

# The mechanism of cross-resistance to proteasome inhibitor bortezomib and overcoming resistance in Ewing's family tumor cells

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**Abstract.** EWS-Flt1 plays important roles in oncogenesis of Ewing's family tumors (EFTs). We have reported that EWS-Flt1 inhibits p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> expressions, which are degraded by the ubiquitin-proteasome pathway. Bortezomib efficiently up-regulated p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> expression, and induced apoptosis accompanied by the expression of cleaved-PARP, DR4 and activated caspase-8 in EFT cells. Since most EFTs deaths result from the tumor being resistant to chemotherapeutic drugs, the effects of novel anti-tumor reagents on drug-resistant tumors were next investigated. The results demonstrated that the drug-resistant EFT clones were cross-resistant to bortezomib probably due to the over-expression of the efflux pumps, P-glycoprotein and MRP1. We further investigated whether the efflux pump inhibitors would modulate the effects of bortezomib. The combination of P-gp-specific or MRP1-specific inhibitors could enhance the anti-tumor effects of bortezomib on the drug-resistant clones. These data suggest that bortezomib might be a substrate of P-gp and MRP1. Although bortezomib would be effective on the primary EFTs, it is necessary to pay attention to the resistance to bortezomib in clinical trials for the advanced cases. The combination of bortezomib and the efflux pump inhibitors might be a promising method as a novel molecular target therapy for advanced EFTs.

## Introduction

Ewing's family tumors (EFTs), including Ewing's sarcoma and primitive neuroectodermal tumor, are the second most frequent malignant bone tumors arising in adolescence and childhood (1). Although the survival rate of patients with EFTs has improved, the prognosis for patients with high risk factors (including metastatic disease at first diagnosis and early relapse) has remained poor (2-4). Therefore, the development of a novel treatment method is needed for patients with high-risk EFTs.

The chromosomal translocation t(11;22) specifically linked to EFTs results in a chimeric molecule fusing *EWS* to the DNA-binding domain encoded by *FLI1* or *ERG* (5-7). The EWS-Flt1 fusion protein possesses a transforming ability which suggests that the fusion protein may play an important role in the oncogenesis of EFTs (7). We have previously reported that the suppression of the EWS-Flt1 fusion protein by antisense oligonucleotides increased the expression of the cyclin-dependent kinase (CDK) inhibitors p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup>, which resulted in the arrest of the G1 phase of the cell cycle in EFT cells (8,9). We also reported that p21<sup>waf1/cip1</sup> is a direct transcriptional target of EWS-Flt1 (10), and that the adenoviral transduction of the *CDKN1B* (*P27KIP1*) gene into EFT cell lines efficiently induced apoptosis in the cells (11).

It is well known that the proteins of p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> are degraded by the ubiquitin-proteasome pathway (12-14). The cell cycle proceeds from the G1 phase to the S phase after the degradation of CDK-inhibitors by the 26S proteasome. The ubiquitin-proteasome pathway is a key regulator of cellular homeostasis by degrading misfolded redundant proteins involved in mediating transcription, cell cycle progression, and apoptosis (14-16).

Proteasome inhibitors have attracted the attention of many oncologists as promising anti-tumor agents. One of the proteasome inhibitors, bortezomib, inhibits chymotrypsin-like activity of the 26S proteasome in mammalian cells, and has a cytotoxic effect on a variety of cancer cell types both *in vitro* and *in vivo*. Previous studies have demonstrated that bortezomib exhibits anti-tumor effects on multiple myeloma via inhibition of the transactivation of NF-κB mediated by IκB accumulation (17,18). It has

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**Abbreviations:** EFTs, Ewing's family tumors; PARP, poly-ADP-ribose polymerase; DR4, death receptor 4; P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; ADR clone, adriamycin-resistant clone

**Key words:** proteasome inhibitor, bortezomib, Ewing's family tumors, multi-drug resistance, apoptosis, p21<sup>waf1/cip1</sup>, p27<sup>kip1</sup>

been reported that bortezomib inhibits the growth of various cancer cells through prevention of the degradation of proteins that regulate the cell cycle (19). Bortezomib has been approved by the US FDA as the first proteasome inhibitor, and it has been used in preclinical and clinical trials for multiple myeloma (20-22), other hematological cancers (23), and solid tumors (24).

It seems that most treatment failures leading to a poor prognosis tend to be associated with the resistance of the tumor cells to chemotherapeutic drugs. It is therefore important to investigate the cross-resistance to novel anti-tumor regents before commencement of clinical trials. Maki *et al* reported the result of a multi-center phase II clinical study of bortezomib for various recurrent or metastatic sarcomas, and demonstrated that bortezomib was not effective against the advanced sarcomas, including 2 cases of EFTs (25). These results suggest the possibility that recurrent or metastatic sarcomas might be resistant to bortezomib. However, it is difficult to interpret the results of the clinical trial, since there have been no fundamental data about the effects of bortezomib on EFT cells and drug-resistant clones of EFTs. In the present study, we analyzed the anti-tumor effects of bortezomib on EFT cells, and further investigated whether bortezomib could reduce the viability of adriamycin-resistant clones of EFTs (ADR clones). We demonstrated that bortezomib inhibited the growth of the parental EFT cells, and that a treatment combining bortezomib and efflux pump inhibitors prevented the growth of drug-resistant EFT cells. This is the first report showing the efficacy of a proteasome inhibitor against drug-resistant EFTs.

## Materials and methods

**Cell lines and reagents.** The human EFT cell lines, WE-68, VH-64 (with wild-type *TP53*), were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. Two adriamycin-resistant cell lines, WE-68/ADR clone and VH-64/ADR clone, were cultured in RPMI-1640 as described previously (26). In addition, SK-N-MC and PNKT-1 (*TP53* null) were cultured in DMEM (Nissui Pharmaceuticals, Tokyo, Japan) containing 10% fetal bovine serum. Balbc3T3 and NIH3T3 cells were used as normal control cell lines, which were cultured in DMEM containing 10% calf serum. These cells were incubated at 37°C humidified atmosphere containing 5% CO<sub>2</sub> in air.

