의학석사 학위논문

ABT-737 induces apoptosis in tamoxifen-resistant MCF7 (MCF7/TamR)

타목시펜 저항성 MCF7 세포주에서 ABT-737의 세포자멸사 유도

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이 논문을 의학석사 학위논문으로 제출함
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위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

ABSTRACT

Introduction: Anti-hormonal therapy, such as tamoxifen, is necessary to reduce recurrence and increase the survival rate of hormone receptor (HR)-positive breast cancer patients. However, many HR-positive breast cancer patients have resistance to anti-hormone therapy. The aim of this study is to confirm the possibility of ABT-737, an anti B-cell lymphoma-2 (Bcl-2) drug, as a treatment to overcome endocrine resistance.

Methods: We conducted experiments using the tamoxifen-sensitive MCF7 (MCF7/TamS), tamoxifen-resistant MCF7 (MCF7/TamR), and ABT-737. Western blot analysis was performed to assess expression levels of Bcl-2 family proteins and caspase-related molecules in the MCF7/TamS and MCF7/TamR. Cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay. In addition, apoptotic cell death was determined by flow cytometry analysis.

Results: The anti-apoptotic Bcl-2 family were overexpressed in MCF7/TamR compared to MCF7/TamS. *(p*<0.01) At all concentrations of ABT-737, cell viability of MCF7/TamR was significantly lower compared to that of MCF7/TamS. (*p*<0.001) The expression of Bcl-2 in MCF7/TamR decreased after the administration of ABT-737. Conversely, the expression of cleaved caspase3, active form of caspase3 increased after the administration of ABT-737. In addition, we confirmed that apoptotic cell death was increased in MCF7/TamR treated with ABT-737.

Conclusions: The activation of the apoptotic pathway using ABT-737 can be a key to overcoming endocrine resistance.

Keyword: ABT-737, Bcl-2, apoptosis, anti-hormone therapy resistance, breast neoplasm

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LIST OF ABBREVIATIONS

HR: Hormone Receptor

HER-2: Human Epidermal growth factor Receptor 2

TMX: Tamoxifen

Bcl-2: B-cell lymphoma-2

BH: Bcl-2 homology

Mcl-1: Myeloid Cell Leukemia-1(Mcl-1)

BAK: Bcl-2 Antagonist Killer

BAX: Bcl-2-associated X protein

MCF7/TamS: Tamoxifen-sensitive MCF7

MCF7/TamR: Tamoxifen-resistant MCF7

FBS: fetal bovine serum

DMEM/F12: Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12

CCK-8: Cell Counting Kit-8

DMSO: dimethyl sulfoxide

PBS: phosphate-buffered saline

SDS: sodium dodecyl sulfate

INTRODUCTION

Breast cancer is a heterogeneous disease with various histological subtypes.[1, 2] Molecular subtypes are distinguished using the hormone receptor (HR) and human epidermal growth factor receptor 2 (HER-2) status.[3, 4] HR-positive breast cancer accounts for 60-70% of all breast cancers and generally has a better prognosis than HR-negative breast cancer.[5-7]

Anti-hormonal therapy, such as tamoxifen (TMX), is a traditional and long-standing therapy in HR-positive breast cancer. Anti-hormonal therapy is necessary to reduce recurrence and increase the survival rate of HR-positive breast cancer patients.[8, 9] Nevertheless, some patients with HR-positive breast cancer may recur after surgery and additional adjuvant anti-hormonal therapy. While about 30% of patients are intrinsic resistant to TMX, many patients who first responded to tamoxifen are also resistant later. Commonly, patients with resistance to anti-hormonal therapy have a poor prognosis.[10] Due to the clinical outcomes of endocrine resistance, new treatment strategies are emerging to overcome this.

B-cell lymphoma-2 (Bcl-2) is the founding member of the Bcl-2 family, regulator proteins that regulate programmed cell death (apoptosis) by controlling pro-apoptotic and anti-apoptotic intracellular signals.[11] Initially, it was believed to act as a oncogene that induces tumor growth, but later it was founded that Bcl-2 instead promotes malignant cell survival by attenuating apoptosis.[12, 13] Members of the Bcl-2 family are grouped, as they contain up to four conserved Bcl-2 homology (BH) regions. The Bcl-2 family consists of three functionally and

structurally distinct subgroups: anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, myeloid cell leukemia-1(Mcl-1), and A1), pro-apoptotic BH3-only proteins (Bid, Bim, Bad, Puma, *etc.*), and essential apoptosis effectors (Bcl-2 antagonist killer (BAK)[13] and Bcl-2-associated X protein (BAX)[14]). Bcl-2 dysregulation results in the overexpression of the anti-apoptotic protein Bcl-2, which alters the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family.[15]

Nowadays, various studies showed that the inhibition of apoptosis-related Bcl-2 family proteins is thought to lead to chemoresistance.[16] These mechanism has been identified in many cancers, including hematological malignancies[17] (such as multiple myeloma, chronic lymphocytic leukemia, acute lymphocytic leukemia, acute myeloid leukemia, myelodysplastic syndrome and myeloproliferative neoplasms) and solid tumors (such as breast cancer[18], lung cancer[19], melanoma[20] and mesothelioma[21]). As such, it can be said that Bcl-2-mediated resistance to intrinsic apoptosis is a distinctive feature of cancer. Therefore, inhibiting the anti-apoptotic Bcl-2 proteins is an attractive strategy in cancer therapy.

