Enhancement of cisplatin sensitivity in human breast cancer MCF-7 cell line through BiP and 14-3-3ζ co-knockdown

TAHEREH KASHKOULINEJAD-KOUHI1, 2, SHAHROKH SAFARIAN1, BLANCA ARNAIZ2 and LAURA SAA3

1Department of Cell and Molecular Biology, School of Biology, College of Science, University of Tehran, Tehran 1417614411, Iran; 2Carbon Bionanotechnology Group and 3Biosensing Group, CIC biomaGUNE, 20014 San Sebastian, Spain

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Abstract. Cisplatin treatment confers the relative resistance to MCF-7 cells as compared to other breast cancer cell lines. One principal reason is that chemotherapeutic agents induce autophagy in these cells to inhibit apoptosis. Binding immunoglobulin protein (BiP), a master regulator of unfolded protein response (UPR) and 14-3-3ζ are two critical proteins upregulated in breast cancer rendering resistance to anticancer drugs. They also play pivotal roles in autophagy with crosstalk with the apoptotic pathways of UPR through certain regulators. Thus, BiP and 14-3-3ζ were selected as the candidate targets to enhance cell death and apoptosis. First, cisplatin resistance was induced and determined by MTT assay and qPCR in MCF-7 cells. Then, the apoptosis axis of UPR was activated by knocking down either BiP or 14-3-3ζ and overactivated by co-knockdown of BiP and 14-3-3ζ. Apoptosis assays were performed using flow cytometry, TUNEL assays utilized confocal microscopy followed by western blot analysis and caspase-3 and JNK activities were investigated to assess the outcomes. Finally, an autophagy assay followed by western blotting was performed to study the effects of co-knockdown genes on cell autophagy in the presence and absence of cisplatin. The present data indicated the enhancement of cisplatin sensitivity in MCF-7 cells co-knocked down in BiP and 14-3-3ζ compared with either gene knockdown. Upregulation of JNK and cleaved-PARP1 protein levels as well as caspase-3 and JNK overactivation confirmed the results. A marked attenuation of autophagy and Beclin1 as well as ATG5 downregulation were detected in co-knockdown cells compared to knockdown with either BiP or 14-3-3ζ. Cisplatin sensitization of MCF-7 cells through double-knockdown of BiP and 14-3-3ζ highlights the potential of targeting UPR and autophagy factors to increase the effect of chemotherapy.

Introduction

Breast cancer is the most commonly diagnosed cancer in women (1). Despite the considerable progress in cancer therapy in preceding years, chemotherapy, radiotherapy, and surgery remain the main approaches to treating breast cancer. However, cancer cells frequently use processes to escape cell death activated owing to chemotherapy (2). Furthermore, resistance occurs in endocrine therapy including tamoxifen for breast cancers, involving the same pathways as chemotherapeutic agents (3-6). In addition, nature-derived anticancer agents such as doxorubicin, actinomycin D, and bleomycin confer resistance to cells. Thus, it is important to develop strategies to oppose this obstacle (7-9).

One main characteristic of cancer is metabolic reprogramming and progressive dependence on glycolysis (10). This reliance on glycolysis leads to the accumulation of impaired glycosylated and misfolded proteins in the lumen of the endoplasmic reticulum (ER stress) and the following unfolded protein response (UPR) (11,12). In addition, the proliferative feature of cancer cells augments ER stress compared to the normal cells (13).

In fact, transformed cells hijack UPR as a survival strategy in the stressful microenvironment of tumors. Several studies have demonstrated the importance of UPR signaling in chemoresistance and tumor growth (14). Clinical indications have revealed that the UPR-driven chemoresistance occurs exclusively in breast cancers (15-18). UPR activation gives rise to chemoresistance by modifying drug targets, increasing efflux pump expression, drug detoxification and activating pro-survival and anti-apoptotic gene expression (19-21).

Numerous studies have suggested that UPR signaling molecules interact with well-known tumor suppressors and oncogenes such as binding immunoglobulin protein (BiP;
also known as GRP78) and 14-3-3ζ. In fact, some cancer cells display constant activation of the IRE-1α-XBP1 pathway (UPR survival pathway) by overexpression of BiP, which is anti-apoptotic (22-25).

Anticancer drugs are an additional source of stress (especially ER stress which promotes UPR) (26), activating the autophagy in tumor cells as a mechanism of resistance to chemotherapy (20,27). Autophagy is a stress-adaptive self-digestive process in which cellular components are enveloped within autophagosomes and degraded by lysosomal hydrolyses to eliminate misfolded proteins, reconstruct ER homeostasis and provide cells with integral nutrients to facilitate cancer cell survival and manage the metabolic stresses caused by anticancer treatments (20).

BiP is the master regulator of the ER stress. In benign breast lesions, the expression levels of BiP are low while as it progresses into breast cancer, BiP expression greatly increases (22,28,29). Solid tumor cells secrete BiP to activate various cellular processes such as tumor cell proliferation, angiogenesis, differentiation of bone marrow-derived mesenchymal stem cells, and the polarization of tumor-associated macrophages (30). An increased level of membrane-resident BiP was discovered in numerous types of cancers, including breast, ovarian, prostate, hepatocellular, gastric, melanoma, fibroblastoma, and renal cancers (31,32). Notably, increased expression of BiP could function in the protection of dormant tumor cells from stress problems, including chemotherapy (33) acting as a chaperone for aggregation-prone misfolded proteins leading to their degradation by autophagy. The mechanisms by which BiP upregulation is involved in chemoresistance is not fully understood. However, it appears that its overexpression serves an anti-apoptotic role by driving the acute requirement of protein synthesis to support different cellular functions such as tumor cell proliferation, migration, and differentiation (34-39). Numerous studies have revealed that BiP expression is linked with resistance of cancer cells to cytotoxic drugs, increased tumor progression, and worse patient prognosis (40), suggesting that its knockdown confers anticancer drug sensitization to the tumor cell (23,41-43). In turn, BiP expression increases due to treatment with several anticancer drugs (23,44-46).

