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Comparative Gene Expression Profiling Reveals Partially Overlapping but Distinct Genomic Actions of Different Antiestrogens in Human Breast Cancer Cells

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Abstract Antiestrogens used for breast cancer (BC) treatment differ among each other for the ability to affect estrogen receptor (ER) activity and thereby inhibit hormone-responsive cell functions and viability. We used high-density cDNA microarrays for a comprehensive definition of the gene pathways affected by 17β-estradiol (E2), ICI 182,780 (ICI), 4OH-tamoxifen (Tamoxifen), and raloxifene (RAL) in ER-positive ZR-75.1 cells, a suitable model to investigate estrogen and antiestrogen actions in hormone-responsive BC. The expression of 601 genes was significantly affected by E2 in these cells; in silico analysis reveals that 86 among them include one or more potential ER binding site within or near the promoter and that the binding site signatures for E2F-1, NF-Y, and NRF-1 transcription factors are significantly enriched in the promoters of genes induced by estrogen treatment, while those for CAC-binding protein and LF-A1 in those repressed by the hormone, pointing to novel transcriptional effectors of secondary responses to estrogen in BC cells. Interestingly, expression of 176 E2-regulated mRNAs was unaffected by any of the antiestrogens tested, despite the fact that under the same conditions the transcriptional and cell cycle stimulatory activities of ER were inhibited. On the other hand, of 373 antiestrogen-responsive genes identified here, 52 were unresponsive to estrogen and 25% responded specifically to only one of the compounds tested, revealing non-overlapping and clearly distinguishable effects of the different antiestrogens in BC cells. As some of these differences reflect specificities of the mechanism of action of the antiestrogens tested, we propose to exploit this gene set for characterization of novel hormonal antagonists and selective estrogen receptor modulators (SERMs) and as a tool for testing new associations of antiestrogens, more effective against BC. J. Cell. Biochem. 98: 1163–1184, 2006.

Key words: estrogen; breast cancer; tamoxifen; raloxifene; ICI 182,780; cDNA microarrays

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Breast cancer (BC) is the most common malignant neoplasm in women [Jemal et al., 2002]. Since years, it is known that development and growth of these tumors are largely influenced by the stimulating action of estrogens toward mammary gland epithelial cells [Beatson, 1896]. The activity of these hormones depend on the presence of specific receptors: estrogen receptors (ERs: Gronemeyer [1991]). Hormone binding to ER induces conformational changes driving dimerization of the protein, enhancing its DNA-binding activity and ability to recruit transcriptional co-regulators and, thereby, regulating the rate of gene transcription (genomic pathway, Nilsson et al. [2001]). Estrogen signaling, however, includes also a "non-genomic pathway" involving the rapid and transient activation of several signal transduction networks, occurring primarily at the periphery of the cell and in the cytoplasm but likely to affect also gene expression [Migliaccio et al., 1996; Kato et al., 2000]. Both these pathways play an important role in regulation of epithelial cell functions by estrogen, and their integration is a key determinant for the actions of these hormones in normal and transformed breast epithelial cells [Nilsson et al., 2001; Gruber et al., 2002].

Estrogen regulation of BC cell proliferation correlates with direct transcriptional activation by ER of cell cycle control genes, including "immediate-early" and D-type cyclins [Weisz and Rosales, 1990; Weisz and Bresciani, 1993; Cicatiello et al., 2004a], and hormonal control of cell survival and differentiated functions occurs via regulation of multiple genomic networks in BC cells [Cicatiello et al., 2004b]. This key role of estrogens in the control of cell growth, viability, and functions led to devising ways for long-term management of BC through endocrine therapy. This is based on administration of antagonist drugs, receptorial antiestrogens, able to compete with the hormone for binding to ERs, thereby reducing stability of the receptor and preventing the allosteric modifications of the molecule that allow establishment of novel intra- and inter-molecular interactions essential for activation of both genomic and non-genomic pathways. These compounds are grouped in two distinct functional classes: pure antiestrogens, including ICI 182,780 (Faslodex) that are thought to be fully devoid of estrogen agonist activities, and mixed agonists/antagonists, endowed instead with graded hormone-like activities, often cell-type specific, and thus able to act also as SERMs. This second class of antiestrogens includes 4OH-tamoxifen and raloxifene (RAL) [MacGregor and Jordan, 1998]. Upon binding to the receptor, SERMs induce different conformational changes of the molecule, causing altered interactions with effector molecules and affecting hormonal signaling and the consequent regulation of gene expression.