Bcl-2 is overexpressed in about 70% of unselected breast cancer, and about 85% of HR-positive breast cancer patients.[22] Also, it has been reported that HR-positive breast cancers frequently overexpress anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1.[23-26] Bcl-2 and Bcl-xL are further elevated upon anti-hormone therapy[18, 27-29], suggesting that HR-positive breast cancers may use anti-apoptotic Bcl-2 family members to drive cell survival and resistance.[30, 31] Therefore, Bcl-2 is likely to be a new therapeutic target in breast cancer that is resistant to anti-

hormone therapy.

To investigate this, we tested the expression of Bcl-2 in MCF7, well known as the ER-positive breast cancer cell line, and its tamoxifen-sensitive (MCF7/TamS)/resistant (MFC7/TamR) cell lines. In addition, each cell line was treated with ABT-737, a representative BH3 mimetic against pro-survival proteins, such as Bcl-2, Bcl-xL, and Bcl-w, to investigate its therapeutic effect.

MATERIALS AND METHODS

Cell culture, materials, and reagents

Three human breast cancer cell lines, including MCF7, Tamoxifen-sensitive MCF7/S0.5 (MCF7/TamS), and Tamoxifen-resistant MCF7/TAMR-1 (MCF7/TamR), were used in this study. MCF7 was obtained from the American Type Culture Collection (Rockville, MB, USA). MCF7/S0.5 and MCF7/TAMR-1 were obtained from Sigma-Aldrich. (Saint Louis, MO, USA). MCF7/S0.5 is a subline cell line derived from the original MCF7 cells.[32] That was gradually adapted to grow in low serum concentration. MCF7/S0.5 subline is parental cell line of tamoxifen resistant MCF7/TAMR-1. MCF7/TAMR-1 was established by extended treatment with 1uM tamoxifen in MCF7/S0.5.[33]

MCF7 was propagated in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS, MPBio, Seoul, Korea) and 1% antibiotics (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). MCF7/TamS and MCF7/TamR were cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12) without phenol red supplemented with 1% FBS, 1% antibiotics. Cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2. MCF7/TamS and MCF7/TamR cells were cultured in medium additionally supplemented with, 2 mM GlutaMAX^{M}-1 and 1-μg/mL insulin. ABT-737 was purchased from Selleck Chemicals (Houston, TX, USA) and solubilized in dimethyl sulfoxide obtained from Thermo Fisher. Reagent of Cell Counting Kit-8(CCK-8) was purchased from Enzo life sciences (Farmingdale, NY, USA).

Drug treatments for breast cancer cells

MCF7/TamS and MCF7/TamR cells were seeded onto 6-well plates at a density of 2X10^5cells/well. After culturing in complete medium for 16 to 18 hours, media was replaced with fresh media with 10% fetal bovine serum-containing dimethyl sulfoxide (DMSO) as a negative control, ABT-737 4 uM, and 8 uM for 24 hours. Cells were lysed in radioimmunoprecipitation lysis buffer (Sigma-Aldrich, St. Louis, USA) with protease inhibitor cocktail (Thermo Fisher). Protein concentrations were determined using a bicinchoninic acid protein assay (Thermo Fisher). Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer for 15 minutes, and the lysates were processed for western blot assay. The experiments were repeated three times.

Western blot analysis

Harvested human breast cancer cells were washed with ice-cold phosphate-buffered saline (PBS) and total protein was extracted using radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Thermo Fisher). Every 50 μg of protein from cell lysate was separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide (PAGE) gel and transferred to nitrocellulose membrane (Thermo Fisher) by electrophoresis. Blots were blocked with 5% nonfat milk in tris buffered saline containing 0.1% Tween 20 at room temperature for 1 hour. The membranes were then incubated with antibodies against Bcl-2 (1:1000, Abcam, Cambridge, UK), Bcl-w (1:1000, Cell signaling Technology, Danvers, MA), Bcl-xL (1:1000, Cell signaling Technology),

Caspase 3 (1:1000, LsBio, Washington DC, USA), Cleaved Caspase 3 (1:1000, Cell signaling Technology), Caspase 8 (1:1000, Cell signaling Technology) and Caspase 9 (1:1000, Cell signaling Technology). The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Following incubation with enhanced chemiluminescence solution (D-Plus™ ECL Pico System, Dongin LS, Seoul, Korea), protein bands were detected by the ImageQuant LAS-4000 system (Fujifilm, Tokyo, Japan). β-actin (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal control. Experiments were repeated three times and the densitometric values of the bands on Western blots obtained by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA) were subjected to statistical analysis. Background in films was subtracted from the optical density measurements.

Cytotoxicity assay

Cells were seeded as triplicates in a 96-well plate at a density of 1.5×10^4 cells/well and treated with ABT-737 at indicated concentrations and time periods. Cell viability was determined using the CCK-8 assay (Enzo Life Sciences, ALX-850-039-0100, Singapore) as per the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution is directly added to each well of the plate and incubated for 2 hours at 37 °C. Absorbance was measured at 450 nm using a microtiter plate reader (SpectraMax Plus 384, Molecular Devices, CA, USA). The mean absorbance of untreated triplicates was used for normalization.