Proteins of the 14-3-3 family play critical roles in several regulatory mechanisms in which they function by binding to the phospho-serine or the phospho-threonine motif in various proteins. They act in signal transduction, apoptosis, DNA repair, and malignant transformation (47-51). 14-3-3ζ is one of the members of this family, and increased expression has been revealed in early stages of several cancers including but not limited to liver cancer, head and neck squamous cell carcinoma, skin cancer, lung cancer and breast carcinoma (52-62).

More than 40% of advanced breast tumor tissues exhibit a high expression of 14-3-3ζ (57). 14-3-3ζ upregulation has been revealed to be directly associated with higher grades of tumors and a more advanced pathologic stage (58). Positive expression of 14-3-3ζ in early stages of cancer, such as hyperplasia, dysplasia and carcinoma, suggest that alteration in its expression is associated to the onset of tumorigenesis (58). Cell survival and anchorage-independent growth are intensified in breast cancer cell lines through 14-3-3ζ overabundance, whereas its knockdown results in cell sensitization to stress-induced apoptosis and decreased tumor xenograft size (54).

Apoptosis signal-regulating kinase 1 (Ask1), a serine-threonine-protein kinase in the JNK-apoptosis pathway of UPR (63-65), can be negatively activated by the phosphorylation of 14-3-3ζ during excess ER stress as well as oxidant stress which is the case in the tumor environment. This association may highly weaken ASK1 potential to activate JNK and apoptosis signaling (66-69).

Cisplatin, a chemotherapeutic agent, is used to treat various malignancies, including lung, ovary and breast. There are several studies on the effects of cisplatin on the MCF-7 cell line (70-73). However, cisplatin exhibits limited therapeutic efficacy. Several studies have reported that cisplatin activates autophagy which acts as a survival factor to counteract cisplatin-induced apoptosis (72). Therefore, more effective therapeutic options are required.

The present study promoted the apoptotic pathway of UPR (ASK1/JNK axis) through BiP knockdown according to previous studies (23,74-76). 14-3-3ζ has been revealed to inactivate ASK1 and prevent apoptosis triggers (66-69). Previous research has revealed that 14-3-3ζ dissociation from ASK1 may accelerate cell death in cancer (66). Thus, in the present study it was hypothesized that the concurrent knockdown of BiP and 14-3-3ζ may have a superior impact on cisplatin sensitization of MCF-7, as a human breast cancer carcinoma cell line, compared to knockdown of either BiP or 14-3-3ζ as previously reported.

Materials and methods

Cell culture and reagents. The MCF-7 breast cancer cell line was acquired from the American Type Culture Collection (ATCC). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml; all from Gibco; Thermo Fisher Scientific, Inc.). The cell line was cultured in a humidified incubator supplying 5% CO₂ at 37˚C. For the experiments, the cells were cultured in serum-free medium. The cell line was cultured at 37˚C in a humidified incubator supplying 5% CO₂. Cells were passaged every 3-4 days and treated in the exponential growth phase. The MCF-7 cells were tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza Group, Ltd.).

Cell transfection. To silence BiP and 14-3-3ζ genes, small interfering (si)RNAs (Table I) against BiP and 14-3-3ζ as well as scrambled siRNA were purchased from GE Healthcare Dharmacon, Inc. (97% purity) and used at a final concentration of 20 nM for MCF-7 cell transfection using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, MCF-7 cells (5x10⁶ cells/well) were plated on 6-well plates containing an antibiotic-free medium. They were incubated overnight at 37˚C. To perform transfection, Lipofectamine RNAiMAX and siRNA (3:1) were added in serum-free medium. The mixture was incubated at room temperature for 20 min to allow the siRNA-lipofectamine complex to be formed. The mixture was then added to the cells in a proper volume of antibiotic- and serum-free DMEM to obtain a final concentration of 20 nM for each siRNA. After incubation for 6 h at 37˚C, DMEM supplemented with serum was added to the cells and were incubated with siRNA/ Lipofectamine at 37˚C for 72 h.
RNA purification and reverse transcription-quantitative PCR (RT-qPCR). To extract total cellular RNA, Ribospin II RNA extraction kit (GeneAll Biotechnology Co., Ltd.) was used following the manufacturer’s instructions. The total RNA concentration and purity were quantified by applying the NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized from 1 µg of total RNA employing RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. RT-qPCR for RNA expression quantification was carried out on Applied Biosystems (StepOnePlus; Thermo Fisher Scientific, Inc.) using QuantiTect SYBR-Green (Qiagen GmbH) with a total reaction volume of 10 µl and the initial denaturation at 95˚C for 15 min, followed by 40 cycles of PCR at 95˚C for 15 sec, 63˚C for 10 sec, 72˚C for 20 sec. Comparative analysis by 2^(-∆∆Cq) was used to compare the expression of target genes (77). Relative expression software tool (REST) was applied for comparison (78), and the statistical analysis of relative expression results in real-time PCR. The related primers (Ki-67, cyclin D1, Rb, p21, BiP and 14-3-3ζ) (Table II) were obtained from Sigma-Aldrich; Merck KGaA. GAPDH served as the reference gene. All reactions were performed in triplicate.

