Abstract. ABT-737, a BH3-mimetic small-molecule inhibitor, binds with very high affinity to Bcl-2, Bcl-xL and Bcl-w, and inhibits their activity. Aurora kinase is one of the serine/threonine kinase family members and is a vital and critical regulator of mitosis and meiosis. In the present study, we investigated the effects and mechanisms of a combined treatment of ABT-737 and VX-680 (Aurora kinase inhibitor) in human breast cancer MDA-MB-435S cells. ABT-737 plus VX-680 induced caspase-dependent apoptosis in the human breast cancer cells. Combined treatment with ABT-737 and VX-680 led to the downregulation of Bcl-2 expression at the transcriptional level and the downregulation of c-FLIP and Mcl-1 expression at the post-transcriptional level. Overexpression of Bcl-2 or c-FLIP could not block the induction of apoptosis caused by the combined treatment with ABT-737 and VX-680. However, overexpression of Mcl-1 partially inhibited the induction of apoptosis. In contrast, the combined treatment with ABT-737 and VX680 had no effect on the apoptosis in normal cells. Taken together, our study demonstrated that combined treatment with ABT-737 and VX-680 induced apoptosis in anti-apoptotic protein (Bcl-2 or c-FLIP)-overexpressing cells.

Introduction

The Bcl-2 superfamily of proteins is classified based on function and Bcl-2 homology (BH)-domain organization, such as anti-apoptotic, pro-apoptotic and BH3-only proteins. Anti-apoptotic Bcl-2 proteins maintain mitochondrial outer membrane permeability by binding to Bax and Bak; thus, overexpression of Bcl-2 results in apoptosis resistance in tumors (1). In contrast, pro-apoptotic Bcl-2 proteins increase mitochondrial outer membrane permeabilization (MOMP), which is related to induction of apoptosis. BH3-only proteins are classified into 2 groups: activators and sensitizers. Activator BH3-only proteins, such as Bid, Bim, and Puma, bind to anti- and pro-apoptotic proteins, whereas sensitizer BH3-only proteins, such as Noxa, bind to anti-apoptotic proteins. When anti-apoptotic proteins bind to BH3-only proteins, activator BH3-only proteins and pro-apoptotic proteins induce MOMP, resulting in the induction of apoptosis (2,3). ABT-737 is a small-molecule BH3 mimic, and directly binds to Bcl-2, Bcl-xL and Bcl-w with a very high affinity, resulted in induction of apoptosis by releasing Bak and Bak. Therefore, ABT-737 induces apoptosis in several types of cancer cells, such as leukemia, myeloma and small-cell lung cancer cells (4-6). In contrast, since ABT-737 has a very low affinity for Mcl-1 (7,8), several types of cancer cells are resistant to ABT-737. Therefore, to enhance the sensitivity of ABT-737, a strategy of combined treatment is needed.

Aurora kinases are serine/threonine kinases, and are important for cell proliferation, cell cycle regulation, mitotic spindle formation, centrosome maturation, and cytokinesis (9,10). Aurora kinase is divided into 3 groups: A, B, and C. Both Aurora kinase A and B are expressed in most normal cells, while Aurora kinase C is only expressed in testicular tissue (11). Recently, several studies found that Aurora kinases are overexpressed in tumors, and the extent of Aurora kinase overexpression is correlated with malignancy and poor prognosis (12-14). Therefore, Aurora kinases are important therapeutic targets. These molecular-targeted strategies inhibit specific molecules, which vary between cancer and normal cells. However, these strategies could be safer and more effective.

In the present study, we investigated whether a combined treatment with ABT-737 and an Aurora kinase inhibitor induces apoptosis, and we aimed to identify the mechanism involved in the combined treatment-mediated apoptosis in human breast carcinoma MDA-MB-435S cells.

Materials and methods

Cells and materials. MDA-MB-435S, Caki, and A549 cells were purchased from the American Type Culture Collection.
Human skin fibroblasts (HSFs) were a gift from Dr T.J. Lee (Yeungnam University, Korea). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 μM HEPES buffer, and 100 μg/ml gentamicin. ABT-737, VX-680 and MLN8237 were purchased from Sellek (Houston, TX, USA). Barasertib was purchased from Tocris (Ellisville, MO, USA). z-VAD was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-death receptor 5 (DR5), anti-Bcl2, anti-Bcl-xL, anti-Mcl-1, anti-cIAP2 and anti-PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-c-FLIP (L) antibody was obtained from Alexis (Lausanne, Switzerland). Anti-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-c-FLIP (L) antibody was obtained from Alexis (Lausanne, Switzerland).

