A Lipid-Modified Estrogen Derivative that Treats Breast Cancer Independent of Estrogen Receptor Expression through Simultaneous Induction of Autophagy and Apoptosis

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Abstract

It is a challenge to develop a universal single drug that can treat breast cancer at single- or multiple-stage complications, yet remains nontoxic to normal cells. The challenge is even greater when breast cancer–specific, estrogen-based drugs are being developed that cannot act against multistaged breast cancer complications owing to the cells differential estrogen receptor (ER) expression status and their possession of drug-resistant and metastatic phenotypes. We report here the development of a first cationic lipid-conjugated estrogenic derivative (ESC8) that kills breast cancer cells independent of their ER expression status. This ESC8 molecule apparently is nontoxic to normal breast epithelial cells, as well as to other noncancer cells. ESC8 induces apoptosis through an intrinsic pathway in ER-negative MDA-MB-231 cells. In addition, ESC8 treatment induces autophagy in these cells by interfering with the mTOR activity. This is the first example of an estrogen structure–based molecule that coinduces apoptosis and autophagy in breast cancer cells. Further in vivo study confirms the role of this molecule in tumor regression. Together, our results open new perspective of breast cancer chemotherapy through a single agent, which could provide the therapeutic benefit across all stages of breast cancer. Mol Cancer Res; 9(3); 364–74. ©2011 AACR.

Introduction

Breast cancer drugs are often based on targeting estrogen receptors (ER) because of ER’s relatively high abundance in most types of breast cancers. The status of this important biomarker provides favorable prognostic features and helps individualize breast cancer therapy (1, 2). Several estrogen-based modulators that rely on mechanisms such as antagonism to estrogen-ER binding, estrogen-mediated transcription, or estrogen-based hybrid DNA-alkylating agents have been developed over time (1, 3–12). But adjuvant therapy is usually warranted in highly aggressive breast cancer phenotypes that are not responsive to conventional single-agent estrogen. Many other selective ER modulators (SERM; e.g., fulvestrant, a lipid-modified analogue of endogenous natural ligand 17β-estradiol; the nonestrogenic molecules tamoxifen and raloxifen) were largely limited to the treatment of ER-positive breast cancer (13–16). However, developing a universal anti-breast cancer estrogenic molecule that is nontoxic to normal cells, yet acts against both ER-positive and ER-negative breast cancers with drug-resistant and metastatic phenotypes, is challenging.

Most anticancer agent–mediated cancer cell killing occurs through induction of apoptosis. Apoptosis is the type I process involved in programmed cell death (PCD), the other mechanism being autophagy (type II). The ability of tumor cells to evade engagement of apoptosis plays a significant role in their resistance to conventional therapeutic regimens. Of the two major pathways of apoptosis (i.e., intrinsic or extrinsic), activation of the mitochondria-associated intrinsic pathway leads to an increase in caspase-9 and caspase-3, resulting in apoptotic cell death (17).

Autophagy, the self-cannibalism in cells, is often triggered for cytoprotective or survival purposes, including facilitating drug resistance in breast cancer cells and protecting cells against proapoptotic insults (18–20). However, further studies also suggest a definitive role for autophagy in cancer and in determining the response of tumor cells (e.g., breast...
tumor cells) to anticancer therapy (21). It has been shown that anti–estrogen treatment of ER-positive breast cancer cells leads to induction of autophagy (22), but to our knowledge there is no evidence of anti–estrogen-mediated involvement of autophagy in ER-negative breast cancer cells. The PI3K-Akt-mTOR signaling pathway, which negatively regulates autophagy, is involved in tumorigenic progression in many cancers and is one of the two mechanisms of induction of autophagy (23).