Flow cytometry analysis

Cells were plated into 6-well plates at a density of 2×10^5 cells/well and treated

with enzalutamide and/or docetaxel at indicated concentrations and time periods. After washing with cold PBS, cells were harvested, centrifugation at 3000 rpm for 5 minutes at 4°C, the cell pellet was incubated with GFP-CERTIFIED® Apoptosis/Necrosis detection solution (Enzo life sciences, Farmingdale, NY, USA). DNA contents of stained cells were analyzed by a flow cytometer (BD FACSCanto™ flow cytometry, BD biosciences, NJ, USA). The early (Annexin V +/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells were quantitated, respectively

Statistical analysis

All statistical analyses and graphs were created using GraphPad Prism software version 7.0 for Windows (GraphPad Software Inc., CA, USA). All tests were 2-sided and a P-value of less than 0.05 was considered statistically significant. All experiments were performed in triplicates or more. All quantitative data were expressed as mean ± standard deviation.

RESULTS

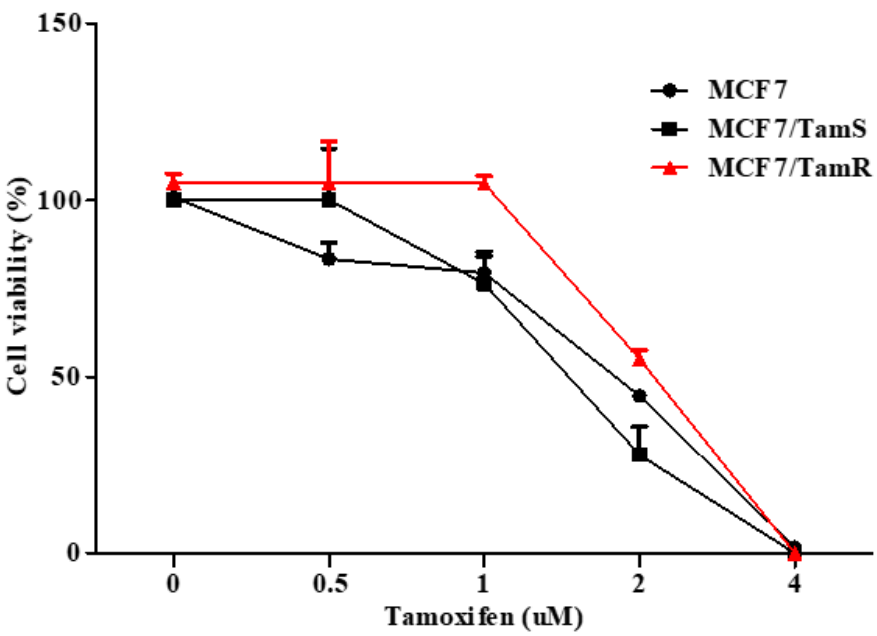
Tamoxifen-resistant MCF7 cell lines (MCF7/TamR) is resistant to treatment with tamoxifen.

CCK-8 assay was performed using MCF7, MCF7/TamS, and MCF7/TamR cells after dose-dependent treatments with various concentration of TMX for 24 hours. At all concentrations, cell viability of MCF7/TamR was higher compared to that of MCF7 and MCF7/TamS. MCF7 and MCF7/TamS showed a response to treatment with TMX at a level of 1uM. On the other hand, almost all MCF7/TamR cells showed resistance to treatment with TMX at a level of 1uM. MCF7/TamR showed significantly higher cell viability after treatment with TMX at a level of 1uM. ($p < 0.05$) (Fig. 1)

Fig.1. The growth curve of MCF7 normal breast cancer cells, MCF7 tamoxifen sensitive (MCF7/TamS) and resistant (MCF7/TamR) cell lines under tamoxifen treatment.

CCK-8 assay was performed using MCF7, MCF7/TamS, and MCF7/TamR cells (1.5×10^4 cells per each cell line) after dose-dependent treatments with the indicated concentration of tamoxifen for 24 hours.

Cell viability (%) was expressed as a standard deviation of three independent experiments. Black circle indicates MCF7 normal breast cancer cells. Black squares indicate MCF7/TamS cells. Red squares indicate MCF7/TamR cells. Statistical significance was assessed by unpaired Student t-test.

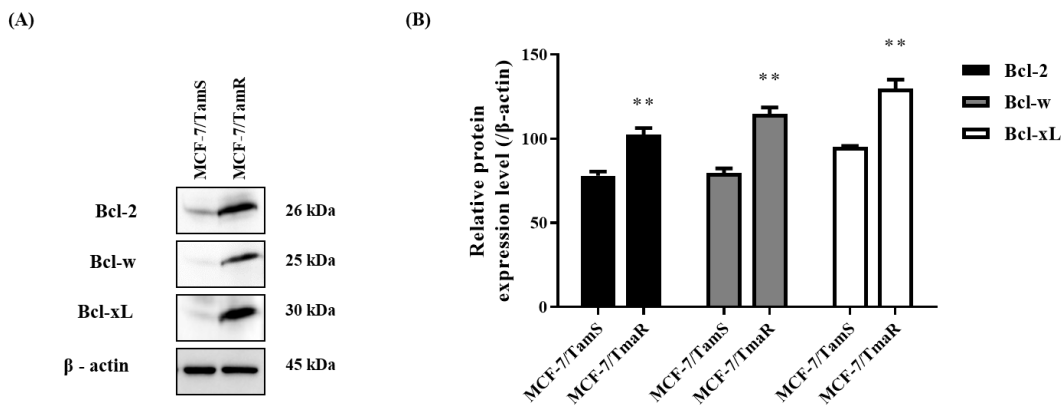


The Anti-apoptotic Bcl-2 family were overexpressed in MCF7/TamR compared to MCF7/TamS.

