respectively. The knockdown of *CASP8* also induced resistance to vincristine and paclitaxel (Fig. 4d, lower graphs). However, *CASP8* siRNA did not affect the sensitivity of the cells to doxorubicin, etoposide, or topotecan (Fig. 4d). These results



+BI 6727	Sub-G1 population
Control	87%
+WT-AKT3	71%
+Myr-AKT3	45%



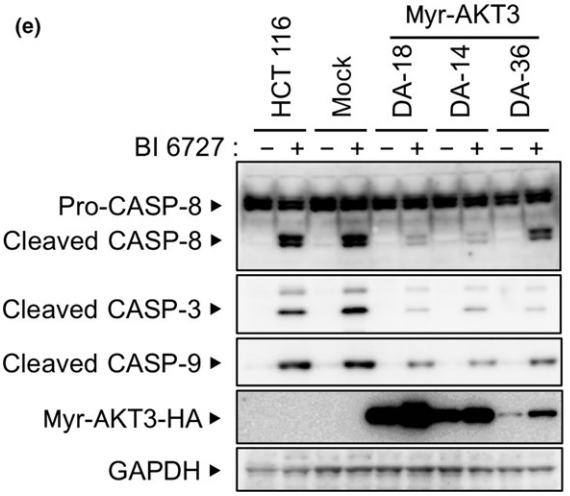


Fig. 6. Resistance to polo-like kinase inhibitors (PLKis) mediated by AKT3. (a) Sensitivity to BI 6727 in WT-*AKT3*-transfected cells. HeLa cells were transiently transfected with a WT-*AKT3*-expressing plasmid. At 24 h after transfection, cells were treated with BI 6727 (0, 3, 10, or 30 nmol/L) for an additional 24 h. The cells were washed, replated, cultured for 14 days in drug-free growth medium, and stained with 0.5% crystal violet. $*P < 0.05$. (b) BI 6727-induced sub-G_1 population in WT- and myristoylated (Myr)-*AKT3*-transfected cell lines. HeLa cells transfected with a WT and Myr-*AKT3*-expressing plasmid were treated with BI 6727 (0 or 20 nmol/L) at 24 h after transfection. After drug treatment for 48 h, the cells were stained with propidium iodide (PI) and the apoptotic sub-G_1 population was examined with flow cytometry. Red and green lines indicate the counts of WT- and Myr-*AKT3*-transfected cells, respectively. (c) Sensitivity to PLKis in the WT-*AKT3*-transfected cell lines. WT-AKT3–hemagglutinin (HA) expression in HCT 116 transfectant cell lines (cl.3-4, cl.4-13, and cl.4-29) was examined by Western blot analysis using an anti-HA antibody (left panels). Stable WT-AKT3-expressing transfectants were treated with PLKis for 96 h and subjected to WST-8 assay. (d) Sensitivity to PLKis in Myr-*AKT3*-transfected cell lines. Myr-AKT3–HA expression in HCT 116 transfectant cell lines (DA-14, -18, and -36) was examined by Western blot analysis using an anti-HA antibody (left panels). Stable Myr-AKT3-expressing transfectants were treated with PLKis for 96 h and subjected to WST-8 assay. (e) BI 6727-induced caspase activation in Myr-*AKT3*-transfected cell lines. Cells were treated with BI 6727 (100 nmol/L) for 48 h. Levels of cleaved caspase (CASP)-3, -8, and -9 were examined by Western blot analysis.

indicate that caspase-8 plays a critical role in PLKi-induced apoptosis in HCT 116 cells.

The BI 2536-resistant cell lines expressed the WT PLK1 protein with no mutation (data not shown). In the course of exploring the resistance mechanisms, we found that AKT3 expression was upregulated and MYC was downregulated in BI 40-1 and BI 40-2 cells (Fig. 5a). Consistent with this, the knockdown of *MYC* expression by siRNA reduced MYC protein for 96 h and conferred resistance to PLKis, BI 2536 and BI 6727 (Fig. 5b). Caspase activation was also suppressed by *MYC* knockdown (Fig. 5c), suggesting that the reduction of MYC protein is involved in the resistance to PLKi-induced apoptosis.

AKT3 confers resistance to PLKis. The phosphorylation of a downstream target of AKT, GSK-3β at Ser-9, was increased in BI 2536-resistant cell lines (Fig. 5a), suggesting that the AKT pathway was activated in resistant cells. The BI 40-1 and BI 40-2 cells, which appeared to have P-GP-independent resistance mechanisms, expressed high levels of AKT3, so we investigated the effects of AKT3 expression on the resistance to PLKis. A colony formation assay showed that the proportion of surviving cells after treatment with 10 nmol/L BI 6727 was higher in *AKT3*-transfected HeLa cells than in control cells (Fig. 6a). A flow cytometric analysis also showed that the transient overexpression of AKT3 reduced the proportions of apoptotic sub-G_1 population cells after treatment with BI 6727 (Fig. 6b). To examine this further, we established WT-*AKT3*-transfected HCT 116 cell lines, cl.3-4, cl.4-13, and cl.4-29 (Fig. 6c), and a growth inhibition assay showed that the WT- and Myr-*AKT3* transfectants showed only marginal levels of resistance to BI 2536, BI 6727, and GSK461364 (Fig. 6c, right graphs). We next examined stable clones DA-14, -18, and -36 that expressed Myr-AKT3, a constitutively active mutant of AKT3. The DA-14 and -18 cells expressed high levels of Myr-AKT3 protein (Fig. 6d, left panels), and showed 4.0- and 3.1-fold higher resistance to BI 6727, respectively, than the control cells (Fig. 6d). They also showed cross-resistance to high concentrations of BI 2536 and GSK461364 (Fig. 6d). The BI 6727-induced cleavage of caspase-8 was reduced in the Myr-AKT3-expressing cells (Fig. 6e). Collectively, these results indicate that the expression of AKT3 suppresses caspase activation and confers resistance to PLKis.