Cell viability assay. Cell viability was quantified by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (≥97% purity; Sigma-Aldrich; Merck KGaA) assay. Basically, cleavage of tetrazolium salts by mitochondrial succinate reductase, which is active in viable cells, leads to formazan dye formation. Cisplatin resistance was induced via culturing the cells in solutions by increasing the concentration of cisplatin (from 0.03 to 15 µM) for 3 months. To identify viability of MCF-7 cells in response to cisplatin (99.9% purity; Sigma-Aldrich; Merck KGaA) exposure, the cells were seeded at a concentration of 8x10^3 cells/well in 96-well plates. They were dedicated time to attaching for 8 h and treated with cisplatin for 48 h. Next, MTT (5 mg/ml) was added to each well, and the cells were incubated for 2 h at 37˚C. DMSO was added to the wells to dissolve the formed formazan crystals. The absorbance was then measured at 570 nm, using a plate reader (Cytation Multi-Mode; BioTek Instruments, Inc.). Cell viability was calculated as the percentage of viable treated cells against the control group. Each treatment of cisplatin or untreated control was performed in triplicate.

Cell lysate and immunoblotting. The MCF-7 cells were seeded into 6-well plates at 5x10^5 cells/well and incubated overnight at 37˚C before being subjected to transfection with BiP siRNA, 14-3-3ζ siRNA and BiP+14-3-3ζ siRNAs for 72 h at 37˚C. The cells were then treated with cisplatin for 48 h. Cold RIPA lysis buffer [150 mM NaCl, 50 mM Tris, 1 mol/l EDTA, Triton X-100, 0.1% sodium dodecyl sulphate, 1 mol/l phenyl methyl sulfonyl fluoride (pH 8.0); Merck KGaA] and fresh protease inhibitor (Sigma-Aldrich; Merck KGaA) were utilized to produce the whole cell lysate. The extracts were sonicated for 15 sec followed by the centrifugation at 8,600 x g for 20 min at 4˚C to discard cell debris. Bradford assay (Bio-Rad Laboratories, Inc.) was administered to assess the protein concentration. Then, the total protein (20 µg) was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich; Merck KGaA). After blocking the membrane with 5% bovine serum albumin TBST [1% Tween-20 in 20 mmol/l Tris-buffered saline (pH 7.6)] at 4˚C for 1 h, the bands were probed with monoclonal anti-BiP.
(1:5,000; product no. SAB5200168; Sigma-Aldrich; Merck KGaA), anti-14-3-3ζ (1:5,000; product code ab110315 from Abcam), anti-c-Jun (1:2,000; product code ab129456), anti-ATG5 (1:5,000; product code ab238092), anti-PARP1 (1:5,000; product code ab110915), anti-cleaved PARP1 (1:2,000; product code ab110315 all from Abcam), anti-c-Jun (1:2,000; product no. 9165 Cell Signaling Technology, Inc.) and anti-Beclin1 (1:5,000; product no. SAB5300513; Sigma-Aldrich; Merck KGaA). GAPDH (1:2000; product code ab125247; Abcam) served as the loading control. The horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; product code ab6721) and rabbit anti-mouse IgG (1:5,000; product code ab6728; both from Abcam) were used as secondary antibodies. The membrane was incubated with secondary antibodies for 1 h at room temperature. The bound antibodies were visualized through an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech; Cytiva) and a Gel Logic 4000 Pro device (Bruker BioSpin Corporation). The quantification of the band intensity was conducted with ImageJ software (1.52c; National Institutes of Health).

Apoptosis assay using flow cytometry. A flow cytometric assay was conducted using an FITC-Annexin V apoptosis detection kit (cat. no. 556547; BD Biosciences) according to the manufacturer's instructions. To perform the apoptosis assay, the cells were seeded overnight at a concentration of 2x10^5 in 24-well plates and transfected with BIP siRNA, 14-3-3ζ siRNA and BiP+14-3-3ζ siRNAs concurrently for a 72-h incubation followed by 2 µM cisplatin treatment for 48 h. Cells were harvested, and centrifuged at 1,500 x g for 5 min at 4°C. The cells were then washed with 1X PBS and 1X binding buffer and were incubated with 5 µl of Annexin V for 15-20 min at room temperature in darkness. After the incubation, the cells were washed and stained with propidium iodide (PI) staining solution on ice for 10 min. The cells were immediately applied to determine different cell populations; early apoptotic cells (PI-/Annexin V +); late apoptotic cells (PI +/Annexin V +) and viable cells (PI-/Annexin V-). Apoptotic cells were assessed as 300 µl of 70% ethanol for 45 min on ice. Then, the cells were then washed twice with PBS. Subsequently, 50 µl of DNA labeling solution (10 µl of TdT reaction buffer, 0.75 µl of TdT Enzyme, 8 µl of Br-dUTP, 32.5 µl of ddH2O) was added to the cells and incubated at 37°C for 1 h. The cells were then washed twice with 300 µl rinse buffer and the antibody solution (5 µl of Anti-BrdU-Red antibody, 95 µl of rinse buffer) was prepared and added after washing the cells and incubated for 30 min at room temperature in the dark. The 300 µl of 7-AAD/RNase solution was added at the end. 4',6-Diamidino-2-phenylindole (DAPI; product no. 4083; Cell Signaling Technology, Inc.) at 1 µg/ml prepared in PBS was applied to stain nucleus. The cells were incubated with DAPI for 5 min at room temperature in the dark before mounting to the coverslips with ProLong Gold Antifade reagent (cat. no. NCM0581708; Thermo Fisher Scientific, Inc.). Confocal microscopy (LSM 510 META; Zeiss AG) was used to observe Red-dUTP-labeled DNA. Fluorescence intensity was assessed in 20 randomly selected microscopic fields of view per sample using a microscope-confocal (magnification, x60). ImageJ software v1.52c was utilized to quantify fluorescence in each image.

