The Bcl-2 silencing with an Antisense Oligonucleotide: Increase early Apoptosis

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Research article

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Abstract

Background

Breast cancer is a heterogenic disease and hormone dependence. Estrogen receptor is positive in more than seventy percent of breast cancer patients. Tamoxifen is an estrogen receptor (ER) antagonist and used as the first line of treatment. Drug resistance is a main reason in failure of cancer treatment and progression of the disease. Combination drug therapy is a method to treatment but is not sufficient. New approaches like molecular therapy reveal new insight to cancer therapy. Studies shown, Bcl-2 gene family inhibitors and ER blockers enhance recovery. Interfering molecules such as antisense can inhibit the expression of Bcl-2 and push the cancer cells to apoptosis. Nevertheless, their effectiveness is low, mostly due to their direct use.

Methods

Our team designed an Antisense Oligonucleotides (ASO). The MCF-7 and the MDA-MB-231 breast cancer cell lines used to evaluate cellular proliferation. Liposome and cationic nano-complex (Niosome) used to increase cellular delivery of ASO and Tamoxifen. We also investigated the cytotoxicity and apoptotic effects of Tamoxifen, naked ASO and Nano-packed ASO.

Results

The ASO functional potency to assess apoptosis and expression of Bcl-2 mRNA compared in different groups. The results indicated, significant down regulation of Bcl-2 gene and inhibition of MCF-7 and MDA-MB-231 cellular proliferation. Flow-cytometry showed early apoptosis in all groups.

Conclusions

The ASO reduced the expression of Bcl-2 gene. It also had the synergistic effect with the Tamoxifen. In all studied groups, it was able to push cancer cells to apoptosis. The cationic nano-complex (Niosome) was more efficient than the liposome in delivering designed oligo antisense Bcl-2 into the cancer cells.

Introduction

The Breast cancer (BC) is the most common type of cancer among women. It is second cause of death after lung cancer in females. According to last statistics estimations that published by International Agency for Research on Cancer (IARS); lung cancer, female breast cancer and colorectal cancer are most common types of cancer that are responsible for one third of the cancer incidence and mortality in worldwide. The estimations indicate breast cancer with approximately 2.1 million diagnoses has burden 11.6% of total cancer incidence. The breast cancer geographical analysis showed Iran located in high
region with nearly 17000 new cases(1). However, breast cancer therapy divided to local and systemic. Local therapy such as surgery or radiation that sometimes is not efficient. Systemic therapies including; chemotherapy, hormone therapy, targeted therapy and immunotherapy(2). Nevertheless, mortality rate is high. New approaches are essential because of high heterogeneity nature of breast cancer and failure of traditionally treatment(3). It seems that molecular therapies open a way to reduce mortality rate in early stages of cancer. Breast cancer can divided to subgroups based on the presence of estrogen receptor (ER), progesterone receptor (PR), HER2, and luminal (A and B), HER2-enriched, basal like and normal breast-like, respectively (4). There are a correlation between these subgroups and survival rate, cancer relapse, place of metastasis and chemotherapy response(5).

About 70% of breast cancers are Estrogen Receptor positive (ER+) and usually treated with Tamoxifen which is an estrogen receptor antagonist rather than aromatize inhibitors(6). Long-time Tamoxifen usage can cause drug resistance (7). In addition, it also could led to some various side-effects in normal tissues that increases susceptibility to endometrial and liver cancers or thrombosis(8). Studies showed, Anti-apoptotic molecules could lead to survival of cancer cells (9). They cause cancer cells avoid from apoptosis and promote tumor progression. They also act as a barrier to chemical cancer therapies. One of them is Bcl-2 family. Increases level of anti-apoptotic Bcl-2 family proteins such as BCL-2; MCL-1 or BCL-XL proteins prevent cancer cells apoptosis. Hence, 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 increased sensitivity of cancer cells to treatment (10–12). Antisense technique is a way to silence anti-apoptotic Bcl-2 protein. Antisense Oligonucleotides (ASO) are short length (18-24 bp), synthetics molecules. They are single-strand, which complementary to the targeted mRNA gene, via Watson-Crick base paring. They can inhibit mRNA processing. Eventually, they lead to suppressing of gene expression through RNase H-mediated mechanism or by modulations of splicing to stop their translations to protein (13). However, they can reduce cancer gene expression but they have some limitations. For example, it degrades rapidly by endo and exo-nucleases, misleading off-target effects and weak cellular uptake. To solve these problems, chemical modification of nucleotides, various modification on backbone or sugar rings of Deoxyribonucleotides with sulfur ion to creating phosphorothionate (PS) linkage and 2'-deoxy residues modifications have been confirmed(14). Nano-packaging is a good way to increase cellular uptake of ASO. Nano-packages Lipofectamine and micelles/niosome can increase cellular uptake and lead to decrease ASO dose (15–17). In this study, we used bioinformatics to predict the second structures of target's mRNA according to minimal delta G energy. It can predict affinity of ASO with target's mRNA. Our team designed an Antisense Oligonucleotides for Bcl-2 mRNA. A biodegradable cationic micelles/niosome and Lipofectamine utilized to evaluate cellular delivery of ASO and Tamoxifen. The Bcl-2 expression as an anti-apoptotic protein evaluated. The MCF-7 and the MDA-MB-231 cell lines selected as Estrogen Receptor-positive and ER-negative cells respectively. Finally, naked ASO, nano-pack ASO and Tamoxifen effects on Bcl-2 expression and cell apoptosis compared in several groups.