Tamoxifen (TAM) is the first antiestrogen introduced in the clinical practice [Ward, 1973; Fisher et al., 1989], where it has shown activity both in reducing tumor burden and in relief of symptoms [Mouridsen et al., 1978], and appears to be effective also in lowering BC risk in postmenopausal women [Fisher et al., 1998]. Unfortunately, TAM is not a pure antagonist but a SERM with partial estrogen-like activity, in particular in the endometrium, where it promotes carcinogenesis [Kedar et al., 1994; Assikis et al., 1996]. This drawback has raised concerns against prolonged TAM administration to patients, especially with preventive aims. Moreover, during long-term hormonal therapy with TAM, loss of hormone-responsiveness develops in most BCs [Gottardis and Jordan, 1988]: the average length of cell responsiveness to hormonal treatment being suggested to be 12 months [Margolese et al., 2000]. To overcome these drawbacks of TAM, new antiestrogen molecules have been devised. Among them, RAL, a SERM with less partial agonistic activity than TAM on breast and endometrium, has proven useful in BC prevention and in the treatment of osteoporosis, for its specific agonist actions on bone, possibly without increasing the risk of endometrial carcinoma (MORE trial, Barrett-Connor et al. [2002]). On the other hand, the pure antiestrogen ICI 182,780 (ICI) shows a full-antagonist activity on breast and endometrium without affecting bone density and serum lipids, and has been successfully used in patients with TAM-refractory BC [Howell et al., 1995] as it shows also growth inhibitory actions in cells that developed TAM-resistant proliferation [Hu et al., 1993].

The mechanistic bases of these different pharmacodynamic properties of the three antiestrogens described above are not fully understood, in particular for what concerns their overall effects in BC cells, and a deeper insight into these aspects is needed to drive the

development of new, more effective, and less toxic drugs of this family. A model of tripartite pharmacology has been proposed for antiestrogens [Katzenellenbogen et al., 1996] in which the final antihormone action depends upon: (1) ER conformational changes induced by the drug and the consequent acquisition of intermediate foldings between the "full active" and the "full inactive" receptor structure [McDonnell et al., 1995; Brzozowski et al., 1997; Shiau et al., 1998], thus altering the pattern of interaction with the transcription machinery [Wijayaratne et al., 1999], (2) the modality of antiestrogen-ER complex binding to the promoter, either direct [Yang et al., 1996] or indirect, through interaction with other trans-activating factors, such as AP-1 [Ambrosino et al., 1993; Cicatiello et al., 2004a], and (3) the co-factors expressed by the cell: the ER-TAM complex has been shown to recruit co-repressors instead of co-activators [Lavinsky et al., 1998; Shang et al., 2000], while an excess of co-activators account for estrogen-like activity of TAM on the endometrium [Katzenellenbogen and Katzenellenbogen, 2002; Shang and Brown, 2002]. The result of this complex network of interactions is a cell- and promoter-specific profile of actions that differs among the various compounds belonging to this class.

An informative way to study the molecular mechanisms underlying these specificities has been shown to consist in testing the transcriptional regulation of estrogen-responsive genes by these drugs [Katzenellenbogen et al., 1996; Levenson and Jordan, 1999; Zajchowski et al., 2000; Shang and Brown, 2002]. Transcription of the estrogen responsive *TGF*-α gene, for example, is regulated positively by TAM [Levenson et al., 1998] and negatively by ICI and RAL [Levenson and Jordan, 1998], suggesting that classification of antiestrogens can be based also on their trans-activating properties on estrogen-responsive genes. Following these lines, a panel of 24 combinations of genes and cells has been recently developed, which discriminates antiestrogen compounds according to their profile of regulation of estrogen-responsive genes [Zajchowski et al., 2000]; this brought these A.s to propose a new functional classification of antiestrogens according to the similarity of the pattern of gene expression generated by each compound, relative to a reference patterns (TAM-like, RAL-like, and pure antagonist (i.e., ICI)-like, for example).