**Western blot analysis.** The cells were suspended in 100 μl of phosphate-buffered saline (PBS), and 200 μl of 95% ethanol was added during a vortex step. The cells were incubated at 4°C for 1 h, washed with PBS and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. The incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by flow cytometry.

**DNA transfection and the luciferase assay.** Transient transfection was performed in 6-well plates. One day before the transfection, MDA-MB-435S cells were plated at ~60-80% confluency. The Bel-2/-2/-254 promoter-luciferase, NF-κB-luciferase, and cAMP response element (CRE)-luciferase plasmid were transfected into the cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The NF-κB-luciferase (plasmid no. 26699) plasmid was purchased from Addgene (Cambridge, MA, USA) (16). The CRE-luciferase plasmid was purchased from Clontech (Palo Alto, CA, USA). To assess the promoter-driven expression of the luciferase gene, the cells were treated with different concentrations of ABT-737 and VX680 alone or in combination. After 48 h, relative survival was assessed and the concentration-effect curves were used to determine the IC_{50} (the half-maximal inhibitory concentration) values for each drug alone and in combination with a fixed concentration of the second agent (15).
Combined treatment with ABT-737 and VX-680 induces apoptosis in breast carcinoma MDA-MB-435S cells. Signaling molecule-target drugs inhibit specific signaling, which is different between cancer and normal cells, and a combination strategy reduces adverse effects, such as toxicity in normal cells. Therefore, we examined whether a combined treatment with sublethal dosages of ABT-737 and VX-680, which inhibits ATP-binding of Aurora kinase A, B and C [17], induces apoptosis in breast carcinoma MDA-MB-435S cells. ABT-737 plus VX-680 markedly induced cell shrinkage and membrane blebbing (Fig. 1A). To determine whether ABT-737 plus VX-680 induces apoptosis, FACS analysis to measure the DNA content and western blotting to detect the cleavage of PARP, a substrate of caspase-3, were performed. VX-680 induced G2/M arrest, but had no effect on apoptosis. However, the combined treatment with ABT-737 and VX-680 increased the sub-G1 population and PARP cleavage (Fig. 1B). In addition, ABT-737 plus VX-680 markedly increased cytoplasmic histone-associated DNA fragmentation (Fig. 1C). Next, to investigate whether the combined treatment with ABT-737 and VX-680 had synergistic effects, MDA-MB-435S cells were treated with varied concentrations of ABT-737 and VX-680. The isobologram analysis revealed that the combined treatment with ABT-737 and VX-680 had synergistic effects (Fig. 1D). Next, to determine whether caspase is involved in the ABT-737 and VX-680-induced apoptosis, we examined caspase activity. ABT-737 and VX-680 increased caspase-3 activity, and a general caspase inhibitor, z-VAD-fmk (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) markedly reduced apoptosis and PARP cleavage (Fig. 1E and F). These data revealed that the combined treatment with ABT-737 and VX-680...
VX-680 induced caspase-dependent apoptosis in the breast carcinoma MDA-MB-435S cells.

Effect of ABT-737 plus VX-680 on other types of cancer cells and on normal cells. We investigated the effects of a combined treatment with ABT-737 and VX-680 on other types of cancer cells (human renal carcinoma (Caki cells), human lung carcinoma (A549 cells)) and normal cells (HSFs). Combined treatment with ABT-737 and VX-680 markedly induced apoptosis and PARP cleavage in the Caki and A549 cells (Fig. 2A and B). When MDA-MB-435S cells were treated with ABT-737 plus VX-680, the cell morphology was altered including cellular shrinkage and blebbing, while the morphology of the HSFs was not altered (Fig. 2C). Furthermore, combined treatment with ABT-737 and VX-680 had no effect on the sub-G1 population in the normal cells (Fig. 2C). Therefore, our results indicate that the combined treatment of ABT-737 and VX-680 induced apoptosis in cancer cells, but not in normal cells.

