Knockdown of the cytoprotective chaperone, clusterin, chemosensitizes human breast cancer cells both in vitro and in vivo

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Abstract
Clusterin is a stress-associated cytoprotective chaperone up-regulated by various apoptotic triggers in many cancers and confers treatment resistance when overexpressed. The objectives of this study were to evaluate clusterin expression levels in human breast cancer and to determine whether antisense oligonucleotides or double-stranded small interfering RNAs (siRNA) targeting the clusterin gene enhance apoptosis induced by paclitaxel. Clusterin immunostaining was evaluated in a tissue microarray of 379 spotted breast cancers. The effect of hormone withdrawal, paclitaxel treatment, clusterin antisense oligonucleotide (OGX-011), and siRNA treatments on clusterin expression was examined in MCF-7 and MDA-MB-231 cells. Northern, quantitative real-time PCR, and Western analyses were used to measure change in clusterin mRNA and protein levels. The effect of OGX-011 or siRNA clusterin treatment on chemosensitivity to paclitaxel was done in both cell lines in vitro, whereas the ability of OGX-011 to chemosensitize in vivo was evaluated in athymic mice bearing MCF-7 tumors. Clusterin was expressed in 62.5% of tumors within the tissue microarray. Clusterin expression increased after estrogen withdrawal and paclitaxel treatment in vitro in MCF-7 cells. OGX-011 or siRNA clusterin decreased clusterin levels by >90% in a dose-dependent, sequence-specific manner and significantly enhanced chemosensitivity to paclitaxel in vitro. When combined, OGX-011 or siRNA clusterin reduced the IC50 by 2-log compared with paclitaxel alone. In vivo administration of OGX-011 enhanced the effects of paclitaxel to significantly delay MCF-7 tumor growth. These data identify clusterin as a valid therapeutic target and provides preclinical proof-of-principle to test OGX-011 in multimodality therapies for breast cancer. [Mol Cancer Ther 2005;4(12):1837–49]

Introduction
Breast cancer is the most common malignancy and second leading cause of cancer mortality of women in the United States with an estimated 211,240 new cases and 40,870 deaths in 2005 (1). Management of metastatic breast cancer includes systemic therapies, such as taxane-based chemotherapeutics, including paclitaxel and docetaxel, which are among the most efficacious cytotoxics. These taxanes improve overall survival, time to progression, and overall response rates in women with metastatic breast cancer (2). Hence, cytotoxic chemotherapy is considered part of the optimal management of women with multiple sites of recurrence, where visceral disease is not easily treated by local modalities or when disease becomes hormone refractory (3, 4).

Current cytotoxic therapies have limited long-term effects in patients with distant metastases with 5-year survival rates of only 15% to 24% due to the inevitable development of chemoresistant disease (1). Furthermore, patients with metastatic breast cancer that fail first-line chemotherapy have only 20% to 30% response with “traditional” second-line chemotherapeutic agents (5). New strategies combining chemotherapy with novel targeted therapies provide new opportunities to further delay time to tumor progression and ultimately improve survival.

Advances in the field of nucleic acid chemistry offer potential strategies to silence gene products mediating tumor progression and treatment resistance. Antisense oligonucleotide–based agents specifically hybridize with complementary mRNA regions of a target gene to form RNA/DNA duplexes and thereby inhibit gene expression. Several antisense oligonucleotide targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (6, 7). Collectively, these studies identify antisense oligonucleotide as a novel class of antineoplastic agents when designed for appropriate molecular targets. However, because numerous genes are involved in tumor progression, simultaneous inhibition of multiple target genes may be necessary to optimally inhibit tumor progression. In fact, combined use of antisense oligonucleotide with other compounds, such as chemotherapeutic agents, has shown synergistic antineoplastic effects in several tumor models (8, 9).
Another novel mechanism of gene down-regulation is the use of RNA interference. When introduced into cells, double-stranded small interfering RNAs (siRNA) can potently suppress gene expression by mechanisms such as mRNA degradation or inhibition of translation. Inhibition of several targets using siRNA has been reported to suppress growth of various cancer cell lines and hence offers a second nucleotide-based approach at suppressing gene products for loss-of-function analyses (10). Furthermore, siRNAs have been shown to enhance sensitivity to different chemotherapeutic agents in vitro (10).