Caspase-3 activity assay. Caspase-3 activity was investigated using the Caspase-3 Activity Detection kit according to the producer's descriptions (product code ab39383; Abcam). To identify caspase-3 activity, the assay was carried out in 96-well plates at a density of 8x10^3 cells/well. Cell lysates were prepared following siRNA transfection and cisplatin treatment. Assays were conducted in 96-well plates. Briefly, 10 µl protein of cell lysate was incubated with 10 µl caspase-3 substrate (Ac-DEVDF-pNA, 2 mM) in 80 µl of reaction buffer [10% glycerol, 1% NP-40, 137 mM NaCl and 20 mM Tris-HCL (pH 7.5)] at 37°C for 5 h. ODs were measured using an ELISA reader (Microlisa Plus; Micro Lab Instruments) at 405 nm. The assay and preparation of the standard curve were performed following the manufacturer's instructions. All experiments were performed in triplicate.

SAPK/JNK kinase assay. JNK activity was performed as described by the manufacturer (product no. 8794; Cell Signaling Technology, Inc.). In short, the cells were seeded in 6-well plates at a concentration of 5x10^5 cells/well and after transfection and treatment with cisplatin were collected under non-denaturing conditions, lysed on ice, and centrifuged at 8,600 x g for 10 min at 4°C. Then, 20 µl phospho-JNK rabbit monoclonal antibody (product code 4306; Cell Signaling Technology, Inc.) linked to agarose beads was incubated with gentle rocking an overnight at 4°C with 200 µl of cell lysates to precipitate the JNK enzyme. Then, necessary buffers, c-Jun substrate (2 µl) and adenosine triphosphate (200 µl) were added, and the reaction mixture was incubated for 30 min at 30°C. The reaction was terminated by adding 25 µl of 4X SDS (>90% purity; Sigma-Aldrich; KGA) sample buffer, and the samples (20 µg) were loaded onto 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich; KGA). The membrane was then subjected to phospho-c-Jun antibody (1:1,000, product no. 12598; Cell Signaling Technology, Inc.) for 1 h at 4°C to determine JNK-induced phosphorylation of c-Jun substrate at Ser63 and Ser73 residues. Then, anti-rabbit...
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

...of the fold change in expression among untreated and different treatments. The data were presented as the means ± SD (standard deviation) of at least three experiments. The statistical analyses were performed with IBM SPSS Statistics software (version 22; IBM Corp.).

**Results**

*Induction of cisplatin resistance in MCF-7 cells.* Cisplatin with increasing concentration was added to the cells twice a week after reseeding. At the end of each month, cell viability was assessed by MTT assay. IC50 values for MCF-7 and resistant MCF-7 cells were 0.65 and 2.8 µM respectively (P<0.001). The IC50 value for resistant cells was approximately four times higher than non-resistant cells (Fig. 1). As the formation of drug resistance to cisplatin in human breast cancer MCF-7 cells is characterized by changes in the expression of proteins involved in the control of apoptosis, the cell cycle, proliferation, and adhesion (80-83) the expression levels of p21, cyclin D1, Rb and Ki-67 were studied to determine whether the expression profiles of these genes are consistent with the profile of the resistant cells. Ki-67, cyclin D1 and Rb exhibited a reduced expression while p21 was upregulated in cis-resistant MCF-7 cells (Fig. 2). These experiments were performed according to a previous study (84) to render cisplatin resistant in MCF-7 cells with which our results were consistent.

*Increased apoptosis in BiP and 14-3-3 ζ co-knockdown cells compared to cells knocked down by either gene.* BiP and 14-3-3 ζ protein levels before and after the induction of cisplatin resistance were analyzed by performing western blotting. An increase in BiP protein expression level (P<0.01) was observed following cisplatin resistance (Fig. 3A) which could be associated with BiP induction during chemotherapy (23,43-45). In addition, 14-3-3 ζ protein was overexpressed after resistance induction (P<0.001) (Fig. 3B) which could be as a result of the protective effects of 14-3-3 ζ against cellular stresses, here exerted through chemotherapy exposure (60,68). Considering this evidence that cisplatin can induce apoptosis, and that...
Figure 3. (A) Western blot analysis of BiP protein before and after cisplatin resistance induction revealed BiP upregulation at the end of 3 months. *P<0.01. (B) Western blot analysis of 14-3-3ζ before and after resistance induction indicated 14-3-3ζ overexpression at the end of 3 months. ***P<0.001. BiP, binding immunoglobulin protein.

