Bcl-2 expression in cell lines breast cancer and death program

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ABSTRACT

Breast cancer is a hormone-dependence and heterogenic disease. Drug resistance is the main reason for the failure of breast cancer treatment. Combinatory medications are methods for treatment but they are not sufficient in action. However, new approaches like molecular therapy reveal a new insight into cancer treatment. Studies show that *Bcl-2* gene family inhibitors and ER blockers cause the improvement of recovery. Interfering molecules such as antisense ones can inhibit the expression of *Bcl-2* and push the cancer cells to apoptosis. Our team designed a new Antisense Oligonucleotide (ASO) based on Antisense oligo G3139. MCF-7 and MDA-MB-231 cell lines were used to evaluate cellular proliferation. Liposomes and cationic nano-complex (Niosome) are used to increase the cellular delivery of ASO and Tamoxifen. We also investigated the cytotoxicity and apoptotic effects of Tamoxifen, naked ASO and Nano-packed ASO. The results indicated significant down-regulation of the *Bcl-2* gene and inhibition of MCF-7 and MDA-MB-231 cellular proliferation. Flow-cytometry showed early apoptosis in all cell groups. The newly designed ASO reduced the expression of the *Bcl-2* gene. It also had a synergistic effect with the Tamoxifen. The cationic nano-complex (Niosome) was more efficient than the liposome in delivering designed oligo antisense *Bcl-2* in the cancer cells.

Introduction

Breast cancer (BC) is the most common type of cancer among women. It is the second cause of death after lung cancer in females. According to the last statistical estimations that were published by the International Agency for Research on Cancer (IARS); lung cancer, female breast cancer and colorectal cancer are the most common types of cancer that account for one-third of the cancer incidence and mortality worldwide. The estimations indicate that breast cancer with approximately 2.1 million diagnoses constitutes 11.6% of the total cancer incidence. However, breast cancer treatment is divided into local and systemic ones. Local treatment such as surgery or radiation is not sometimes efficient. Systemic treatments include chemotherapy, hormone therapy, targeted therapy, and immunotherapy. Nevertheless, the mortality rate is high. New approaches are essential because of the high heterogeneity nature of breast cancer and the failure of traditional treatment. It seems that molecular treatments open a way to reduce the mortality rate in the early stages of cancer.

Breast cancer can be divided into subgroups based on the presence of estrogen receptor (ER), progesterone receptor (PR), HER2, and luminal (A and B), HER2-enriched, basal and normal breast-like, respectively (1-6). There is a correlation among these subgroups and survival rate, cancer relapse, place of metastasis, and chemotherapy response (7,8).

About 70% of breast cancers are Estrogen Receptor positive (ER⁺) and usually treated by Tamoxifen, which is an estrogen receptor antagonist rather than aromatize inhibitors. A long-time Tamoxifen usage can cause drug resistance (9). In addition, it could also lead to various side effects in normal tissues that increase susceptibility to endometrial and liver cancers or thrombosis (10,11). Studies show that anti-apoptotic molecules could lead to the survival of cancer cells (12, 13). They cause the cancer cells to avoid apoptosis and promote tumor progression. They also act as a barrier to chemical cancer treatments. One of them is the *Bcl-2* family which increases the level of anti-apoptotic *Bcl-2* family proteins such as *BCL-2*; *MCL-1* or *BCL-XL* proteins, which prevent cancer cell apopto-

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sis. Hence, the anti-apoptotic *Bcl-2* protein promotes cancer cells to survive and allows them to resist. Therefore, the anti-apoptotic *Bcl-2* protein (*Bcl-2*) silencing could enhance apoptosis and increase the sensitivity of cancer cells for treatment. The antisense technique is a way to silence anti-apoptotic *Bcl-2* protein (14-17). Anti-sense Oligonucleotides (ASO) are short in length (18-24 bp) and synthetic molecules. They are single-strand, which is complementary to the targeted mRNA gene, via Watson-Crick base pairing. They can inhibit mRNA processing. Eventually, they lead to suppression of gene expression through the RNase H-mediated mechanism or by modulations of splicing to stop their transformations to protein (18, 19). Nano-packaging is a good way to increase cellular uptake of ASO. For example, Nano-packages of Lipofectamine and micelles/niosome can increase cellular uptake and lead to ASO dose reduction. Lipofectamine as a common transfection reagent leads to an increase in the transfection efficiency of ASO into the cell. It contains lipid subunits that can form liposomes in an aqueous environment and generate a complex with negatively charged nucleic acid molecules like ASO. This complex can overcome the electrostatic repulsion of the cell membrane.

Niosomes (nonionic surfactant vesicles) are considered as novel drug delivery systems. Because the drug that is transferred into the cell with niosomes has better solubility and stability. Therefore, using Niosomes leads to more doses of ASO in the cell. In addition, they can release drugs such as ASO molecules in a targeted and controlled manner. (20-22).

In this study, we used bioinformatics to predict the second structures of targeted mRNA according to minimal delta G energy. It can predict the affinity of ASO with targeted mRNA (23-29). Our team designed a new Antisense Oligonucleotide for *Bcl-2* mRNA. A biodegradable cationic micelles/niosome and Lipofectamine were utilized to evaluate the cellular delivery of ASO and Tamoxifen. The *Bcl-2* expression was evaluated as an anti-apoptotic protein. The MCF-7 and the MDA-MB-231 cell lines were selected as Estrogen Receptor(ER) positive and ER-negative cells, respectively. Finally, naked ASO, nano-pack ASO, and Tamoxifen impacts on *Bcl-2* expression and cell apoptosis were compared in several groups.