Tumor progression and drug resistance results, in part, from increased expression of cell survival genes that collectively regulate the apoptotic “rheostat” of cancer cells. Clusterin is a disulfide-linked heterodimeric glycoprotein (11) that is present in most human tissues and body fluids and is implicated in a wide variety of physiologic and pathologic processes, including tissue remodeling, lipid transport, phagocyte recruitment signaling, complement cytolysis inhibition, and apoptosis (12).

High levels of clusterin are associated with numerous tumors, including breast (13), prostate (14), lung (10), lymphoma (15), ovarian (16), and renal cell carcinoma (17). In breast cancer, clusterin expression, at both protein and RNA levels, has been correlated with breast cancer size, negative estrogen, and progesterone receptor status and with progression from primary carcinoma to metastasis to lymph nodes (13). Clusterin expression is strongly up-regulated after the addition of antiestrogens, including tamoxifen (18) and toremifene (19), treatment with vitamin D analogues (20), and exposure to X-ray radiation (21). Similarly, clusterin overexpression has also been shown to be associated with the anti-HER2 antibody trastuzumab (Herceptin) treatment resistance through the inhibition of apoptosis (22). In the present study, we identify clusterin to be prevalently expressed in human breast cancer and report for the first time that down-regulation of clusterin using sequence-specific antisense oligonucleotides and siRNA enhances paclitaxel chemosensitivity in both estrogen receptor (ER)–positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cell lines. Collectively, these findings identify clusterin as a valid therapeutic target in strategies employing novel multimodality therapy for patients with advanced cancer.

Materials and Methods

Tissue Microarray Case Series
This study group comprised 379 women, with ages ranging from 28 to 87 years (mean age, 61 years), with primary invasive breast cancer who underwent surgery for breast cancer between 1974 and 1995 at Vancouver General Hospital. These were consecutive cases, and the presence of invasive breast carcinoma was the only selection criterion in this study. One hundred twenty-six patients had histologically confirmed lymph node metastasis and 217 patients had tumors <20 mm and 149 had tumors ≥20 mm. Adjuvant therapy varied substantially during the period 1974 to 1995, and no information on individual adjuvant treatment was available. Ethical approval was obtained from the institutional ethical review board to perform this study.

Tissue Microarray Immunostaining
Tissue microarrays were designed by spotting two 0.6-mm tissue cores per case, obtained from formalin-fixed, paraffin-embedded archival tumor blocks, and constructed using a Beecher microarrayer. Sections were deparaffinized and rehydrated through xylene and ethanol and then transferred to the 0.02% Triton for permeabilization. Slides in citrate buffer (pH 6) were heated in the steamer for 30 minutes. After cooling for 30 minutes and 3 × 5-minute wash in PBS, the slides were incubated in 3% bovine serum albumin for 30 minutes. The slides were successively transferred to 3% H2O2 for 10 minutes and then incubated overnight with goat anti-human clusterin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at the concentration of 1:600 in 1% bovine serum albumin. The next day, the primary antibody was washed extensively with PBS and the LSAB+ kit (DAKO, Carpinteria, CA) was used as detection system. The chromogen NovaRed (Vector Laboratories, Burlingame, CA) was applied for 2 minutes and counterstaining was done with H&E (Vector Laboratories). After ethanol rehydrating, a cover glass was applied with Cytoseal, a xylene-based mounting medium (Stephen Scientific, Riverdale, NJ). Negative control slides were processed in an identical fashion to that above, with the substitution of 1% bovine serum albumin for the primary antiserum. Photomicrographs were taken with a Leica DMLS microscope coupled to a digital camera (PhotometricsCoolSNAP, Roper Scientific, Inc., Glenwood, IL).

Scoring of Clusterin Staining
The staining intensity of malignant tissue was evaluated and scored by two pathologists (L.F. and D.H.). Specimens were graded from 0 to +3 intensity representing the range from no staining to high staining. The overall percentage of cancer cells showing staining (0–100%) was also indicated. All comparisons of staining intensities and percentages were made at ×200 magnification. Any discrepancies were resolved with a multihead microscope.