Figure 4. (A) RT-quantitative qPCR of BiP expression in cisplatin-resistant MCF-7 cells transfected with scrambled siRNA or siRNA against BiP or BiP+14-3-3ζ. Data are expressed as the mean ± SD normalized to GAPDH. Results were averaged from three independent experiments measured in triplicate. The significance of differences was calculated using one-way ANOVA. **P<0.01. (B) Western blot detection of BiP in cisplatin-resistant MCF-7 cells transfected with scrambled siRNA, siRNA against BiP or BiP+14-3-3ζ. The intensity of the bands was calculated using ImageJ software (National Institutes of Health). GAPDH served as the loading control. (C) RT-PCR of 14-3-3ζ expression in cisplatin-resistant MCF-7 cells transfected with scrambled siRNA, siRNA against 14-3-3ζ and BiP+14-3-3ζ. Data are expressed as the mean ± SD normalized to GAPDH. Results were averaged from three independent experiments measured in triplicate. The significance of differences was calculated using one-way ANOVA. **P<0.01. (D) Western blot detection of 14-3-3ζ in cisplatin-resistant MCF-7 cells transfected with scrambled siRNA, siRNA against 14-3-3ζ and BiP+14-3-3ζ. The intensity of the bands was calculated using ImageJ software. GAPDH served as the loading control. BiP, binding immunoglobulin protein; si, small interfering.
BiP or 14-3-3ζ positive expression possibly leads to cisplatin resistance in breast cancer cells through anti-apoptotic pathways, apoptosis induction in cisplatin-resistant MCF-7 cells knocked down in BiP, 14-3-3ζ and BiP+14-3-3ζ was assessed. To achieve the results, the cells were transfected with BiP or 14-3-3ζ siRNA and co-transfected with BiP and 14-3-3ζ siRNAs. Quantitative PCR and western blot analysis revealed that downregulation of mRNA and protein levels of BiP and 14-3-3ζ occurred when the genes were inhibited or co-inhibited in the cells (Fig. 4A-D). These results revealed that there was no conflict between the two siRNAs, when they were utilized in co-transfection of the cells.

Flow cytometric results revealed that co-knockdown with both siRNAs led to moderately increased apoptosis in the absence of cisplatin, which was ~1.8% in the co-inhibited cells, 0.35% in BiP knockdown cells, and 0.75% in 14-3-3ζ knockdown cells (normalized with scrambled). Data are expressed as the mean ± SD. The significance of differences was calculated using one-way ANOVA and post hoc, Tukey’s test. *P<0.05 and **P<0.01. BiP, binding immunoglobulin protein; si, small interfering.

Figure 5. Quantification of apoptosis. BiP and 14-3-3ζ co-knockdown slightly increased the apoptosis of cisplatin-resistant MCF-7 cells when cisplatin is absent. Flow cytometric analysis revealed a mild increase in apoptosis induction of cisplatin-resistant MCF-7 cells transfected with siBiP+si14-3-3ζ compared with either siBiP or si14-3-3ζ in the absence of cisplatin. The quantification of data is presented in the graph. Data were averaged from three independent experiments. Apoptosis percentage was calculated by the sum of the two quadrants on the right in each FACS figure. Co-knockdown with both siRNAs led to moderately augmented apoptosis in the absence of cisplatin, which was ~1.3% in co-inhibited cells, 0.2% in BiP-knockdown cells and 0.4% in 14-3-3ζ knockdown cells (normalized with scrambled). Data are expressed as the mean ± SD. The significance of differences was calculated using one-way ANOVA and post hoc, Tukey’s test. *P<0.05 and **P<0.01. BiP, binding immunoglobulin protein; si, small interfering.
assay was performed. The TUNEL assay demonstrated consistent results with the flow cytometric data confirming a considerable increase in the apoptosis of co-knockdown cells in the presence of cisplatin (Figs. 7 and S1).

These results strongly supported the rationale that the overexpression of 14-3-3ζ in MCF-7 cells may prevent the intense UPR-induced apoptosis, and its downregulation along with BiP could decrease drug resistance more potentially in comparison to the knockdown of each protein.

Co-knockdown of BiP and 14-3-3ζ markedly increases the protein levels of cleaved PARP1 and JNK as well as caspase-3 and JNK activity in comparison with knockdown of each gene alone. Apoptosis involves the mitochondrial (intrinsic) and death receptor-mediated (extrinsic) pathways, using caspases as the principal players of apoptosis in a series of distinct steps (85). The present results indicated that the overexpression of 14-3-3ζ in MCF-7 cells can prevent intensive apoptosis induced by cisplatin in the BiP-knockdown cells, suggesting that co-knockdown of BiP and 14-3-3ζ may increase the expression of JNK and caspase-3 activity to induce apoptosis. To this end, a caspase-3 activity assay followed by cleaved PARP1 western blotting were carried out. PARP1 is a downstream target of caspase-3 and is cleaved in the DEVD214 site leading to the formation of 24 kDa and 89 kDa fragments. Thus, the presence of cleaved PARP1 is an indicator of caspase-3 activity (86). The results revealed increased caspase-3 activity (P<0.01) (Fig. 8) and cleaved PARP1 (P<0.05) levels (Fig. 9A and C) in co-knockdown cells as compared with knockdown ones by either gene. Then, the JNK protein level as well as its activity in co-knockdown and knockdown cells were examined. As revealed in Fig. 9A-C, the levels of JNK protein (P<0.01) and phosphorylated c-Jun (P<0.01) increased to a greater extent in co-knockdown cells compared with knockdown cells with either BiP or 14-3-3ζ siRNA, suggesting that the reduced protein levels of 14-3-3ζ

