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ORIGINAL ARTICLE

Apatinib Sensitizes Human Breast Cancer Cells against Navitoclax and Venetoclax Despite Up-regulated Bcl-2 and Mcl-1 Gene Expressions

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OBJECTIVE
Defects in apoptotic cell death which restrict the success of conventional cytotoxic therapies have pivotal roles in a number of pathological conditions including cancer. However, a novel drug class targeting pro-survival Bcl-2 protein family members has been developed with the understanding of the structures and interactions of Bcl-2 proteins. Within this new class, Bcl-2/Bcl-xL inhibitor Navitoclax and Bcl-2 specific inhibitor Venetoclax have been shown to demonstrate strong anticancer activities on several types of cancers. But their low affinity to other anti-apoptotic proteins limits their clinical usage. Here, we investigated the cytotoxic and apoptotic effects of Navitoclax/Venetoclax and their combinations with specific tyrosine kinase inhibitor Apatinib on estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cell lines.

METHODS
MTT assay was used for the evaluation of the inhibition of cancer cell proliferation. ELISA test and Quantitative real-time PCR assay was performed to determine the role of caspase-3, Bak, Bax, Bcl-2, Bcl-xL and Mcl-1 proteins in the inhibition of cell proliferation triggered by the tested agents.

RESULTS
We found that aggressive MDA-MB-231 cell line was more sensitive to all tested agents. Apatinib significantly enhanced Navitoclax/Venetoclax mediated inhibition of cell viability in both cancer cell lines despite up-regulation in the expression levels of Bcl-2 and Mcl-1 genes. We further demonstrated significant Bak/Bax and caspase-3 expression in less aggressive MCF-7 cells.

CONCLUSION
Our findings have impacts on Navitoclax/Venetoclax plus Apatinib based therapy for breast adenocarcinoma. On the other hand, further studies should be conducted to elucidate the mechanisms underlying synergistic effects of Navitoclax/Venetoclax plus Apatinib combinations.

Keywords: Apatinib; Apoptosis, Breast adenocarcinoma; Cytotoxicity; Navitoclax; Venetoclax.

Introduction

Cancer, widely known as the "disease of the age" is a complex genetic disorder defined as the conversion of a normal/healthy cell to a malignant one as a result of various genetic changes such as mutations seen in onco- and tumor suppressor genes, and developing resistance to death, especially apoptosis.[1,2] The con-

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nection between the induction of apoptotic cell death and elimination of potential malignant cells, hyperplasia, and tumor progression dates back to the early 1970s.[3] Today, it is known that escape from apoptosis is crucial in the transformation process to malignancy [4] and therefore the focus is on new approaches targeting apoptosis regulation in cancer treatment. [5-7] Apoptosis is the primary type of programmed cell death induced when DNA damage is beyond repair. The induction of apoptosis takes place through two main pathways, namely the extrinsic and intrinsic pathways.[8] In the case of intrinsic pathway, various stimuli directly affect their targets within the cell and a series of events are initiated by the mitochondria. Basically, all these stimuli cause the mitochondrial permeability transition pores to open, a decrease in mitochondrial transmembrane potential, and consequently, the release of pro-apoptotic factors to the cytosol to induce cellular death. The regulation of mitochondrial pores is mainly governed by the Bcl-2 family proteins consisting of a series of evolutionarily conserved pro- and anti-apoptotic members that share Bcl-2 homology (BH) domains. Among the pro-apoptotic members, BH3-only proteins are the ones which share only a small region of homology, the third Bcl-2 homology (BH3) domain, with other Bcl-2 family members. The so-called BH3-only proteins exert their pro-apoptotic functions both by neutralizing the effects of other anti-apoptotic relatives and activating pro-apoptotic members with multi-BH domains, which make them valuable in breaking the resistance of cancer cells against apoptotic death. Nevertheless, many types of tumors, particularly those that are resistant to treatment, express one or more of anti-apoptotic members at very high levels and also carry mutations that inhibit the induction of BH3-only proteins.[9] On the other hand, upon understanding the structures and interactions of many Bcl-2 protein family members, it has become possible to develop new generation BH3 mimetics that may be alternative to traditional chemotherapy.[10-15]