Bortezomib (Millennium Pharmaceuticals, Cambridge, MA) was individually purchased through RHC Corporation (HI, USA). Bortezomib was dissolved in sterile saline at a concentration of 1 mg/ml (for *in vivo* study) or 100 µM (for *in vitro* assay) and stored at -20°C until use. Verapamil and MK-571 were purchased from Sigma chemical (St. Louis, MO) and Alexis Biochemicals (Lausen, Switzerland), respectively.

**20S proteasome activity assay.** 20S proteasome activity was measured as described previously (27). After treatment with various concentrations of bortezomib for 8 h, protein samples were harvested in lysis buffer [50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM PMSF, 0.5 mM DTT], and subjected to a proteasome activity assay using

N-Succinyl-Leu-Leu-Val-Tyr 7-amido-4-methylcoumarin (Sigma) as a substrate for 20S proteasome in a reaction buffer that consisted of 20 mM HEPES (pH 8.0), 0.5 mM EDTA (pH 7.6), and 0.5 g/l SDS. Recombinant 20S proteasome (Calbiochem, San Diego, CA) was used as a control. A Twinkle LB970 fluorescence spectrophotometer was used to detect signals (Berthold Technologies, Tokyo, Japan).

**Cell viability and chemosensitivity assays.** Cells (3x10<sup>4</sup>) were seeded into 24-well plates. After cell cultivation for 24 h, various concentrations of bortezomib were added to the media. The cells were incubated for 48 h, and the number of viable cells was subsequently counted using a Coulter Hematology Analyzer (Beckman Coulter, Fullerton, CA). For the chemosensitivity assay to evaluate the cross-resistance of WE-68 and WE-68/ADR or VH-64 and VH-64/ADR to bortezomib, 1x10<sup>4</sup> cells were seeded into 96-well plates. After a 48 h incubation with various concentrations of bortezomib in the presence or absence of 10 µM verapamil or 30 µM MK-571, the number of viable cells was measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI), according to the manufacturer's protocol. The chemosensitivity assay was carried out in triplicate and repeated at least 3 times.

**Western blot analysis.** Cell lysates from bortezomib-treated cells were prepared and subjected to a Western blot analysis as described previously (28). Briefly, 20 µg of each protein sample were electrophoresed on a 4-12% gradient polyacrylamide gel (Invitrogen) and transferred onto a nitrocellulose membrane (Amersham, Arlington Heights, IL). The membranes were immunoblotted with primary antibodies for p21<sup>waf1/cip1</sup> (Pharmingen, San Diego, CA), p27<sup>kip1</sup> (Transduction Laboratories, Lexington, KY), p53 (Santa Cruz Biotechnology, Santa Cruz, CA), PARP (Roche, Indianapolis, IN), DR4 (Upstate, Lake Placid, NY), phospho-IκBα-Ser32/36 (Cell Signaling, Danvers, MA), cIAP1, cIAP2 (Santa Cruz) and actin (Chemicon, Temecula, CA) for 1.5 h at room temperature. After several washes, the filters were treated with mouse or rabbit-horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1.5 h. The immunoreactivity on the blots was detected using an enhanced chemiluminescence (ECL) detection system (Amersham).

**RT-PCR.** Cells were treated with bortezomib for various times, and the total RNA was prepared using an RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA extracted from EFT cells treated with bortezomib was used for the reverse transcription reaction using a SuperScriptII reverse transcriptase (Invitrogen). PCR was performed in a final volume of 50 µl for 25 (18S) or 30 (mdr-1 and mrp-1) cycles using the following primers: ribosomal 18S (forward: 5'-TTACCAAAAGTGGCCCACTA-3' reverse: 5'-GAAAGATGGTGAAGTATGCC-3'), mdr-1 (forward: 5'-CCCATCATGCAATAGCAGG-3', reverse: 5'-GTTCAAACCTTCTGCTCCTGA-3'), and mrp-1 (forward: 5'-GGACCTGGACTTCGTTCTCA-3', reverse: 5'-CGTCCAGACTTCTTCATCCG-3'). Each PCR cycle consisted of a denaturation step at 94°C for 1 min or 30 sec, a primer-annealing step at 55°C

for 30 sec, and an extension step at 72°C for 30 sec or 1 min. The PCR product sizes for 18S, mdr-1, and mrp-1 were 345, 157 and 292 bp, respectively. The PCR products were analyzed in 1.5% agarose gel.

**Xenograft murine model.** WE-68 cells ( $1 \times 10^7$  viable cells/mouse) were subcutaneously inoculated into athymic nu/nu mice. Approximately two weeks after the tumor inoculation, bortezomib suspended in 100  $\mu$ l of sterile saline was administered intraperitoneally at 1.0 mg/kg (n=6) or 1.5 mg/kg (n=6) twice a week for three weeks and the same amount of saline was used as a control (n=6). Caliper measurements of the longest perpendicular tumor diameters were performed every other day to calculate the tumor volume using the following formula:  $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$ . All animal experiments were carried out in accordance with the Institutional Guidelines for Animal Experiments of Kyushu University.

**TUNEL assay.** TUNEL staining was performed according to the manufacturer's instructions using Apoptosis Detection Kit (Takara Bio, Tokyo, Japan). The tumor samples resected from mice xenografts were fixed in 10% formaldehyde for 24 h. H&E staining was also performed on tumor sections.

**Statistical analysis.** Tumor volume data were analyzed using ANOVA and the post hoc Dunnett's t-test, for which  $p < 0.05$  was deemed significant using a two-tailed test.