Effect of ABT-737 plus VX-680 on apoptosis-related proteins. Next, we investigated the expression levels of the Bcl-2 family, the inhibitor of apoptosis (IAP) family and death receptors in ABT-737 and VX-680-treated cells. As shown in Fig. 3A, ABT-737 and VX-680 alone had no effect on apoptosis-related proteins, while the combined treatment with ABT-737 and VX-680 reduced c-FLIP, Bcl-2 and Mcl-1 protein expression. Furthermore, downregulation of Bcl-2 mRNA was detected, while the mRNA expression levels of c-FLIP and Mcl-2 were not altered in the ABT-737 plus VX-680-treated cells (Fig. 3B). To further confirm the mechanism of Bcl-2 downregulation in ABT-737 plus VX-680-treated cells, we examined whether the combined treatment with ABT-737 and VX-680 inhibits Bcl-2 promoter activity. As shown in Fig. 3C, ABT-737 plus VX-680 markedly reduced Bcl-2 promoter (Bcl-2/-3254) activity. Furthermore, we investigated whether the combined treatment with ABT-737 plus VX-680 inhibits NF-κB and CRE-associated transcriptional activities, which are important to Bcl-2 mRNA expression (18-20). NF-κB and CRE transcriptional activities were markedly reduced in the ABT-737 plus VX-680-treated cells (Fig. 3D). These results indicated that NF-κB and CRE transcriptional activities are involved in ABT-737 plus VX-680-mediated downregulation of Bcl-2 expression.

Effect of Bcl-2, c-FLIP and Mcl-1 overexpression on ABT-737 plus VX-680-mediated apoptosis. Combined treatment with ABT-737 and VX-680 downregulated Bcl-2, c-FLIP and Mcl-1 protein expression within 9, 18 and 24 h, respectively (Fig. 4A). To investigate whether downregulation of Bcl-2, c-FLIP and Mcl-1 is associated with ABT-737 plus VX-680-induced apoptosis, MDA-MB-435S cells were transiently transfected with Bcl-2, c-FLIP and Mcl-1. As shown in Fig. 4, overexpression of Bcl-2 or c-FLIP had no effect on the ABT-737 plus VX-680-mediated apoptosis, but overexpression of Mcl-1 partially blocked apoptosis in the ABT-737 plus VX-680-treated cells. These data revealed that combined treatment...
with ABT-737 and VX-680 induced apoptosis in the Bcl-2- or c-FLIP-overexpressed cells.

Effect of inhibition of Aurora kinase A and B on apoptosis in VX-680-treated cells. In the present study, we used VX-680, which inhibits both Aurora kinase A and B. To identify which Aurora kinase plays an important role in ABT-737-induced apoptosis, we tested more specific inhibitors of Aurora kinase A (MLN8237) and Aurora kinase B (barasertib). As shown in Fig. 5, both inhibitors of Aurora kinase A (MLN8237) and Aurora kinase B (barasertib) induced an increase in the sub-G1 population and PARP cleavage in the ABT-737-treated cells (Fig. 5A and B). Therefore, these results revealed that inhibition of both Aurora kinase A and B could be involved in the apoptosis in ABT-737-treated cells.

Discussion

In the present study, we demonstrated that combined treatment with ABT-737 and VX-680 induced apoptosis in breast carcinoma MDA-MB-435S cells through downregulation of Bcl-2, c-FLIP and Mcl-1 expression. ABT-737 and VX-680 alone did not alter the expression levels of Bcl-2, c-FLIP and Mcl-1. However, combined treatment with ABT-737 and VX-680 induced the downregulation of Bcl-2 expression at the transcriptional level and downregulation of c-FLIP and Mcl-1 at the post-transcriptional level. Furthermore, ABT-737 plus VX-680 had no effect on the apoptosis of normal cells. Therefore, our results revealed that combined treatment with ABT-737 and VX-680 is an effective strategy to induce apoptosis in cancer cells.