In this study, we aimed to assess the cytotoxic and apoptotic effects of clinically available navitoclax (NTX) and venetoclax (VTX), which are members of BH3 mimetics, and also their combination with apatinib (APTB) on estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cell lines. Previous studies showed that both BH3 mimetics have therapeutic properties on some hematological malignancies, but various solid tumors develop resistance to these drugs due to especially the increased expression of Mcl-1, which is an anti-apoptotic member of the Bcl-2 protein family.[16-18] The APTB combination with BH3 mimetics in the scope of present work is a powerful inhibitor of the signal transduction pathway dependent on vascular endothelial growth factor (VEGF). VEGF is shown as a major angiogenic factor in human cancers and is associated with failures in treatment.[19] As a matter of fact, it has been known for a long time that serum and plasma VEGF levels of metastatic breast cancer patients are also quite high. [20] Since VEGF is also known to up-regulate Mcl-1 protein,[21] it has been thought that the combined application of the VEGF inhibitor APTB with the so-called BH3 mimetics may offer a potential alternative approach to traditional chemotherapy in breast cancer treatment.

Materials and Methods

Cell Culture

The human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-231) were obtained from American Type Culture Collection (ATCC, USA). Both cell lines were cultured with high-glucose DMEM supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 20% fetal bovine serum (FBS) in a humidified atmosphere of 95% air with 5% CO_2 at 37°C.

Cell Proliferation Assay

The effects of NTX and VTX, and also their combination with APTB on the proliferation of breast cancer cell lines were detected by MTT assay. Briefly, MCF-7 and MDA-MB-231 cells were seeded into 96-well plates at a density of $1x10^4$ per well and allowed to attach for 24 h before treatment. The cells were exposed to various concentrations of NTX, VTX and APTB (0–100 µM) alone or in combination for 24 h (The sole treatment of APTB was carried out to determine non-toxic concentrations of the inhibitor for further combined applications). After incubation period, MTT solution (5 mg/ml PBS) was added and the plates were located in an incubator with 5% CO_2 at 37 °C. After 4 h, MTT-formazan crystals were dissolved in DMSO and then cell growth was assessed by measuring the absorbance at 570 nm. Cell viability was expressed as percentage survival, with 100% survival taken as that observed in related control cells which were treated with 0.1% and 0.2% DMSO for alone and combined treatments, respectively.

Mcl-1 Protein Levels

Samples were assayed for Mcl-1 levels by using a commercially available ELISA kit (RayBiotech, Inc., Nor-

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cross, GA, USA). Briefly, 100 µl of each standard or sample were added to appropriate wells of the plate pre-coated with an antibody specific for human Mcl-1. The plate was incubated for 2.5 h at room temperature, washed, and then all wells received 100 µl biotinylated antibodies. Following 1 h incubation at room temperature, plate was washed, 100 µl Streptavidin solutions were added to each well and incubated for 45 min at room temperature. After repetition of the washing step, 100 µl of TMB One-Step Substrate reagent was added to each well for color development and incubated for 30 min at room temperature. The reaction was stopped by adding 50 µl Stop solution to each well and the optical density was measured at 450 nm. A Mcl-1 standard curve was generated to quantitate the amount of Mcl-1 in ng/ml.

RNA Isolation and Quantitative Real Time PCR (qRT-PCR)

RNA extraction from the samples was performed by using RNeasy Plus Universal RNA Isolation Kit following the manufacturer's protocol with slight modifications (Qiagen, Redwood City, CA, USA). The RNA concentration was determined using a NanoDrop (MaestroNano micro-volume Spectrophotometer, USA) and the RNA was reverse transcribed using a RevertAid Reverse Transcriptase (ABM).[22] cDNA was stored at -80^{0}C for further use.

qRT-PCR analysis was carried out using SYBR Green qPCR Master Mix (GM, Taiwan) in an Exicycler 96 Real Time Quantitative Thermal Block PCR System (Bioneer, Daejeon, Korea). Beta-actin *(ACTB)* gene was chosen from the group of housekeeping genes to normalize gene expression. The primer sequences for the target genes and amplification conditions were given in Table 1.