![Image of Figure 6](image-url)

**Figure 6.** Quantification of apoptosis. Flow cytometric analysis revealed apoptosis of MCF-7 cells transfected with scrambled siRNA or siRNA against BiP or 14-3-3ζ or siBiP+si14-3-3ζ in the presence of cisplatin treatment (2 µM). Apoptosis significantly increased in the cells co-knocked down with siBiP+si14-3-3ζ compared to the cells knocked down with siBiP or si14-3-3ζ in the presence of cisplatin. The quantification of data is presented in graph. Apoptosis percentage was calculated by the sum of the two quadrants on the right in each FACS figure. This apoptosis induction was ~13% in BiP- and 15% in 14-3-3ζ-knockdown cells, and 32% in co-inhibited cells (normalized with cis-scrambled). *P<0.01 for the differences between co-knockdown and single-knockdown apoptosis. Data were averaged from three independent experiments. Data are expressed as the mean ± SD. The significance of differences was calculated using one-way ANOVA and post hoc, Tukey's test. **P<0.01 and ***P<0.001. BiP, binding immunoglobulin protein; cis, cisplatin; si, small interfering.
and BiP may play a key role in the overactivation of JNK and caspase-3 as pro-apoptotic proteins.

**BiP and 14-3-3ζ co-knockdown attenuates autophagy in the presence of cisplatin.** Cisplatin counteracts breast cancer apoptosis by autophagy induction. BiP and 14-3-3ζ play important roles in the autophagy (76,88). The overexpression of BiP aids in cancer cell survival by increasing autophagy as a mechanism of resistance to chemotherapy (76), whereas 14-3-3ζ overexpression reduces the rate of autophagy (88). Thus, the effect of BiP/14-3-3ζ double-knockdown on autophagy in absence as well as the presence of cisplatin was investigated. A decrease in autophagy in BiP-knockdown and an increase in 14-3-3ζ-knockdown cells in the absence of cisplatin were observed which is based on previous studies (76,88-90). The co-knockdown cells exhibited a slight increase in autophagy, while the pattern of autophagy was different in the presence of cisplatin (Fig. 10). The present data revealed a reduction in autophagy in all knockdown cells but more significantly in co-knockdown cells, suggesting cisplatin can markedly decrease the autophagosome formation in MCF-7 in BiP and 14-3-3ζ double-downregulation (P<0.001) compared to targeting either gene (P<0.05) (ImageJ quantification).

**Figure 7.** Representative images from TUNEL assay revealing apoptosis of cisplatin-resistant MCF-7 cells transfected with siBiP, si14-3-3ζ, and siBiP+si14-3-3ζ in the presence of cisplatin treatment (2 µM). DAPI was used to stain the nucleus. Scale bar, 20 µm. BiP, binding immunoglobulin protein; cis, cisplatin; si, small interfering.

**Figure 8.** Caspase-3 activity assay revealed that caspase-3 was significantly activated following cisplatin treatment in co-knockdown cells compared to single knockdown (fold change; **P<0.01, ***P<0.001). BiP, binding immunoglobulin protein; cis, cisplatin; si, small interfering.
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To further confirm the autophagy results, the protein levels of ATG5 with and without cisplatin treatment in different transfected cells was investigated. ATG5 is essential for autophagosome formation. ATG5 knockdown or knockout can lead to downregulation or inhibition of autophagy, suggesting that ATG5 plays a central role in autophagy (91). The results revealed a reduction in ATG5 protein level in co-knockdown cells compared to single-knockdown cells while the protein levels of Beclin-1 exhibited no change in the western blots. Cleaved PARP1 which is an indicator of apoptosis promoted by caspase-3 activity decreased in the co-knockdown cells compared to the single-knockdown cells. JNK could regulate the crosstalk between autophagy and apoptosis through phosphorylation of Bcl-2 (92). The stress-induced activation of JNK (here exerted by BiP knockdown and enhanced via 14-3-3ζ downregulation), can phosphorylate Bcl-2 leading to dissociation of Bcl-2 and Beclin1 (92). Beclin1, a central protein in autophagosome formation, cross-regulates apoptosis and autophagy which can be cleaved by caspase-3 in two cleavage sites during apoptosis. It can activate apoptosis in apoptotic-competent cells by inactivating autophagy (92). For this reason, the effects of apoptosis induced through the effects of BiP and 14-3-3ζ double-knockdown on Beclin1 expression were investigated.