Materials and Methods

Cell culture

The breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from the Pasteur Institute of Iran (NCBI codes: C135, and C578, respectively). They were cultured at RPMI 1640 with 10% FBS and incubated at 5% CO_2 and 37 °C. The cells were passed when their confluences reached about 95% and the cell medium was altered every 36hr regularly according to the duplicate time of the cells.

Antisense Oligonucleotide design

The Antisense Oligonucleotides (ASO) were designed according to the published nucleotide sequence of *Bcl-2* mRNA in the Ensembl database (https://asia.ensembl.org/Homo_sapiens). The secondary structure of *Bcl-2* mRNA was predicted by the minimum free energy (MFE) approach (http://rna.tbi.univie.ac.at//cgibin/RNAWebSuite/RNAfold.cgi) and based on antisense oligo G3139. The sequence of ASO was 5´-GTTCTCCCAGCGTGCGCCATCC-3'. All bases are phosphorothioate. Six first bases (GTTCTC …..) and six last bases (….. CCATCC) also have 2'-O-(2-methoxyethyl).We bought it from the Gorgon Gene Inhibition Biotechnology Company (Iran).

Primers designed for RT-PCR analysis

The PCR primers were designed according to the published nucleotide sequence of *Bcl-2* and *GAPDH* mRNA in the RefSeq database (https://ncbi.nlm.nih.gov/RefSeq), and the Oligo Primer analysis software version 7. The sequences of *Bcl-2* primers were forward, 5´-GACGACTTCTCCCGCCGCTAC-3', and reverse, 5´-TCCCCCAGTTCACCCCGTCC-3´. The sequences of *GAPDH* primers were forward, 5´-CCTGCCGTCTAGAAAAACCTGCCAA-3´, and reverse, 5´-CAGCGTCAAAGGTGGAGGAGTGGG -3´. Amplicons' sizes were 177 bp and 196bp, respectively.

RNA isolation and cDNA synthesis

Total RNA was isolated from MDA-MB-231 and MCF-7 cell lines using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. In addition, the isolated RNAs were treated with DNase1 (Yekta Tajhiz Azma Company, Iran) and then, the integrity and quality of total mRNA were ascertained by gel electrophoresis and the 260/280 ratio spectrophotometer, respectively. The first strand of cDNA was synthesized by using the cDNA synthesis kit of Yekta Tajhiz Company (YTA; Iran) according to the manufacturer's instructions.

RT-PCR analysis

The quantitative Real-time RT-PCR (RT-qPCR) was used to investigate the expression of *Bcl-2* relative to a housekeeping gene (*GAPDH*) in different conditions. It was carried out by using the ABI Step One Real-Time Instrument (Applied Bio-systems, USA). All the reactions were performed in triplicate and in a total volume of about 12 µl containing 1-2 µl cDNA, 0.5 µl each primer (5 pmol), 5 µl master-mix RealQ plus SYBR Green PCR Master Mix, (Ampliqon; Denmark) and 4-5 µl water. The RT-PCR machine conditions were 10 min at 95°C; 45 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72 °C for 30 sec.

Tamoxifen preparation

The Tamoxifen citrate salt is an estrogen receptor antagonist. The methanol was used to prepare the Tamoxifen due to the low solubility of Tamoxifen in aqueous medium (Supplemental Figure 1).

Lipofectamine preparation

The Lipofectamine 2000 (Invitrogen) was used in the form of a complex with the ASO based on 1:1 (w/w). The Lipofectamine (100 µg/ml) was allowed to be complexes with antisense oligonucleotides (ASO) (25,125,250,500 and 1000 nM) in serum and antibiotic-free medium before dilution and addition to cells.

Nano-complex (Micelles/Niosome) preparation

The nano-complex (micelles/niosome) was prepared based on our previous research with some modifications. The cationic nano-complex comprising polyethylenimine (PEI), tween80, and squalene was synthesized. Tween

80 & squalene were in a molecular ratio of 1:1 and PEI concentration was according to the ratio of PEI to ASO (23, 24). Electron microscopy was used to investigate the morphology of the micelles/niosome. The zeta potential, size, and polydispersity index (PDI) of the vesicles in PBS (pH 7.4) were analyzed by DLS (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK) using an argon laser beam at 633 nm and a 90° scattering angle (Table 1).

ASO and nano-complex (micelles/Niosome) preparation

The tween80 and squalene were dissolving in chloroform and methanol (ratio 3:2). This emulsion was rotated in a vacuum rotary evaporator for 1h at 45°C until a thin film formed. The thin film was incubated in a 37°C shaker overnight and then, it was dissolved in PBS and sonicated for 30 mins. Afterwards, it was filtered by 22-micron filters to purify the lipid particles. Finally, ASO was added to the lipid nano-size particles with molar ratio of 1:1. The final emulsion was rotated for 30 seconds and incubated for 30 minutes at 37°C to enhance the electrostatic interaction.

ASO, nano-complex (Micelles/Niosome) and Tamoxifen preparation

It was the same as ASO and micelles/niosome preparation but had a difference in the Tamoxifen loading process. The Tamoxifen loading process was formulated by using the nano-precipitate technique; 3 ml of chloroform and 2 ml of methanol (in which 2 mg Tamoxifen was dissolved) were introduced to 2 ml aqueous phase drop containing surfactant (Tween 80) and helper lipid (Squalene). The gel retardation assay has shown the ability of ASO packaging by PEI to form PEI/ASO complexes. PEI/ASO was prepared at various ratios. The resulting PEI/ASO various products were subjected to 2.5 % agarose gel electrophoresis and were visualized by ethidium bromide staining (data are not shown).

