Gene Expression Profiles with Activation of the Estrogen Receptor α-Selective Estrogen Receptor Modulator Complex in Breast Cancer Cells Expressing Wild-Type Estrogen Receptor

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ABSTRACT

Selective Estrogen Receptor Modulators (SERMs) are a new class of drugs that bind to estrogen receptor (ER) and elicit agonistic or antagonistic responses, depending on the target tissue. We have developed an in vitro system in which some SERMs (4-hydroxytamoxifen and resveratrol) demonstrate estrogenic response through wild-type (wt) ER, whereas others (raloxifene and GW7604) remain antiestrogenic. This system mimics the tamoxifen-resistant phenotype in clinic, when resistant tumors contain wtER. We used Atlas cDNA arrays to study gene expression profiles after ER activation by different SERMs in MDA-MB-231 human breast cancer cells stably transfected with wtER. Cells were treated with estradiol, four different SERMs, and the pure antiestrogen ICI 182,780. The obtained expression data were analyzed using GeneSpring software. Real-time reverse transcription-PCR was used to verify the array data. Our results showed that treatment with various compounds altered the expression of a diverse group of genes, revealing sets of overlapping genes that may represent a complex network of genes of interrelated signal transduction pathways.

Sets of “agonistic” and “antagonistic” genes were identified on the basis of the known response to different SERMs. Further analysis of selected sets of genes revealed functionally related group of genes in each set, encoding proteins that were related to cell proliferation, survival, and apoptosis. Flow cytometry data indicated an antiapoptotic activity in cells treated with agonists versus apoptotic activity in cells treated with antagonists. A model for estradiol-like (survival) and antiestrogen-like (apoptosis) activities of SERMs on the basis of their gene expression profiles is suggested.

INTRODUCTION

SERMs are a new class of drugs that have an important impact in breast cancer treatment and prevention and hold promise for preventing several other disorders in women (1).

One of the drugs, tamoxifen, is the most widely used hormonal therapy for all stages of breast cancer (2, 3). In breast tissue, tamoxifen binds to the ER and competitively blocks the action of estrogen (4); in other tissues (cardiovascular system, bone, and uterus), however, tamoxifen itself has estrogen-like effects. In the clinical setting, tamoxifen therapy, besides reducing breast cancer incidence (5), can also produce a favorable lipid profile and maintain bone density, providing multiple potential benefits to cancer patients (6, 7). At the same time, in the uterus tamoxifen acts as a partial estrogen and can therefore increase the risk of endometrial carcinomas (8). Another undesirable effect of tamoxifen is development of drug resistance (9).

Drug resistance to tamoxifen can be manifested in many ways (9, 10), one of which is tamoxifen-stimulated tumor. Clinical data show that some women who have breast tumor relapse while on tamoxifen therapy have tumor regression after tamoxifen withdrawal (11–13), suggesting that tumor recurrence may result from cells that are growth-stimulated by tamoxifen. Although there is no possibility that the exact proportion of tamoxifen-stimulated tumors can be determined from the existing clinical data, some studies demonstrated ~20% of withdrawal responses after cessation of tamoxifen as first-line therapy for advanced disease (13). This real clinical phenomenon is the basis for the development of aromatase inhibitors that are superior to tamoxifen for the treatment of advanced disease (14) and the basis for the current clinical use of aromatase inhibitors after tamoxifen failure (15). Another drug of the SERM group, Ral, exhibits high affinity for the ER. It has been shown to prevent bone loss in postmenopausal women (16), act as an antiestrogen in mammary tissue (17), and reduce estrogenic actions in the uterus (18).

The clinical success of tamoxifen and Ral has encouraged the search for novel SERMs for applications as multifunctional drugs. The tamoxifen-derivative GW5638 is an antiestrogen with actions in vivo that classify it as a SERM with less estrogenic activity than tamoxifen in the rat uterus but agonist activity in bone (19, 20). GW5638 is a prodrug that is converted to its active metabolite, GW, used in this study. Another compound, used in this study is Res, a natural phytoalexin found in grapes and other food products. It is categorized as a phytoestrogen because it can bind and activate ER (21). Recent studies suggested that Res possesses mixed agonist/antagonist activity of the ER (22–25).