Discussion

Polo-like kinase plays key roles in the cell cycle and its overexpression is observed in a wide range of cancer cells. Inhibition of PLK causes mitotic arrest and apoptosis, so PLK is considered a target for cancer chemotherapies.[7,23] Several PLKis have been developed[12] and one PLKi, BI 6727, has been evaluated in clinical trials for various cancers, including AML,[14,24–26] as a monotherapy or in combination with other agents.

Many tyrosine kinase inhibitors have been used in various clinical settings,[27] and the emergence of drug-resistant cells is a critical problem.[15] On-target mutations, bypath mechanisms, and drug efflux pumps have been identified as typical mechanisms of drug resistance.[15] Moreover, resistance to apoptosis is also considered to be involved in chemoresistance.[28] A precise analysis of the resistance mechanisms to PLKis and an exploration of predictable biomarkers should improve the therapeutic efficacy of PLKis. In this study, we established new cell lines resistant to BI 2536, one of the earliest-identified inhibitors of PLK, and investigated the pharmacological biomarkers involved in the undetermined mechanisms of PLKi resistance.

Our BI 2536-resistant cell lines showed cross-resistance to other PLKis (BI 6727 and GSK461364) and also to doxorubicin and vincristine. Doxorubicin and vincristine are typical substrates of the multidrug efflux pump P-GP and, as expected, P-GP was overexpressed in the BI 2536-resistant lines. Previous studies have shown that P-GP overexpressing cells show resistance to BI 2536, BI 6727, and GSK461364,[16–18] and we confirmed the involvement of P-GP in our BI 2536-resistant cell lines. However, P-GP expression did not correlate with the levels of resistance to BI 6727, suggesting that mechanisms other than P-GP were present in our clones. Therefore, P-GP expression is one of the determinants of the therapeutic efficacy of BI 6727, but not the only one.

Intriguingly, in two highly BI 2536-resistant cell lines, BI 40-1 and BI 40-2, the P-GP inhibitor MS-209 was insufficient to reverse the cells' resistance to PLKis. We found that MYC was downregulated and AKT3 was upregulated in these cells. BI 2536 and BI 6727 are dual inhibitors of both PLK1 and bromodomain containing 4 (BRD4).[29,30] The high concentration of BI 2536 represses *MYC* transcription by inhibiting BRD4, and AKT-mediated phosphorylation of GSK-3β at Ser-9 represses GSK-3β activity.[31] As GSK-3β-mediated MYC phosphorylation at Thr-58 destabilizes MYC protein,[32] we guess that AKT3 upregulation might inhibit GSK-3β, and counteract with BI 2536-induced MYC depletion, by which AKT3-expressing cells might be able to proliferate under BI 2536 treatment and therefore be selected as BI 2536-resistant cells. Moreover, increasing the drug concentration during selection resulted in emergence of different types of resistant clone, so that the drug resistance could be caused in different manners, depending on drug treatment conditions.

Concerning the downstream targets of AKT in our five BI 2536-resistant cells, phosphorylation levels of Ser-9 on GSK-3β, a hallmark of AKT activation, were not completely paralleled with the degree of PLKi resistance in five BI 2536-

resistant cells. We speculate that undermined pathway might participate in high levels of Ser-9 phosphorylation on GSK-3β in BI 10-1-5, BI 10-1-10, and BI 20-1 cells, because Ser-9 of GSK-3β is phosphorylated by multiple protein kinase, including AKT, protein kinase A, protein kinase C, p70 S6 kinase, and other kinases.[33]

In addition, MYC-knockdown cells showed different sensitivities to PLKi, BI2536, BI 6727, and GSK461364. BI 2536 and BI 6727 potently inhibit PLK1 as well as PLK2 and PLK3, and the inhibitor-induced G_2/M arrest was followed by strong induction of apoptosis.[13] In contrast, GSK461364 has selectivity for PLK1 compared with PLK2 and PLK3, and the high concentration of GSK461364 induces G_2 delay rather than mitotic arrest.[34] Thus, pharmacological phenotypes of GSK461364 seem to be different from those of BI 2536 and BI 6727, especially regarding apoptosis-inducing effects at higher concentrations.

The strong expression of MYC sensitizes cells to cytotoxic anticancer drugs and mediates various types of apoptosis, suggesting that MYC has pro-apoptotic activity.[35] Conversely, the activation of the AKT pathway contributes to cell survival by phosphorylating various apoptosis-associated molecules.[36,37] Therefore, the downregulation of MYC and the upregulation of AKT3 would increase the possibility of evading the apoptosis induced by PLKis. Several studies have reported that MYC activates FAS death signaling and caspase-8-initiated apoptosis,[38] and that AKT suppresses both MYC- and FAS-mediated apoptosis.[39,40] These findings indicate that the AKT pathway potentially antagonizes MYC-dependent apoptosis. Consistent with these studies, we have shown that PLKi-induced apoptosis depends on MYC and caspase-8, but that AKT3 antagonizes this apoptosis (Figs 4,5). As apoptosis resistance is thought to be involved in drug resistance,[28] our results suggest that the downregulation of MYC and the upregulation of AKT3 might influence the effectiveness of PLKis. Overall, our findings indicate that the expression levels of P-GP, MYC, caspase-8, and AKT3 are candidate biomarkers of cell sensitivity to PLKis.

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Disclosure Statement

The authors have no conflict of interest.

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