Comparative genome-scale analyses of gene expression are quite useful to depict the changes induced by one or more antiestrogen in BC cells [Soulez and Parker, 2001; Inoue et al., 2002; Levenson et al., 2002a, 2002b]. To this aim, we thus used high-density cDNA microarrays to identify transcriptional programs regulated by a short-term treatment of the human hormone-responsive BC cell line ZR-75.1 with TAM, RAL, or ICI, in the absence or presence of E2, with the main aims to gain new insights on the genomic pathways regulated by one or more of these compounds and, at the same time, to create an informative set of "antiestrogen-responsive" genes useful for the classification of new antiestrogens [Levenson et al., 2002a] as well as to predict the clinical effects of the different drugs.

MATERIALS AND METHODS

Cell Culture and RNA Purification

ZR-75.1 cells were propagated in DMEM medium supplemented with 5% FBS and antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin and 250 ng/ml Amphotericin-B). For synchronization, cells were plated at 20% confluence and maintained for 4 days in a steroid-free medium (phenol red-free DMEM medium with 5% FBS pre-treated with dextran-coated charcoal and antibiotics). The cells were stimulated with 10^{-10} M 17β-estradiol [E2] for 72 h; in the last 24 h of treatment, both E2-treated and synchronized cells were incubated with a fixed concentration (2×10^{-8} M) of 4-hydroxy-tamoxifen (4OHT) or RAL or ICI, while control cells were left, respectively, in E2-added and steroid-free medium.

Total RNA was extracted as described earlier [Cicatiello et al., 2000], and poly(A)+ RNA was isolated with the Dynabeads method (Oligo $(dT)_{25}$; Dynal, Oslo, Norway), resuspended in DEPC-treated water, quantitated, and tested by agarose gel electrophoresis. Estrogen and antiestrogens effects on cell proliferation were also tested by cytofluorimetric analysis.

cDNA Labeling and Microarray Hybridization

cDNA microarray analysis was carried out on human UniGEM V 2.0 glass arrays (Incyte Genomics, St. Louis, MO), encompassing

9,128 cDNA elements, corresponding to 8,286 unique UniGene clusters. Two-hundred nanograms of poly (A)+ RNA were used to synthesize cDNA fluorescently labeled with either Cy5 (treated samples) or Cy3 dye (reference RNAs). Competitive hybridization on microarrays glasses was performed after mixing labeled cDNA from each experimental sample with the relative reference sample: untreated cells for the E2-untreated group, E2-stimulated cells for the E2-treated group. Three independent hybridizations were performed for each sample pair; in one of the three triplicates a dye swap was carried out.

Microarray Data Analysis

Selection of informative genes and normalization. Data from microarray scanning was first filtered, by discarding data from the elements of the array, which did not pass the Incyte PCR quality controls, showing multiple bands or no amplification. Then intra-array and inter-array scaling were performed. Standard intra-array scaling was carried out for each array according to a whole-chip approach, to minimize intrinsic fluorescence differences between the two dyes and to allow inter-array normalization, which was then performed to make data from different arrays directly comparable for permutation-based statistical analysis. E2-treated and E2-untreated group were thereby normalized independently from each other. A normalization coefficient was calculated for each array by computing the ratio of the total fluorescence intensity of the chip to the mean of all the values of total fluorescence within the array group, and data of each element of each array were scaled according to the relative coefficient. Subsequently, data showing a signal to background ratio lower than 3.0 were discarded. Genes (6,379) were selected as informative for the treated versus untreated group, 6,378 for the antiestrogen-treated versus estrogen-treated group. The groups were partially overlapping, and encompassed a total of 6,452 elements of the array.