Tumor Cell Lines
MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD). Cells were maintained in DMEM (Invitrogen Life Technologies, Rockville, MD).
Clusterin siRNA and Antisense Oligonucleotide

siRNAs were purchased from Dharmaco Research, Inc. (Lafayette, CO) using the siRNA sequence (5'-GCAGCA-GAGUUCUCAUCAU-3') corresponding to the human clusterin initiation site and a scramble control (5'-CAGCGCUGACACAGUUCUCAU-3'). Antisense oligonucleotide sequences were manufactured by ISIS Pharmaceuticals (Carlsbad, CA) and supplied by OncoGenex Technologies (Vancouver, British Columbia, Canada). The antisense oligonucleotides were second-generation 21-mer antisense oligonucleotides with a 2-O-(2-methoxy)ethyl modification. The antisense oligonucleotide clusterin sequence corresponding to the human clusterin initiation site was 5'-CAGCGCAGAGTCATCATCAT-3' and designated OGX-011 (OncoGenex Technologies), whereas the scramble control sequence was 5'-CAGCGCCTGACAACAGA-TTTCAT-3'.

Treatment of Cells with siRNA and Antisense Oligonucleotide

Oligofectamine (Invitrogen Life Technologies, Inc., Carlsbad, CA) was used to enhance transfection of cells. MCF-7 or MDA-MB-231 cells (8 x 10^5) were treated with 5, 25, or 50 nmol/L clusterin siRNA after preincubation for 20 minutes with 4 µg/mL Oligofectamine in 5 mL serum-free Opti-MEM (Invitrogen Life Technologies). Four hours after starting the incubation, 2.5 mL DMEM/15% FCS was added to produce a final concentration of 5% FCS. Cells were treated once and then either harvested using 0.25% trypsin 48 hours after transfection for RNA extraction or lysed in radioimmunoprecipitation assay buffer plus protease inhibitors 72 to 120 hours after transfection for protein isolation. MCF-7 or MDA-MB-231 cells were treated with 10, 50, 100, or 500 nmol/L OGX-011 or control oligonucleotide after preincubation for 20 minutes with 4 mg/mL Oligofectamine in serum-free Opti-MEM. Four hours after starting the incubation, DMEM/15% FCS was added as described above. Cells were treated once daily for 2 successive days and then harvested 24 hours following the final treatment for RNA extraction and 48 hours following final treatment for protein isolation.

Northern Blot Analysis

Total RNA was isolated from MCF-7 or MDA-MB-231 cells using Trizol/chloroform extraction. For cDNA synthesis, reverse transcription-PCR was carried out at 37°C for 1 hour using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and 2 µg extracted RNA per sample. Applied Biosystems 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was used for real-time monitoring of PCR amplification of the cDNA following the Taqman Universal PCR Master Mix protocol. The amplification of clusterin cDNA was done using primers and Taqman probes (Nucleic Acids Protein Services Unit, Biotechnology Laboratory, University of British Columbia) consisting of 5'-GAGUCUUCAUCAU-3' (antisense) for clusterin and 5'-ATATTGGCAGGTGTTTTTGTA-3' (sense) for glyceraldehyde-3-phosphate dehydrogenase. Density of bands for clusterin was normalized against that of glyceraldehyde-3-phosphate dehydrogenase.

Western Blot Analysis

siRNA or antisense oligonucleotide transfected MCF-7 or MDA-MB-231 cells were harvested 72 hours after transfection by lysis in radioimmunoprecipitation assay buffer and protease inhibitors and shearing with a 26-gauge needle. Protein (30 µg) was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and analyzed by Western blotting with 1:400 goat anti-human clusterin antibody. Loading levels were normalized using 1:2,000 anti-vinculin antibodies (Sigma Chemical Co., St. Louis, MO) and densitometric analysis.