## Results

**Inhibition of the proliferation of EFT cells by bortezomib both in vitro and in vivo.** We have previously reported that a low expression level of p27<sup>kip1</sup> protein was related to a poor prognosis for EFT patients, and that the over-expression of p27<sup>kip1</sup> with an adenoviral vector significantly inhibited the proliferation of EFT cells and induced apoptosis (11). We have also reported that the knockdown of EWS-Flt1 with RNAi technique increased the expression of p27<sup>kip1</sup> protein in EFT cells (29). Since it has been reported that proteasome inhibitors induce the accumulation of p27<sup>kip1</sup> in various tumor cells, we analyzed the effect of bortezomib, a proteasome inhibitor, on various EFT cell lines. The cells were incubated with various concentrations of bortezomib for 48 h, and the number of viable cells was subsequently counted. Bortezomib dose-dependently inhibited the proliferation of WE-68, VH-64, SK-N-MC, and PNKT-1 (Fig. 1A). The IC<sub>50</sub> for each cell line was 12 nM, 12 nM, 5 nM and 5 nM, respectively. Bortezomib significantly inhibited the growth of EFT cells at nanomolar concentrations. We also studied the *in vivo* anti-tumor activity of bortezomib using a xenograft model of WE-68 cells. Nude mice were inoculated with  $1 \times 10^7$  cells of WE-68 in the right flank. Approximately 2 weeks after the tumor inoculation, bortezomib was administered intraperitoneally at 1.0 mg/kg or 1.5 mg/kg twice a week for 3 weeks. Bortezomib significantly inhibited the tumor growth in nude mice in a dose-dependent manner (Fig. 1B, left panel). The administration of bortezomib did not result in any signs of toxicity or weight loss in the treated mice (Fig. 1B, right panel). These results demonstrated the

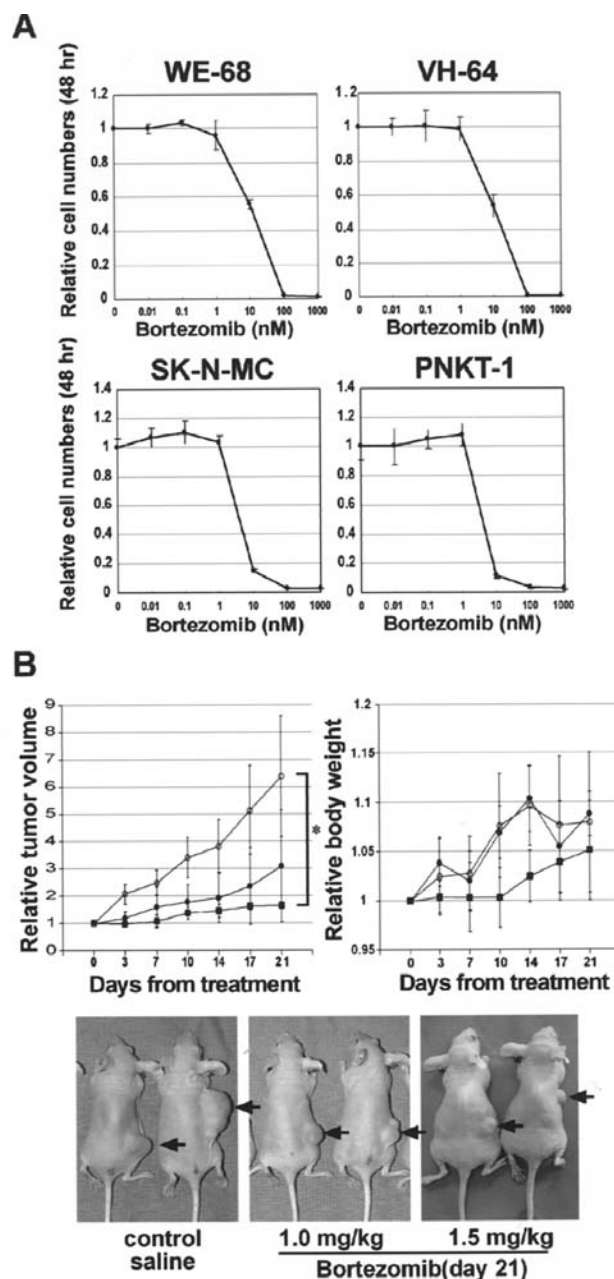


Figure 1. Bortezomib inhibited the viability of EFT cell lines *in vitro* and *in vivo*. (A) The EFT cells were seeded on 24-well plates ( $3 \times 10^4$  cells/well). After treatment with various concentrations of bortezomib, the cells were incubated for 48 h and the number of viable cells was subsequently counted. The concentrations of bortezomib at which the cell survival was reduced to 50% (IC<sub>50</sub>) in WE-68, VH-64, PNKT-1 and SK-N-MC were 12 nM, 12 nM, 5 nM, and 5 nM, respectively. (B) WE-68 cells ( $1 \times 10^7$  cells/mouse) were subcutaneously inoculated into the right flank of nude mice. Bortezomib (1.0 mg/kg or 1.5 mg/kg) was administered intraperitoneally 6 times for 3 weeks. The control mice were administered saline. We calculated the tumor volume using the following formula:  $4\pi/3 \times (W/2)^2 \times (L/2)$ . The body weight of each mouse was measured while injecting bortezomib or saline. The lower panel shows the gross appearance of the tumors in the treated mice 21 days after the first treatment.

potent anti-neoplastic effects of bortezomib on EFT cells both *in vitro* and *in vivo*.

**Suppression of the proteasome activity and accumulation of proteasome-target proteins in EFT cells by bortezomib.** We next examined the effects of bortezomib on 20S proteasome