Transfection of the cell lines

Approximately $5×10^5$ cells of two breast cancer cell lines (MDA-MB-231 & MCF-7) were incubated in RPMI-1640 with 10% FBS for 24h. Then, the media was replaced and the cells were transfected by 125 nM, 250 nM, 500 nM and 1000 nM of Tamoxifen/ ASO/ Lipofectamine/ ASO, Lipofectamine/ ASO, Lipofectamine and Tamoxifen/ Niosome/ ASO and Niosome /ASO, Niosome and Tamoxifen, respectively. They were incubated again for 4h at 37°C. Each sample had a normal control.

Cellular analysis

The cells were cultured in a 96-well plate. The labeled scrambled sequence with 5'-Cy3 was formulated in ASO - PEI- Lipid complex. The internalization was observed by confocal fluorescent microscopy (Nikon, Japan, Figure 6).

MTT assay

The MTT {3-[4, 5-dimethylthiazol-2-yl]-2, 5-dipheyl tetraolium bromide} powder (Sigma Aldrich Company, Germany) was used to evaluate cells viability based on NADPH-dependent cellular oxidoreductase enzyme. NADPH-dependent cellular oxidoreductase enzyme revives the tetraolium dye (MTT) to its insoluble formazan, which has a purple color. After an incubation period, 100-μl DMSO (dimethyl sulfoxide, Sigma Aldrich Company, Germany) was added to dissolve the insoluble formazan and its absorbance was measured at 570 nm. The MCF7 and MDA-MB-231 cell lines were seed at a density of $12×10^3/200$ μL into 96-well chamber slides and treated with desired concentrations of agents including Tamoxifen, ASO, and micelles/niosome. Various concentrations of Tamoxifen/ASO/Lipofectamine/Niosome/ASO and Lipofectamine/ASO and Lipofectamine and Tamoxifen/ ASO and Niosome/ASO and Niosome and Tamoxifen were added to each cell's media, respectively. Cell viability was analyzed by spectrophotometer after 24, 48, and 72hr.

Apoptosis analysis

Normal, apoptotic, and necrotic cells can be distinguished by using the Annexin V-FITC/Propidium Iodide assay kit according to the manufacturer's instructions (MabTag's Company, Germany). The flow-cytometry technique was used to assay the cells Apoptosis by Annexin V-FITC/Propidium iodide staining according to the manufacture's instrument (MabTag's; Germany). Data was analyzed by Flowjo v software (BD Company, USA). In other words, Flow-cytometry was used to assess the cell viability after the addition of drugs such as Tamoxifen/ ASO /Lipofectamine /ASO and Lipofectamine /ASO, Lipofectamine and Tamoxifen/ Niosome/ ASO and Niosome/ ASO, Niosome, and Tamoxifen.

Statistical analysis

Expression data analysis was carried out by using the LinReg software version 11.0 (http://LinRegPCR.nl) and the Relative Expression Software Tool (REST©) according to the manufacturer's instructions, respectively. The graphs were designed by Graph Pad Prism 8 software. All the data were expressed as the means ± SEM (standard error of the mean). A one-way ANOVA and t-test were performed to determine the statistically significant differences among the groups.

Results

The MCF-7 and MDA-MB-231 cell lines were selected as Estrogen Receptor-positive and ER-negative cells, respectively in this study.

The Tamoxifen cytotoxicity and apoptosis in MCF-7 cell line

The cytotoxicity of the MCF-7 cell line was evaluated by Tamoxifen at concentrations of 1, 2, 5, 10, 20, 50, and 100 μM in intervals of one, two, and three days. The MTT assay was used to measure its cytotoxicity (Supplemental Figure 2). The cell death increases as time and dose increase. IC_{25} of 48 hr was selected for the following study. About 26.6% of the cells entered into the initial phase of apoptosis at IC_{25} {Tamoxifen (2μM)} were compared to the control sample (Figure 1).

The ASO cytotoxicity and apoptosis in MCF-7 cell line

The MCF-7 cell line was transfected with different ASO concentrations (100 to 1200 nM, Supplemental Figure 3). IC_{50} levels were measured at 24 and 48 hours. After 48 hours, almost half of the cells were destroyed at 800 nM. While gene expression assay after 48 hours, at 125 and 250 nM of ASO, the *Bcl-2* mRNA expression reduced by

about 30 to 40%, respectively. Expression decrease was up to 50% at 500 nM of ASO. The expression reduction was quite obvious and close to 70% at 1000 nM of ASO. The lower concentrations did not show significant results (P-value < 0.05). The ASO 500 nM was selected for further study. Reverse microscopy was used to investigate the cell morphology indicating the onset of apoptotic activity, while we used Annexin V-FITC/PI staining to show the modes of cell death stage. About 60% of the cells that entered into the initial phase of apoptosis were compared to the control sample at 500 nM of ASO (Figure 2).