Statistical Analysis

The data are presented as the mean±S.E.M of three experiments. The differences in variance were analyzed statistically using a one-way analysis of variance (ANOVA) test by Graphpad prism 5.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a *post hoc*.

Results

The Effects of BH3 Mimetics and Their Combinations with Apatinib on the Proliferation of Human Breast Cancer Cell Lines

Firstly, the effects of the BH3 mimetics and APTB alone on the cell viability of human breast cancer cells were investigated. For this purpose, MCF-7 and MDA-MB-231 cell lines were exposed to the drugs in the concentration range of 0-100 µM for 24 h and the results were presented in Figure 1 and 2, respectively. As can be seen from these figures, APTB was found non-toxic for both cell lines at the concentrations of 2.5 and 5 µM and MDA-MB-231 cells were more sensitive than MCF-7 cell line to higher concentrations of the inhibitor. The results displayed that while the dose range of NTX with increased cytotoxicity was determined as 0-50 and 0-5 µM on MCF-7 and MDA-MB-231 cells, respectively, it was 0-50 µM for VTX on both cell lines.

Table 1 Primer sequences and amplification conditions

Gene	NCBI reference sequence	Primer sequence (5′ → 3′)	Annealing temperature (°C)
CASP3	NM_004346	F: GCA GCA AAC CTC AGG GAA AC R: TGT CGG CAT ACT GTT TCA GCA	61
BAX	NM_004346	F: AGA GGA TGA TTG CCG CCGT R: CAA CCA CCC TGG TCT TGG ATC	57.5
BAK	NM_001188	F: GGC AGA CTT CAC TGG GACC R: TTG CCC CGA AGC CAT TTTTC	60
BCL2	NM_000633	F: GGT GGG GTC ATG TGT GTG G R: CGG TTC AGG TAC TCA GTC ATC C	62
BCLXL	NM_001191	F: AGC TTG GAT GGC CAC TTAC R: TCG GCT GCT GCA TTG TT	54
MCL1	NM_021960	F: CCG CCC TAA AAC CGT GAT AAA GGA R: TGG CCA AAA GTC GCC CTC	61
*ACTB	NM_001101	F: GCC GCC AGC TCA CCA T	59

*ACTB was the housekeeping gene used for normalization of gene expressions

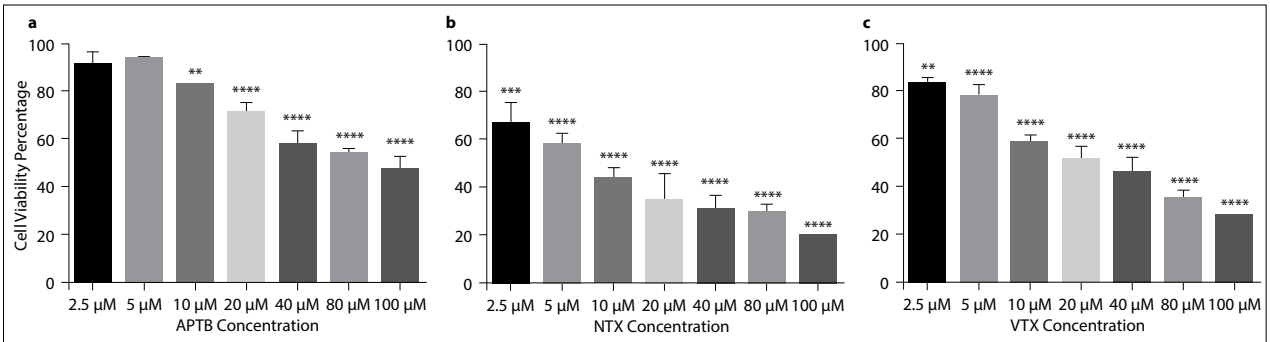

Fig. 1. The effects of APTB (a), NTX (b) and VTX (c) on cell proliferation of human breast cancer cell line MCF-7. The cells were treated with 0.1% DMSO as control or three different agents at various concentrations for 24 h and cell viability percentages were calculated compared with the control group. Data with error bars show the mean±S.E.M of three experiments. **=p<0.01; ***=p<0.001; ****=p<0.0001 denote significant differences between control and other studied groups by Tukey's multiple range tests.