In this study, we developed a cationic, lipid-conjugated, estrogenic molecule that, in contrast to other estrogenic drugs and SERMs, induced cell death in both ER-positive and ER-negative breast cancer cells. This estrogenic drug-mediated killing of ER-negative breast cancer cells prompted us to conduct an in-depth mechanistic study in ER-negative MDA-MB-231. We found that the apoptosis was induced through upregulation of the Bax/Bcl-2 ratio, leading to activation of initiator caspase-9 and effector caspase-3. In this study, we also found that ESC8 treatment evoked the upregulation of autophagy via inhibition of the mTOR pathway and that activation of autophagy promoted the cell death of MDA-MB-231 cells. We observed a decrease in phosphorylation of mTOR at Ser-2448 and a simultaneous increase in phosphorylation of Akt1/2/3 at Ser-473 on ESC8 challenge. We assume that ESC8 treatment inhibited mTOR activity, thereby increasing phosphorylation of Akt in a feedback loop similar to that of rapamycin treatment. The molecule was also tested in vivo to show a significant reduction of tumor aggression. To our knowledge, this is the first example of a concomitant induction of estrogenic drug-mediated autophagy and apoptosis in breast cancer through regulation of PI3K-Akt-mTOR signaling and uncovering of this pathway, which we believe is novel, for promoting tumor cell death may have therapeutic implications in the treatment of breast cancer.

Materials and Methods

Antibodies
Anticaspase-3 (#9662), caspase-8 (#9746), caspase-9 (#9502), p-mTOR (#29271), mTOR (#2972), p-p70S6K (#9234), Bcl-2 (#2876), Bcl-xL (#2877), Beclin-1 (#3738), and LC-3B (#2775) antibodies were purchased from Cell Signaling Technology; antimouse β-actin antibody was purchased from BD-Pharmingen; anti-p-Akt-1/2/3 (Ser-473; sc-7985), anti-Akt-1 (sc-1618), anti-Bax (sc-6236), anti-Bid (sc-11423), and anti-cytochrome c (sc-13156) antibodies were purchased from Santa Cruz Biotechnology.

Compound synthesis and characterization
A detailed synthesis of ESC8 is described in the Supplementary section.

Cells and cell culture conditions
Detailed cell culture conditions have been provided in the Supplementary Materials and Methods section.

Isolation and maintenance of rat hepatocytes
Hepatocytes were isolated from adult male Wistar rats (250 g). A detailed procedure is available in the Supplementary Data.

Isolation of normal human breast epithelium cells
Normal human breast epithelial cells were isolated from human normal breast epithelial tissue of donor breast cancer patient and obtained as a kind gift from Indo-American Cancer Center, Hyderabad, India. A detailed procedure is available in the Supplementary Data.

Preparation of samples and sample treatment
The compounds were dissolved in cell culture–grade dimethyl sulfoxide (DMSO) to get a primary stock. The stock was progressively diluted with DMSO to get secondary stocks. Finally, the working concentrations of the derivatives were obtained by adding the secondary DMSO stocks in 10% FBS containing cell culture medium. For controls, cells were treated with only DMSO containing serum medium and volume of DMSO was equal to the volume of DMSO in which the drugs were dissolved and added to the serum containing medium before the treatment. The amount of DMSO in working solutions never exceeded more than 0.2% with respect to the serum containing culture medium. For the viability studies, 100 μL of cell culture solutions containing respective concentrations of compounds are given to cells presathed with PBS. For flow cytometric analysis, 1.5 mL of culture media containing a respective concentration of ESC8 was added to each well of 6-well plates.

Cytotoxicity study
Cytotoxicities of the compounds were evaluated by the MTT reduction assay. A detailed procedure is available in Supplementary Data.

Annexin V staining
The Annexin V—FITC (fluorescein isothiocyanate)-labeled apoptosis detection kit (Sigma Chemical Co.) was used to detect and quantify apoptosis by flow cytometry as per manufacturer’s instructions. In brief, cells (1 × 10⁶ cells/well) were seeded in 6-well plates and cultured overnight in 10% serum media. The next day, cells were treated with 10 μmol/L ESC8 for 16 hours, harvested in PBS, and collected by centrifugation for 5 minutes at 1,000 rpm. Cells were then resuspended at a density of 1 × 10⁶ cells/mL in 1× binding buffer (HEPES buffer 10 mmol/L & pH 7.4, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, and 1.8 mmol/L CaCl₂) and stained simultaneously with FITC-labeled annexin V (25 ng/mL) and propidium iodide (PI; 50 ng/mL) according to the manufacturer’s protocol (Sigma Chemical Co.). Cells were analyzed using a flow cytometer (Becton Dickinson), and data were analyzed with CellQuest software.