Quantitative Real-time PCR

Total RNA was isolated from MCF-7 or MDA-MB-231 cells using Trizol/chloroform extraction. For cDNA synthesis, reverse transcription-PCR was carried out at 37°C for 1 hour using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and 2 µg extracted RNA per sample. Applied Biosystems 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was used for real-time monitoring of PCR amplification of the cDNA following the Taqman Universal PCR Master Mix protocol. The amplification of clusterin cDNA was done using primers and Taqman probes (Nucleic Acids Protein Services Unit, Biotechnology Laboratory, University of British Columbia) consisting of 5'-GAGUCUUCAUCAU-3' (antisense) for clusterin and 5'-ATATTGGCAGGTGTTTTTGTA-3' (sense) for glyceraldehyde-3-phosphate dehydrogenase. Density of bands for clusterin was normalized against that of glyceraldehyde-3-phosphate dehydrogenase.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The in vitro growth inhibitory effects of siRNA clusterin or OGX-011 in combination with paclitaxel on MCF-7 and MDA-MB-231 cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, 3 x 10^5 to 5 x 10^5 cells were plated per well in 96-well plates and allowed to attach overnight. The next day, cells were treated with clusterin siRNA, OGX-011, or scramble control (20–50 nmol/L) for 4 hours daily for 1 day (siRNA) or 2 days (antisense oligonucleotide). Cells were then treated with various concentrations of paclitaxel ranging from 0 to 500 nmol/L. After 72 hours of incubation, 10 mg/mL dimethylsulfoxide 2-yl 2,5-diphenyltetrazolium bromide (20 µL; Sigma) in PBS was added to each well and incubated at 37°C for 4 hours. The formazan crystals were dissolved in DMSO followed by determination of the
absorbance with a microplate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 560 nm. Absorbance values were normalized to that of values obtained for the vehicle-treated cells to determine the cell proliferation compared with control. Every 24 hours over a period of 4 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were carried out. Each assay was done in triplicate.

Changes in Clusterin Expression after Estrogen Withdrawal or Paclitaxel Treatment

In vitro
To determine whether estrogen withdrawal or paclitaxel treatment altered clusterin expression, cells were treated with either charcoal-stripped serum (Hyclone Laboratories, Logan, UT) to remove steroids, including estrogen, or 100 nmol/L paclitaxel. After 48 hours of treatment, cells were then treated with 50 nmol/L OGX-011 as described previously. Protein was harvested 48 hours after the final treatment of OGX-011.

Effects of OGX-011 on MCF-7 Tumor Growth In vivo

MCF-7 cells (1 × 10⁶) were inoculated s.c. with 0.1 mL Matrigel (Becton Dickinson Labware, Mississauga, Ontario, Canada) in the flank region of 6- to 8-week-old female athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) via a 27-gauge needle under halothane anesthesia. All mice were treated with slow-release 17β-estradiol pellets (1.7 mg pellet/90-day release) from Innovative Research of America (Sarasota, FL) the day before tumor inoculation to facilitate tumor growth. When MCF-7 tumors reached 1,000 mm³, usually 3 to 4 weeks after injection, mice were randomly selected for treatment with paclitaxel plus OGX-011 or scrambled antisense oligonucleotide. Each experimental group consisted of 10 mice. After randomization, 10 mg/kg OGX-011 or scrambled antisense oligonucleotide was injected i.p. once daily for 42 days. Paclitaxel (0.5 mg) was administrated i.v. thrice weekly from days 7 to 14 and days 21 to 28. Tumor volume measurements were done once weekly and calculated by the formula: length × width × depth × 0.5236 (23). Data points were expressed as average tumor volume levels ± SE. All animal procedures were done according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

Mice tumors were harvested on day 42, and half of each tumor was formalin fixed and paraffin embedded or flash frozen in liquid nitrogen and then treated with radioimmunoprecipitation assay buffer to obtain total cell lysate. Western blot analysis was done on the total cell lysate to assess total clusterin levels. Protein (30 µg) was separated by 10% PAGE, transferred to 0.45-µm Immobilon-P transfer membrane, and analyzed by Western blotting with 1:400 goat anti-human clusterin antibody. Loading levels were normalized by blotting with 1:2,000 anti-vinculin for densitometric analysis.

A tissue microarray using the above-described protocol was made using five MCF-7 tumors treated with OGX-011 and an equal number treated with scramble antisense oligonucleotide. This tissue microarray was stained for clusterin using the protocol described above. To determine

<table>
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<th>Clusterin Intensity</th>
<th>Total</th>
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<tr>
<td>0</td>
<td>142 (37.5%)</td>
</tr>
<tr>
<td>1</td>
<td>136 (35.9%)</td>
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<tr>
<td>2</td>
<td>63 (16.6%)</td>
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<td>3</td>
<td>38 (10.0%)</td>
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![Figure 1. Immunohistochemical staining of clusterin in a breast cancer tissue microarray. Tissue microarrays were immunostained for clusterin and scored by two pathologists. Specimens were graded from 0 to +3 intensity representing the range from no staining to heavy staining. Three representative images at ×40 magnification show high (A), moderate (B), and low (C) clusterin staining.]
effect of treatment on apoptotic rates, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining (Apoptosis Detection kit, Chemicon, Temecula, CA) and caspase-3 staining (Cell Signaling, Beverly, MA) of the tissue microarray was scored by two pathologists as a percentage of the total number of cells.

