S6K1 inhibition enhances the apoptotic cell death of breast cancer cells in response to Bcl-2/Bcl-xL inhibition by the downregulation of survivin

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Abstract. Breast cancer cells possess a deregulated apoptotic pathway with increased expression levels of anti-apoptotic B-cell lymphoma-2 (Bcl-2) family proteins and ribosomal S6 kinase 1 (S6K1) protein activity. Therefore, combined interference of anti-apoptotic Bcl-2 family and S6K1 protein expression may be a reasonable therapeutic strategy for the treatment of patients with breast cancer. In the present study, it was identified that the administration of a combination of ABT263 [navitoclax; a Bcl-2/Bcl-extra large (Bcl-xL) inhibitor] and PF4708671 (an S6K1 inhibitor) markedly increased apoptotic cell death in the BT474 breast cancer cells compared with the administration of either agent alone. Furthermore, the downregulation of Bcl-2/Bcl-xL and S6K1 with small interfering RNA induced a significant increase in cell death compared with RNA interference of either agent alone. Notably, combination treatment with ABT263 and PF4708671 decreased the expression level of survivin protein, with this ectopic expression of survivin attenuating cell death. Thus, the present study determined that the combined inhibition of Bcl-2/Bcl-xL and S6K1 may be a good strategy for treating patients with breast cancer.

Introduction

All types of cancer cell are prone to resisting apoptotic cell death; therefore, knowledge of the mechanisms of resistance to apoptosis may lead to the development of novel therapeutic strategies (1). Changes in the apoptotic susceptibility of cancer cells may contribute to enhanced resistance to conventional anticancer therapies, such as radiation and cytotoxic agents, as well as to neoplastic development (2). Altered expression of B-cell lymphoma-2 (Bcl-2) family members has been proposed as a possible mechanism for the development of resistance to certain cytotoxic antineoplastic agents. The Bcl-2 family of proteins is composed of pro- and anti-apoptotic members that regulate cellular proliferation and death, respectively, via inter- and intra-family interactions (3,4). In particular, Bcl-2 has emerged as an important clinical prognostic marker in breast cancer (5,6), being overexpressed in ~75% of cases of breast cancer (approaching 85% for estrogen-positive breast cancer) (6). ABT263 (navitoclax) is a small molecule that binds with high affinity to Bcl-2 and Bcl-extra large (Bcl-xL), and antagonizes their anti-apoptotic functions, thereby inducing apoptosis in various cancer cell types (7,8). However, this molecule is limited by its lack of efficacy, and its significant toxicity and resistance (9).

Ribosomal S6 kinase 1 (S6K1) is the downstream effector of mammalian target of rapamycin complex 1 (mTORC1), a protein complex that regulates essential cellular functions, including cell survival, proliferation, metabolism, migration and angiogenesis (10). The S6K1 gene is amplified in numerous breast cancer cell lines (11-13), and S6K1 gene amplification or protein expression has been associated with a poor prognosis in breast cancer (14). Thus, the targeting of S6K1 in combination with ABT263 may present an effective strategy for the treatment of patients with breast cancer.

Therefore, the present study aimed to examine the combinatorial apoptotic effects of ABT263 (a Bcl-2/Bcl-xL inhibitor) and PF4708671 (an S6K1 inhibitor) treatment on BT474 breast cancer cells.

Materials and methods

Cell culture and reagents. The breast cancer BT474 and MCF7 cell lines were obtained from the American Type

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Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium and Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen Life Technologies). PF4708671 was purchased from Sigma-Aldrich (St. Louis, MO, USA), ABT263 (navitoclax) from Selleckchem (Houston, TX, USA) and Sorafenib from Bayer Healthcare Pharmaceuticals (New Haven, CT, USA).

Assessment of cell death. The BT474 cells were treated with 0, 0.5, 1 and 2 μM ABT263, or 0, 5, 10 and 20 μM PF4708671. The treated cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Biosciences, San Jose, CA, USA) for the assessment of apoptotic cell death, as previously described (15). As a positive control, MCF7 cells were treated with 5 μM sorafenib and deprived of glucose for 24 h.