The molecular mechanisms responsible for tissue selectivity of SERMs are unknown. A significant step toward resolving the issue was made when crystal structures of the ligand binding domain of ER with estrogens and antiestrogens became available (26, 27). It is now clear that ER, when occupied by estrogens, has a distinct conformation that is different from conformations when occupied by antiestrogens. In addition to the “on” and “off” conformations, intermediate conformations are possible that determine the contact with different adapter proteins producing a spectrum of ER agonist/antagonist activities (28, 29). Clearly, the shape of an ER-SERM complex will dictate how or if any other protein in a transcription unit will bind and subsequently activate gene transcription. The identification and characterization of genes regulated by different SERMs through an ER signaling pathway will identify potential targets for future therapeutic applications.

We have developed an in vitro system for functional analysis of...
clinically relevant antiestrogens. In MDA-MB-231 breast cancer cell lines, stably transfected with wtER, some SERMs demonstrate an estrogenic response, whereas others remain antiestrogenic (30–36). In particular, 4OHT, a potent tamoxifen metabolite used in our experiments, caused a significant estrogenic response, which was evidenced by changes in expression levels of E2-regulated genes and cell growth response (30–32). Similarly to 4OHT, Res acted as an agonist on the activation/repression of selected endogenous estrogen-responsive genes as well as on the cell growth response. Two other SERMs, Ral and GW, along with the pure antiestrogen ICI, acted as antiestrogens in this system (33–36).

We report here differential gene expression profiles in breast cancer cells expressing wtER after treatment with E2, 4OHT, Ral, GW, Res, and ICI using Atlas cDNA expression arrays (Clontech). Comprehensive analysis of array data allowed us to identify “agonistic” and “antagonistic” genes and to propose SERM-induced differential signaling pathways.

**MATERIALS AND METHODS**

**Tissue Culture.** The stable transfectants (S30) used in this study were constructed from the ER-negative MDA-MB-231 human breast cancer cells as described previously (37). The level of ER in these cells is comparable with the level of ER in MCF-7 cells. Cell were maintained in phenol red-free MEM with 5% charcoal-dextran-treated calf serum, supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM l-glutamine, 6 ng/ml bovine insulin, 100 mM nonessential amino acids, and 500 μg/ml G418. All of the tissue culture solutions were from Life Technologies, Inc. (Gaithersburg, MD).

**Compounds.** E2, Res, and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). 4OHT and ICI were obtained from ICI Pharmaceuticals (Macclesfield, England). Ral was a generous gift from Eli Lilly Research Laboratories (Indianapolis, IN). GW was a generous gift from Dr. Timothy Willson (Glaxo Wellcome, Inc., Durham, NC). The structures of the compounds were published previously (25, 34). All compounds used in the experiments were dissolved in 100% ethanol and added to the medium in 1:1000 dilutions for a final ethanol concentrations no higher than 0.2%. VP-16 was diluted in DMSO for a final concentration of 50 μM.

**Array-based Expression Profiling.** We used Atlas Human cDNA expression arrays (Clontech, Palo Alto, CA), a positively charged nylon membrane that is spotted in duplicate with 200–600-bp cDNA fragments representing 588 known cancer-related genes and 9 housekeeping genes. These known genes are divided into six functional categories: category A, oncogenes, tumor suppressor genes, and cell cycle regulatory genes; category B, stress response, ion channel and transport, and intracellular signal transduction modulators and effectors; category C, apoptosis-related, DNA synthesis, and repair; category D, transcription factors and DNA binding proteins; category E, receptors, cell surface antigens, and cell adhesion genes; and category F, cell-cell communication genes.