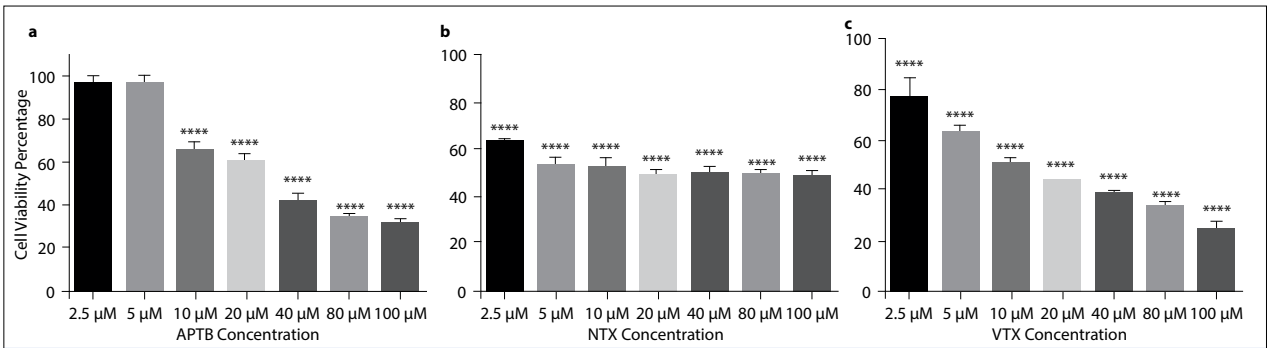

Fig. 2. The effects of APTB (a), NTX (b) and VTX (c) on cell proliferation of human breast cancer cell line MDA-MB-231. The cells were treated with 0.1% DMSO as control or three different agents at various concentrations for 24 h and cell viability percentages were calculated compared with the control group. Data with error bars show the mean±S.E.M of three experiments. ****=p<0.0001 denotes significant differences between control and other studied groups by Tukey's multiple range tests.

Interestingly, more aggressive MDA-MB-231 cells showed resistance to NTX at higher concentrations and cell viability could not be reduced to below 48% by increasing the mimetic concentration. Importantly, it should be stated that relatively low concentrations of both mimetics, 2.5 and 5 µM, could not reduce cell viability of both two cell lines to less than 50% compared to the their control groups.

To obtain more effective results in this relatively low concentration range (0-5 µM), NTX and VTX were combined with the non-toxic concentrations of APTB (Figs. 3, 4). While MCF-7 cell proliferation could not be decreased below 50% with these combined treatments, it was acquired for MDA-MB-231 cell line with NTX plus APTB applications. In the case of MCF-7, there were no significant differences between the groups with increased NTX plus constant APTB concentrations or vice versa. On the other hand, approximately 58% cell viability was determined in 0.5 µM NTX plus APTB treatments as with alone 5 µM NTX treatment (Fig. 3a). These findings are significant in terms of getting similar results with much lower concentration of the mimetic. Unlike from NTX–APTB combined applications, it was found that the most cytotoxic 5 µM VTX plus 5 µM APTB treatment had also significantly lower levels of cell viability than 5 µM VTX plus 2.5 µM APTB group ($p<0.05$) (Fig. 3b). When it comes to MDA-MB-231 cell line, it was clearly seen that 2.5 µM APTB combinations did not provide significant improvement to the toxicity of VTX. However, when 2.5 µM VTX were combined with 5 µM APTB, cell viability was significantly decreased from about 77% to about 63% ($p<0.05$). The effectiveness of the so-called combined application was not able to significantly increase by increasing VTX concentration to 5 µM (Fig. 4b). Finally, Figure 4a shows that cell viability was about 45%