MCF7/TamR showed a higher expression of anti-apoptotic protein including Bcl-2, Bcl-w, and Bcl-xL than MCF7/TamS. (Fig. 2A) We performed a quantitative analysis based on the expression level of loading control(β-actin). The relative protein expression levels of Bcl-2, Bcl-w, and Bcl-xL were 78±4.4, 79.8±4.5, and 95.2±1.2 in MCF7/TamS and 102.5±6.9, 114.8±6.6, and 129.7±9.5 in MCF7/TamR, respectively. Anti-apoptotic proteins were expressed significantly higher in MCF7/TamR than in MCF7/TamS. (p<0.01, Fig. 2B)

Fig.2. The protein expression of Bcl-2 families on MCF7/TamS and MCF7/TamR breast cancer cells.

Western blot analysis for Bcl-2 families using MCF7/TamS and MCF7/TamR cell lines. (A) After lysing harvested cells (2X10^5 cells per each cell line), an equal amount of cell lysate extracted from two cell lines using radioimmunoprecipitation assay buffer was subjected to western blot analysis using specific antibodies. Signal intensity was normalized to that of β-actin as a loading control. (B) Quantification analysis of Bcl-2 families protein significantly increased than MCF7/TamS cell lines. In (B), data show means±SD (n=3). ***p*<0.01 compared with MCF7/TamS by GraphPad Prism t-test.



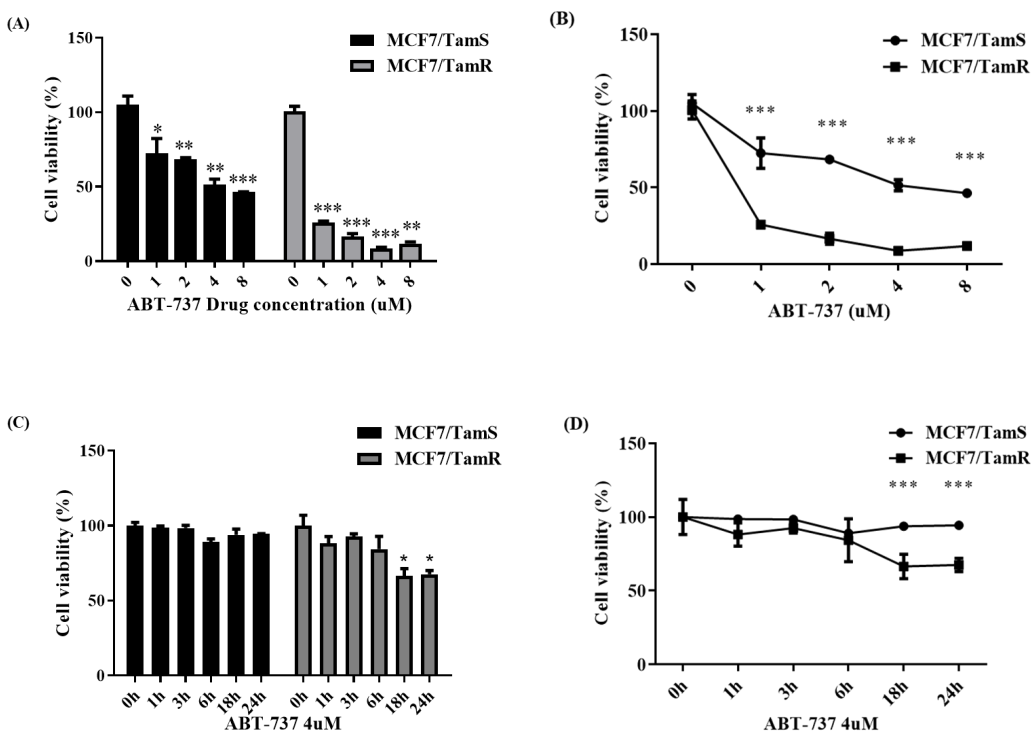
MCF7/TamR has a higher sensitivity to treatment with ABT-737 than MCF7/TamS.

Cell viability by ABT-737 in MCF7/TamS and MCF7/TamR at various concentration and treatment times was tested by CCK-8 assay. Cell viabilities of MCF7/TamS were 72.5±17.1%, 68.3±2.1%, 51.4±6.3%, and 46.3±0.4% at concentrations of 1uM, 2uM, 4uM, and 8uM of ABT-737 for 24 hours, respectively. In comparison, cell viabilities of MCF7/TamR were 25.8±2.1%, 16.5±3.6%, 8.7±0.9%, and 11.8±1.9% at concentrations of 1uM, 2uM, 4uM, and 8uM of ABT-737 for 24 hours, respectively. (Fig. 3A) At all concentrations, cell viability of MCF7/TamR was significantly lower compared to that of MCF7/TamS. (Fig. 3B, all $p< 0.01$).