In breast cancer cells, Aurora kinases are overexpressed, thus targeting to Aurora kinases is a good strategy by which to induce apoptosis. Pan-Aurora kinase inhibitor (VX-680) alone did not induce apoptosis, whereas VX-680 induced G2/M arrest (Fig. 1B). Combined treatment with ABT-737 and VX-680 markedly induced apoptosis through caspase activation (Fig. 1E). As shown in Fig. 3A, ABT-737 plus VX-680 markedly reduced c-FLIP, Bcl-2, and Mcl-1 protein expression. Firstly, downregulation of Bcl-2 is modulated at the transcriptional levels. Several transcription factors regulate Bcl-2 transcriptional level. Among them, p53 negatively modulates Bcl-2 expression (21-23). We investigated whether combined treatment with ABT-737 and VX-680 downregulates Bcl-2 mRNA by p53. However, pifithrin-α (p53 inhibitor) had no effect on the downregulation of ABT-737 plus VX-680-mediated Bcl-2 expression, and combined treatment with ABT-737 and VX-680 reduced Bcl-2 expression in wild-type p53 HCT116 human colon carcinoma cells and p53-null HCT116 cells (data not shown). Therefore, we ruled out the possibility of p53-mediated modulation. NF-κB and CRE are associated with Bcl-2 mRNA expression (18-20). We found
Figure 4. Effect of Bcl-2, c-FLIP and Mcl-1 overexpression on ABT-737 plus VX-680-mediated apoptosis. (A) MDA-MB-435S cells were co-treated with 0.1 μM ABT-737 and 10 μM VX-680 for the indicated time periods. Bcl-2, c-FLIP and Mcl-1 protein levels were determined by western blotting. MDA-MB-435S cells were transiently transfected with the Bcl-2 expression plasmid (B), c-FLIP expression plasmid (C), Mcl-1 expression plasmid (D) and the control plasmid pcDNA3.1 using Lipofectamine as prescribed by the manufacturer. After 24 h of incubation, the transfected cells were treated with 0.1 μM ABT-737 plus 10 μM VX-680 for 24 h. Apoptosis was analyzed as the sub-G1 fraction by FACS analysis. *P<0.01 compared to ABT-737 plus VX-680-treated MDA-MB-435S/vector cells.

Figure 5. Effect of Aurora kinase inhibitors on the apoptosis in ABT-737-treated cells. MDA-MB-435S cells were treated with the indicated concentrations of VX-680, MLN8237, and barasertib in the presence or absence of 0.1 μM ABT-737 for 24 h. (A) Cell cycle and apoptosis were analyzed by FACS analysis. (B) The protein expression levels of PARP and actin were determined by western blotting. The level of actin was used as a loading control.
that combined treatment with ABT-737 and VX-680 markedly decreased NF-κB and CRE transcriptional activities (Fig. 3D). These results indicate that the ABT-737 plus VX-680-mediated inhibition of NF-κB and CRE transcriptional activities is probably involved in the downregulation of Bcl-2 expression. Next, to identify the importance of Bcl-2 in ABT-737 plus VX-680-mediated apoptosis, MDA-MB-435S cells were transiently transfected with the Bcl-2 expression plasmid. As shown in Fig. 4B, although Bcl-2 was overexpressed, the combined treatment with ABT-737 plus VX-680 markedly induced apoptosis. ABT-737 binds to Bcl-2, Bcl-xL and Bcl-w with a very high affinity, resulting in induction of apoptosis. ABT-737 inhibited the anti-apoptotic function of Bcl-2, thus the population of apoptotic cells was not changed. Secondly, ABT-737 plus VX-680 reduced Mcl-1 and c-FLIP expression at the post-transcriptional level. Ubiquitin-dependent proteasomal degradation is a major mechanism of post-transcriptional regulation (24), and Mcl-1 and c-FLIP are also mainly degraded by the ubiquitin-proteasome pathway. Therefore, further experiments are needed to identify the relationship of proteasome activation in the downregulation of Mcl-1 and c-FLIP. As shown in Fig. 4D, overexpression of Mcl-1 attenuated ABT-737 plus VX-680-induced apoptosis. Since ABT-737 has a low affinity for Mcl-1, Mcl-1-overexpressing cells are resistant to ABT-737-induced apoptosis. In contrast, when c-FLIP was overexpressed, ABT-737 plus VX-680 induced apoptosis (Fig. 4C). Song et al. reported that ABT-737 (>5 µM) induces DR5 expression in renal carcinoma cells (25). Induction of DR5 expression overcomes endogenous c-FLIP expression (25). However, in the present study, ABT-737 did not induce DR5 expression (Fig. 3A), while ABT-737 plus VX-680 induced apoptosis in the c-FLIP-overexpressing cells. Further experiments are needed to identify the mechanism of ABT-737 plus VX-680-mediated apoptosis in c-FLIP-overexpressing cells.

Taken together, our results revealed that the combination treatment with ABT-737 and VX-680 induced apoptosis in breast cancer cells, and overcame resistance by overexpression of Bcl-2 and c-FLIP.

References