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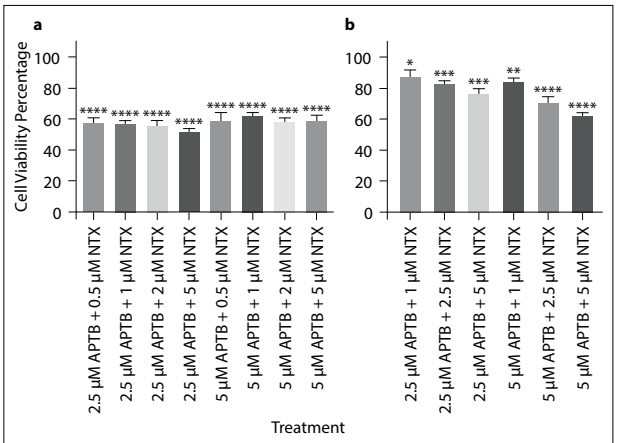

Fig. 3. The effects of NTX (a) and VTX (b) plus APTB on cell proliferation of human breast cancer cell line MCF-7. The cells were treated with 0.2% DMSO as control or NTX/VTX plus APTB combinations at various concentrations for 24 h and cell viability percentages were calculated compared with the control group. Data with error bars show the mean±S.E.M of three experiments. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001 denote significant differences between control and other studied groups by Tukey's multiple range tests.

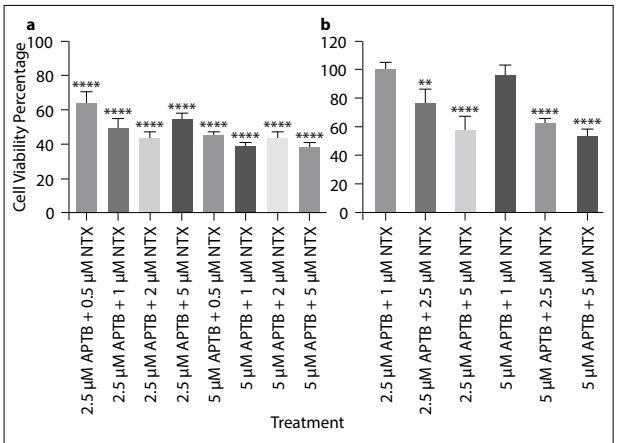

Fig. 4. The effects of NTX (a) and VTX (b) plus APTB on cell proliferation of human breast cancer cell line MDA-MB-231. The cells were treated with 0.2% DMSO as control or NTX/VTX plus APTB combinations at various concentrations for 24 h and cell viability percentages were calculated compared with the control group. Data with error bars show the mean±S.E.M of three experiments. **=p<0.01; ****=p<0.0001 denote significant differences between control and other studied groups by Tukey's multiple range tests.

in 0.5 µM NTX plus 5 µM APTB treatment and this value could not be significantly decreased by increasing NTX concentration as in the case of VTX. When all above-mentioned results were evaluated together, the apoptotic effects of 0.5 µM NTX plus 2.5 µM APTB, 0.5 µM NTX plus 5 µM APTB and 5 µM VTX plus 5 µM APTB on MCF-7, and 0.5 µM NTX plus 5 µM APTB and 2.5 µM VTX plus 5 µM APTB combinations on MDA-MB-231 cell line were further investigated compared to the sole applications.

The Effects of BH3 Mimetics and Their Combinations With Apatinib on the Mcl-1 Protein Levels of Human Breast Cancer Cell Lines

As stated above, similar or better results in cytotoxicity assessment with much lower concentrations of BH3 mimetics could be obtained by combining these mimetics with non-toxic dosages of APTB. To enlighten the role of Mcl-1 protein levels in these synergic cytotoxic effects of the so-called combinations, the changes in these protein levels were determined with ELISA kit and compared with alone treatments. As is known, Mcl-1 is one of the anti-apoptotic members of the Bcl-2 protein family which regulates mitochondrial pore formation and among the factors that are held responsible for the resistance of solid tumors to BH3 mimetics.[9] The results were presented in Figure 5. Among comparable groups, the only significant difference was found between control and 5 µM VTX and between 2.5 and 5 µM APTB groups of MCF-7 cells (p<0.05). Unfortunately, significant decreases in Mcl-1 protein levels could not be attained with the application of combined treatments in both cell lines.