The nano-complex (Micelles/Niosome) cytotoxicity

The two cell lines (the MCF-7 and MDA-MB-231) were transfected with the micelles/niosome. Its construct
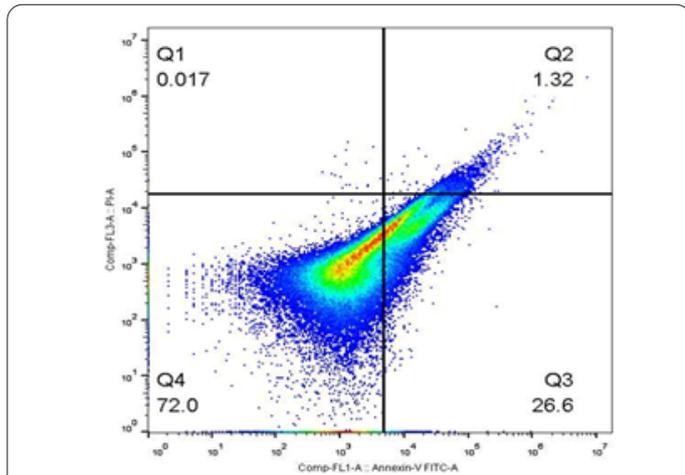
Figure 1. The effects of tamoxifen on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of tamoxifen for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/ PI+) are in the Q1.
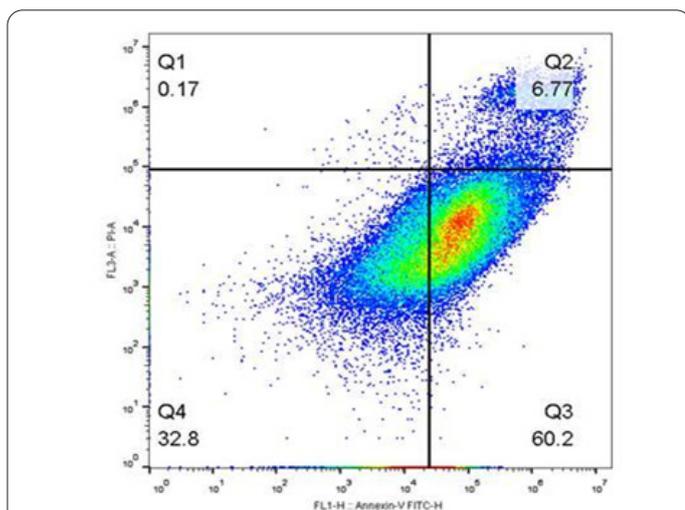
Figure 2. The effects of ASO on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO for 48 hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

was 13 μl of 1040 μM stock that was mixed with PBS in the ratio of one to 100. The average micelles/niosome sizes were 100 nm. The MTT assay was used to measure the bioactivities of the cell lines. The percentage of cell viability was equal to the control samples.

The ASO, Micelles/Niosome, PEI cytotoxicity, and apoptosis in MCF-7 cell line

Different concentrations of Poly-Ethylen-Imine (PEI) and the micelles/niosome were assessed by MTT to investigate their toxicity effects on the MCF-7 cell line. More than 85% of cells were viable at 1mM of PEI and the micelles/niosome. Then, ASO was added. Different concentrations of (PEI, Micelles/Niosome and ASO) i.e. 100 to 1200 nM were prepared. Then, The MCF-7 cell line was transfected by these concentrations and IC_{50} levels were measured at different times (Supplemental Figure 4). Almost half of the cells were destroyed at 240 nM after 48 hours. The expression level was measured. The *Bcl-2* mRNA was significantly down-regulated (Figure 3). About 85.2% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 4) at 500 nM of ASO, Micelles/Niosome, and PEI.
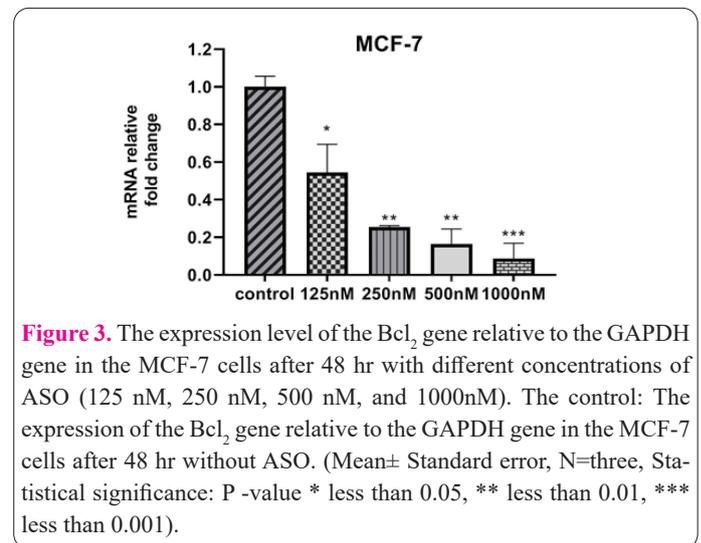
Figure 3. The expression level of the Bcl_2 gene relative to the GAPDH gene in the MCF-7 cells after 48 hr with different concentrations of ASO (125 nM, 250 nM, 500 nM, and 1000nM). The control: The expression of the Bcl_2 gene relative to the GAPDH gene in the MCF-7 cells after 48 hr without ASO. (Mean± Standard error, N=three, Statistical significance: P -value * less than 0.05, ** less than 0.01, *** less than 0.001).
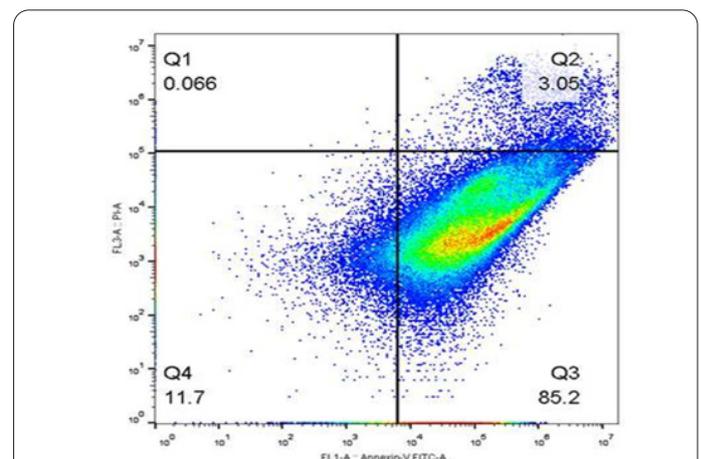
Figure 4. The effects of ASO, Niosome and PEI on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO, Niosome and PEI for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