Statistical analysis. Statistical analysis was performed according to the protocol developed by Tusher et al. [2001], that distinguishes casual fluctuation of fluorescence values from significant variations in gene expression, according to the distance (Δ) of observed relative difference (d_i) between treatment and control from the expected d_i, calculated for each gene as the mean value of d_i computed for all possible inter-chip permutations. Permutations were performed, in each case, with data from triplicate determinations versus three references. Delta value was selected when corresponding to a false discovery rate (FDR) ranging between 0.02% and 5%.

Selection of regulated genes. For antiestrogen-regulated genes, a double cut-off method was used, in order to make selection of both "regulated" and "non-regulated" genes as stringent as possible. We considered antiestrogen-responsive the genes with a fold change (FC) (treatment to reference ratio) equal or greater than |1.7|, for both activation and inhibition. This cut-off is higher than what originally proposed by the authors of SAM, who selected their treatment-responsive genes according to a FC cut-off of 1.5 [Tusher et al., 2001]. For estrogen-responsive genes, a cut-off value of |1,4| was instead assigned, thanks to the most favorable Δ value obtained with these datasets. According to these criteria, 497 genes were defined as estrogen-responsive, and 373 as antiestrogen-regulated, among which 52 genes were not influenced by the hormone.

Functional annotations. Unique UniGene clusters (www.ncbi.nlm.nih.gov/UniGene) were extracted for all cDNAs of regulated genes. Chromosomal localization, gene description and other functional informations were derived by LocusLink (www.ncbi.nlm.nih.gov/LocusLink) as well as other databases (www.ensembl.org; bioinformatics.weizmann.ac.il/cards/). Functional classification of genes was carried out according to the Gene Ontology Consortium nomenclature [Ashburner et al., 2000]; (www.geneontology.org), using SOURCE (genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch), and Onto-Express softwares [Khatri et al., 2002; vortex.cs.wayne.edu:8080]. Functional annotation was based on GO tags found in at least four genes and enriched with respect to the starting dataset (P-value ≤ 0.05).

In silico promoter analysis. Three different ERE annotation sources were used to search for the presence of putative EREs in the estrogen- and antiestrogen-regulated loci. First of all, we searched for the presence of our genes in the RRE database [Lazzarato et al., 2004]. In this database are annotated human genes containing, in their −2,000/+500 region, at

least one ERE, also conserved within rat and mouse orthologs.

The second approach consisted in comparing our list of genes within the data set of ERE containing genes published by Bourdeau et al. [2004].

Finally, promoter analysis was performed with the PRIMA tool, developed by Elkon et al. [2004]. Given target and background sets of promoters, PRIMA performs statistical tests aimed at identifying TFs whose binding sites are significantly more abundant in the target set than in the background set. PRIMA uses position weight matrices (PWMs) as models for regulatory sites that are bound by TFs. Some 400 PWMs that represent human or mouse TF binding sites were obtained from the TRANSFAC database [Matys et al., 2003]. The entire collection of genes that were expressed in our cell line (6,452) was used as background set for PRIMA analysis. For each treatment we generated two target sets, comprising genes that were either up- or downregulated by the respective treatment. PRIMA default parameters were used in all runs. The analyzed promoter regions span from 1,000 bp upstream to 200 bp downstream the putative transcription start site. PRIMA was also used to identify other TFs signatures associated with the different classes of estrogen- and antiestrogen-regulated genes.

RESULTS

Transcriptional and Mitogenic Response of ZR75.1 Cells to Estrogen and Antiestrogens

In order to gain new insights in the mechanisms by which antiestrogens block E2 action in human BC cells, the effects of growth-inhibiting concentration of 4OHT (the active metabolite of TAM), RAL, and ICI, which have been used as standards for classification of antiestrogens [McDonnell et al., 1995; Levenson and Jordan, 1999] were studied on the hormone-dependent ZR-75.1 cell line. These are hormone-responsive human BC cells that represent a reliable model to study estrogen-dependent proliferation, since estrogen deprivation induces G_1 arrest of these cells, while its addition to the culture medium stimulates cell cycle re-entry [Caristi et al., 2001].