The Effects of BH3 Mimetics and Their Combinations With Apatinib on Pro- and Anti-Apoptotic Gene Regulation of Human Breast Cancer Cell Lines

Transcriptional regulation of pro-apoptotic caspase-3 (Cas3), Bak and Bax, and anti-apoptotic Bcl-2, Bcl-xL and Mcl-1 genes in two different human breast cancer cell lines which were exposed to BH3 mimetic and APTB alone or in combination at the concentrations determined as mentioned above were investigated. As very well known, all studied genes except Cas3 are the members of the Bcl-2 gene family. The effector protein Cas3 can be considered as the intersection and the last step of different apoptotic cell death cascades. The effects of the agents and their combinations on the transcriptional regulation of the specified genes in MCF-7 and MDA-MB-231 cell lines are presented in Tables 2 and 3, respectively. From the obtained results, it was seen that to combine 0.5 µM NTX with 2.5 µM APTB

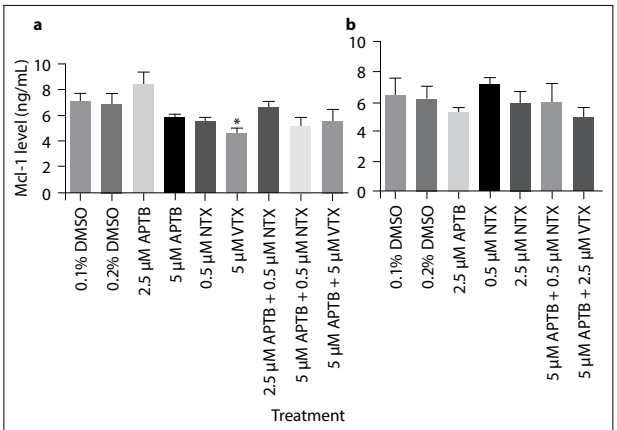

Fig. 5. The effects of alone APTB, alone BH3 mimetic and combined BH3 mimetic plus APTB treatments on Mcl-1 protein levels of human breast cancer cell lines MCF-7 (a) and MDA-MB-231 (b). The cells were treated with 0.1 or 0.2% DMSO as control groups or agents at various concentrations for 24 h and Mcl-1 protein levels were calculated. Data with error bars show the mean±S.E.M of three experiments. *=p<0.05 denotes significant differences between 0.1% DMSO control and other studied group by Tukey's multiple range tests.

caused significant induction in the expression of pro-apoptotic adaptor Bak and Bax proteins when compared with both control group and alone 0.5 μM NTX and 2.5 μM APTB applications (p<0.0001) in MCF-7 cell line. However, this combined treatment also significantly induced anti-apoptotic Bcl-2 and Mcl-1 expression compared with control. When 0.5 μM NTX or 2.5 μM APTB was applied alone, either there was no significant change or significantly lower induction by comparison with the so-called combined treatment (p<0.0001) were observed in the expression levels of these anti-apoptotic genes, respectively. On the other hand, when we consider the interactions between pro- and anti-apoptotic relatives of Bcl-2 protein family,[9] this situation can be interpreted as the response against increased Bax and Bak protein expression. Surprisingly, to increase APTB concentration from 2.5 to 5 μM in the combined application also gave rise to induction in the expression of Bcl-2 and Mcl-1 proteins without any induction in pro-apoptotic ones. Differently from NTX plus APTB combination, 5 μM APTB combination of 5 μM VTX could significantly increase Cas3 expression besides significant induction in the expression of Bak and Bax. As indicated in Table 2, although higher induction in Bak and Bax expression were observed in 5 μM VTX when compared to combined treatment, Cas3 expression did not significantly increase. So, it would not be wrong to say that VTX and APTB combination showed synergistic effect in terms of apoptotic death of human breast cancer cell MCF-7. Unfortunately, the highest induction in the anti-apoptotic gene expression was also found for this combination, which can be commented as a parameter reducing the effectiveness of this dual drug administration. Contrary to the promising results seen on MCF-7, no significant increases in pro-apoptotic gene expression have been observed in combined applications performed on MDA-MB-231 cell line (Table 3). Conversely, the sharpest increase in Bcl-2 gene expression and significant down regulation in Cas3 were determined in this cell line that treated with 5 μM APTB + 0.5 μM NTX. The significant down regulation of Mcl-1 gene in the same combined group also could not be seen as an improved resistance to survival due to the similar results that obtained both in the alone 5 μM APTB and 0.5 μM NTX treated groups. Although, significant up-regulation of Bcl-xL gene in 0.5 μM NTX group was repressed by the combination with 5 μM APTB, it is clear that higher sensitivity of