The ASO, Lipofectamine 2000, Tamoxifen and Micelles/Niosome apoptosis in the MCF-7 cell line

Four mixtures were made by ASO, Lipofectamine 2000, Tamoxifen, and micelles/niosome. They were ASO and Lipofectamine 2000 / ASO and Tamoxifen / ASO, Tamoxifen and Lipofectamine 2000 / ASO, Tamoxifen and micelles/niosome. The MCF-7 cell lines were transfected by these four-construct separately. The *Bcl-2* mRNA expression was reduced in all groups. The cells transfected by ASO and Lipofectamine 2000 (ratio 1:1), about 80.6% of the cells entered into the initial phase of apoptosis compared to the control sample. Another group of the cells was transfected by ASO and Tamoxifen (20mM), about 78.3% of the cells entered into the initial phase of apoptosis compared to the control sample and another group of the cells were transfected by ASO, Tamoxifen, and Lipofectamine 2000, about 83.2% of the cells entered into the initial phase of apoptosis compared to the control sample. The final group of the cells was transfected by ASO, Tamoxifen, and micelles/niosome (13µg/ml), about 92.4% of the cells entered into initial phase of apoptosis compared to the control sample (Figure 5).

The Tamoxifen cytotoxicity and apoptosis in the MDA-MB-231 cell line

The cytotoxicity of the MDA-MB-231 cell line was evaluated as previously noted for the MCF-7 cell line (Supplemental Figure 5). At IC_{25}, about 11.8% of the cells entered into initial phase of apoptosis compared to the control sample (Supplemental Figure 6).

The ASO cytotoxicity and apoptosis in the MDA-MB-231 cell line

The MDA-MB-231 cell line was transfected in different ASO concentrations (100 to 1200 nM, Figure 7). IC_{50} levels were measured at 24 and 48 hours. After 48 hours, almost half of the cells were destroyed at 950 nM. While gene expression assay at 125 nM of ASO, the *Bcl-2* mRNA expression reduced after 48 hours, by about 30 % (Figure 8). At 250nM of ASO, expression reduction was up to 50%. At 500nM of ASO, expression reduction was nearly 70%. At 1000nM of ASO, expression reduction was obvious and close to 80%. The lower concentrations did not show significant results (P-value < 0.05). The ASO 500 nM was selected for further study. Reverse microscopy was used to investigate the cell morphology indicating the onset of apoptotic activity, while we used Annexin V-FITC/PI staining to show modes of cell death stage. At 500 nM of ASO, about 69% of the cells entered into the initial phase of apoptosis compared to the control sample
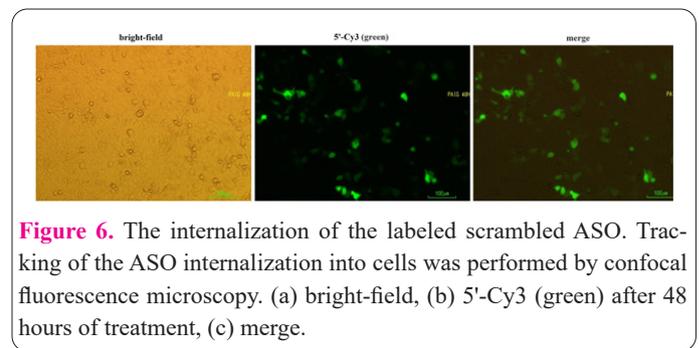
Figure 6. The internalization of the labeled scrambled ASO. Tracking of the ASO internalization into cells was performed by confocal fluorescence microscopy. (a) bright-field, (b) 5'-Cy3 (green) after 48 hours of treatment, (c) merge.
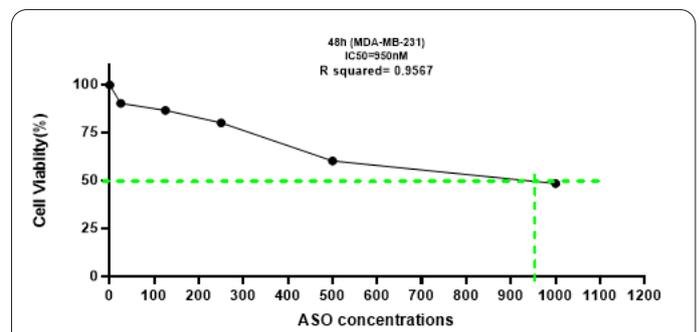
Figure 7. The effects of ASO on the apoptosis of MDA-MB-231 cells.
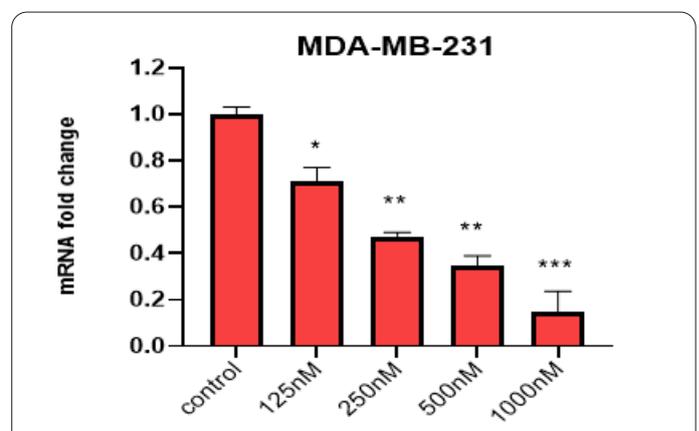
Figure 8. The expression level of the Bcl_2 gene relative to the GAPDH gene in the MDA-MB-231 cells after 48 hr with different concentrations of ASO (125 nM, 250 nM, 500 nM, and 1000nM). The control: The expression of the Bcl_2 gene relative to the GAPDH gene in the MDA-MB-231 cells after 48 hr without ASO. (Mean± Standard error, N=three, Statistical significance: P -value * less than 0.05, ** less than 0.01, *** less than 0.001).