DAPI staining
MDA-MB-231 cells grown on the cover slip were first treated with DMSO or ESC8 (5 μmol/L) for 16 hours.
Cells were then washed with PBS and fixed with 3% paraformaldehyde for 15 minutes at room temperature and again washed thrice with PBS. Cells were then stained with DAPI (4,6-Diamidino-2-phenylindole; 1 μg/mL, Sigma) for 30 minutes. Stained nuclei were visualized using microscope, Axiovert 200M (Carl Zeiss, Inc.) and photographed. Apoptotic cells were morphologically identified by cytoplasmic and nuclear shrinkage.

**siRNA knockdown**

MDA-MB-231 cells were treated with 100 nmol/L of control/Atg5 siRNA using Dharmafect-1 (Dharmacon). After 48 hours, cells were trypsinized and 5,000 cells were plated in 96-well plates and kept overnight. Then cells were treated with either DMSO or ESC8 (5 μmol/L) for 16 hours and cell viability assay was done as described in Supplementary Data.

**Autophagic flux assay**

Detection of autophagic flux, the specific processing of autophagy protein LC3 with or without a lysosomal protease inhibitor was determined by Western analysis. MDA-MB-231 cells were treated with or without both pepstatin A and E46d (10 μmol/L; ref. 24) along with ESC8 (1 or 5 μmol/L) for 16 hours and LC3-II level was detected by Western blot analysis.

**Visualization of monodansylcadaverine-labeled vacuoles**

MDA-MB-231 cells were grown on the cover slip, were first treated with DMSO or ESC8 (5 μmol/L) for 16 hours. Autophagic vacuoles were then labeled with monodansylcadaverine (MDC) by incubating cells with 0.05 μmol/L MDC in PBS for 10 minutes at 37°C. After incubation, cells were washed thrice with PBS and fixed with 3% paraformaldehyde for 15 minutes at room temperature and again washed thrice with PBS. Cells were then permeabilized with 0.05% Triton-X for 15 minutes at room temperature, washed with PBS, and followed by mounting in Vectashield (Vector Labs) without DAPI. Confocal microscopy was performed using a Zeiss LSM 510 confocal laser scan microscope.

**Cytosolic extract preparation**

Cytosolic extracts were prepared using MDA-MB-231 cells after ESC8 treatment for 16 hours following a standard protocol with modifications (25, 26). Cells were washed in cold PBS, suspended in buffer A [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 2 mmol/L MgCl2, 0.1 mmol/L EDTA, 10 μg/mL aprotinin, 3 mmol/L DTT (dithiothreitol), and 0.1 mmol/L phenylmethylsulfonyl fluoride] and incubated for 15 minutes on ice. Cells were then lysed with 10% Igepal and then centrifuged at 14,000 rpm for 5 minutes. Clear supernatants containing the cytosolic proteins were collected and stored at −70°C. Cytochrome c levels in cytosolic extracts were detected by Western blot analysis.

**Western blot analysis**

Western blot analysis was performed to detect the levels of Bcl-2, Bax, Bid, Arg5-Arg12 complex, Beclin-1 and LC-3 expressions, Akt-1/2/3, mTOR, p70S6K phosphorylations, and caspase activation in ESC8-treated MDA-MB-231 cells. Cells were washed with PBS and lysed with RIPA (radioimmunoprecipitation assay) buffer supplemented with a protease inhibitor cocktail after 16 hours of treatment. Supernatant was collected by centrifugation at 13,000 rpm for 10 minutes. Samples were then subjected to SDS-PAGE and then transferred to polyvinyl difluoride membranes and immunoblotted. Antibody-reactive bands were detected by enzyme-linked chemiluminescence (Amersham) and quantified by laser densitometry. These experiments were repeated at least 3 times.