**Methods And Materials**
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 were incubated at 5% CO2 and 37°C. The cells were passage when their confluences reached about 95% and cell medium was alter every 36h regularly according to the duplicate time of the cells.

Antisense Oligonucleotides design

The Antisense Oligonucleotides (ASO) was design 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 predicted by minimum free energy (MFE) approach (http://rna.tbi.univie.ac.at//cgibin/RNAWebSuite/RNAfold.cgi). The sequences of ASO was 5'-GTTCTCCCAGCGTGCGCCATCC-3'. We bought it from the Gorgon Gene Inhibition Biotechnology Company (Iran).

Primers design for RT-PCR analysis

The PCR primers were design 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'-GACGACTTCTCCGCGCTAC-3', and reverse, 5'-TCCCCAGTTCACCCCGTCC-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 treated with DNase1 (YT8054, Iran) and then the integrity and quality of total mRNA was ascertained by gel electrophoresis and the 260/280 ratio spectrophotometer respectively. The first strand cDNA synthesized by using the cDNA synthesis Kit Yektatajhiz Company (YTA; Iran) according to the manufacturer's instructions.

RT-PCR analysis

The Real-time RT-PCR was use to investigate the expression of Bcl-2 relative to a housekeeping gene (GAPDH) in different conditions. It was carrying 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 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. Due to the low solubility of Tamoxifen in aqueous medium, the methanol used to prepare the Tamoxifen (see Additional file 1).

**Lipofectamine preparation**

The Lipofectamine 2000 (Invitrogen) used in the form of complex with the ASO based on 1:1 (w/w). The Lipofectamine (100 µg/ml) was allowed to complex 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 (25). The cationic nano-complex comprising polyethylenimine (PEI), tween80, squalene was synthesizing. Tween 80 & squalene was in molecular ratio 1:1 and PEI concentration was according to ratio of PEI to ASO (1). Electron microscopy were use to examine the morphology of the micelles/niosome. The zeta potential, size, and Polydispersity index (PDI) of the vesicles in PBS (pH 7.4) 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 rotate in vacuum rotary evaporator for 1h at 45°C until, thin film formed. Thin film was incubated in 37°C shaker for overnight and then it was dissolved in PBS and was sonicated for 30min. Afterward, to purify lipid particles, it was filtered by 22 micron filters. Finally, ASO was adding to the lipid Nano size particles with molar ratio of 1:1. To enhance electrostatic interaction, the final emulsion was rotate for 30 seconds and incubated for 30 minutes at 37°C.

**ASO, Nano-complex (Micelles/Niosome) and Tamoxifen Preparation**

It was same as ASO and micelles/niosome preparation, but had difference in Tamoxifen loading process. Tamoxifen loading process was formulating using by nano-precipitate technique; 3ml of chloroform and 2 ml of methanol (in which 2 mg Tamoxifen was dissolve) were introduce to 2ml 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 at various ratios was prepared. The resulting PEI/ASO various products subjected to 2.5 % agarose gel electrophoresis and were visualize by ethidium bromide staining (Data not shown).

**Transfection of the cell lines**

Approximately 5×10⁵ 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 replace and the cells were transfected with
125nM, 250nM, 500nM and 1000nM of Tamoxifen / ASO / Lipofectamine / ASO and Lipofectamine / ASO, Lipofectamine and Tamoxifen / Niosome / ASO and Niosome / ASO, Niosome and Tamoxifen respectively. They were incubates again for 4h at 37°C. Each sample had a normal control.

**Cellular analysis**

The cells were culture in a 96-well plate. The labeled scrambled sequence with 5’-Cy3 was formulating in ASO - PEI - Lipid complex. The internalization observed by confocal fluorescent Microscopy (Nikon, Japan).