Figure 5. The effects of ASO and Lipofectamine on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO and Lipofectamine for 48hr (A). The effects of ASO and Tamoxifen on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO and Tamoxifen for 48hr (B). The effects of ASO, Tamoxifen and Lipofectamine on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO, Tamoxifen and Lipofectamine for 48hr (C). The effects of ASO, Tamoxifen and Niosome on the apoptosis of MCF-7 cells. Cells were treated with the selected concentration of ASO, Tamoxifen and Niosome for 48hr (D). Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

Figure 9. The effects of ASO on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in Q2 and necrotic cells (Annexin V–/PI+) are in Q1

(Figure 9).

The ASO, Micelles/Niosome, PEI cytotoxicity, and apoptosis in the MDA-MB-231 cell line

Different concentrations of PEI, micelles/noisome, and ASO (100 to 1000 nM) were prepared as much as previously noted for the MCF-7 cell line (Figure 10). The MDA-MB-231 cell line was transfected and IC_{50} levels were measured. After 48 hours, almost half of the cells were destroyed at 320nM. The expression level was measured. The *Bcl-2* mRNA is significantly down-regulated (Figure 11). The morphological changes of the MDA-MB-231 cell line by reverse microscopy indicate the onset of apoptotic activity. At 500 nM of ASO, micelles/noisome, and PEI, about 82.9% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 12).

The ASO, Lipofectamine 2000, Tamoxifen, and Micelles/Niosome apoptosis in the MDA-MB-231 cell line

Four mixtures were made with ASO, Lipofectamine 2000, Tamoxifen, and micelles/niosome. They were ASO and Lipofectamine 2000/ASO and Tamoxifen/ASO, Tamoxifen and Lipofectamine 2000/ASO, Tamoxifen and micelles/niosome. The MDA-MB-231 cell lines were transfected by these four-construct separately. The *Bcl-2* mRNA expression was reduced in all groups. The cells transfected by ASO and Lipofectamine 2000 (ratio 1:1), about 80.9% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 13). Another group of the cells was transfected by ASO and Tamoxifen (20mM), about 69.6% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 14). Moreover, another group of the cells were transfected with ASO, Tamoxifen, and Lipofectamine 2000, about 84.6% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 15). The final group of the cells was transfected by ASO, Tamoxifen and micelles/niosome (13μg/ml), about

Figure 10. The effects of ASO on the apoptosis of MDA-MB-231 cells.
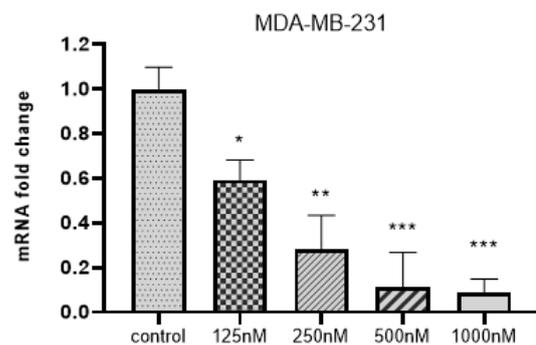
Figure 11. The expression level of the Bcl_2 gene relative to the GAPDH gene in the MDA-MB-231 cells after 48 hr with different concentrations of ASO, Niosome and PEI complex (Mean± Standard error, N=three, Statistical significance: P value *less than 0.05, **less than 0.01, ***less than 0.001). The control: The expression of the Bcl_2 gene relative to the GAPDH gene in the MDA-MB-231 cells after 48 hr without ASO, Niosome and PEI complex. (Mean± Standard error, N=three, Statistical significance: P -value * less than 0.05, ** less than 0.01, *** less than 0.001).
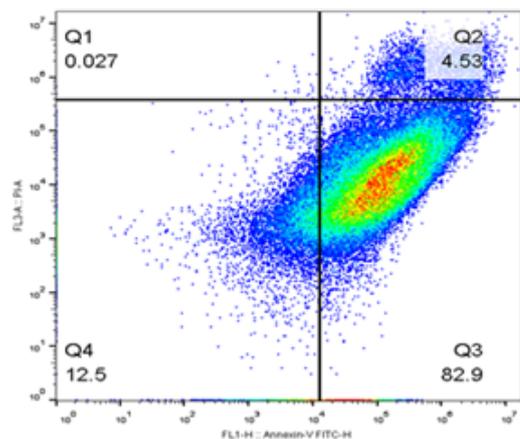
Figure 12. The effects of ASO, Niosome and PEI on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO, Niosome and PEI for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