**Animal xenograft model**

Five million MDA-MB-231 cells were orthotopically implanted in the right lower mammary fat pad of each of 6- to 7-week-old female Balb/C SCID mice (NCI-Frederick). When the average tumor sizes were 30 to 35 mm³, 3 groups of 5 mice each were segregated into the following treatment groups: (a) untreated group injected with 5% glucose solution intraperitoneally, (b) ESC8 (10 mg/kg mice)-injected group, (c) ESC8 (10 mg/kg/mice)-injected group in which, injections were started when tumor size reached about 130 to 135 mm³. Tumor sizes were measured twice a week. Only 2 alternate day injections were given to groups a and b, whereas 4 consecutive day injections were given to group c. All the experiments were done under the animal protocol approved by the Mayo Clinic’s Institutional Animal Care and Use Committee.

**Histologic study**

MDA-MB-231 tumors were removed and fixed in neutral buffered 10% formalin at room temperature for 24 hours before embedding in paraffin and sectioning. Sections were deparaffinized and then subjected to TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining for apoptosis according to the manufacturer’s instructions (DeadEnd Colorimetric TUNEL system, Promega). The TUNEL-positive nuclei were counted in the entire section. Thereafter, 10 fields of vision were photographed, the total number of nuclei was counted, and the average number of nuclei per unit area was calculated. Photographs of the entire cross-section were digitized using an Olympus camera (DP70). The apoptosis index was calculated as the number of positive nuclei divided by the total number of nuclei.

**Statistical analysis**

Data were expressed as mean ± SD and statistically analyzed by the 2-tailed, unpaired, Student’s t test using Microsoft Excel. Data were considered significant when P < 0.05 for animal studies and P < 0.01 for cell culture–based studies.
Results

Synthesis and anticancer effect of ESC8

We synthesized ESC8 by inserting an 8-carbon (C8), twin-chain, cationic lipid at the 17β-position of estradiol (ES; Fig. 1A). Our previous study indicated that any lipid-based substitution at the cyclopentyl-ring (ring d), especially at the 17β-position, largely retains its selective ability to target ER-expressing cancer cells when associated with a liposomal delivery system (27). The detailed synthesis of

![Image of ESC8 structure](https://www.aacrjournals.org/molcanres/article-pdf/9/3/367/3346662/molcanres10-0526v1.pdf)