**Statistical Analysis**

Results were expressed as the mean ± SE. Statistical analysis was done by an unpaired two-tailed t test (GraphPad Instat 3, San Diego, CA). \( P \leq 0.05 \) was considered significant.

**Results**

Clusterin Expression in Human Breast Cancer

As illustrated in Fig. 1, immunohistochemistry of the human breast cancer tissue microarray revealed clusterin expression in 63% of tumors (237 tumors) similar to studies reported previously (13). A statistically significant association between prognosis and intensity of staining or nuclear staining was not found (data not shown). No differences were observed between nuclear and cytoplasmic clusterin levels.

![Graph](image)

Figure 2. A, clusterin knockdown in MCF-7 cells treated with OGX-011 or RNA interference clusterin. MCF-7 cells were treated with 5, 25, or 50 nmol/L either siRNA5 (one treatment) or 2′-O-2-methoxyethyl–modified clusterin (CLU) antisense oligonucleotide (ASO; OGX-011, two treatments on successive days) for 4 h at 37°C. Total RNA was extracted and analyzed by Northern blot with a 1.7-kb human clusterin probe. Values were normalized for loading by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization and expressed as gene expression relative to Oligofectamine-only control (O only). B, Western blot analysis. Clusterin siRNA or OGX-011 transfected MCF-7 cells were harvested at day 5 after transfection and protein (30 μg) was analyzed by Western blot with anti-human clusterin antibody (1:400). Loading levels were normalized by blotting with 1:2,000 anti-vinculin and expressed as percent expression relative to scramble control. *, \( P \leq 0.05 \); **, \( P \leq 0.01 \); ***, \( P \leq 0.001 \).
Sequence-Specific and Dose-Dependent Inhibition of Clusterin Expression Using OGX-011 and siRNA

To study the functional role of clusterin expression in MCF-7 and MDA-MB-231 cells, antisense oligonucleotide (OGX-011) or siRNA clusterin induced inhibition of clusterin expression was determined by Northern analysis (Figs. 2A and 3A) and Western blot analysis (Figs. 2B and 3B). As shown in Fig. 2A, treatment of MCF-7 cells with antisense oligonucleotide significantly reduced clusterin mRNA levels by up to 53% (OGX-011) and 63% (RNA interference) in a dose-dependent manner, whereas clusterin mRNA expression was not significantly suppressed by scramble oligonucleotide. More potent dose-dependent suppression of protein expression was observed using Western blot analysis (OGX-011 or siRNA clusterin transfected MB231 cells were harvested at day 3 after transfection and protein (30 μg) was analyzed by Western blot with anti-human clusterin antibody (1:400 dilution). Loading levels are normalized by blotting with 1:2,000 anti-vinculin and expressed as percent expression relative to Oligofectamine only control. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
and B), illustrating that the capacity to suppress clusterin is independent of ER status. Dose-dependent and sequence-specific suppression of secreted form of clusterin using OGX-011 or siRNA was also confirmed in MCF-7 and MDA-MB-231 cell lines using quantitative real-time PCR (data not shown).

**Increased Clusterin Expression after Estrogen Withdrawal or Paclitaxel Treatment In vitro in MCF-7 Cells**

Western analysis detected three bands in cells (60, 55, and 45 kDa) treated with paclitaxel and estrogen withdrawal and only one band (55 kDa) in cells treated with OGX-011 (Fig. 4). As described previously in irradiated MCF-7 cells, the 55-kDa (nuclear clusterin) band corresponds to a truncated nuclear form that arises via alternative splicing at the second ATG site and induces death signals through interaction with the DNA helicase Ku70 (21). Increased nuclear clusterin levels also increase after treatment with chemotherapeutics in other tumor models (24, 25). The 60- and 45-kDa bands (secreted form of clusterin) correspond to the full-length secreted form of clusterin as described previously (21). Consistent with previous reports in prostate (26), lung (10), and renal cell (27) cancers, treatment-induced stress using paclitaxel or estrogen withdrawal increases secreted form of clusterin levels in ER-positive MCF-7 cells (Fig. 4). After combination therapy with OGX-011, secreted form of clusterin levels are significantly reduced, whereas nuclear clusterin levels are increased, where nuclear clusterin may act as a proapoptotic signal. These observations are consistent with the targeting of OGX-011 to the first ATG site to inhibit translation of full-length, secreted clusterin but not the alternatively spliced, nuclear clusterin.