First, we defined the minimal concentration of E2 required to exert a full biologic effect (stimulation of the transcription and cell proliferation) in ZR-75.1 cells. To this aim, hormone-starved cells transiently transfected with pERE-TK-luc carrying an estrogen responsive luciferase reporter gene, were treated with different concentrations of E2. As shown in Figure 1A, full reporter gene induction is achieved under these consitions by 10^{-10} M E2, in agreement with what reported by MacGregor and Jordan [1998]. The ability of G_1-arrested ZR-75.1 cells to show cell-cycle progression in response to E2 was also analyzed under the same conditions (Fig. 1B). A 24-h stimulation with estrogen led to transition of about 50% of the cells in S phase, indicating a robust response to the mitogenic stimulus exerted by the hormone.

Next, the effects of different antiestrogens on ZR-75.1 cell proliferation and ER-mediated *trans*-activation were investigated. To this end, the cells were treated with antiestrogens in steroid-free medium, to assess the direct effects of each compound on the parameters under investigation, or in the presence of E2, to measure antiestrogen ability to influence estrogen signaling. Results show that all three antiestrogens were able to prevent activation of the ERE-TK-luc reporter by E2, each according to its known relative biological potency (ICI = RAL > 4OHT). The same was true for the ability of the drugs to interfere with cell-cycle progression in estrogen-stimulated cells, with the notable exception of 4OHT that appears much less effective in preventing G_1 to S transition in hormone-stimulated cells, possibly for a partial agonistic cell cycle effect in these cells (Fig. 1C). Based on the results of these tests, 2×10^{-8} M antiestrogen and 10^{-10} M E2 was used for all subsequent experiments, as indicated.

cDNA Microarray Analysis of Gene Expression Profiles Induced by Antiestrogens in ZR-75.1 Cells

The effect of 4OHT, RAL, and ICI on estrogen-independent and -dependent gene expression patterns was then examined by cDNA microarray analysis in hormone-starved or -stimulated ZR-75.1 cells, respectively. Our aim was to define the estrogen-antagonist as well as -agonist and -independent actions of the three drugs on BC cells trascriptome. ZR-75.1 cells were grown in steroid-free medium for 4 days, before addition of the same (control) or medium containing 10^{-10} M E2 (+E2) and further incubation for 72 h, to allow a comprehensive