Figure 1. A, structure of ESC8. Anticancer effect of ESC8. B, MCF-7 and MDA-MB-231 cells were treated with ES, C8 control, ES + C8 control, or ESC8 for 48 hours at a concentration 10 μmol/L (of each component) followed by determination of viabilities of cells using MTT assay. C and D, viability studies in MCF-7, and in MDA-MB-231 cells in presence of following compounds, ESC8 (●), 2OMe-ES (■), 4OH-Tam (▲), Epi (○), Tam (△) at the indicated concentrations for 48 hours. ESC8, 5 μmol/L, treatment for MCF-7 and 5 and 10 μmol/L ESC8 treatments for MDA-MB-231. *, P values are < 0.005 compared with other drugs treatments. At 10 μmol/L dose in MCF-7; *, P < 0.005 between ESC8 treatment and other drugs except 4OH-Tam. E, representation of viabilities of ESC8-treated (10 μmol/L, 48 hours) breast cancer cells (MCF-7, T47D, ZR-75-1, MDA-MB-231, and MDA-MB-435S) and noncancer cells (COS-1, NBE, hepatocytes, and CHO). F, comparison of the viabilities of ESC8-treated (10 μmol/L, 4 hours, left for 44 hours) cells in presence and absence of the pretreatment of ICI (ICI182780, an ER antagonist, 1 μmol/L, 2 hours; *, P < 0.001 for ICI182780 treated vs. untreated MCF7 cells). Figures are representative of a minimum of 4 separate experiments with similar results. For NBE cells, the experiments were done only three times.
ESC8 is provided in the Supplementary Data. Cationic lipids containing twin carbon chains show natural affinity toward negatively charged cellular membrane making them excellent agents to deliver bioactive molecules, such as DNA, inside the cells. We hypothesized that they may enhance the uptake of small molecule ligands also. In accordance with our hypothesis, cotreatment of cationic lipid moiety and estrogen-induced increased toxicity in comparison to individual treatments (Fig. 1B). We also presumed that a chemical conjugation would be more effective than cotreatment due to proximity and better cooperative effect. This presumption indeed seems to be true as the conjugated moiety showed additional level of anticancer effect (Fig. 1B). In an attempt to justify our hypothesis, we tested the anticancer effect of ESC8, an estrogenic molecule, in different kinds of breast cancer cells. The anticancer effect of ESC8 in ER-positive, MCF-7 and ER-negative, MDA-MB-231 cells were compared with the anticancer effects of the estrogenic drug 2-methoxyestradiol (2OMe-ES) and nonestrogenic anti-breast cancer agents such as 4-hydroxytamoxifen (4OH-Tam), tamoxifen (Tam), and epirubicin (Epi). As indicated in Figure 1C and D, ESC8 was clearly the most efficient killer of both ER-positive and ER-negative cells. We found lower IC50 for ESC8 (MCF-7 3.0 μmol/L and MDA-MB-231 3.2 μmol/L) compared with 4OH-Tam (4.3 and 7.7 μmol/L) and 2OMe-ES (10.5 and 18.0 μmol/L) under the experimental conditions. Figure 1E shows the effect of ESC8 on other breast cancer and noncancer cells. Five breast cancer cells and four noncancer cells were tested. The other tested breast cancer cells were T47D (dural, ER+), ZR-75-1 (dural, ER−), and MDA-MB-435S (dural, ER−). The noncancer cells were COS-1, CHO, freshly isolated rat hepatocytes, and normal breast epithelia (NBE, isolated from human breast cancer patients). Figure 1E clearly shows that ESC8 induced killing of all breast cancer cells but was unable to induce toxicity to any of the normal or healthy cells at the treated concentration. Moreover, use of ER antagonist, ICI182780, was able to abrogate the anticancer effect of ESC8 in ER-positive MCF-7 cells but had no effect in ER-negative MDA-MB-231 (Fig. 1F). Therefore, ESC8-mediated toxicity via ER antagonism can be ruled out for MDA-MB-231. In conclusion, ESC8 was clearly the most potent anticancer agent in comparison to other breast cancer-specific estrogenic, nonestrogenic, and ER-antagonizing molecules and—most importantly—ESC8 induced killing of these cells irrespective of ER expression status.

ESC8 induces apoptosis in breast cancer cells

To ascertain the induction of apoptosis in cancer cells following ESC8 treatment, we first studied the Annexin V/PI binding profile of ESC8-treated MCF-7 and MDA-MB-231 cells using flow cytometry analysis. As shown in Figure 2A, the ESC8 treatment (10 μmol/L, 16 hours) led to the accumulation of 70% of Annexin- and PI-positive MCF-7 cells. In MDA-MB-231, 40% apoptotic (Annexin and PI positive) cells were accumulated. In normal cells, COS-1 and isolated rat hepatocytes, ESC8 could not induce any significant apoptosis. Clearly, ESC8 induced apoptosis in both ER-positive/negative breast cancer cells. MDA-MB-231 cells were also stained with DAPI after 5 μmol/L ESC8 treatment to show the cytoplasmic and nuclear shrinkage due to induction of apoptosis (Supplementary Fig. S1a). Thus, it became a point of interest to ascertain the actual pathway through which apoptosis induction was triggered, especially in ER-negative cells. ESC8, an estrogen-like molecule, was naturally expected to have an anticancer effect on ER-positive cells, at least, via possible ER antagonism but similar reason could not be expected in ER-negative cells.

Effect of ESC8 on MDA-MB-231 apoptosis: initiation of intrinsic pathway and increased caspase activity

Apoptosis provides a conceptual framework to link cancer genetics with cancer therapy. The intrinsic apoptotic pathway hinges on the balance of activity between pro- and antiapoptotic members of the Bcl-2 family, which act to regulate the permeability of mitochondrial membranes and subsequently induce apoptosis. The cells were treated with increasing doses of ESC8. The 10 μmol/L dose of estradiol derivative ESC8 increased the Bax (an apoptosis promoter) to Bcl-2 (an apoptosis inhibitor) protein ratio in MDA-MB-231 cells after 16 hours of treatment (Fig. 2B). ESC8 treatment caused significant upregulation of the level of cytochrome c in cytosolic extracts of ESC8-treated cells (Fig. 2B). We also found that a 1 and 5 μmol/L dose of ESC8 were sufficient for the induction of activated caspase-9 and caspase-3 activities (Fig. 2C), respectively, in MDA-MB-231 cells after 16 hours of treatment, consistent with the increase in Annexin V/PI staining (Fig. 2A). However, no change in the levels of activated caspase-8 (data not shown) and Bid (Fig. 2B) were detected in MDA-MB-231 cells after ESC8 treatment. The data clearly indicate that the induction of ESC8-mediated apoptosis in MDA-MB-231 was due to activation of caspases such as initiator caspase-9 and effector caspase-3.