Cell viabilities of MCF7/TamS were 98.7±2.0%, 98.5±2.9%, 89.1±3.6%, 93.9±6.7%, and 94.5±0.3%, respectively, after 1, 3, 6, 18, and 24 hours of ABT-737 4uM administration. In comparison, cell viabilities of MCF7/TamR were 88.2±7.8%, 92.6±34%, 84.4±14.5%, 66.5±8.3%, and 67.5±4.5%, respectively, after 1, 3, 6, 18, and 24 hours of ABT-737 4uM administration. (Fig. 3C) At 18 and 24 hours after administration of ABT-737 4uM, cell death was significantly higher in MCF7/TamR than in MCF7/TamS. ($p<0.05$, Fig. 3D)

Fig.3. Effect of ABT-737 on MCF7/TamS and MCF7/TamR breast cancer cells determined by CCK-8 assay.

(A, B) The ABT-737 dose-dependent growth-inhibitory effects of apoptotic cell death in MCF7/TamS and MCF7/TamR cell lines. **(C, D)** The effect of time-dependent growth-inhibition by ABT-737 4uM treatment of MCF7/TamS and MCF7/TamR cell lines. Cells were treated with the indicated concentration of ABT-737 on 24 hours. Data represent the averages of three different experiments, each performed in triplicate. The data are presented as the mean ± SD. * p<0.05, ** p<0.01, *** p<0.001 vs. the negative control group.



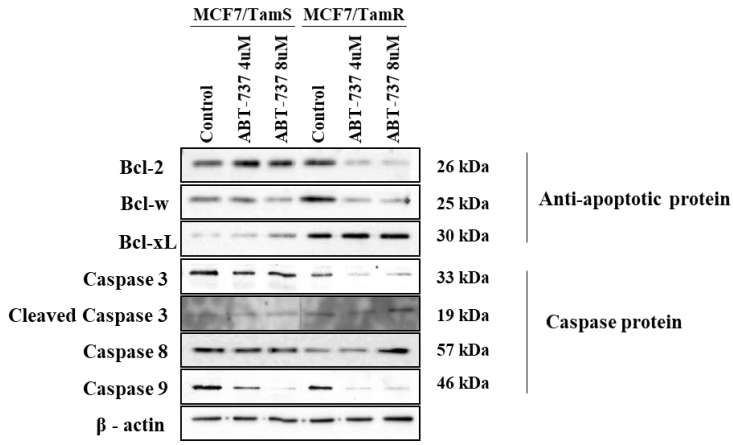
ABT-737 triggers caspase-3 dependent apoptotic cell death.

The expression of Bcl-2 in MCF7/TamR decreased after the administration of ABT-737. Conversely, the expression of cleaved caspase 3, active form of caspase 3 increased after the administration of ABT-737. (Fig. 4A) In a quantitative analysis based on the expression level of loading control (β-actin), the relative protein expression levels of Bcl-2 in MCF7/TamR were 1.17±0.30, 0.26±0.06, and 0.21±0.03 at concentration of 0 (control), 4uM, and 8uM of ABT-737, respectively. And the protein expression levels of cleaved caspase 3 relative to caspase 3 in MCF7/TamR were 0.99±0.0, 3.48±1.02, and 2.48±0.79 at concentration of 0(control), 4uM, and 8uM of ABT-737, respectively. (Fig 4B) When treated with ABT-737, Bcl-2 was significantly reduced ($p<0.01$, Fig. 4B) and cleaved caspase3 was significantly increased. ($p<0.05$, Fig. 4B)

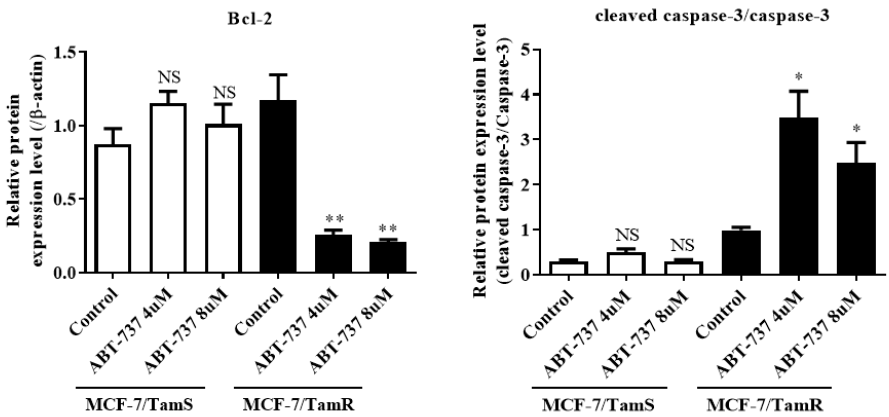
Fig.4. Western blot analysis for anti-apoptotic, pro-apoptotic genes, and caspase genes using tamoxifen sensitive and resistant cell lines after ABT-737 monotherapy.