Western blot analysis. The cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS] supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentrations were then measured using the Bradford method (16). The cell lysates were separated via SDS-PAGE and transferred to nitrocellulose membranes, followed by immunoblotting with specific primary and horseradish peroxidase-conjugated secondary antibodies. Antibodies specific for cleaved poly(ADP-ribose) polymerase (PARP; rabbit polyclonal; 1:1,000; cat no. 9541), p-S6 at Ser240/244 (rabbit monoclonal; 1:2,000; cat no. 4838), S6K1 (rabbit polyclonal; cat no. 9202; 1:1,000) and survivin (rabbit monoclonal; 1:1,000; cat no. 2808) were obtained from Cell Signaling Technology (Beverly, MA, USA), an antibody for Bcl-xL (mouse monoclonal; 1:1,000; cat no. 610746) was obtained from BD Biosciences (Franklin Lakes, NJ, USA), and an antibody for β-actin (mouse monoclonal; 1:5,000; cat no. A5316) was obtained from Sigma-Aldrich. The immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrates (Thermo Scientific Pierce, Rockford, IL, USA).

small interfering (siRNA) and transfection. Bcl-2 (cat no. sc-29214), Bcl-xL (cat no. sc-43630) and control (cat no. sc-37007) siRNAs were purchased from Santa Cruz Biotechnology, Inc. (San Jose, CA, USA). S6K1 (cat no. SI00301721) and control siRNAs (cat no. 1022076) were purchased from Qiagen, Inc. (Valencia, CA, USA). Transient 24-h siRNA transfections were performed with Lipofectamine® RNAiMAX reagent, according to the manufacturer's instructions (Invitrogen Life Technologies). The BT474 cells were transiently transfected with the indicated siRNAs for 24 h.

Measurement of cell viability. The cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide to a colored product, as previously described (15). Images of cell populations were captured microscopically with an Olympus CKX41 microscope (Olympus Corporation, Tokyo, Japan).
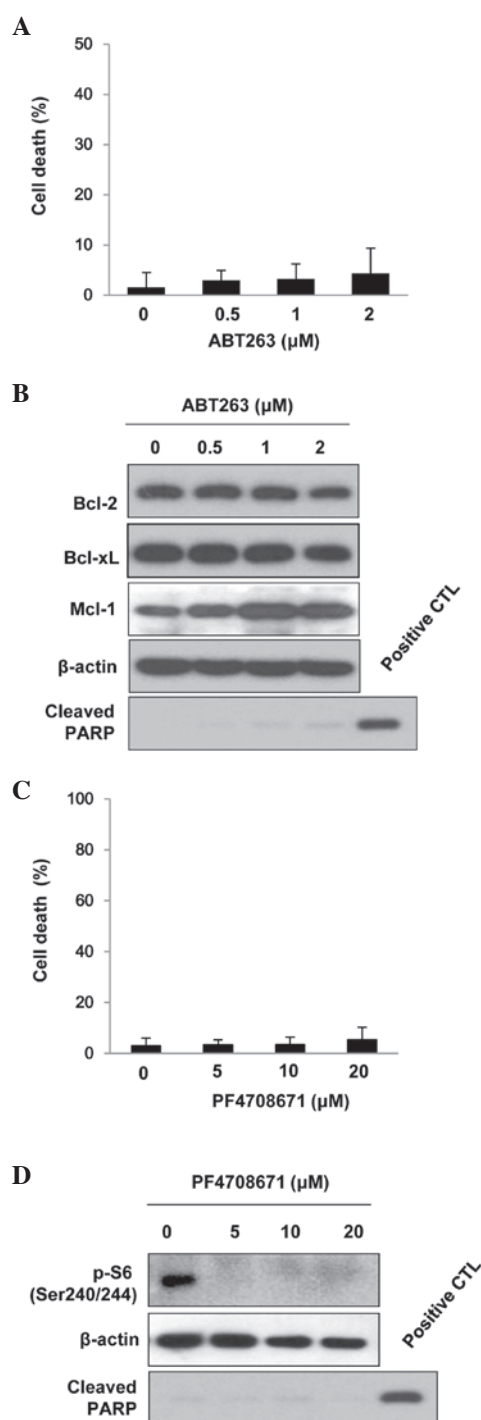