Cisplatin considerably decreases Beclin1 protein level in co-knockdown cells. JNK could regulate the crosstalk between autophagy and apoptosis through phosphorylation of Bcl-2 (92). The stress-induced activation of JNK (here exerted by BiP knockdown and enhanced via 14-3-3ζ downregulation), can phosphorylate Bcl-2 leading to dissociation of Bcl-2 and Beclin1 (92). Beclin1, a central protein in autophagosome formation, cross-regulates apoptosis and autophagy which can be cleaved by caspase-3 in two cleavage sites during apoptosis. It can activate apoptosis in apoptotic-competent cells by inactivating autophagy (92). For this reason, the effects of apoptosis induced through the effects of BiP and 14-3-3ζ double-knockdown on Beclin1 expression were investigated. Thus, the expression levels of Beclin1 in the absence and presence of cisplatin were assessed. The present results indicated a significant decrease of Beclin1 protein in co-knockdown cells compared to cells knocked down with either siRNA in absence.
the presence of cisplatin (P<0.05, P<0.01) (Fig. 9A and C). This finding indicated that cisplatin may change the cell fate from autophagy to apoptosis in knockdown cells by reducing Beclin1. No difference in the expression level of Beclin1 was observed in the absence of cisplatin (Fig. 9A and C).

Discussion

BiP belongs to the HSP70 family which has pivotal functions in stress during oncogenesis. In addition to contributing to the protein folding and impeding protein aggregation, BiP functions as a regulator of ER stress signaling. In normal and non-stressed cells, BiP binds to three sensors in the ER membrane, IRE-1, PERK and ATF6, rendering them inactive. Whereas under physiological stress, following which ER function is disturbed, BiP is dissociated from the sensors, rendering them active to send signals to the nucleus and trigger UPR (63). This protects cells and tissues against pathological conditions, such as arteriosclerosis, neurotoxic stress and myocardial infarction. To highlight the importance of BiP for survival of stressed cells such as in cancer, one study revealed that the mice with heterozygous BiP, which have half of the expression level of BiP than the wild-type (WT), exhibited equivalent growth status with WT mice, while they had markedly lower tumor development (93).

BiP has been revealed to be increased in cancer metastasis, and for this reason its downregulation diminishes tumor cell invasion in vitro and in vivo (93). In cancer cells, following the accumulation of misfolded proteins and homeostasis perturbation, BiP dissociates from IRE-1 which then dimerizes and promotes the survival pathway of UPR, increasing the transcription of chaperones and quality control proteins. Through BiP knockdown, ER stress persists by virtue of BiP depletion, leading to IRE-1 oligomerization. This induces ASK1/JNK activation and signals apoptosis (63).

14-3-3ζ overexpression occurs in an early stage of breast cancer and contributes to the transformation of human mammary epithelial cells and the progression of breast cancer (47-51). There are several lines of evidence that have revealed that 14-3-3ζ binds to ASK1 and inactivates it (66-68). Zhang et al indicated that ASK1 associates with 14-3-3ζ through phosphoSer697 (66). They determined that the dissociation of 14-3-3ζ/ASK1 augments ASK1-induced apoptosis through JNK and caspase-3 activation and that the high expression of 14-3-3ζ halts ASK1-induced apoptosis in several cell lines.

Jiang et al indicated the cisplatin counteracts breast cancer (MCF-7 and MDA-MB-231 cells) apoptosis by autophagy induction (72). Since BiP and 14-3-3ζ have roles in autophagy and JNK and Beclin1 crosstalk between apoptosis and autophagy, we examined the impact of the double-knockdown of these two genes on cisplatin treatment of MCF-7 cells.

Conversely, it has already been well-established that as a result of aberrant protein folding in the ER environment, cancer cells activate autophagy, a stress-adaptive self-degradative process (94). In fact, cancer cells rely on autophagy for survival more than normal cells. For instance, PERK (one sensor of the UPR pathway)-eIF2α phosphorylation is essential to decrease protein synthesis to activate autophagy (95,96). It was previously reported that the activation of autophagy and cell survival upon ER stress can be achieved by the IRE-1α-JNK pathway, which has been suggested to act in the interplay between cancer cell death and as a process of chemotherapy resistance (97). Autophagy promotes cancer cell survival during chemotherapy to deal with metabolic stresses induced by chemotherapeutic drugs (20). In addition, in breast cancer cells, resistance to endocrine therapy including fulvestrant and tamoxifen is the outcome of the dynamic network between UPR activation, apoptosis and autophagy (3-5).

JNK plays a role in the regulation between apoptosis and autophagy through the phosphorylation of Bcl-2. Activating JNK disrupts Bcl-2 and Beclin1 interaction. Beclin1 is the cross-regulator of autophagy and apoptosis. Moreover, caspase-3 can cleave Beclin1 to change the cell fate from...
autophagy to apoptosis (98,99). The C-terminal fragment of Beclin1 can then move to the mitochondria and induce mitochondrial membrane permeability and apoptosis (100).

Taking all these into consideration, we assumed that 14-3-3ζ combined with BiP may be a more effective co-target to enhance cisplatin sensitivity compared to targeting either gene. Cisplatin-induced resistant MCF-7 cells were generated by a three-month cisplatin treatment in constantly increasing concentrations. A viability MTT assay before and after the induction of resistance was performed. The results indicated that the IC₅₀ of cells increased by 4-fold in the MCF-7-resistant cells when compared to the MCF-7 cells. The cellular adaptation was investigated by ascertaining the expression levels of four critical genes (Ki-67, cyclin D1, Rb and p21), the expression of which changed following cisplatin resistance consistent with a previous study (84). The Ki-67 protein is a marker for cell proliferation, present during all active phases of the cell cycle (G₁, S, G₂, and mitosis), but is absent in resting (quiescent) cells (G₀) (80) cyclin D1 is overexpressed in breast carcinoma (81) and has been shown to be required for progression through the G₁ phase of the cell cycle to induce cell migration (101) and angiogenesis (102). Hyperphosphorylation of Rb promotes proliferation and plays a role in tumor progression (82) and p21 protein is a cyclin-dependent kinase inhibitor (CKI) that is capable of inhibiting all cyclin/CDK complexes (83).