(A) After lysing harvested cells ($2X10^6$ cells per each cell line), an equal amount of cell lysate extracted from MCF7/TamS and MCF7/TamR cell lines using radioimmunoprecipitation assay buffer was subjected to Western blot analysis using specific antibodies. Signal intensity was normalized to that β-actin as a loading control. **(B)** Quantification analysis of typical protein for each gene types significantly increased (Caspase-3) or decreased (Bcl-2 and Bim) than MCF7/TamS cell lines. Data represent the mean±SD. *p<0.05, **p<0.01, ***p<0.001.

(A)


MCF7/TamS
MCF7/TamR
Control
ABT-737 4uM
ABT-737 8uM
Control
ABT-737 4uM
ABT-737 8uM
Bcl-2
Bcl-w
Bcl-xL
Caspase 3
Cleaved Caspase 3
Caspase 8
Caspase 9
β - actin
26 kDa
25 kDa
30 kDa
33 kDa
19 kDa
57 kDa
46 kDa
Anti-apoptotic protein
Caspase protein

(B)

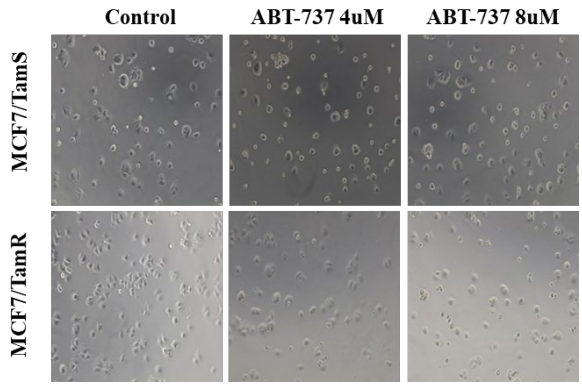

Bcl-2
Relative protein expression level (/β-actin)
1.5
1.0
0.5
0.0
NS
NS
**
**
Control
ABT-737 4uM
ABT-737 8uM
Control
ABT-737 4uM
ABT-737 8uM
MCF-7/TamS
MCF-7/TamR
cleaved caspase-3/caspase-3
Relative protein expression level (cleaved caspase-3/Caspase-3)
5
4
3
2
1
0
NS
NS
*
*
Control
ABT-737 4uM
ABT-737 8uM
Control
ABT-737 4uM
ABT-737 8uM
MCF-7/TamS
MCF-7/TamR

Apoptotic cell death in MCF7/TamR treated with ABT-737 is also confirmed in cell microscopy and apoptosis analysis by flow cytometry.

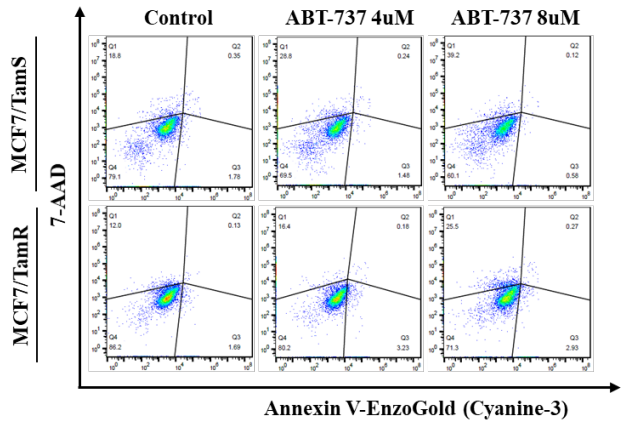
We could observe the increase in cell death in MCF7/TamR than in MCF7/TamS when administrated with ABT-737 in contrast microscopy. (Fig. 5A) ABT-737 treatment promoted more early and late apoptosis compared with control cells. (Fig. 5B) In total, Apoptotic populations increased an approximately 3-folds in ABT-737 8uM compared to control in MCF-7/TamR cells. ($p<0.01$, Fig. 5C)

Fig.5. Cell microscopy and apoptosis analysis by flow cytometry in MCF7/TamS and MCF7/TamR breast cancer cells by treatment of ABT-737. Phase contrast microscopy (×100) (A). The MCF7/TamS and MCF7/TamR cells were exposed to 4 uM or 8 uM of ABT-737, or staurosporine, detached from the culture dishes by gentle trypsinization and analyzed by FACS for 7AAD uptake and PE-annexin V staining at 6h of incubation (B). Bar graphs (C) show a quantitative summary of FACS analysis from apoptotic cell death (7AAD/PE annexin double-positive) data. The graphs were analyzed from 3 to 6 independent experiments. Results are expressed as mean±SD. Statistical difference to control cells was assessed by the GraphPad prism t-test. * $p<0.05$, ** $p<0.01$ vs. the control group.