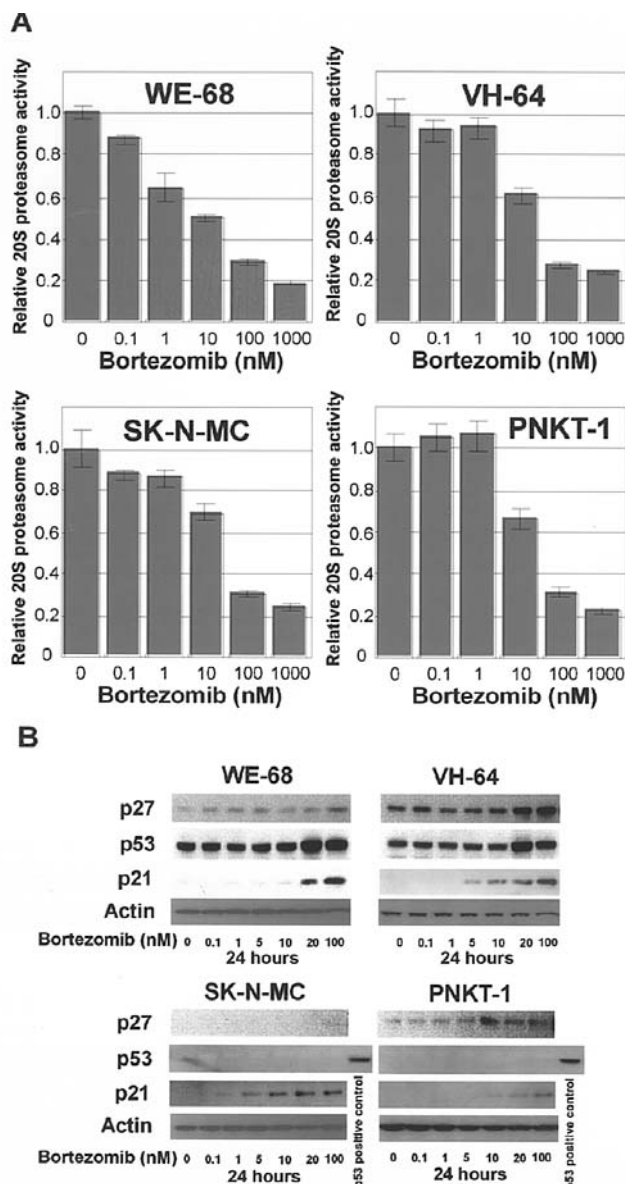


Figure 2. Bortezomib decreased the activity of the 20S proteasome in EFT cells. (A) After treatment with various concentrations of bortezomib, the protein lysates were harvested and 20S proteasome activity in each sample was measured. The proteasome activity in all EFT cells tested was inhibited by bortezomib in a dose-dependent manner. (B) A Western blot analysis for p27<sup>kip1</sup>, p53, and p21<sup>waf1/cip1</sup> expression in EFT cells. The protein levels of p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> were dose-dependently up-regulated in all cell lines by treatment with bortezomib, whereas the p53 expression was increased only in WE-68 and VH-64, the cell lines with wild-type p53.

activity in EFT cells. The treatment with bortezomib significantly decreased 20S proteasome activity in the cells in a dose-dependent manner (Fig. 2A). We have previously shown that the EWS-Flt1 fusion protein directly inhibits the transactivation of the *CDKN1A* (*P21*) gene, and promotes degradation of p27<sup>kip1</sup> in EFT cells (10,11). Ling *et al* also reported that bortezomib-induced cell cycle arrest might be associated with the accumulation of p21<sup>waf1/cip1</sup> in human non-small cell lung cancer cells (30). Therefore, we tested the effects of bortezomib on the expression of cell cycle regulating proteins (including p21<sup>waf1/cip1</sup>) in EFT cells. Western blot analysis revealed that the protein expression levels of p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> were dose-dependently increased by

treatment with bortezomib. In the p53-functional cell lines (WE-68 and VH-64), the accumulation of p53 by bortezomib treatment was detected in a dose-dependent manner (Fig. 2B). These data indicated that the suppression of proteasome activities by bortezomib resulted in the accumulation of the proteins that are usually degraded by proteasome, including p21<sup>waf1/cip1</sup>, p27<sup>kip1</sup>, and p53, in EFT cells.

*Inhibition of the cell cycle progression and induced apoptosis in EFT cells by bortezomib.* We conducted flow cytometry analysis to monitor the alteration of the cell-cycle distribution by bortezomib in EFT cells. The changes in the cell cycle in VH-64 cells as a result of various concentrations of bortezomib are shown in Fig. 3A. Treatment with bortezomib for 24 h did not cause the accumulation of cell populations within the G0-G1 fraction of the cell cycle (Fig. 3A and B). However, the accumulation of cells in the sub-G1 fraction in the cell cycle, an indicator of apoptosis, was detected in all EFT cell lines tested without obvious induction of G1 or G2 cell-cycle arrest (Fig. 3A and C). To confirm the induction of bortezomib-mediated apoptosis in EFT cells, we carried out Western blot analysis to demonstrate the expression of cleaved PARP and caspase-8. The cleaved forms of PARP and caspase-8 were detected in all cell lines treated with bortezomib (Fig. 3D). The detection of caspase-8 activation suggested that bortezomib might induce apoptosis through the death receptor pathway in EFT cells. To determine whether bortezomib induces apoptosis *in vivo*, TUNEL staining was performed on the tumor sections from the murine xenograft models of EFT treated with bortezomib. Although most of the tumor cells were TUNEL-negative in the untreated controls, the EFT cells in the tumors treated with 1.5 mg/kg of bortezomib were TUNEL-positive (Fig. 3E). However, the expression of the cleaved PARP was not observed in the normal fibroblast cells treated with bortezomib (Fig. 3F). The IC<sub>50</sub> for NIH3T3 and Balb3T3 fibroblasts of bortezomib were 67 and 225 nM, respectively, and were significantly higher than the IC<sub>50</sub> for EFT cells. These results showed that bortezomib selectively inhibited the proliferation of EFT cells both *in vitro* and *in vivo* via the induction of apoptosis. We therefore concluded that bortezomib selectively induce apoptosis in EFT cells.