Figure 1. (A and B) ABT263 and (C and D) PF4708671 had no effect on BT474 cell death when applied individually. BT474 cells were treated with the indicated concentrations of ABT263 or PF4708671 for 24 h. (A and D) Cell death was determined using flow cytometry following Annexin V/propidium iodide staining. Data are presented as the means of triplicate samples with error bars indicating the standard deviation from the mean. (B and C) Protein expression levels were estimated using western blot analysis. As a positive CTL, MCF7 cells were treated with 5 μM sorafenib and deprived of glucose for 24 h. Bcl-2, B-cell lymphoma-2; Bcl-xl, B-cell lymphoma-extra large; PARP, poly(ADP-ribose) polymerase; CTL, control.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. RNA isolation and RT-PCR analysis were conducted as previously described (17). The following primers were used:
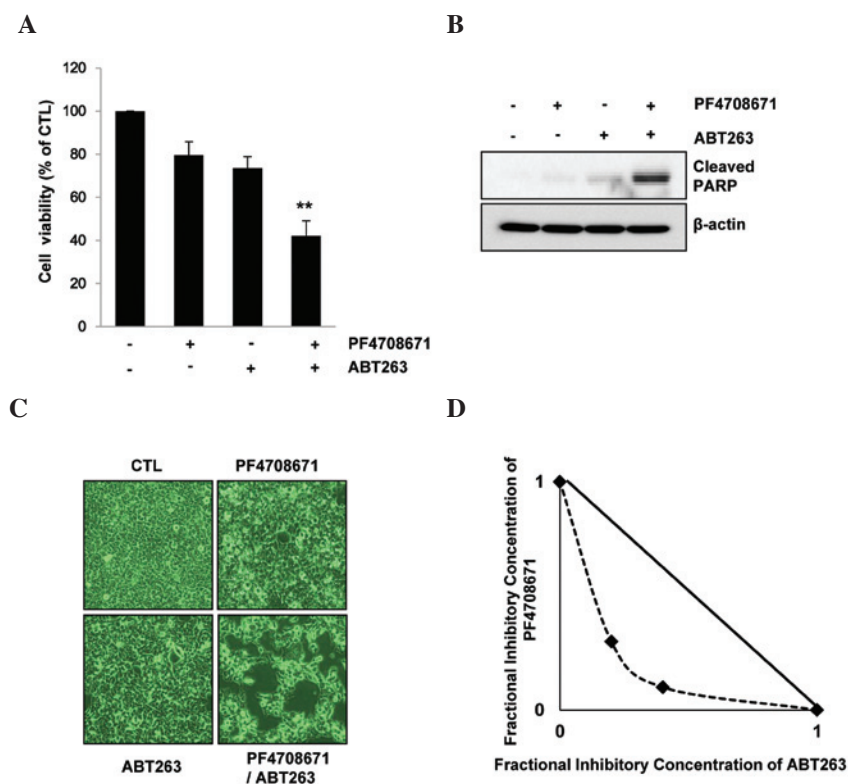
Figure 2. Combined treatment with PF4708671 and ABT263 synergistically induces cell death. (A-C) BT474 cells were treated with or without 1 μM ABT263 and/or 10 μM PF4708671 for 24 h. (A) Cell viability was determined by performing a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Data are presented as the means of triplicate samples, with error bars indicating the standard deviation from the mean. (B) Protein expression levels of cleaved PARP were analyzed by western blotting. (C) Images of cell populations for each treatment group were obtained by fluorescence microscopy. (D) Synergy between the effects of PF4708671 and ABT263 was assessed by an isobologram analysis. **P<0.01 vs. PF4708671- or ABT263-treated group. CTL, control; PARP, poly(ADP-ribose) polymerase.

Survivin, sense, 5'-GGACCACCG CATCTCTAC-3' and anti-sense, 5'-CAGCCTTCCAGCTCCTTG-3', with a 156-bp product (15); and β-actin, sense, 5'-GGATTCCTA TGTGGGCGACAG-3' and anti-sense, 5'-CGCTCGGTGAG-GATCTTCATG-3', with a 438-bp product (15).

Isobologram analysis. The effects of two-drug combinations were assessed by isobologram analysis. The cells were treated with different concentrations of ABT263 (0-20 μM) and PF4708671 (0-40 μM). Combinations resulting in 25±1% cytotoxicity were expressed as a percentage of each single drug, alone, producing an equivalent level of cytotoxicity [fractional inhibitory concentrations (FIC)=concentration of each drug in the combination/concentration of each drug alone]. When the sum of the FIC was 1, the combination was additive and the graph was expressed as a straight line; when the sum was <1, the combination was synergistic and the graph demonstrated a concave shape; and when the combination was >1, the combination was antagonistic and the graph demonstrated a convex shape.