**OGX-011 and siRNA Clusterin Chemosensitize MCF-7 and MDA-MB-231 Cells to Paclitaxel**

To assess whether clusterin suppression alters chemosensitivity of breast cancer cells, MCF-7 and MDA-MB-231 cells were treated with paclitaxel plus OGX-011 or siRNA clusterin. Cells were treated for 2 days with 250 nmol/L OGX-011 antisense oligonucleotide or scrambled control for 1 day with 20 nmol/L clusterin siRNA or scramble control and subsequently incubated with increasing concentrations of paclitaxel. Growth rates of MCF-7 or MDA-MB-231 cells were measured using the MTT assay after 2 days of treatment (Fig. 5). Figure 5A shows a >2-log decrease in the IC₅₀ of paclitaxel in OGX-011 or siRNA clusterin treated
Targeting Clusterin in MCF-7 and MDA-MB-231 Cell Lines

OGX-011 Decreases Clusterin Expression In vivo in MCF-7 Xenografts

Western blot analysis (Fig. 7) of xenograft tissues illustrates that secreted clusterin expression was significantly reduced in mice treated with OGX-011. Levels of nuclear clusterin (55 kDa) were elevated as well, mirroring the effects of combination therapy of OGX-011 and paclitaxel in vitro (Fig. 4). Immunostaining of MCF-7 tumors spotted onto a tissue microarray supported the Western analysis with significantly lower clusterin staining in OGX-011-treated tumors (Fig. 8). These data confirm that systemically given OGX-011 can suppress target clusterin expression in vivo, consistent with prior preclinical studies (28) and a recent report of a phase I presurgery trial in men with localized prostate cancer (29).

OGX-011 Increases Paclitaxel-Induced Apoptosis of MCF-7 Xenografts

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling TUNEL assay and caspase-3 immunostaining were done in five MCF-7 xenografts from each treatment group to assess whether OGX-011-induced suppression of secreted clusterin with increased nuclear clusterin enhanced apoptotic rates in vivo. OGX-011-treated tumors have higher rates of apoptosis when compared with tumors treated with paclitaxel plus scrambled controls as shown in the TUNEL assay scoring (Fig. 9B) and caspase-3 scoring (Fig. 10B). These results suggest the delay in tumor progression in the OGX-011 plus paclitaxel group resulted from increased apoptosis induced by the chemosensitizing effects of clusterin suppression.

Discussion

Despite significant advances in the management of breast cancer, metastatic disease remains incurable. With currently available treatments, median survival of patients with metastatic disease is only 2 years (30). Interestingly, 10% of women have metastatic disease at time of diagnosis of breast cancer (31), whereas up to 40% with early-stage disease will experience recurrence (32).

Whereas traditional systemic therapies integrate chemotherapy at all stages of disease, many tumors become chemoresistant and progress. Extensive research into the molecular events mediating progression of breast cancer, coupled with the need to discover additional treatments in the setting of chemoresistant disease, is expanding the paradigm of targeted therapy in advanced cancer. Examples of cancer genotyping coupled with specific targeted therapies include use of estrogen and progesterone status with hormonal therapies like tamoxifen and aromatase inhibitors (33, 34), and HER status with anti-HER antibodies like trastuzumab or small-molecule inhibitors like gefitinib or erlotinib (35).

Rational combination strategies employing cytotoxic agents with chemosensitizing targeted therapies promise to increase response rates and prolong time to tumor progression. Studies of mechanisms mediating chemo-resistance have focused on the regulation of drug transport and metabolism, such as the up-regulation of the toxin

MCF-7 cells. Similar chemosensitization was found in the MDA-MB-231 cell lines (Fig. 5B). These results show that down-regulation of clusterin using either antisense oligonucleotide or siRNA significantly chemosensitizes breast cancer cells to paclitaxel.