*Increase in the expression of death receptor 4 protein in bortezomib-treated EFT cells.* It has been reported that the anti-tumor effects of bortezomib on multiple myeloma cells are mediated by the inhibition of NF-κB activity via IκB accumulation (17,18). NF-κB is present in the cytoplasm of the cell in its inactive state, thereby forming a complex with the inhibitory IκBα proteins. The activation of NF-κB occurs via the phosphorylation of IκBα at Ser32 and Ser36, resulting in the release and nuclear translocation of active NF-κB. In the present study, a Western blot analysis using anti-phospho-IκBα-Ser32/36 antibody revealed an accumulation in the phospho-IκBα by bortezomib treatment. However, the expression of cIAP1 and cIAP2 (inhibitor of apoptosis protein), which acts as anti-apoptotic factors induced by NF-κB, were not affected (Fig. 4A). Therefore, the induction of apoptosis in EFT cells by bortezomib might not be mediated by the NF-κB pathway. To elucidate the

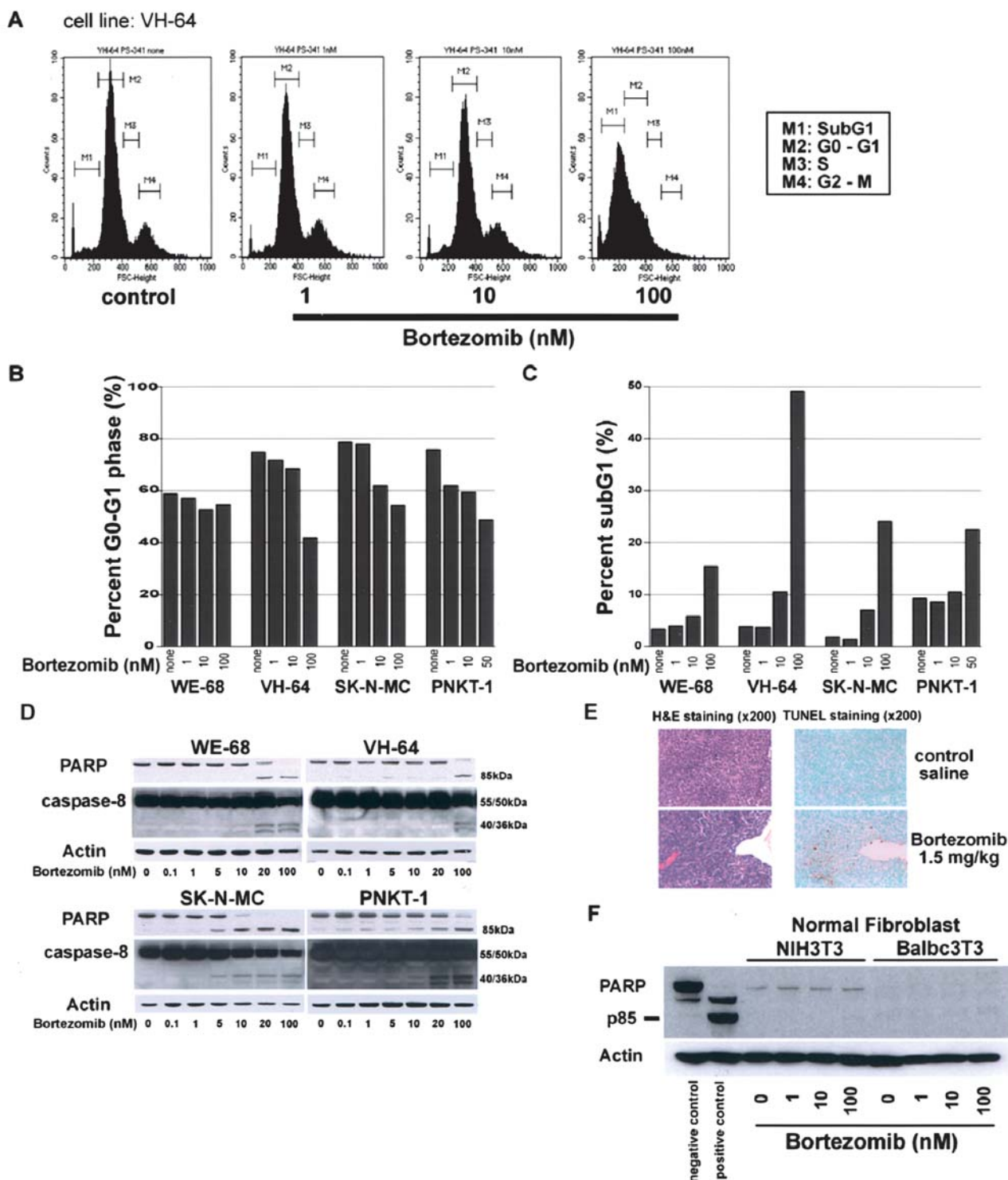


Figure 3. Bortezomib induced apoptosis in the EFT cell lines. EFT cells were exposed to various concentrations of bortezomib for 24 h. Then, the cells were processed for propidium iodide staining and flow cytometry analysis. (A) The cell-cycle distributions of VH-64 cells treated with various concentrations of bortezomib. The percentages of events in each phase of the cell cycle (M1, sub-G1; M2, G0-G1; M3, S; M4, G2-M) were calculated using CellQuest software. (B) The bar graph indicates the percentage of the population within the G0-G1 phase in the cell cycle. Treatment with bortezomib did not result in the accumulation of the cell population in the G0-G1 fraction. (C) The populations in the sub-G1 fraction with a DNA content less than 2 n, which is indicative of apoptosis, were dose-dependently increased by treatment with bortezomib in the EFT cells. (D) EFT cells were exposed to various concentrations of bortezomib for 24 h, and protein samples were collected and subjected to a Western blot analysis. The cleaved PARP was detected in all the EFT cell lines. In addition, an active form of caspase-8 was detected at concentrations from 5 to 100 nM of bortezomib. (E) The tumor tissue sections resected from mice who received 3 injections of bortezomib were subjected to TUNEL staining and H&E staining (original magnification, x200). The number of TUNEL-positive EFT cells increased due to bortezomib treatment (1.5 mg/kg), whereas most of the tumor cells from the control mice were TUNEL-negative. (F) To estimate the cytotoxicity of bortezomib in normal fibroblasts (NIH3T3 and Balbc3T3), a Western blot analysis using anti-PARP antibody was conducted. The cleaved PARP could not be detected in either of the fibroblastic cells at any concentration of bortezomib.

mechanism by which bortezomib induces apoptosis in EFT cells, we analyzed the expression of the death receptor 4

(DR4) in the cells. WE-68, VH-64, SK-N-MC, and PNKT-1 cells were incubated with 100 nM bortezomib for 4-20 h, and