Statistical analysis. Data are expressed as the mean ± standard deviation of three independent experiments. A Student's t-test was performed to analyze differences between groups. All statistical analyses was performed using SigmaPlot software (version 11; Systat Software Inc., San Jose, CA, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

Individual treatment with ABT263 or PF4708671 has no effect on cell death. Initially, the effects of ABT263 on BT474 on breast cancer cell apoptosis were examined. Apoptosis assays were performed using Annexin V/PI staining and western blot analysis of cleaved PARP expression. As indicated in Fig. 1A and B, treatment with ≤2 μM ABT263 did not induce marked apoptotic cell death in the BT474 cells. Furthermore, protein expression levels of myeloid cell leukemia-1 [Mcl-1 (a Bcl-2-related protein)] were upregulated upon treatment with ABT-263, however, the expression levels of Bcl-2 and Bcl-xL did not change (Fig. 1B).

BT474 breast cancer cells are characterized by the overexpression of S6K1 (18). PF4708671 treatment suppressed S6K1 activity in the BT474 cells, as evidenced by the decreased phosphorylation of S6 at Ser240/244 (Fig. 1C). However, PF4708671 (≤20 μM) resulted in no significant cell death (Fig. 1D). These data indicate that ABT263 or PF4708671 treatment alone is not sufficient to cause apoptosis.

Combined treatment with ABT263 and PF470867 synergistically induces cell death. The present study examined the effect of combined treatment with ABT263 and PF4708671 on the induction of cell death. Cell viability was reduced by 20% following treatment with 10 μM PF4708671 and by 27% following treatment with 1 μM ABT263. However, combination treatment with the two agents resulted in a
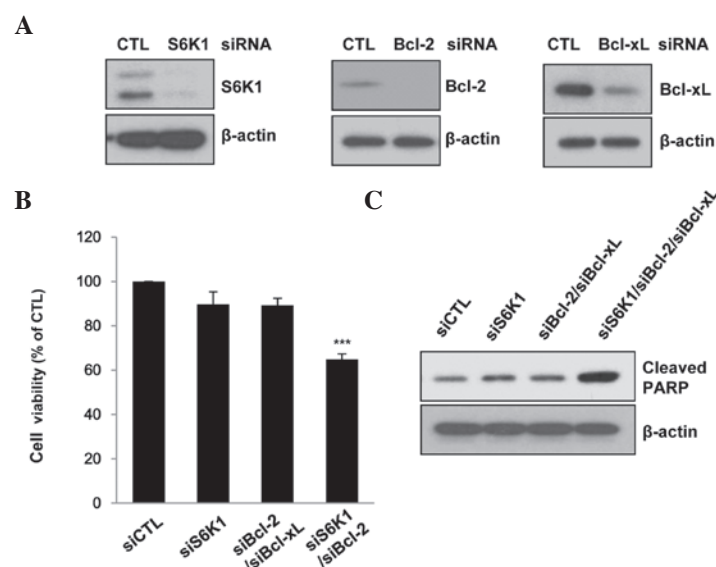
Figure 3. Silencing of S6K1 and Bcl-2/Bcl-xL induces cell death. (A-C) The BT474 cells were transiently transfected with the indicated target genes for 24 h. (A and B) Expression levels of the indicated proteins were analyzed by western blotting and (C) cell viability was measured by performing a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Data are presented as the means of triplicate samples, with error bars indicating the standard deviation from the mean.[***]$P<0.001$ vs. siS6K1- or siBcl-2/Bcl-xL-treated group. CTL, control; S6K1, ribosomal S6 kinase 1; si, small interfering; Bcl-2, B-cell lymphoma-2; Bcl-xl, B-cell lymphoma-extra large; PARP, poly(ADP-ribose) polymerase.
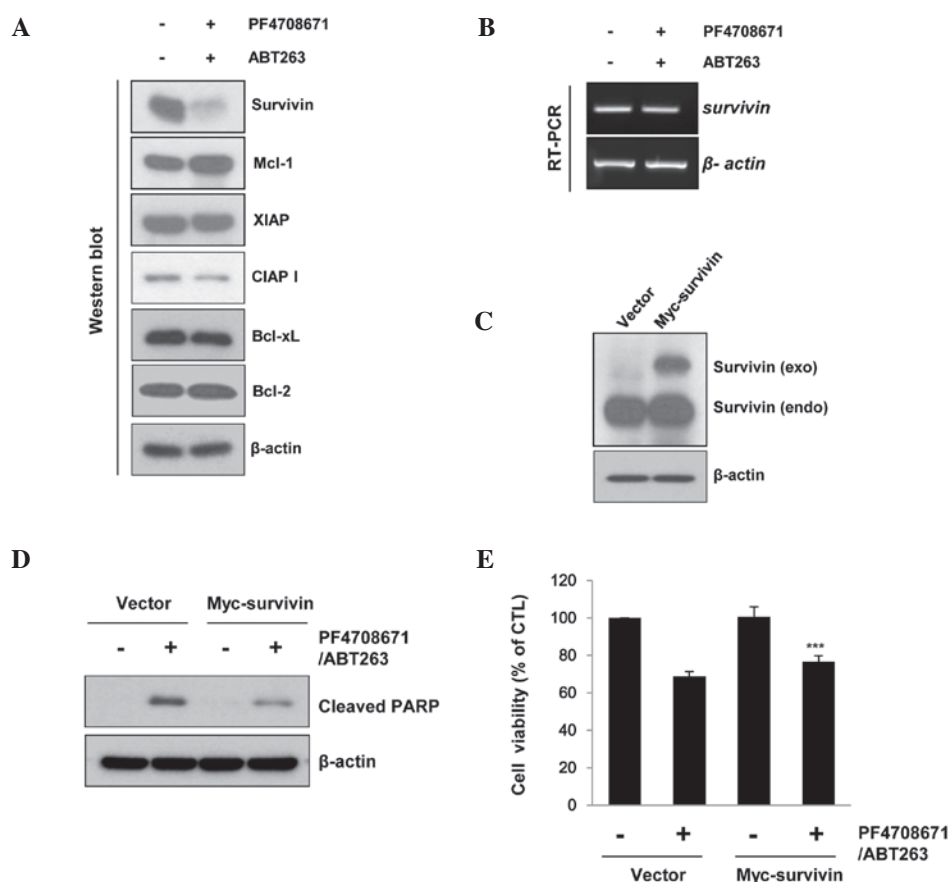