The labeling and hybridization procedures were conducted as specified by the manufacturer (Clontech). We isolated total RNA from cells after 24-h treatment with different compounds or ethanol (vehicle) using Trizol Reagent (Life Technologies, Inc.). RNA were converted to P32-labeled cDNAs using gene-specific primers and hybridized to array membranes according to the protocol. Two independent RNA populations (n = 2) for each experimental condition were arrayed in three hybridizations (n = 3) using at least two fresh (unstripped) membranes.

**Array Quantitation, Data Processing, and Statistical Analysis.** Signals were detected by phosphorimaging analysis using a Molecular Dynamic Storm phosphorimagier (Molecular Dynamics, Sunnyvale, CA). Initial analysis was performed using AtlasImage 1.5 software (Clontech). Each spot was defined by aligning the image to the grid template. Composite arrays based on averaging three independent Atlas Array experiments for each compound were created. After background correction, normalization was done for each gene against GAPDH. We compared several methods of normalization and found use of the GAPDH values to be optimal. Comparisons were done between composite array of cells treated with compound versus composite array of vehicle-treated control cells. Two parameters were closely monitored and used for comparison: signal ratio and signal difference. A ratio of 2 and higher (for up-regulation) and a ratio of 0.5 and lower (for down-regulation) between background-corrected normalized gene expression levels were considered to be significant. However, this refers only to those genes for which background-corrected signal is above zero in vehicle-treated control cells. Therefore, the heterogeneity of levels of expression for different gene influences the outcome of data analysis by AtlasImage 1.5 software. However, we are interested in the differences between levels of expression for a given gene independent of its own level of expression. For genes with low expression levels in vehicle-treated control cells, the changes in expression can be dramatic after treatment with compound. These changes, from no expression in vehicle-treated cells to any expression in treated cells, could be of particular interest and should not be overlooked.

There are two categories of changes in gene expression patterns: up-regulation and down-regulation. There are two subsets for each of these categories: subset 1, a significant changes calculated as a fold increase (>2) and b, “undefined” fold representing changes from zero in untreated control cells to detectable signal in treated cells; and subset 2, a, significant changes as a fold decrease (<0.5) and “zero” fold representing changes from detectable signal in treated cells to zero in untreated control cells. Therefore, a distinguishing characteristic in formulating a comparison between two arrays is whether or there was signal detected for a given gene in both arrays. Which type of changes is more indicative of biologically relevant information is yet to be determined.

To calculate P values in our expression data, we performed a t test using the null hypothesis that nothing changes in each experiment. Under the null hypothesis, expression levels would be the same between treated and control cells, and the fold change would be equal to 1. Therefore, each fold ratio greater than one can be viewed as a measure of change. To test for changes irrespective of the specific drug within a group, we view each fold ratio in this test as an individual observation and disregard the fact that there are from different
treatments. The average and SD was calculated using fold increases from the three treatments, \( P \) was calculated using 2 degrees of freedom and a \( t \)-distribution. Genes with low \( P \) and consistent change were considered to have significant induction because of treatment within agonists or antagonists.6

It is critical to preprocess the data set into a form suitable for approaching different statistical and visualization methods. Before grouping, multiple expression data sets for analysis, normalization was performed to account for systematic differences across data sets. Microsoft Excel, Access (Microsoft Corp., Seattle, WA) and GeneSpring (Silicon Genetics, Redwood City, CA) software were used for generating lists of selected genes and for different statistical and visualization methods.

**Real-Time Quantitative PCR.** We used the same mRNA pools for both microarray and real-time PCR. mRNA quantitation was performed using ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), which uses the 5′ nucleic activity of Taq DNA polymerase to generate a real-time quantitative DNA analysis assay. Briefly, gene-specific oligonucleotides probes with 5′ fluorescent and 3′ quencher dyes (TaqMan probes) and primers were designed using Primer Express software (PE Applied Biosystems). We used TaqMan Gold RT-PCR kit (PE Applied Biosystems) for preparation of one-step PCR Mix. RT-PCR was performed using 5 ng of total RNA. We used a multiplex PCR assay. In this case, two sets of primers and probes with different quencher dyes were added to each individual PCR, thus allowing the coamplification of both gene of interest and internal control (GAPDH) cDNA in a single well and therefore eliminating variation introduced attributable to differences in cDNA loading. Three replicates were run for each sample. Amplification consisted of a 30-min reverse transcription step at 48°C, immediately followed by 40 cycles of a two-step PCR reaction: 95°C for 15 s and 60°C for 1 min. The results were analyzed by using 7700 system.