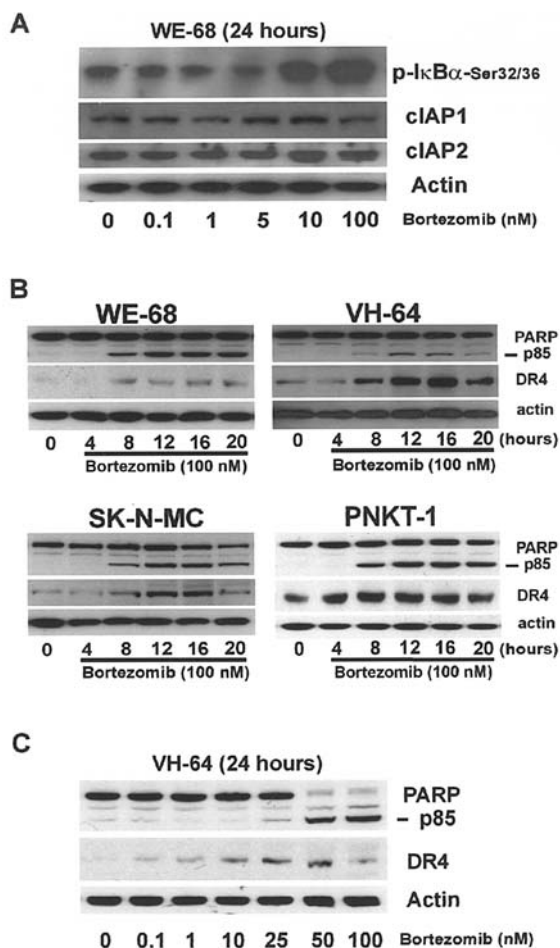


Figure 4. The expression of the DR4 (death receptor 4) protein in EFT cells treated with bortezomib. (A) WE-68 cells were incubated with bortezomib (0.1–100 nM) for 24 h, and the protein samples from the cells were processed for Western blot analysis using phospho-IkB $\alpha$ -Ser32/36, cIAP1, cIAP2, and actin antibodies. Although the phosphorylated IkB $\alpha$  was up-regulated by bortezomib treatment, the expression levels of cIAP1 and cIAP2 were not affected by bortezomib. (B) EFT cells were incubated with 100 nM bortezomib for 4 to 20 h, and the whole cell lysates were subsequently collected and subjected to Western blot analysis using anti-PARP, anti-DR4, and anti-actin antibodies. The time-course detection of DR4 was parallel to the induction of the cleaved PARP in EFT cells treated by bortezomib. (C) VH-64 cells were incubated with bortezomib (0.1–100 nM) for 24 h, and the whole cell lysates were harvested. The up-regulation of DR4 expression was observed in a dose-dependent manner followed by the induction of the cleaved PARP.

the whole cell lysates were subjected to Western blot analysis using anti-PARP, anti-DR4, and anti-actin antibodies. The up-regulation of the expression of DR4 was detected at 8 h after bortezomib treatment when the cleaved PARP was observed (Fig. 4B). The dose-dependent up-regulation of DR4 expression by bortezomib was also observed in VH-64 cells (Fig. 4C).

**Cross-resistance to bortezomib of drug-resistant EFT cells expressing P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1).** Most treatment failures of the tumors result from the resistance of the tumor cells to the chemotherapeutic drugs. One of the factors associated with the multi-drug resistance is an efflux pump, including P-gp and MRP1 (31). Adriamycin, vincristine, actinomycin D, and etoposide which are key anti-tumor drugs of EFTs become

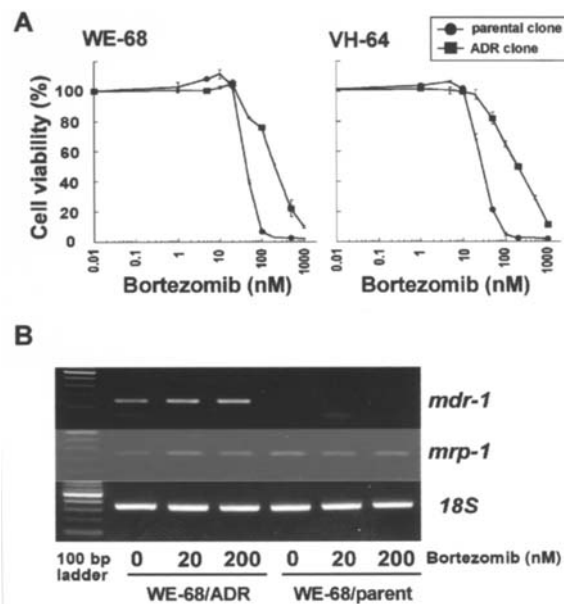


Figure 5. The cross-resistance of the drug-resistant EFT clones to bortezomib. (A) The parental and ADR clones of WE-68 and VH-64 (circles, parental clones; squares, ADR clones) were treated with various doses of bortezomib for 48 h. Thereafter, the cell viability was assessed using CellTiter-Glo Luminescent Cell Viability Assay system. The cell viability assay demonstrated the cross-resistance of both ADR clones to bortezomib. The data represent the means of three separate experiments performed in triplicate. (B) To evaluate the effects of bortezomib on the expression of mdr1 (P-gp) mRNA or mrp1 mRNA in WE-68/ADR and WE-68/parent cells, RT-PCR was performed as described in Materials and methods. The treatment of bortezomib did not modulate the mRNA expression of the mdr1 and mrp1 genes in either cell line. 18S rRNA was used as an internal control.

the substrate of P-gp and MRP1. We previously established multidrug-resistant EFT cell lines (WE-68/ADR and VH-64/ADR), both of which over-expressed P-gp and MRP1, as models of drug-resistant EFTs (26). The ADR clones (WE-68/ADR and VH-64/ADR) exhibited the strong cross-resistance to adriamycin and histone deacetylase inhibitors, including FK228 and apicidin, although the parental cells were highly sensitive to these drugs. Therefore, we investigated whether bortezomib could demonstrate inhibitory effects on the cell viability of ADR clones as well as on parent cell lines. The cell viability assay demonstrated that the drug-resistant WE-68/ADR and VH-64/ADR cells exhibited cross-resistance to bortezomib (Fig. 5A). The fold resistance to bortezomib of each ADR clone (IC<sub>50</sub> for ADR clones/IC<sub>50</sub> for parental clones) was 5- or 8-fold, respectively. Since Fujita *et al* reported that bortezomib could inhibit the mRNA expression of both mdr-1 (P-gp) and mrp-1 (MRP1) (32), we examined the effects of bortezomib on the expression of P-gp and MRP1 genes in EFT cells. However, the RT-PCR analysis revealed that mRNA expression of mdr-1 and mrp-1 was not affected by bortezomib in either WE-68/ADR or the parental clones (Fig. 5B).