Figure 4. Downregulation of survivin expression by PF4708671 and ABT263 induces cell death. (A and B) BT474 cells were treated with 10 μM PF4708671 and 1 μM ABT263 for 24 h. (C-E) BT474 cells were transiently transfected with empty or myc-survivin vector for 24 h, prior to treatment with 10 μM PF4708671 and 1 μM ABT263 for 24 h. (A, C and E) Protein expression levels were determined by western blotting and (B) mRNA expression levels were determined by RT-PCR. (D) Cell viability was analyzed by performing a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. The data are presented as the means of triplicate samples and the error bars indicate the standard deviation from the mean. [***]$P<0.001$ vs. vector/PF4708671/ABT263-treated group. Mcl-1, myeloid cell leukemia-1; XIAP, X-linked inhibitor of apoptosis; cIAP1, cellular inhibitor of apoptosis 1; Bcl-2, B-cell lymphoma-2; Bcl-xl, B-cell lymphoma-extra large; RT-PCR, reverse transcription-polymerase chain reaction; CTL, control; PARP, poly(ADP-ribose) polymerase.

significant reduction in cell viability (~60%; P<0.01) (Fig. 2A) and the induction of apoptotic cell death, as examined by PARP cleavage and microscopy images of apoptosis (Fig. 2B and C). Furthermore, isobolographic analysis clarified the synergy between ABT263 and PF4708671 (Fig. 2D). These data indicate that combined treatment with ABT263 and PF4708671 synergistically induces cell death in BT474 breast cancer cells.

Silencing of S6K1 and Bcl-2/Bcl-xL induces cell death. To further investigate the effect of the targeting of S6K1 and Bcl-2/Bcl-xL on BT474 cell death, the inhibitory effects of siRNAs targeting S6K1 and Bcl-2/Bcl-xL were investigated. The siRNAs caused marked knockdown of the target genes (Fig. 3A). The combined siRNA knockdown of S6K1 and Bcl-2/Bcl-xL significantly inhibited cell viability and induced PARP cleavage (P<0.001). By contrast, S6K1 or Bcl-2/Bcl-xL siRNA alone did not induce PARP cleavage (Fig. 3B) and exhibited marginal inhibitory effects on cell viability (Fig. 3C). These data indicate that the synergistic targeting of S6K1 and Bcl-2/Bcl-xL may induce BT474 cell death.