ESC8 induces autophagy

The role of autophagy in cancer is a topic of intense debate. Many anticancer agents have been reported to induce autophagy, suggesting tumor cell death by these agents (21). As a matter of interest, we chose to see whether ESC8 also falls in the aforementioned category of agents that induce autophagy as a mediator of cancer cell death. As part of our investigation, we checked the status of autophagy induction in MDA-MB-231 cells. MDA-MB-231 cells were treated with 3 different doses of ESC8 for 16 hours. A cytosolic form of LC3 (LC3-I) associates to phosphatidylethanolamine to form a LC3-phosphatidylethanolamine conjugate (LC3-II). This is recruited to autophagosomal membranes, and the total turnover of LC3-II reflects the autophagic turnover in the cell (21, 28). Figure 3A clearly shows the significant upregulation of LC3B-II, starting with a 1 μmol/L dose of ESC8, indicating a substantial increase in autophagy. Interestingly, no significant changes in the
expression levels of autophagy-related proteins, such as Atg5-Atg12 complex with anti-Atg5 or anti-Atg12 antibody and Beclin-1 (Fig. 3A), were detected. In addition, when shows that upon ESC8 treatment MDC (a specific marker for autolysosomes; ref. 29) labeled autophagic vesicles were induced in MDA-MB-231 cells (Supplementary Fig. S1b).

At the late stage of autophagy, LC3-II itself is degraded by lysosome. Therefore, autophagic flux assay is recently being performed to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels with or without the treatment of lysosomal protease inhibitors (30). ESC8 treatment in combination with pepstatin A and E64d showed an increase in LC3-II level compared with ESC8 treatment alone in MDA-MB-231 cells, indicating the accumulation of LC3-II due to the blockade of LC3-II degradation in lysosomes (Fig. 3B), which further suggests an autophagic response caused by ESC8.

Next, we were interested in evaluating whether autophagy is responsible for cell death caused by the exposure to ESC8. MDA-MB-231 cells were pretreated with Atg5

Figure 2. ESC8 induces apoptosis in breast cancer cells. A, Annexin V (An) binding and PI uptake in cells treated with ESC8 were measured using flow cytometry. MCF-7, MDA-MB-231, COS-1, and hepatocyte cells were treated with ESC8 (10 μmol/L) for 16 hours or kept untreated and stained with annexin V and PI for further flow cytometric analysis. At a 10 μmol/L concentration, significantly higher levels of Annexin/PI staining were recorded in ESC8-treated MCF-7 and MDA-MB-231 cells (*, P < 0.05 for both An and An + PI positive, MDA-MB-231 and MCF7 cells with ESC8 treatment vs. control and *, P < 0.001 between An + PI-positive cancer cells and noncancer cells). The data shown are representative of 3 independent experiments with similar findings. B, the 10 μmol/L dose of estradiol derivative ESC8 increased the Bax (an apoptosis promoter) to Bcl-2 (an apoptosis inhibitor) protein ratio in MDA-MB-231 cells after 16 hours of treatment. ESC8 treatment also upregulated the cytosolic level of cytochrome c. Bid expression did not change with ESC8 treatments. C, the 1 and 5 μmol/L doses of ESC8 were sufficient for the induction of activated caspase-9 and caspase-3 activities, respectively, in MDA-MB-231 cells after 16 hours of treatment, consistent with the increase in Annexin V/PI staining. β-Actin was used as a loading control. The relative fold expressions of protein levels have been indicated as required. Figures represent 3 separate experiments with similar results.
siRNA to block the induction of autophagy and then treated with 5 μmol/L of ESC8. After 16 hours of ESC8 treatment, cell survival was measured by MTT assay. After Atg5 knockdown, ESC8 could not induce LC3-II formation (Fig. 3C) and survival of MDA-MB-231 cells was significantly increased (P < 0.01; Fig. 3D) as evidenced from the MTT assay result. Atg5 knockdown has no effect on the Annexin V/PI staining of the MDA-MB-231 cells compared with control siRNA-treated cells upon ESC8 treatment (data not shown).