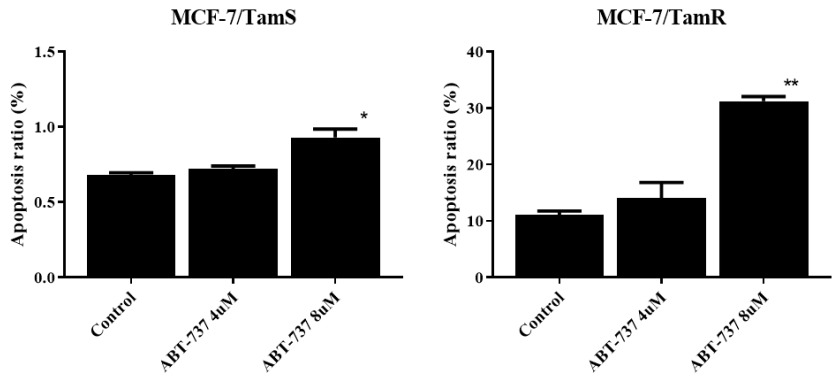
(A)


Control
ABT-737 4uM
ABT-737 8uM
MCF7/TamS
MCF7/TamR

(B)


Control
ABT-737 4uM
ABT-737 8uM
MCF7/TamS
MCF7/TamR
7-AAD
Annexin V-EnzoGold (Cyanine-3)

(C)


MCF-7/TamS
MCF-7/TamR
Apoptosis ratio (%)
1.5
1.0
0.5
0.0
40
30
20
10
0
*
**
Control
ABT-737 4uM
ABT-737 8uM

DISCUSSION

Overcoming resistance to anti-hormonal therapy in patients with HR-positive breast cancer is a crucial issue. Various mechanisms, including pharmacologic mechanism, loss or modification in ER expression, cross-talk with receptor tyrosine kinases (RTKs), alteration of cell cycle regulators, and the expression change of apoptotic pathway molecules, have been proposed to have resistance to anti-hormonal therapy in HR-positive breast cancer.[10, 34]

There have been many studies targeting various mechanisms to overcome resistance to anti-hormonal therapy in HR positive breast cancer. Moreover, several drugs, such as Phosphatidylinositol 3-kinase (PI3K) inhibitor, mammalian target of rapamycin (mTOR) inhibitor, cyclin-dependent kinase (CDK) 4/6 inhibitor, and poly ADP ribose polymerase (PARP) inhibitor, have already been used clinically in the treatment of recurrent patients after adjuvant hormone therapy.[34] However, resistance to anti-hormonal therapy in patients with HR-positive breast cancer is still a problem to overcome and further study of various targets is needed. In this regard, inhibition of Bcl-2 may be another target for the treatment of HR positive breast cancer.

Since Bcl-2 is an anti-apoptotic protein, it can be said that inhibiting Bcl-2 decreases cell viability by activating the apoptosis. Apoptosis is a major form of programmed cell death. It can be initiated via the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by membrane receptors that respond to death signals. The intrinsic pathway is controlled by members of the Bcl-2 family

controlling pro-apoptotic and anti-apoptotic intracellular signals. Apoptosis initiated through the intrinsic and extrinsic pathway is carried out by enzymes called caspases. Activation of caspase-3 among various caspase is essential component in apoptotic cell death.[11, 35, 36]

Many studies reported that the inhibition of apoptosis-related Bcl-2 family proteins is thought to lead to chemoresistance.[16] Therefore, targeting the anti-apoptotic Bcl-2 proteins can be considered as one of the attractive therapeutic strategy in chemo-resistant cancer. A previous study has reported that caspase-3 apoptotic foci in tumors are increased in mice bearing triple negative breast cancer (TNBC) xenografts overexpressing Bcl-2 after treatment with combination therapy of docetaxel and ABT-737, a representative anti Bcl-2 drug.[25] Another paper has reported that paclitaxel plus ABT-737 treatment not only activates caspase-3 and caspase-9 but also activates caspase-8 in the MDA-MB-468 TNBC cell line.[37]

Moreover, changes in the expression of the various pro-apoptotic and anti-apoptotic proteins that make up the Bcl-2 family not only cause resistance to chemotherapy, but also affect to anti-hormonal therapy. In breast cancer, Bcl-2 is overexpressed in approximately 75% of tumors (approaching 85% for ER-positive disease).[38] HR-positive breast cancers frequently overexpress anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1.[23-26] Also, Bcl-2 and Bcl-xL are further elevated upon anti-hormonal therapy[18, 27-29], suggesting that HR-positive breast cancers may use anti-apoptotic Bcl-2 family members to drive cell survival and treatment resistance.[30, 31] Based on these existing studies, we tried

to find a clue to overcoming endocrine resistance through inhibition of the anti-apoptotic protein Bcl-2 and activation of the apoptotic pathway.

In this study, we used human breast cancer cell lines, including MCF7/TamS and MCF7/TamR to confirm the relationship between tamoxifen resistance and Bcl-2 expression and the therapeutic effect of ABT-737. The MCF7 is the most famous HR-positive breast cancer cell line and MCF7/TamR we used is a proven TMX -resistant MCF7 that is commercially available. Although MCF7/TamR is a commercially available cell line, we again confirmed TMX resistance through repeated experiments. (Fig. 1) ABT-737 is a representative BH3 mimetics and inhibits its function by binding to anti-apoptotic Bcl-2 families such as Bcl-2 or Bcl-xL.