Downregulation of survivin expression by PF4708671 and ABT263 induces cell death. The present study invesitgated the mechanisms involved in ABT263- and PF4708671-induced cell death. Combined treatment with ABT263 and PF4708671 appeared to have no effect on the expression of Bcl-2-related proteins, such as Bcl-2, Bcl-xL and Mcl-1. Notably, survivin protein, unlike inhibitor of apoptosis (IAP) family members, such as X-linked IAP (XIAP) and cellular IAP 1 (cIAP1), was significantly decreased in the presence of ABT263 and PF4708671 (Fig. 4A). However, combined treatment with ABT263 and PF4708671 did not modulate survivin mRNA expression levels (Fig. 4B), indicating that the observed changes in survivin expression following ABT263 and PF4708671 exposure did not involve transcriptional regulation.

To further clarify the role of survivin in ABT263 and PF4708671-induced cell death, empty or myc-survivin vectors were transiently transfected into treated BT474 cells prior to receiving combined ABT263 and PF4708671 treatment. The expression of myc-tagged survivin in the BT474 cells significantly suppressed the cell cytotoxicity induced by PF4708671 and ABT263 (P<0.001; Fig. 4C and D), and attenuated the PARP cleavage induced by these inhibitors (Fig. 4E). These data indicate that the cell death induced by PF4708671 and ABT263 is at least in part mediated by the downregulation of survivin protein.

Discussion

The impairment of apoptosis is a hallmark of cancer and is key in the resistance to anticancer therapy. Tumors deploy diverse strategies to limit or evade apoptosis, frequently involving perturbation of the intrinsic Bcl-2 apoptotic pathway (19). As the overexpression of the pro-survival protein Bcl-2 is common in breast cancer (6), Bcl-2 may serve as a potential target for the treatment of this disease. ABT263 is an orally bioavailable small molecule inhibitor of Bcl-2 family proteins that binds with high affinity to Bcl-2 and Bcl-xL, and antagonizes their anti-apoptotic function (20). In the present study, ABT263 (≤2 μM) treatment resulted in upregulated Mcl-1 expression, in agreement with a previous report (21). However, ABT263 (≤2 μM) did not induce

apoptotic cell death in BT474 breast cancer cells (Fig. 1). Preclinically, ABT263 has demonstrated limited activity in the majority of solid tumors containing breast cancer (22-24). However, when used in combination with other agents, such as rapamycin, rituximab, rituximab-cyclophosphamide, doxorubicin, vincristine and prednisolone, and bortezomib, ABT263 appeared to enhance their efficacy in leukemia and lymphoma models (23).

Our previous study indicated that phosphorylated S6K1, a downstream effector of mTORC1, may be effectively applied as a predictive marker for breast cancer cells (25). Indeed, S6K1 is frequently overexpressed in breast cancer cells (19). In the present study, although PF4708671 (an S6K1 inhibitor) suppressed S6K1 activity in the BT474 cells, PF4708671 (≤20 μM) did not result in cell death (Fig. 1), indicating that PF4708671 alone is not sufficient to cause apoptosis. However, it was identified that combination treatment with ABT263 and PF4708671 synergistically induced BT474 cell death (Fig. 2). Furthermore, the combined inhibition of Bcl-2, Bcl-xL and S6K1 using siRNAs induced marked apoptosis (Fig. 3). These data indicate that ABT263 and PF4708671 in combination may be particularly effective treatment strategy for breast tumors with high levels of Bcl-2 and S6K1 expression.

ABT263 and PF4708671 combination treatment had no effect on the expression on anti-apoptotic Bcl-2 family proteins, such as Bcl-xL and Bcl-2, or IAP family proteins, such as XIAP and cIAP. However, ABT263 and PF4708671 notably induced decreases in survivin protein expression without affecting its mRNA expression. Furthermore, the overexpression of survivin attenuated the cell death induced by ABT263 and PF4708671. These results indicate that survivin may be involved in regulating the apoptosis induced by ABT263 and PF4708671.

In conclusion, data obtained in the present study indicates that ABT263 or PF4708671 treatment alone cannot induce apoptosis in breast cancer BT474 cells. However, combined treatment with ABT263 and PF4708671 appears to extensively induce cell death. Furthermore, the downregulation of survivin by ABT263 and PF4708671 treatment may induce cell death. Therefore, Bcl-2 and S6K1, which are frequently activated in breast cancer, may serve as potential targets in the treatment of patients with breast cancer.

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