88.6% of the cells were entered into the initial phase of apoptosis compared to the control sample (Figure 16).
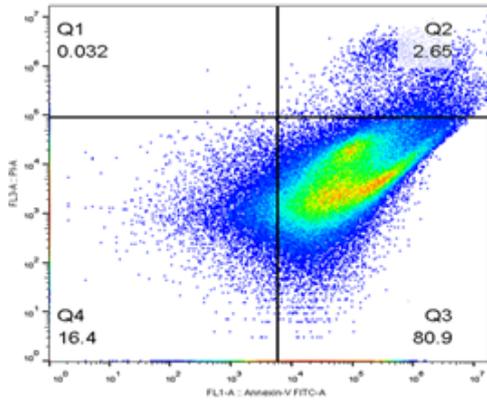
Figure 13. The effects of ASO and Lipofectamine on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO and Lipofectamine for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.
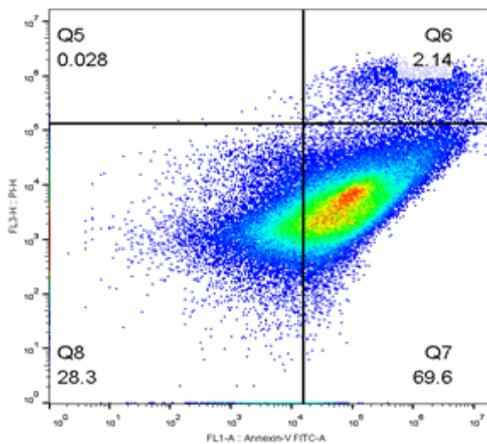
Figure 14. The effects of ASO and Tamoxifen on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO and Tamoxifen for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

The ASO, Lipofectamine 2000, Tamoxifen, and Micelles/Niosome apoptosis in the MDA-MB-231 cell line

Four mixtures were made with ASO, Lipofectamine 2000, Tamoxifen, and micelles/niosome. They were ASO and Lipofectamine 2000/ASO and Tamoxifen/ASO, Tamoxifen and Lipofectamine 2000/ASO, Tamoxifen and micelles/niosome. The MDA-MB-231 cell lines were transfected by these four-construct separately. The *Bcl-2* mRNA expression was reduced in all groups. The cells transfected by ASO and Lipofectamine 2000 (ratio 1:1), about 80.9% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 13). Another group of the cells was transfected by ASO and Tamoxifen (20mM), about 69.6% of the cells entered into the initial phase of apoptosis compared to the control

sample (Figure 14). Moreover, another group of the cells were transfected with ASO, Tamoxifen, and Lipofectamine 2000, about 84.6% of the cells entered into the initial phase of apoptosis compared to the control sample (Figure 15). The final group of the cells was transfected by ASO, Tamoxifen and micelles/niosome (13µg/ml), about 88.6% of the cells were entered into the initial phase of apoptosis compared to the control sample (Figure 16).

Discussion

Breast cancer is one of the most common causes of death in women. Several treatments are used to treat breast cancer, such as surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy and immunotherapy (1-
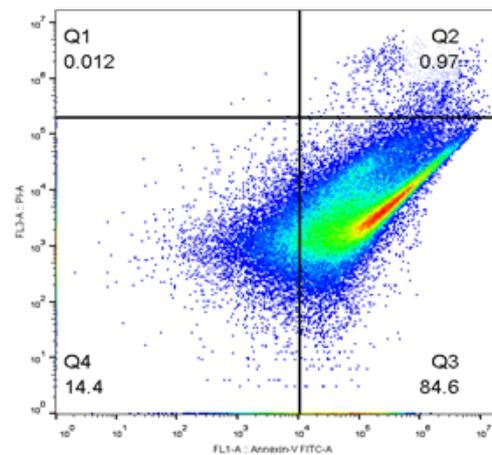
Figure 15. The effects of ASO, Tamoxifen and Lipofectamine on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO, Tamoxifen and Lipofectamine for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.
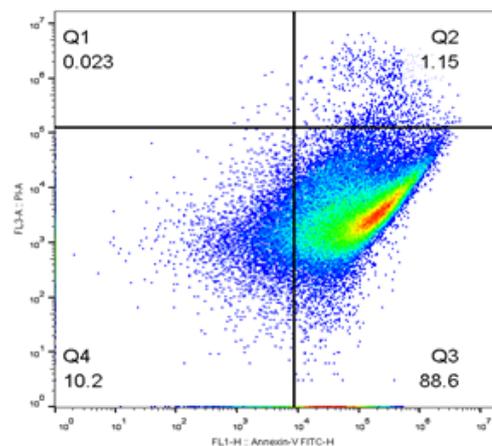
Figure 16. The effects of ASO, Tamoxifen and Niosome on the apoptosis of MDA-MB-231 cells. Cells were treated with the selected concentration of ASO, Tamoxifen and Niosome for 48hr. Cells were analyzed in independent triplicates of each combination. FL1 channel was used to detect Annexin V-FITC and FL3 indicated PI signals. The viable cells (Annexin V–/PI–) are in the Q4. Early apoptotic cells (Annexin V+/PI+) are in the Q3. Late apoptotic cells (Annexin V+/PI+) are in the Q2 and necrotic cells (Annexin V–/PI+) are in the Q1.

4).

Most types of breast cancer are estrogen receptor-positive (ER+) and usually treated with tamoxifen, but long-term use of tamoxifen can cause drug resistance, endometrial cancer, liver cancer, or thrombosis. Several studies show that molecular therapies can inhibit the growth of cancer cells in the early stages. Moreover, they show that anti-apoptotic proteins can increase cancer cells life. In other words, destroying the anti-apoptotic protein can cause the destruction of cancer cells (5-13).