Several previous studies have reported that the proteins of anti-apoptotic Bcl-2 families, such as Bcl-2, Bcl-w, Bcl-xL, were overexpressed on TMX-resistant breast cancer cell line or xenograft model.[18, 39, 40] Similarly, MCF7/TamR showed higher expression of the anti-apoptotic protein than MCF7/TamS in our study. (Fig. 2A) Since Bcl-2 protein is expressed in a large number of HR-positive breast cancer patients, another study has shown that targeting Bcl-2 in HR-positive breast cancer cell line and xenograft enhances the efficacy of anti-hormone therapy.[18] Because the anti-apoptotic proteins were overexpressed in MCF7/TamR rather than MCF7/TamS, we could predict that the effect of ABT-737 would be better in MCF7/TamR than MCF7/TamS. So, we tried to confirm the effect of ABT-737 in the MCF7/TamR. We measured cell viability after treating MCF7/TamS and MCF7/TamR by varying the ABT-737 concentration and

treatment time. As a result, more cell death occurred than MCF7/TamS in MCF7/TamR at each ABT-737 concentration and treatment time, and the highest therapeutic effect was found when treated with ABT-737 8 uM for 24 hours. (Fig. 3)

As described above, since Bcl-2 is an anti-apoptotic protein, it can be inferred that cell death through Bcl-2 inhibition is due to activation of the apoptosis pathway. To investigate this, we analyzed expressions of caspase 3 and cleaved caspase 3, which are a well-known marker of apoptosis, and Bcl-2 expression after treatment with ABT-737 on the cell line. As a result, the expression of Bcl-2 was decreased, and the expression of cleaved caspase 3, an active form of caspase 3, was increased when MCF7/TamR was treated with ABT-737. (Fig. 4) Also, we confirmed by flow cytometry that cell death by ABT-737 was due to the apoptosis pathway. As in the protein expression analysis, in the FACS analysis using annexin V and 7-AAD, it was verified that apoptosis was significantly increased when MCF7/TamR was treated with ABT-737. (Fig. 5)

HR-positive breast cancer cells acquire resistance to anti-hormonal therapy in a various of way. It may be the result of the interaction of various pathways that cancer cells acquire hormone resistance. Therefore, there are many limitations to explain the mechanism of resistance acquisition by the Bcl-2 related apoptosis pathway alone. In addition to Bcl-2, there are various substances involved in apoptosis. And analysis of the interaction of Bcl-2 protein with signal pathways other than the apoptotic pathway will also be required.

Although this study has a limitation as an experiment using only one type of TMX resistance cell line, these results show that activation of the apoptosis pathway through inhibition of anti-apoptotic factors can be a way to overcome treatment resistance. Indeed, one study showed in xenograft animal models that targeting Bcl-2 with BH3 mimic ABT-199 in HR-positive breast cancer reduces side effects to TMX and increases the effect of PI3K/mTOR inhibitors.[18] However, few studies have targeted Bcl-2 in endocrine resistant breast cancer. In that sense, this study provides another clue to overcome endocrine resistance.

This study has a limitation that it is the result of using only MCF-7/TamR among several known tamoxifen-resistant cell lines. In addition, there is a limitation that the possibility of ABT-737 as an agent to overcome TMX-resistant was confirmed only *in vitro*. In the future, additional *in vivo* tests using xenograft model will be required. There is also a need for studies that correlate endocrine resistance with Bcl-2 expression in clinical setting.

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국문 초록

서론: 호르몬 수용체 양성 유방암에서 타목시펜과 같은 항호르몬 치료는 호르몬 재발을 줄이고 생존율을 높이는데 매우 중요한 치료이다. 하지만 많은 호르몬 수용체 양성 유방암 환자들은 항호르몬 치료에 저항성을 갖는다. 본 연구의 목적은 호르몬 치료에 대한 저항성을 극복하기 위한 치료제로서, 항 Bcl-2 약제인 ABT-737의 가능성을 확인하고자 하는 것이다.

방법: 우리는 타목시펜 민감성 MCF7(MCF7/TamS)와 타목시펜 저항성 MCF7(MCF7/TamR) 세포주, 그리고 ABT-737 약제를 이용하여 실험을 진행하였다. MCF7/TamS와 MCF7/TamR에서 Bcl-2 family 단백과 caspase-연관 단백질을 분석하기 위해 Western blot을 시행하였다. 세포 생존능 분석은 Cell Counting Kit-8(CCK-8)를 이용하였으며, 유세포 분석을 이용하여 세포자멸사를 확인하였다.

결과: 항-세포자멸사 Bcl-2 family 단백질들은 MCF-7/TamS 세포주보다 MCF7/TamR 세포주에서 과발현 되었다. (p<0.01) ABT-737의 모든 농도에서 MCF7/TamR 세포주의 세포들은 MCF7/TamS 세포주에 비해 통계적으로 유의하게 낮은 세포 생존능을 보였다. (p<0.001) ABT-737 주입 후에 MCF7/TamR 세포주에서 Bcl-2 발현은 줄어들었으며, 반대로 caspase 3의 활성화 형태인 cleaved caspase 3의 발현은 증가하였다. 또한 ABT-737 치료

이후에 MCF7/TamR 세포주에서는 세포자멸사의 비율이 증가하는 것을 확인 할 수 있었다.

결론: ABT-737에 의한 세포자멸사 경로의 활성화는 호르몬 치료에 대한 저항성을 극복하는데 중요한 역할을 할 수 있다.

주요어: ABT-737, Bcl-2, 세포자멸사, 항호르몬 치료, 유방 신생물

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