Table 2 The expression level of the selected genes in MCF-7 cell line

	2.5 μM APTB	5 μM APTB	0.5 μM NTX	5 μM VTX	2.5 μM APTB + 0.5 μM NTX	5 μM APTB + 0.5 μM NTX	5 μM APTB + 5 μM VTX
BAK	1.32±0.42	-1.01±-1.01	1.28±0.29	**2.25±0.16**	**2.77±0.01**	1.81±0.15	**2.18±0.05**
BAX	1.13±0.26	1.74±0.12	1.16±0.45	**3.03±0.13**	**3.06±0.01**	1.31±0.37	**2.01±0.05**
CAS3	1.04±0.26	-1.86±-0.08	-1.59±-0.45	1.4±0.38	1.17±0.19	1.18±0.17	**2.74±0.11**
BCL2	**2.03±0.02**	-1.01±-0.17	-1.06±-0.29	1.93±0.11	**3.8±0.003**	**3.41±0.01**	**6.83±0.001**
BCLXL	1.18±0.22	1.06±0.25	-1.39±-0.33	1.66±0.07	1.67±0.28	1.73±0.28	**2.5±0.14**
MCL1	**2.09±0.29**	1.15±0.38	1.29±0.13	1.83±0.26	**2.72±0.33**	**2.58±0.15**	**4.01±0.05**

Data were presented as means±S.E.M of four experiments. The results were normalized according to ACTB expression. The values that differ significantly from control were highlighted in bold (There was no significance difference between control groups and therefore the expression levels were compared with the means of two control groups)

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Table 3 The expression level of the selected genes in MDA-MB-231 cell line

	5 µM APTB	0.5 µM NTX	2.5 µM VTX	5 µM APTB + 0.5 µM NTX	5 µM APTB + 2.5 µM VTX
BAK	**-2.31±-0.14**	**2.17±0.18**	1.43±0.38	-1.07±0.21	-1.05±0.20
BAX	**2.08±0.05**	**-3.03±-0.07**	-1.84±-0.17	1.04±0.10	-1.42±-0.30
CAS3	-1.34±-0.26	1.05±0.27	-1.21±-0.34	**-2.22±-0.01**	1.08±0.40
BCL2	**5.1±0.01**	1.07±0.36	**2.14±0.09**	**7.05±0.003**	**3.17±0.02**
BCLXL	1.3±0.25	**2.06±0.17**	1.31±0.28	-1.05±-0.29	-1.57±-0.29
MCL1	**-2.27±-0.08**	**-2.13±-0.01**	1.32±0.30	**-2.15±-0.07**	-1.28±-0.24

Data were presented as means±S.E.M of four experiments. The results were normalized according to ACTB expression. The values that differ significantly from control were highlighted in bold (There was no significance difference between control groups and therefore the expression levels were compared with the means of two control groups)

more aggressive MDA-MB-231 cells to the combined tested agents rather than MCF-7 is independent from the factors investigated.