**Evaluation of Apoptosis.** We used TACS Annexin V-FITC detection kit (R&D Systems, Minneapolis, MN) to evaluate apoptosis, which is based on the use of Annexin V-FITC conjugates for flow cytometry of cell surface changes that occur early in the apoptotic process. Briefly, cells were seeded in six-well plates at 2 \( \times 10^5 \) cells/well in phenol red-free MEM containing 5% charcoal-stripped calf serum as described above. After treatment with compounds and VP-16 for 24 h, cells were detached with EDTA, harvested, washed with cold PBS, and counted (3 \( \times 10^7 \)/100 \( \mu \)l). After incubation with Annexin V-FITC and propidium iodide for 15 min incubation in the dark, samples were analyzed by flow cytometry using a fluorescence-activated cell sorter (EPICS XL-MCL; Beckman Coulter, Miami, FL). Analyses of flow cytometry results were done using System II Software (Beckman Coulter).

**RESULTS**

As the first step toward identification of “agonistic” and “antagonistic” genes differentially expressed in response to SERMs in breast cancer, we identified gene expression profiles in MDA-MB-231 human breast cancer cells transfected with vteR after treatment with E2, SERMs (4OHT, Ral, Res, and GW), and pure antiestrogen ICI. Seven sets of data were generated for this cell line after 24 h of treatment: gene expression profile for cells treated with vehicle ethanol (control); with \( 10^{-9} \)M E2; with \( 10^{-6} \)M 4OHT; with \( 10^{-6} \)M Ral; with \( 5 \times 10^{-8} \)M E2; with \( 10^{-8} \)M GW; and with \( 10^{-6} \)M ICI. In each case, the concentrations of compounds used were based on studies published previously (30–35).

**Differential Gene Expression in Cells Treated with Different Compounds: “Agonistic” and “Antagonistic” Genes.** Similarities and differences between gene expression profiles of two given samples can be visualized using scatterplots. In a scatterplot, each point represents the expression value of a gene in two experiments, one plotted on the X-axis (untreated control) and the other one on the Y-axis (compound-treated; Fig. 1). Although the transcript levels

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6 Complete data sets for expression profiles are available at http://www.math.mtu.edu/~ Igor/Gene_index.html.
from the vast majority of genes remain unaltered [genes lined up on the identity line (diagonal)], there are outliers in each experimental condition when cells were treated with different compounds. Most of outliers are up-regulated genes in cells treated with compounds compared with untreated control cells.

The criteria for differential gene analyses were chosen based on prior knowledge of responsiveness (e.g., growth inhibition by E2 and endogenous gene expression modulation) of the cells to different compounds; 4OHT had estrogenic effects on these cells, whereas Ral and ICI were antiestrogens (30–34). In addition, Res had the same effects on growth of these cells and expression of selected target genes and growth factors (hepatoma-derived growth factor and insulin-like growth factor binding proteins 1 and 3) expression might indicate activation of survival pathways on response to hormone-induced stress. Up-regulation of MAP kinase p38 after treatment with these compounds supports this observation.

When the same method was applied to antagonist-induced genes, 24 genes were selected as overlapping among cells treated with Ral, ICI, and GW (Fig. 2B). These genes can be categorized as genes that control cell proliferation and apoptosis. Although these genes have diverse functions, the striking observation is that a large number of genes from this group implicate stress-induced apoptotic pathways (RANTES, MLK3, protease-activated receptor 1, EMAF2, caspase-4, and c-Jun).