**Efflux pump inhibitors enhance the effects of bortezomib on the drug-resistant EFT cells.** The modulation of the effects of bortezomib on ADR clones by verapamil (a P-gp specific inhibitor) and MK-571 (an MRP1 inhibitor) were next investigated using cell viability assay. The co-treatment with verapamil or MK-571 partially augmented the inhibitory



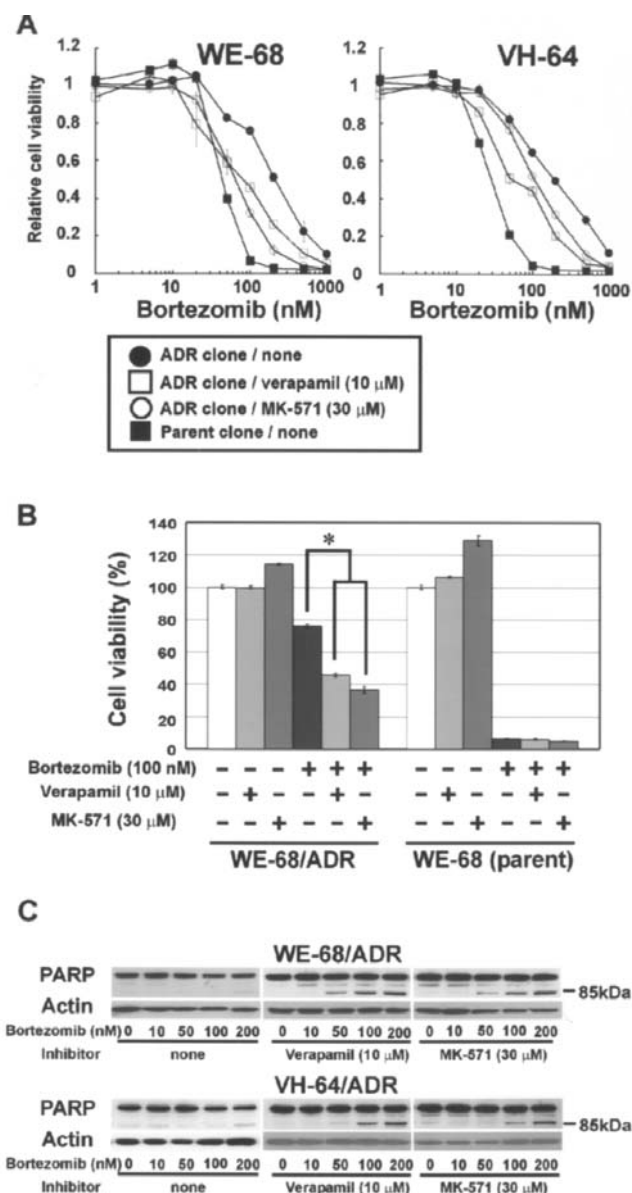


Figure 6. The P-gp- and MRP1-specific inhibitors could enhance the effects of bortezomib on the multi-drug resistant EFT cells. (A) The parental and ADR clones of EFTs were incubated with various concentration of bortezomib for 48 h with or without of 10  $\mu$ M verapamil or 30  $\mu$ M MK-571. Filled circle, ADR clones treated with bortezomib alone; open square, ADR clones co-treated with verapamil; open circle, ADR clones co-treated with MK-571; filled square, parental clones treated with bortezomib. (B) WE-68/ADR cells or the parental cells were incubated with 100 nM bortezomib for 48 h in the presence or absence of 10  $\mu$ M verapamil or 30  $\mu$ M MK-571. White bar, none; black bar, treatment with bortezomib alone; gray bar, co-treatment with verapamil; dark gray bar, co-treatment with MK-571. The anti-tumor effects of bortezomib were enhanced by co-treatment with verapamil and MK-571 in the ADR clones of WE-68. The statistical differences between each sample ( $p < 0.001$ ) were studied by ANOVA with post-hoc and are represented by an asterisk. (C) After the drug-resistant EFT cells were incubated with or without the efflux pump inhibitors and bortezomib for 24 h, the whole cell lysates were subjected to Western blot analysis using anti-PARP and anti-actin antibodies. In both the WE-68/ADR and VH-64/ADR cells, treatment with bortezomib combined with verapamil or MK-571 induced expression of the cleaved PARP.

effects of bortezomib on the growth of WE-68/ADR and VH-64/ADR cells (Fig. 6A and B). We also examined the combination effects of the efflux pump inhibitors with bortezomib on the induction of cleavage of PARP. The results

revealed that verapamil and MK-571 could enhance the induction by bortezomib of the expression of the cleaved PARP in both WE-68/ADR and VH-64/ADR cells (Fig. 6C). These data suggest that bortezomib might be the substrate of P-gp and MRP1 in the drug-resistant EFT cells, and that the efflux pumps might play important roles in the cross-resistance to bortezomib of ADR clones. Therefore, the inhibition of the efflux pump activities might be an effective method to overcome the cross-resistance to bortezomib of the drug-resistant EFT cells.