OGX-011 Treatment Enhances Paclitaxel Chemotherapy In vivo

We next evaluated the effects of clusterin antisense oligonucleotide OGX-011 treatment on the growth of MCF-7 tumors in vivo (Figs. 6 and 7). Female nude mice bearing MCF-7 tumors (750 mm³) were randomly selected for paclitaxel plus either OGX-011 or scrambled controls. Mean tumor volume was similar in all groups before therapy. Figure 6 shows that OGX-011 treatment, compared with scrambled control, significantly enhanced the effects of paclitaxel in vivo, reducing mean MCF-7 tumor volume by >75% by 5 weeks after initiation of treatment (P ≤ 0.01). Xenografts treated with scrambled controls responded to the first cycle of paclitaxel but did not decrease in size after the second. In contrast, mice treated with OGX-011 responded significantly after each cycle of paclitaxel treatment. Under the experimental conditions described, no adverse effects were observed.
transport pump MDR1 (36). Chemoresistance may also develop from alterations in the apoptotic machinery secondary to increased expression of antiapoptotic genes regulating various cell survival pathways. Survival proteins up-regulated after apoptotic triggers that function to inhibit cell death include antiapoptotic members of the bcl-2 protein family, clusterin, heat shock proteins, and survivin (37, 38).

Clusterin expression is rapidly up-regulated in various tissues undergoing apoptosis, including normal and malignant breast and prostate tissues following hormone withdrawal (26, 39), renal tubular cells after oxidative stress (40), and thymocytes under negative selection (41). Previous studies have also linked clusterin expression with induction and progression of many cancers, including breast cancer (13, 42). Interestingly, although initially considered an androgen-repressed gene and marker of apoptosis after castration in the involuting rat prostate gland (43), clusterin up-regulation following castration is inhibited when apoptosis is attenuated by pretreatment with calcium channel blockers (44). Under these conditions, clusterin levels remained unchanged, confirming that clusterin is an apoptosis-related gene rather than androgen-repressed gene (44, 45). Furthermore, clusterin up-regulation following androgen ablation in xenograft tumor models accelerates progression to the androgen-independent phenotype and renders cells resistant to various apoptotic stimuli, including taxane chemotherapy (44, 46).

Clusterin exhibits chaperone-like activity, inhibiting stress-induced protein precipitation in a manner analogous to small heat shock proteins, and its promoter contains a 14-bp element recognized by the transcription factor heat shock factor 1 (47). Recent preclinical studies provide proof-of-principle evidence that targeting cell survival genes, such as clusterin or bcl-2, with antisense oligonucleotides enhances apoptosis induced by conventional chemotherapy (46, 48) and has led to clinical trials testing antisense oligonucleotide therapy in combination with chemotherapy at several institutions (49, 50).

Previous studies with the ER positive breast cancer cell line MCF-7 report that clusterin expression is strongly up-regulated after treatment with antiestrogens such as toremifene (18) or tamoxifen (19), vitamin D analogues (20), and radiation (21). Recent studies have shown that targeted therapy against HER-2 increases clusterin expression in breast cancer cells (22), and that OGX-011 increases apoptotic rates after treatment with the anti-HER-2 antibody trastuzumab.

Our present study confirms that secreted clusterin levels increase after estrogen withdrawal and paclitaxel treatment in the MCF-7 cells. We also show that OGX-011 inhibits the up-regulation of secreted clusterin induced by estrogen withdrawal or paclitaxel treatment. The chemosensitizing effect of OGX-011 in both MCF-7 and MDA-MB-231 cell lines illustrates the significant cytoprotective role of siRNA clusterin. Even more intriguing is the ability of combination therapy of OGX-011 and chemotherapy to simultaneously suppress secreted clusterin levels while increasing levels of nuclear clusterin in vitro and in vivo, where nuclear clusterin may act as a proapoptotic signal. Similar up-regulation of nuclear clusterin after treatment with chemotherapy has also been shown in osteosarcoma and human colon cells (24, 25), but no combination therapies targeting clusterin have been shown previously to simultaneously reduce secreted clusterin and increase nuclear clusterin.
Increased expression of secreted clusterin has been associated with chemoresistance, radioresistance, and hormone resistance (10, 51), making secreted clusterin an attractive target for antitumor therapeutics. However, it is crucial that any targeting of secreted clusterin should have minimal effect on nuclear clusterin. Although the role of nuclear clusterin remains unclear, this isoform is associated with cell cycle arrest and cell death induction (21). We show that OGX-011 seems to be specific for secreted clusterin; treatment of OGX-011 with paclitaxel causes suppression of secreted clusterin with increased nuclear clusterin levels in MCF-7 xenografts. Furthermore, we also show that combination therapy of OGX-011 with paclitaxel is associated with increased apoptotic rates and delayed tumor progression in vivo.