**MTT assay**

The MTT {3-[4, 5-dimethylthiazol-2-yl]-2, 5-dipheyl tetrazolium bromide} powder (Sigma Aldrich Company, Germany) was use to evaluate cells viability based on NADPH-dependent cellular Oxidoreductase enzyme. NADPH-dependent cellular Oxidoreductase enzyme revives the Tetrazolium dye (MTT) to its insoluble Formazan, which has a purple color. After an incubation period, it was added 100 µl DMSO (Dimethyl sulfoxide, Sigma Aldrich Company, Germany) to solve insoluble Formazan and its absorbance was measure at 570 nm. The MCF7 and the MDA-MB-231 cell lines were seed at a density of 12×10^3 / 200 µL into 96 well chamber slides and were treat 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 added to the each cell’s media respectively. After 24, 48 and 72h cells viability analyzed by spectrophotometer.

**Apoptosis analysis**

Normal, apoptotic and necrotic cells can distinguish by using the Annexin V-FITC/Propidium Iodide assay kit according to the manufacturer’s instructions (MabTag’s Company, Germany). Flow-cytometry technique used to assay the cells Apoptosis by Annexin V-FITC/Propidium iodide staining according manufacture’s instrument (MabTag’s; Germany). Data analyzed by Flowjo v software (BD Company, USA). In other words, Flow cytometry was use to assess 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](http://LinRegPCR.nl)) and the Relative expression software tool (REST©) according to the manufacturer’s instructions respectively. The graphs designed by Graph pad Prism 8 software. All data expressed as the means ± SEM (standard error of the mean). A one-way ANOVA and t-test performed to determine the statistically significant differences among groups.
Results

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

The Tamoxifen cytotoxicity and apoptosis in the MCF-7 cell line

The cytotoxicity of the MCF-7 cell line were evaluated with Tamoxifen at concentration of 1, 2, 5, 10, 20, 50 and 100 μM in intervals of one, two and three days. The MTT assay used to measure its cytotoxicity (see Additional file 2). The cells death increases with increase time and dose. IC$_{25}$ of 48 hr selected for following study. At IC$_{25}$ (Tamoxifen (2μM)) about 26.6% of the cells entered into initial phase of apoptosis compared to the control sample (Fig 1).

The ASO cytotoxicity and apoptosis in the MCF-7 cell line

The MCF-7 cell line was transfected with different ASO concentrations (100 to 1200 nM, see Additional file 3). IC50 levels measured at 24 and 48 hours. After 48 hours, almost half of the cells destroyed at 800 nM. While gene expression assay after 48 hours, at 125 and 250 nM of ASO, the Bcl-2mRNA expression reduced about 30 to 40% respectively. At 500 nM of ASO, decrease expression was up to 50%. At 1000 nM of ASO, decrease expression was quite obvious and close to 70%. The lower concentrations did not show significant results (P-value < 0.05) (Fig. 2). The ASO 500 nM selected for further study. The Reverse microscopy was used to examine cell morphology indicating the onset of apoptotic activity, while to show modes of cell death stage, we used Annexin V-FITC/PI staining. At 500 nM of ASO, about 60% of the cells entered into initial phase of apoptosis compared to the control sample (Fig. 3).

The Nano-complex (Micelles/Niosome) cytotoxicity

The two cell lines (the MCF-7 and The MDA-MB-231) were transfected with the micelles/niosome. Its construct was 13 μl of 1040 μM stock that mixed with PBS in a ratio of one to 100. The average micelles/niosome sizes were 100 nm. The MTT assay used to measure the bioactivities of the cell lines. The percentage of cell viability was equal to the control samples. Almost 100% of the cells were biological active.

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

Different concentrations of Poly-Ethylen-Imine (PEI) and the micelles/niosome were assessing by MTT to investigate the toxicity effects of them on the MCF-7 cell line. At 1mM of PEI and the micelles/niosome, more than 85% of cells were viable. Then, ASO added. Different concentrations (PEI, Micelles/Niosome and ASO) 100 to 1200 nM were prepared. Then, The MCF-7 cell line was transfected with these concentrations and IC50 levels measured at different times (see Additional file 4). After 48 hours, almost half of the cells destroyed at 240 nM. The expression level measured. The Bcl-2 mRNA was significantly down regulated (Fig. 4). At 500 nM of ASO, Micelles/Niosome and PEI, about 85.2% of the cells entered into initial phase of apoptosis compared to the control sample (Fig. 5).
The ASO, Lipofectamine 2000, Tamoxifen and Micelles/Niosome apoptosis in the MCF-7 cell line