Furthermore, BiP and 14-3-3ζ protein expression levels were assessed before and after resistance. The results revealed an increase in BiP and 14-3-3ζ expression levels after cisplatin resistance induction, which could be a result from the fact that chemotherapy induces BiP (23,43-45) and the fact that 14-3-3ζ plays protective roles during cellular stresses promoted for example during chemotherapy (59,68). This protective role of 14-3-3ζ was confirmed when it was silenced by siRNA, revealing, it sensitized the cells to stress-induced apoptosis (59,68). The resistant MCF-7 cells were cultured and utilized in the next experiments. To validate the downregulation of BiP and 14-3-3ζ, qPCR was used revealing ~60% decrease in the mRNA levels in the single and co-transfected cells. To support the PCR results, the reduction of the protein levels was confirmed for BiP and 14-3-3ζ using western blot analysis.

Then, the MCF-7 cells were transfected with either BiP or 14-3-3ζ siRNA or both and apoptosis in the absence and presence of cisplatin was investigated. Based on the flow cytometric and confocal microscopic results, BiP and 14-3-3ζ double-knockdown could increase the sensitivity of resistant MCF-7 cells exposed to cisplatin treatment compared to downregulation of either gene. Furthermore, the activities of caspase-3 and JNK were assessed and the results revealed higher expression in the combined BiP with 14-3-3ζ group compared to single knockdown of BiP or 14-3-3ζ and these results were confirmed with phosphorylated c-Jun and cleaved PARP1 protein levels. These results revealed that apoptosis may be enhanced in the double-knockdown of BiP and 14-3-3ζ compared to targeting each gene alone.

The knockdown effects of either gene in autophagy is paradoxical, with BiP knockdown preventing autophagy formation (76) and 14-3-3ζ downregulation inducing autophagy (88). Thus, the effects of cisplatin were assessed in the three types of knockdown cells to determine whether autophagy changes after BiP/14-3-3ζ co-knockdown. The results revealed a slight increase of autophagy in the co-inhibited cells in the absence of cisplatin and a significant decrease in the two single-knockdown (BiP or 14-3-3ζ) and co-knockdown (BiP+14-3-3ζ) cells in the presence of cisplatin. This reduction was more prominent in the latter case, supporting the critical impact of BiP and 14-3-3ζ on autophagy in resistant MCF-7 cells following cisplatin treatment. Collectively, this indicated that co-knockdown of BiP and 14-3-3ζ leads to autophagosome attenuation in the presence of cisplatin in MCF-7 cells. The autophagy-related results were confirmed by assessing the protein levels of Beclin1 and ATG5 in the absence and presence of cisplatin which revealed a significant decrease in their levels in the co-knockdown compared to the knockdown cells. This was consistent with a previous study which demonstrated that activation of IRE-1/JNK can promote apoptosis by inactivating autophagy (92) and BiP/14-3-3ζ double-knockdown could enhance this pathway.

MCF-7, an HR-positive and HER2-negative cell line, is not a perfect chemotherapy model as are triple negative cell lines (103). The main obstacle for these cancers is that the chemotherapeutic agents including cisplatin increase autophagy. Autophagy is one of the chemotherapy resistance mechanisms in breast cancer (72). Thus, certain processes involved in chemotherapy resistance need to be tackled to overcome this problem. The ability of a protein to perform its function depends on its potential to interact with other proteins. Thus, a system that can circumvent a potential chemotherapy resistance component, may influence other associated proteins. For example, it is well known that PIK3CA, with which Beclin1 interacts, is mutated in some breast cancers related to overactivation of PI3K/Akt signaling pathways. The mutations in PIK3CA confer resistance to cisplatin in some cancers (104). Therefore, if Beclin1 is downregulated, it could disrupt the whole complex to prevent the overexpressed signals in cancer. A model from the STRING interaction network (79) which revealed some of the proteins interacting with Beclin1 which may be affected by Beclin1 downregulation is presented in Fig. 11.
In conclusion, cisplatin resistance occurs relatively often in breast cancer carcinoma and the MCF-7 cell line, and for this reason finding a method to overcome this obstacle of chemotherapy tolerance is critical. This resistance may occur as a consequence of autophagy induced by cisplatin. Thus, detecting the pivotal factors involved in autophagy may give rise to improved treatment. BiP/GRP78 and 14-3-3ζ proteins play roles in autophagy, and their overexpression leads to chemotheraphy resistance. The dissociation of these proteins from their partners (BiP from IRE-1 and 14-3-3ζ) from ASK1) relay UPR-apolotic signal in a series of pathways. By targeting both genes concurrently, the MCF-7 cells were markedly sensitized to cisplatin. The results indicated that this sensitization was related to attenuation of autophagy.

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Availability of data and materials
All data generated and analyzed during this study are included in this article.

Authors’ contributions
TKK designed and performed the experiments, prepared the figures and/or tables, analyzed the data and wrote the manuscript. SS supervised the research, designed the experiments, analyzed the data, wrote the study and reviewed drafts of the study. BA reviewed drafts of the manuscript and supported the research techniques for flow cytometry and its analysis. LS reviewed drafts of the paper and technically supported the research for western blot experiments. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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