Taken together, our data (Figs. 2 and 3) indicated that ESC8 led to simultaneous induction of apoptosis and autophagy to kill the ER-negative breast cancer cells. Finally, we wanted to understand which pathway might be involved in this regulation.

Effect of ESC8 on Akt and mTOR phosphorylation

The PI3K/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-signaling pathways has been implicated in autophagy and apoptosis regulation (31–33). First, we examined whether PI3K-Akt pathway inhibition could have any effect on ESC8-mediated killing. Pretreatment of MDA-MB-231 cells with PI3K-Akt pathway inhibitor LY294002 (LY) followed by treatment with ESC8 clearly and considerably inhibited the ESC8-mediated toxicity in the cells (Fig. 4A). Further Western blot results show that a 1 μmol/L ESC8 treatment blocked the phosphorylation of mTOR at Ser-2448 (Fig. 4B). Inhibition of mTOR phosphorylation resulted in decreased phosphorylation of p70S6 kinase at Thr-389 (Fig. 4B), the downstream effector of mTOR and indicating the inhibition of mTOR kinase activity upon ESC8 treatment. In contrast, a 10 μmol/L dose of ESC8 in MDA-MB-231 cells induced phosphorylation of Akt1/2/3 at Ser-473 (Fig. 4B). To replicate these anticancer effects in a preclinical setup, we further developed an orthotopic breast cancer model with MDA-MB-231 cells.

Effect of ESC8 on tumor regression and tumor cell apoptosis

The naked molecule ESC8 was injected intraperitoneally into mice containing a mammary pad tumor xenograft of MDA-MB-231 cells. The 10 mg/kg ESC8 injection began when average tumor volumes were about 30 to 40 mm3. In another group of mice, 10 mg/kg of ESC8 was injected intraperitoneally when the average tumor volume reached 130 mm3. Figure 5A clearly indicates the tumor inhibitory effect of ESC8, even at a limited number of injections. The
significant inhibitory effect of ESC8 was pronounced even when the tumor size was bigger. To further confirm the mechanism of the observed tumor-suppressive activities, we examined the effect of ESC8 on MDA-MB-231 tumor cell apoptosis in vitro with the TUNEL assay (Fig. 5B and C). The average number of TUNEL-positive cells in 10 randomly selected microscopic fields in both control and ESC8-treated groups were calculated. A significant increase in the number of apoptotic cells was observed in the group treated with ESC8 ($P < 0.001$, compared with the control group).

**Discussion**

Breast cancer, the leading cause of death in women worldwide, is associated with ER and its functional activity in 60% to 70% of cancer cases. Conventional treatments of early-stage or ER-responsive, advanced-stage breast cancer rely on either hormone ablation or antagonizing ER through the use of small molecule drugs. The use of conventional therapeutics for advanced breast cancer becomes wasteful, non-effective and, in some cases, exacerbates the situation through the induction of thrombosis and endometrial carcinoma—especially for tamoxifen-treated cases (34, 35).

The complexity in the advanced or post-ER-reliance stage of cancer, which includes the failure to activate apoptotic signals, heralds the transformation of primary cancer cells into a drug-resistant and metastatic phenotype. In a recent study, we could selectively manipulate glucocorticoid receptor (GR), a nuclear hormone receptor (NHR), in cancer cells using a cationic liposomal formulation associated with GR–ligand dexamethasone for selective access to the GR response element in cancer cells’ nuclei (36). In trying to replicate a similar observation with breast cancer cell–associated NHR, ER using its endogenous ligand, ES, we found to our surprise that ES in mere association with an 8-carbon, twin-chain, quaternary ammonium cationic lipid could efficiently kill both ER-positive and ER-negative breast cancer cells.