Eliminating or reduction of the genes that produce anti-apoptotic proteins is a great approach. siRNA and antisense are two approaches that are commonly used to silence genes, For example, in the antisense technique, the designed oligonucleotides stop protein production by RNase-H mechanism or by interfering in the mRNA processing of the target gene (14-17).

Studies show that *Bcl-2* gene expression is increased in more than 70% of breast cancers. This increased expression can lead to more growth of cancer cells and a lack of treatment response. For this purpose, in this study, the *Bcl-2* gene was selected as the target gene and a new antisense oligo-nucleotide based on G3139 antisense was designed for its mRNA (18, 20). For better stability of this new oligo-nucleotide, we designed it to contain phosphorothionate as two wings. To enhance the cellular uptake of the designed oligonucleotide in accordance with other studies, lipofectamine nano-carrier and cationic micelle/nisome nano-carrier were used to transport this new oligonucleotide and tamoxifen into the cells (21-29).

To investigate the performance of designed antisense oligo-nucleotide compared to tamoxifen in cell death and reduction of *Bcl-2* gene expression, different groups of cells were prepared. These cell groups were named with the names of the materials used in them.

To evaluate the effectiveness of antisense oligo-nucleotide and tamoxifen in all mentioned cell groups, Real-Time RT PCR and Flow-cytometry techniques were used and their results were compared (30).

In other words, first, at different concentrations of ASO, the Real-time RT-PCR was used to investigate the expression of the *Bcl-2* gene relative to the GAPDH gene in the MCF-7 and the MDA-MB-231 cell groups, respectively (Figure 3 and Supplemental Figure 8). Then Flow-cytometry was used in seven mentioned cell groups.

In general, it can be concluded that in all stages of research and investigation, the newly designed ASO could reduce the expression of the *Bcl-2* gene (Figure 3). It is clear that by reducing the expression of the *Bcl-2* gene, cancer cells may respond to the treatment easier or they may enter the apoptosis phase earlier. It can be expected that a more appropriate treatment response will obtained.

Flow-cytometry like Real-time RT-PCR showed us remarkable results as follows: In the first cell group of study (Tamoxifen alone cell group), Tamoxifen was able to induce about 26.6% of the MCF-7 cells and about 11.8% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 1 and Supplemental Figure 6).

In the second cell group of study (Antisense oligo-nucleotide alone cell group), the newly designed ASO was able to induce about 60% of the MCF-7 cells and about 69% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples at 500 nM of ASO,

respectively (31) (Figure 2 and Figure 9).

In the third cell group of study (Antisense oligo-nucleotide and tamoxifen cell group), the newly designed ASO and tamoxifen were able to induce about 78.3% of the MCF-7 cells and about 69.6% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 5B and Figure 14).

In the fourth cell group of study (Antisense oligo-nucleotide with lipofectamine nano-carrier cell group), the newly designed ASO encapsulated in lipofectamine nano-carrier was able to induce about 80.6% of the MCF-7 cells and about 80.9% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 5A and Figure 13).

In the fifth cell group of study (Antisense oligo-nucleotide and tamoxifen with lipofectamine nano-carrier cell group), the newly designed ASO and tamoxifen encapsulated in lipofectamine nano-carrier were able to induce about 83.2% of the MCF-7 cells and about 84.6% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 5C and Figure 15).

In the sixth cell group of study (Antisense oligo-nucleotide with cationic micelle/nisome nano-carrier cell group), the newly designed ASO encapsulated in cationic micelle/nisome nano-carrier was able to induce about 85.2% of the MCF-7 cells and about 82.9% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 4 and Figure 12).

In the seventh cell group of study (Antisense oligo-nucleotide and tamoxifen with cationic micelle/nisome nano-carrier cell group), the newly designed ASO and tamoxifen encapsulated in cationic micelle/nisome nano-carrier were able to induce about 92.4% of the MCF-7 cells and about 88.6% of MDA-MB-231 cells into the early phase of apoptosis compared to the control samples, respectively (Figure 5D and Figure 16).

It can be concluded that combination therapy appears to be better than single-drug therapy. In addition, the use of nano-carriers compatible with the cellular environment, which are responsible for transporting the drug into the cells, can help increase the effect and better performance of the drug. In this study, it was observed that the combination of tamoxifen and the newly designed antisense oligo-nucleotide with cationic micelle/nisome nano-carrier has the best performance. In other words, it was able to make about 90% of cancer cells enter the early phase of apoptosis. In addition, it seems that the cationic micelle/nisome nano-carrier has a better performance in transporting tamoxifen and the antisense oligonucleotide into the cells compared to the lipofectamine nano-carrier.

Finally, the combined therapeutic approaches based on drug and gene therapy seem to represent a new perspective in cancer treatment. In this study, it was observed that the newly designed antisense oligo-nucleotide reduced the expression of the target gene to an acceptable amount. This reduction in expression was greater than the effect of the anticancer drug alone (tamoxifen). In addition, it also had a synergistic effect with the Tamoxifen.

In the current study, the newly designed ASO reduced the expression of *Bcl-2* gene production. It also had a synergistic effect with the Tamoxifen. The cationic nano-complex (Niosome) was more efficient than the liposome in delivering designed oligo antisense *Bcl-2* in the cancer

cells. However it needs more research work in this issue to draw firm conclusions.

Competing interests

The ethics code is http://ethics.research.ac.ir/IR.GOUMS. REC.1398.067 in the medical research committee of Golestan University of Medical Sciences. Gorgan, Iran.

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