Next, we identified saturated pools of “agonistic” (E2-like response) and “antagonistic” (AE-like response) genes. Fig. 3 shows list of genes specific for E2-like (A) only and AE-like (B) only pathways. Interestingly, there are five genes whose expression is up-regulated with all treatments (Fig. 3C) compared with vehicle-treated control cells. To validate Atlas data, we used a quantitative real-time PCR method to analyze a subset of genes up-regulated by agonists and antagonists. Overall, changes in mRNA levels were overestimated by Atlas microarray method compared with the PCR method, although we observed a general and consistent agreement between the two methods (Fig. 4). At this moment, we do not have any exact explanation for this diversity in the sensitivities of these two methods; however, our preliminary data indicate reduced efficiency of cDNA synthesis and/or amplification for heterogeneous RNA samples isolated from the cells.

To determine whether apoptosis might occur as a result of treatment with E2 and/or AE in these cells, we assessed apoptotic activities using the ability of cells undergoing apoptosis to bind Annexin V and exclude propidium iodide with subsequent analysis by flow cytometry. Cells were treated for 24 h with E2, 4OHT, Ral, ICI, and VP-16, a topoisomerase II inhibitor known as an inducer of apoptosis in many cell lines (38, 39). As can be seen in Fig. 5, the proportion of cells

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7 B. Chen, unpublished data.
undergoing apoptosis after treatments with E2 and 4OHT were considerably less than in cells treated with Ral and ICI. Because of high variability between individual experiments, statistical evaluation showed significant differences only between ICI- and E2-treated cells. However, a clear trend consistent with normalized data presented in the figure was observed in each individual experiment.

**DISCUSSION**

We used cDNA Atlas arrays to evaluate the gene expression profiles after the activation of ER by different ligands in breast cancer cells expressing ectopic wtER. Studies on changes in gene expression profiles of parental cells (MDA-MB-231) treated with SERMs in comparison with profiles of the ER transfectants treated with SERMs would give valuable information about SERM-specific but ER-independent effects. However, our goal was to identify an “agonistic” versus “antagonistic” set of genes using our unique in vitro system in which E2, 4OHT, and Res have agonist activities (28–32) and Ral, ICI, and GW have antagonist activities (33–36). There are several interesting features of our findings: (a) there are more up-regulated genes after treatments than down-regulated genes; (b) the number of activated genes after treatment with SERMs are higher than after treatment with E2; and (c) gene expression patterns of cells after treatment with pure antiestrogen ICI are not dramatically different than after treatments with SERMs. The latter observation was surprising and indicates the activation of large number of genes through ER-independent mechanisms.

The action of E2, 4OHT, and Res (E2-like) and Ral, GW, and ICI (AE-like) in cells is reflected by the variation of a set of genes shown in Fig. 3A and B. These two distinct sets of coexpressed genes provide views of the activities of specific signaling and/or regulatory pathways in each case. We have interpreted our gene array findings to propose hypothetical E2-like and AE-like pathways activated in the stably transfected breast cancer cells (Fig. 6).

**E2-like Pathways.** Two interrelated survival pathways are activated (Fig. 6A). SHP-2 are adapter molecules that are involved in the signal transduction of ligand-activated tyrosine kinase receptors (40–42). SHP-2 mediates the association of interleukin 7Rα with PI-3K (43). The intracellular kinase Akt (also called protein kinase B) affects multiple cellular functions initiated by integrins, cytokines, and hormones through PI-3K (44, 45), and the PI-3K-Akt pathway is known as a cell survival signaling pathway (46). Another pathway, activated by SHP-2 and well recognized as estrogen-activated pathway, is Src/p21<sup>WNT</sup>/extracellular signal-regulated kinase pathway (47). Interestingly, it has been also demonstrated that this pathway can be activated by the agonist-activated progesterone receptor (48). In addition, activation of the Src family kinases or Ras/MAP kinase/extracellular signal-regulated kinase pathways can be linked to survival but not proliferation (49, 50). Two transcription factors up-regulated in response to agonists (Fig. 3A) belong to zinc finger protein subfamilies; ZNF91 is a member of KRAB zinc-finger proteins of potent transcriptional repressors (51), and ZNF148, a Krup-
pel-type zinc finger protein, participates in negative cell growth regulation (52).