## Discussion

Recent evidence suggests that the proteasome inhibitors might be promising anti-neoplastic agents. The potent anti-tumor effects of bortezomib, a proteasome inhibitor also known as PS-341, have been reported on hematological malignancies and solid tumors. We have previously demonstrated that the antisense oligodeoxynucleotide against *EWS-Flil* effectively inhibits the expression of the fusion gene and the proliferation of human EFT cells by the induction of G1 arrest in the cell cycle and the up-regulation of the CDK inhibitors p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> (8,9). We have also reported that the *EWS-Flil* fusion protein directly suppressed the expression of p21<sup>waf1/cip1</sup> (10), and that patients with p27<sup>kip1</sup>-negative EFTs showed significantly worse outcomes than patients with p27<sup>kip1</sup>-positive tumors. These findings indicate that a low expression level of p27<sup>kip1</sup> clearly correlates with a poor prognosis in patients with EFTs (11). These observations brought us to speculate that bortezomib might have an inhibitory effect on EFT cells since both p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> are targets of the ubiquitin-proteasome pathway.

On the contrary, a recent phase II clinical study revealed that the effect of bortezomib to be limited on recurrent or metastatic sarcomas, including two cases of EFTs (25). However, since no basic study regarding the effects of bortezomib on EFT cells has yet been reported and the number of patients with EFTs was too small in the clinical trial, it is difficult to conclude the efficacy of bortezomib on EFTs. We thus investigated the effects of bortezomib on EFT cells and further the drug-resistant clones of EFTs.

We first elucidated the basic effects of bortezomib on the parental EFT cells, and found that bortezomib could decrease EFT cell viability at a nanomolar level of concentration and induced apoptotic cell death in EFT cells both *in vitro* and *in vivo*. The anti-tumor effects of bortezomib showed selectivity for EFT cells, because bortezomib did not exhibit growth inhibition in Balbc3T3 or NIH3T3 fibroblasts at the concentration that is effective on EFT cells. Previous studies have demonstrated that bortezomib exhibits anti-neoplastic effects via the accumulation of I $\kappa$ B $\alpha$  and subsequent inhibition of NF- $\kappa$ B activation in multiple myeloma cells (17,18). In this study, we observed the up-regulation of phosphorylated I $\kappa$ B $\alpha$  by bortezomib treatment. However, we did not observe a clear modulation of the expression of cIAP1 and cIAP2, which are located downstream of NF- $\kappa$ B. Our data indicated that the induction of apoptosis in EFT cells was accompanied by the activation of caspase-8, which is the key caspase in the death receptor pathway. We also observed that the induction of DR4 expression was detected in both time- and

dose-dependent manner. We therefore, concluded that bortezomib might induce apoptotic cell death through the death receptor pathway in EFT cells, and the anti-tumor effects of bortezomib on EFT cells might not be mediated by the activation of NF- $\kappa$ B. Further investigation is needed to elucidate the precise mechanisms by which bortezomib induces apoptosis in EFT cells.

The growth inhibitory effects of bortezomib were potent in EFT cells with dysfunctional p53 (SK-N-MC and PNKT-1) and functional p53 (WE-68 and VH-64). It is well known that p53 is ubiquitinated by MDM2 and is also degraded by the ubiquitin-proteasome pathway (33). Therefore, bortezomib could naturally increase the expression level of p53 in WE-68 and VH-64 cells. It is also notable that treatment with bortezomib up-regulated the expression of p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> in all EFT cell lines tested. Although the expression of p21<sup>waf1/cip1</sup> is induced by p53, the increase in p21<sup>waf1/cip1</sup> expression by bortezomib is not mediated by the accumulation of p53 protein in EFT cells with dysfunctional p53. Because p21<sup>waf1/cip1</sup> protein is degraded via the ubiquitin-proteasome pathway, the inhibition of the proteasome activity by bortezomib might increase the protein expression level of p21<sup>waf1/cip1</sup> regardless the status of p53 in EFT cells. However, it remains unclear whether the accumulation of p27<sup>kip1</sup> and p21<sup>waf1/cip1</sup> might directly lead to the induction of apoptosis in EFT cells.

In the clinical application of a novel anti-tumor drug, the advanced cases with recurrent and/or metastatic tumors would be selected for the trial. It is noteworthy, that such advanced tumors have become resistant to various anti-cancer drugs. Although bortezomib showed potent anti-tumor effects on EFT cells, it is very important to investigate whether bortezomib would have the same effects on the drug-resistant EFT cells as the parental clones before clinical application of the drug to the advanced cases with EFTs. The major cause of the drug resistance is attributed to efflux pumps (including P-gp and MRP1) that can reduce the intracellular drug concentration. Since the key drugs used in chemotherapy for EFTs, including adriamycin, vincristine, etoposide, and actinomycin D (2), are known to be substrates of P-gp and/or MRP1 (34,35), the drug resistance of EFTs might also be related to these efflux pumps. In the present study, the drug-resistant EFT clones expressing P-gp and MRP1 exhibited the cross-resistance to bortezomib. Therefore, the results of the phase II clinical study of bortezomib for the advanced sarcomas were not surprising (25).

Our data suggest the reason for the insufficient effects of bortezomib on the advanced stages of EFTs to probably be due to the over-expression of the efflux pumps in the tumor, thus resulting in the cross-resistance of the tumor to the proteasome inhibitor. The mRNA expression of P-gp in MCF7/ADR (multidrug resistant breast cancer cell line) has been reported to decrease after treatment with bortezomib (32). In our study, however, a decrease in the gene expression of either P-gp or MRP1 was not detected in ADR clones of EFTs. These observations indicate the necessity of developing a method to inhibit the activities of the efflux pumps in tumor cells for the successful clinical application of bortezomib to the drug-resistant EFTs. When we challenged ADR clones with verapamil or MK-571, the resistance to

bortezomib was partly overcome. We therefore, suggest that a combination of bortezomib with P-gp inhibitor and MRP1 inhibitors might effectively reverse the resistance of EFTs to bortezomib, and that this combined regimen would be beneficial in the clinical use of proteasome inhibitors for patients with high-risk EFTs.

Our results demonstrated that bortezomib effectively induces apoptosis in EFT cells via the death receptor pathway both *in vitro* and *in vivo*. Bortezomib therefore, shows promising potential in the treatment of primary EFTs, although the effects of bortezomib on recurrent or metastatic EFTs might be limited. The co-treatment of P-gp inhibitors and MRP1 inhibitors with bortezomib has the potential to be a novel therapeutic strategy for treatment of the advanced EFTs.

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