An initial objective of this study was to characterize clusterin expression levels in human breast cancer using a tissue microarray of invasive cancers. These data confirm that clusterin is present in >60% of invasive breast cancer. To clarify the functional role of clusterin in breast cancer, we then tested whether suppression of clusterin using antisense oligonucleotide (OGX-011) or siRNA could enhance the cytotoxic effects of paclitaxel in either ER-positive (MCF-7) or ER-negative (MDA-MB-231) breast cancer cell line. Our results show that OGX-011 and siRNA inhibited clusterin levels in both cell lines. Pretreatment of MCF-7 and MDA-MB-231 cells with OGX-011 antisense oligonucleotide or siRNA reduced the IC50 of paclitaxel by >2 log. Consistent with these in vitro results, synergistic effects of combined use of clusterin antisense oligonucleotide or siRNA plus paclitaxel was also observed in vivo. Systemic administration of the second-generation clusterin antisense oligonucleotide, OGX-011, plus paclitaxel suppressed MCF-7 tumor growth compared with treatment with control oligonucleotides plus paclitaxel. Detection of increased apoptosis after combined OGX-011 plus chemotherapy by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling analysis and caspase-3 immunohistochemistry suggests that delayed tumor progression after combined therapy resulted from enhanced chemotherapy-induced apoptosis rather than decreased cell proliferation.

These data, along with previous reports in other tumor models, provide preclinical proof-of-principle supporting clinical trials of OGX-011 plus chemotherapy in breast and other human cancers. A phase I trial, NCIC IND.153, was recently completed with OGX-011 (29). This trial had a unique design in that patients with localized prostate cancer were given the 2'-O-(2-methoxy)ethyl antisense oligonucleotide before radical prostatectomy; thus, a pharmacodynamic end point (i.e., inhibition of clusterin expression) could be evaluated for each patient and dose level. OGX-011 was given as a 2-hour i.v. infusion over 2 hours thrice in week 1 and then weekly until surgery on day 35. Relevant concentrations of OGX-011 were achieved that inhibited expression of clusterin in human cancer
tissue in a dose-dependent fashion, a notable first in the literature. Concentrations of OGX-011 associated with preclinical effect were measured in tumor tissue and a biologically effective dose of 640 mg was identified based on 90% suppression in clusterin. Side effects included fever and chills in the first week of infusions and transient myelosuppression and elevations of liver enzyme tests that normalized despite continued therapy. A second phase I trial combined increasing doses of OGX-011 with docetaxel in patients with metastatic breast, non–small cell lung, and hormone-refractory prostate cancers established a phase II dose for OGX-011 of 640 mg in combination with weekly or

Figure 9. Apoptotic index increases in MCF-7 xenografts treated with paclitaxel + OGX-011. A, tissue microarrays spotted with MCF-7 tumors were immunostained using a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay by enzymatically labeling the free 3′-OH terminal generated on DNA fragments of 180 to 200 bp. B, mean scores were determined as a percentage of the total number of cells.

Figure 10. Caspase-3 increases in MCF-7 xenografts treated with paclitaxel + OGX-011. A, tissue microarrays spotted with MCF-7 tumors were immunostained using a caspase-3 antibody. B, mean scores were determined as a percentage of the total number of cells.
Targeting Clusterin in MCF-7 and MDA-MB-231 Cell Lines

every 3 weeks of docetaxel (52). Three phase II trials of OGX-011 in combination with chemotherapy are now under way in patients with prostate, breast, and lung cancers.

The plethora of information unraveling multiple molecular mechanisms of breast cancer tumor induction and progression has revealed that breast cancer is a heterogeneous disease with multiple alterations at the genomic and proteomic levels. These differences identify many crucial redundant cell survival mechanisms that allow breast cancer to adapt and proliferate after treatments, such as hormonal therapy and chemotherapy. Thus, optimal cancer management will require targeting multiple mechanisms of cellular survival. The present study supports the development of targeted strategies employing clusterin antisense oligonucleotide in combination with conventional cytotoxic therapies for advanced breast cancer.

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Molecular Cancer Therapeutics

Knockdown of the cytoprotective chaperone, clusterin, chemosensitizes human breast cancer cells both in vitro and in vivo

Alan So, Shannon Sinnemann, David Huntsman, et al.


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