On the basis of the above observations, we hypothesized that instead of mere association, if we chemically conjugate this 8-carbon cationic lipid to an estrogen molecule, we might get enhanced anti-breast cancer activity. We found that ESC8 could initiate ER expression status nonspecific, anti-breast cancer activity, but with minimal toxicity to the noncancer cells. The observation is in contrast to previous evidence in the literature in which anticancer molecules with ES-moiety or ES-targeting antagonists, such as tamoxifen or Faslodex, show selective activity against ER-positive breast cancer cells. Our data clearly indicate that ESC8 is like any other common estrogenic drug-type molecule that is prone to induce cell death in ER-positive cells. At least in ER-positive breast cancer cells, the cationic molecule in the ER-bound state would have proximity to the estrogen-responsive promoter region of genomic DNA, thereby possibly damaging the DNA through irreversible electrostatic interaction. In ER-negative breast cancer cells, ESC8 exerts its effect possibly by manipulating PI3K/Akt/mTOR pathway.

Our subsequent studies revealed ESC8-mediated prominent induction of apoptosis in both ER-positive and ER-negative cancer cells. On investigating the specific reason for the activation of apoptosis in ER-negative MDA-MB-231 cells, we found significant apoptosis induction through the mitochondria-mediated intrinsic pathway in which caspase-3 formation was triggered by an elevated level of caspase-9 on ESC8 treatment. We also noticed that autophagy was highly induced but with no significant change in the level of
autophagy-related proteins such as Atg5-Atg12 and Beclin-1. Because one of the autophagy regulators is the PI3K-Akt-mTOR pathway, we investigated the effect of ESC8 on the protein levels of p-Akt-1/2/3 (Ser-473), p-mTOR (Ser-2,448), and p-p70S6K (Thr-389). ESC8 treatment inhibited mTOR and p70S6K phosphorylations; however, we observed an increase in phosphorylation of Akt at Ser-473 with the 10 μmol/L dose of ESC8. Although increased Akt phosphorylation is prosurvival, this finding resembles the rapamycin-mediated mTOR inhibition, which accompanies elevation of the phospho-Akt level in a negative feedback loop. Previous literatures have shown that rapamycin-induced Akt phosphorylation is dependent on PI3K and IGF signaling pathway (37, 38). Therefore, mTOR is a possible target of ESC8 and we think that ESC8 in combination with Akt Inhibitor would be better option for breast cancer therapy. Additional in-depth studies are currently being done to further look into the novel mechanism.

Our observation of simultaneous induction of mitochondria-assisted apoptotic signal and autophagy induction followed by inhibition of mTOR activity contradicts the previous literature, which showed that inhibition of mTOR fortifies cells against proapoptotic stimuli with the concomitant induction of autophagy (18, 19). Here, in ESC8-mediated killing of breast cancer cells, autophagy accompanies apoptosis, and our data also clearly prove that under these circumstances, the induction of autophagy is not cytoprotective in nature.

In conclusion, we report the development of a new class of highly efficient anti–breast cancer agents that contain a novel combination of estradiol and cationic lipid moieties. This estrogenic molecule, in contrast to other available estrogenic drugs, exhibits highly efficient anticancer activity in all ER-positive and ER-negative primary and advanced breast cancer cells. Hence, this anticancer agent is the first estrogenic drug that, with this special chemicostructural trait, shows unusual coinduction of autophagy and apoptosis in that it kills even ER-negative breast cancer cells. This unique structural trait provides the potential scope to treat even multiple-staged breast cancer using a single drug-based therapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

B.S. Reddy thanks UGC (University Grants Commission) and S. Ray, K. Pal, G. Sudhakar, and K.R. Ray thank CSIR, Govt. of India, for their respective doctoral fellowships. The authors also thank Santanu Bhattacharya and Jim Tarara, Mayo Clinic for helping with MDC staining and confocal microscopy, respectively. D. Mukhopadhyay is a scholar of the American Cancer Society.

References


Molecular Cancer Research

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doi:10.1158/1541-7786.MCR-10-0526

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