Four mixtures 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 MCF-7 cell lines transfected with these four-construct separately. The Bcl-2 mRNA expression reduced in all groups. The cells transfected with ASO and Lipofectamine 2000 (ratio 1:1), about 80.6% of the cells entered into initial phase of apoptosis compared to the control sample. Another group of the cells transfected with ASO and Tamoxifen (20mM), about 78.3% of the cells entered into initial phase of apoptosis compared to the control sample and another group of the cells transfected with ASO, Tamoxifen and Lipofectamine 2000, about 83.2% of the cells entered into initial phase of apoptosis compared to the control sample. Final group of the cells transfected with ASO, Tamoxifen and micelles/niosome (13µg/ml), about 92.4% of the cells entered into initial phase of apoptosis compared to the control sample (Fig. 6).

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

The cytotoxicity of the MDA-MB-231 cell line evaluated as same as previously noted for the MCF-7 cell line (see Additional file 5). At IC$_{25}$, about 11.8% of the cells entered into initial phase of apoptosis compared to the control sample (see Additional file 6).

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

The MDA-MB-231 cell line was transfected with different ASO concentrations (100 to 1200 nM, see Additional file 7). IC$_{50}$ levels measured at 24 and 48 hours. After 48 hours, almost half of the cells destroyed at 950 nM. While gene expression assay after 48 hours, at 125 nM of ASO, the Bcl-2 mRNA expression reduced, about 30 %(see Additional file 8). At 250nM of ASO, decrease expression was up to 50%. At 500nM of ASO, decrease expression was nearly 70%. At 1000nM of ASO, decrease expression was obvious and close to 80% .The, lower concentrations did not show significant results (P-value < 0.05). The ASO 500 nM selected for further study. The Reverse microscopy was used to examine cell morphology indicating the onset of apoptotic activity, while to show modes of cell death stage, we used Annexin V-FITC/PI staining. At 500 nM of ASO, about 69% of the cells entered into initial phase of apoptosis compared to the control sample (see Additional file 9).

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

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

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

Four mixtures 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 transfected with these four-construct separately. The Bcl-2 mRNA expression reduced in all groups. The cells transfected with ASO and Lipofectamine 2000 (ratio 1:1), about 80.9% of the cells, entered into initial phase of apoptosis compared to the control sample (see Additional file 13). Another group of the cells transfected with ASO and Tamoxifen (20mM), about 69.6% of the cells, entered into initial phase of apoptosis compared to the control sample (see Additional file 14). Moreover, another group of the cells transfected with ASO, Tamoxifen and Lipofectamine 2000, about 84.6% of the cells, entered into initial phase of apoptosis compared to the control sample (see Additional file 15). Final group of the cells transfected with ASO, Tamoxifen and micelles/niosome (13µg/ml), about 88.6% of the cells entered into initial phase of apoptosis compared to the control sample (see Additional file 16).

**Discussion**

A determined inactivated gene is a good way to understand how and why the gene works. The knockouts methods are time-consuming and costly process. An alternative method is gene silencing. It may do at different levels (transcript, splice, translate and post translate). For example, at the mRNA level, the RNA transcript of a desire gene targeted instead of the gene itself. However, it prevents expression of the target gene, or leads to transcript cleavage and resulting in mRNA degradation. Therefore, expression of the target gene stops. Sometimes, it may create a therapeutic effect. Two main strategies may do it, siRNA and Antisense technology. Although, siRNA is a potential therapeutic strategy, it has entered a lower therapeutic phase than antisense due to its cross-sectional structure and low efficiency in penetrating tissues and entering the cells. Second strategy is the Antisense technology. It used as a method to reduce or suppress gene expression at the mRNA level in the target genes. In this study, the Bcl-2 selected. The Bcl-2 mRNA expression increases in more than 70% of breast cancers. It can lead to further growth of cancer cells. An oligo antisense used to intervene at the mRNA level. An important problem in oligo-antisense-based therapies is the stability of oligonucleotides at the target site in the cells. To solve this problem, some investigators used somatic changes of oligonucleotides-based structures such as replacement of phosphate bond with Phosphorothionate, addition of a sulfur atom, base changes, terminal changes, and changes in ribose sugar in carbon No. 2 by methyl group. In this research, we designed an oligo-antisense and involving Phosphorothionate as two wings. Another important problem in oligo-antisense-based therapies is the transfer of oligonucleotides to the target site. We used Lipofectamine 2000 for transport of Bcl-2 mRNA oligo-antisense into the cells. We also applied cationic micelles/niosome as a carrier. Tamoxifen selected as a therapeutic drug to evaluate the effect of our ASO. The designed oligo-antisense functional potency to assess apoptosis and Bcl-2 mRNA reducing
expression compared in different groups as Tamoxifen, Naked Antisense, Antisense and Tamoxifen, Antisense and Lipofectamine, Antisense and Tamoxifen and Lipofectamine, Antisense and micelles/niosome, Antisense and Tamoxifen and micelles/niosome. The Real-Time RT PCR and Flow cytometry used to evaluate their effectiveness. On average, in two cell lines of breast cancer, Tamoxifen entered about 27% of the cells into the initial phase of apoptosis compared to the control sample. While, for the naked Antisense was 65%, the Antisense and Tamoxifen was 74%, the Antisense and Lipofectamine was 81%, the Antisense, Tamoxifen and Lipofectamine was 84%, the Antisense and micelles/niosome was 84%, the Antisense, Tamoxifen and micelles/niosome was 90%.