Discussion

The Bcl-2 protein family controls the mitochondrial pathway of apoptotic cell death depend on the levels and interactions of its pro- and anti-apoptotic members. On the other hand, the findings obtained until now show us that several anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xL and Mcl-1 are expressed at high levels in many cancer types including breast adenocarcinoma.[23-27] Hence, it is crucial to specifically target these anti-apoptotic proteins in terms of developing novel and effective treatments with fewer side effects. In this context, the mimetics of BH3-only proteins are thought to be powerful agents against cancer.[9] NTX, which can target Bcl-2/Bcl-xL, and Bcl-2 specific VTX are among the BH3 mimetics which were proved to be active in lymphoid malignancies and studies which are being conducted on for other types of tumors.[28-31] Of course, it should be remembered that the impression of any BH3 mimetic can extend beyond its targeted protein.[32-34] Nevertheless, other non-targeted anti-apoptotic members, such as Mcl-1, are held responsible for the resistance of especially solid tumors to these drugs and focus is on combined studies.

In the current study, we analyzed the cytotoxic and apoptotic effects of NTX/VTX and their combination with APTB on two different breast cancer cell lines with different aggressiveness for the first time. We observed that 0–5 µM APTB alone was non-toxic on both MCF-7 and MDA-MB-231 cell lines and the latter one was more sensitive to the agent in its higher doses within 24 h. In a recent study of Gao et al., supportive cytotoxicity results were shown on MDA-MB-231 cells for 72 h.[35] It was determined that APTB exerted dose-dependent inhibition of MCF-7 cell growth at the concentrations higher than 2 µM after 48 h treatment, which is also compatible with our data.[36] According to the literature, APTB shows its cytotoxic effect on cancer cells by blocking the phosphorylation of VEGFR-2 and thereby restraining several signaling pathways.[37] On the other hand, as stated previously, it was found that there has been correlation between VEGF and Mcl-1 expressions on certain hematological malignancies,[21,38] which give rise to thought that BH3 mimetics plus APTB can show synergistic effects in terms of inhibition of solid tumor cell proliferation. As a matter of fact, we found that to combine NTX/VTX with non-toxic concentrations of APTB significantly reduced the mimetic concentrations required to achieve similar or higher cytotoxic effects when compared to their sole applications. In other words, APTB combination sensitized the cells against the so-called BH3 mimetics in correlation with their aggressiveness. Tutusaus et al., indicated the synergistic effect of NTX plus sorafenib, a multikinase inhibitor, on hepatocellular carcinoma depend on the changes in the Bcl-2 protein profile.[39] In another recent study, tyrosine kinase inhibitors were shown to drive prostate cancer apoptosis when they were combined with Bcl-xL/Bcl-2 inhibitors by increasing Mcl-1 degradation.[40] However, we observed significant up-regulation in Bcl-2 expression in both MCF-7 and MDA-MB-231 cell lines, which were exposed to the combined treatments. Mcl-1 expression was also significantly up-regulated in MCF-7 cells. For these reasons, we could not explain the synergistic cytotoxic effects of NTX/VTX plus APTB over the changes in Bcl-2 and Mcl-1 gene expression against human breast cancer cells. After all, the more sensitivity of aggressive MDA-MB-231 cells

to the so-called combined applications and significant Bak/Bax and Cas3 expression in less aggressive MCF-7 cells can be seen as encouraging results. So, we can suggest that VTX/NTX plus APTB in their indicated low concentrations might be used efficaciously on human breast cancer cells but further studies are needed to clearly elucidate the observed synergistic cytotoxic effects.

Conclusion

Our results suggested that NTX/VTX and APTB showed cytotoxic effects on breast cancer cell lines depending on cell aggressiveness. Additionally, in this study, it was demonstrated that APTB sensitized breast cancer cells to low doses of NTX/VTX again in correlation with the aggressivity. Hopefully, these combinations can provide new options for novel approaches to the treatment of breast cancer. However, the underlying mechanism(s) in these synergistic cytotoxic effects should be elicited with further studies.

Limitations of the Study

Within the scope of this study, expression levels of a limited number of genes could be examined. In order to elucidate the exact mechanisms leading to the cytotoxic effects of the studied applications, more target behaviors should be investigated.

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