**AE-like pathways.** AE-activated pathways in these cells include genes involved in the control of cell proliferation and stress-induced apoptosis (Fig. 3B and Fig. 6B). The MLK-3 can activate stress-activated protein kinase/c-Jun NH2-terminal kinase 1 apoptotic pathways when induced by various agents (53, 54). It has been demonstrated recently that tamoxifen induces apoptosis in ER-negative breast cancer cells through the JNK1 and caspase-3 pathways (55). Production of the chemokine RANTES activates an apoptotic pathway and activation of caspase-9 and caspase-3 in melanoma cells (56). In addition, RANTES production and transcriptional regulation is regulated by other factors involved in apoptosis (57, 58). EMAP2, caspase-4, and copper-zinc SOD1 have been shown previously to participate in cell modifications induced by stress and apoptosis. EMAP2 is an antitumor cytokine that suppresses primary and metastatic tumor growth and induces stress-related apoptosis in endothelial cells (59), whereas caspase-4 is involved in Fas-mediated apoptosis (60). Protease-activated receptor 1 is a G-protein-coupled cell surface receptor that participates in thrombin-induced caspase-mediated apoptosis (61). In contrast, SODs, an antioxidant, has a protective effect against toxic oxygen radicals that are implicated in the induction and mediation of apoptosis and DNA damage (62). Interestingly, the examination of the effects of different hormones on mRNA levels of SOD-1 showed that prolactin and placental lactogen induce a remarkable increase, progesterone has no effect, whereas estrogen and dexamethasone inhibit SOD-1 mRNA levels in primary granulose cells and transformed luteal cells (63). We found that levels of SOD-1 were increased by antiestrogens Ral, ICI, and GW in our breast cancer cells.

Both RANTES and MLK3 regulate signals mediated by the stress-responsive stress-activated protein kinase/c-Jun NH2-terminal kinase 1 pathway to lead to the activation of c-Jun that plays a fundamental role in growth control. Evidence has accumulated to implicate activated protein-1 (Fos/Jun) transcription factor complexes as both positive and negative modulators of distinct apoptotic pathways in many cell types (64). Moreover, stimulation of other genes, such as cyclin B1 and cyclin-dependent kinase-like kinase, normally involved in the cell cycle and growth control and known to be regulated by estradiol (65), has been implicated in DNA damage-induced apoptosis in vitro and in vivo (66–69). Finally, it has been shown that topoisomerase II also functions during apoptotic cell death (70–72).

Five genes are overlapping between the two categories (E2-like, AE-like) of genes (Fig. 3C). The cyclin-dependent kinase inhibitor p21Cip1/WAF1 is known to play a role in regulating the cell cycle by inducing G1 arrest and blocking entry into S-phase. The fact that its expression level was highly induced by E2, 4OHT, and Res might reflect the eventual growth-inhibitory effect of the compounds on these cells (36, 37). Induction of p21Cip1/WAF1 by estrogen in a breast epithelial cell line transfected with the ER has been suggested as a possible mechanism for a negative regulatory role of estrogen (73). Another gene from this group, MAP kinase p38, appears to play a role in both apoptotic and survival pathways. The ZNF9 transcription factor plays a role in sterol-induced regulation of cell viability and growth (74) and is up-regulated by sterols (75).

In conclusion, our results lead us to propose the following plausible model for how breast cancer cells have different agonistic/antagonistic responses to SERMs. Treatment with compounds induces stress. Sets of genes are activated in response to stress that might lead to the growth arrest state. In case of an E2-like response, survival pathways are activated that results in resistance to apoptosis. On the other hand, after a transient growth arrest state, treatment with antagonists leads to activation of various apoptotic pathways.

It is evident that biological responses to estrogens and antiestrogens reflect the existence of a complex network of genes that can comprise
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