Conclusions

The designed ASO reduced the expression of *Bcl-2*. It was greater than the effect of the anticancer drug alone (Tamoxifen). It also had the synergistic effect with it. Tamoxifen entered about 27% the cells into the initial phase of apoptosis while ASO and Tamoxifen was 74%. The effectiveness of designed ASO would be different with various carriers. ASO and Lipofectamine entered about 81% the cells into the initial phase of apoptosis while ASO and micelles/niosome was 84%. The micelles/niosome efficiency was better than the Lipofectamine for transport of ASO into the cancer cells. It seems combination therapeutic approaches based on drug and gene therapy reveal a new vision in treatment of cancer.

Declarations

- Ethical Approval and Consent to participate

The ethics code is [http://ethics.research.ac.ir/IR.GOUAMS.REC.1398.067](http://ethics.research.ac.ir/IR.GOUAMS.REC.1398.067).

- Consent for publication

All authors agree to publish this study.

- Availability of supporting data

All data and supporting data are available.

- Competing interests

There is no conflict of interest between the authors.

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Dr. Ali Akbar Saffar Moghadam received the grant.

- Authors' contributions

The **Mohsen Sedaghat Janaghard** conducted the experiments. He also wrote the initial draft of the article.

The **Vahid Erfani-Moghadam** was adviser in the field of nano-carriers. He also helped in the article.

The **Ali Akbar Saffar Moghadam** was supervisor. He designed the study and wrote the article. He also is corresponding author.

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**Tables**

**Table 1**: Size, zeta potential and Polydispersity index of micelles/niosome with different components based on DLS.

| Treat                  | Particle size (nm) | PDI   | Zeta potential (mV) |
|------------------------|--------------------|-------|---------------------|
|                        | Micelles           | Noisome |                     |
| (T:S) 1040µM           | 16.8±1.0 (0.99%)   | 136.3±11.2 (0.01%) | 0.542 | -9.0     |
| (T:S) 1040µM:PEI*      | 15.1±0.8 (%99)     | 128.7±10.3 (%01)  | 0.504 | -8.1     |
| (T:S) 1040µM:PEI       | 16.8±1.0 (99%)     | 136.3±11.2 (0.01%) | 0.542 | -4.1     |
| PEI                    | 88.5±8.2 (100%)    |       | 0.875   | +0.6     |
| (T:S) 1040µM:PEI:ASO   | 21.5±1.6 (96%)     | 116.4±22.5 (0.4%) | 0.231 | -4.3     |
| (T:S) 1040µM:PEI:Tam   | 125.5±23.4 (100%)  |       | 0.287   | -10.8    |
| (T:S) 1040µM:PEI:ASO:Tam | 10.0±0.5 (0.07%) | 150.5±9.2 (0.93%) | 0.607 | -7.0     |

**Figures**
Figure 1

The Tamoxifen apoptosis in the MCF-7 cell line.

Figure 2

The Bcl-2 expression level in the MCF-7 cell line after 48 hr with different naked ASO concentrations
(Mean± Standard error, N=three, Statistical significance: P value * less than 0.05, ** less than 0.01, ***
less than 0.001).  

**Figure 3**

The ASO apoptosis in the MCF-7 cell line.
Figure 4

The Bcl-2 expression level in the MCF-7 cell line after 48 hr with different 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 5

The ASO, Niosome and PEI apoptosis in the MCF-7 cell line.
Figure 6

A: The ASO and Lipofectamine apoptosis in the MCF-7 cell line. B: The ASO and Tamoxifen apoptosis in the MCF-7 cell line. C: The ASO, Tamoxifen and Lipofectamine apoptosis in the MCF-7 cell line. D: The ASO, Tamoxifen and Niosome apoptosis in the MCF-7 cell line.

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