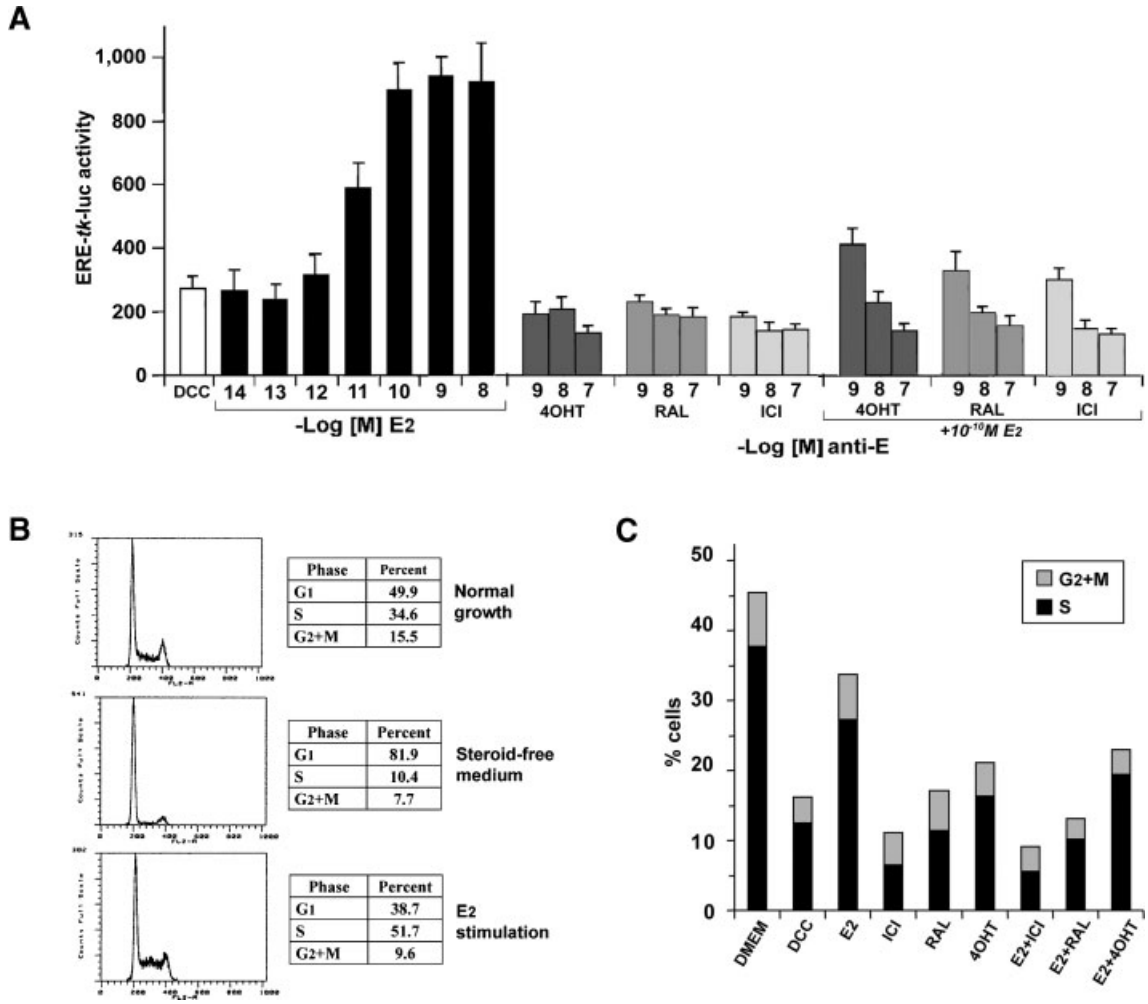


Fig. 1. Transcriptional and proliferative response of ZR-75.1 breast cancer cell line to 17β-estradiol and to antiestrogens. **A**: ZR-75.1 cells were transiently transfected with a reporter plasmid carrying the luciferase gene under the control of a estrogen responsive minimal promoter (ERE-TKluc). Cells were plated in steroid-free medium (phenol red-free medium containing 5% charcoal-stripped fetal bovine serum) and, 12 h after transfection, treated with different concentrations of E2 for 24 h. The luciferase activity was assayed before (DCC, **lane 1**) and after treatment. An average of three independent experiments is reported. **B**: ZR-75.1 cells were synchronized by starvation (growth in steroid-free medium for 4 days) and then treated for 24 h with the indicated concentrations of 17β-estradiol (E2, **lanes 2–8**) or of the antiestrogens 4-hydroxy-tamoxifen (4OHT, **lanes 9–11** and **18–20**), raloxifene (RAL, **lanes 12–14** and **21–23**), or ICI 182,780 (ICI, lanes **15–17** and **24–26**). B: Progression of the cell cycle was evaluated through cytofluorimetric analysis in three different conditions: asynchronous growth in ''red medium'' (with phenol red, ''normal growth''), synchronized cells (steroid-free medium) and treatment with E2 (E2 stimulation). The cell cycle profiles and the percentage of cells in each phase of the cell cycle are reported. **C**: ZR-75.1 cells were synchronized in steroid-free medium (DCC) for 4 days and then stimulated with 10^{-10} M estradiol (E2) for 72 h. In the last 24 h, 2×10^{-8} M antiestrogens ICI, RAL, or 4OHT were added to the cell culture medium. The three antiestrogens were administrated both in steroid-free medium and in E2-stimulated cells. Cytofluorimetric analysis was performed, and the percentage of cells in G_2/M and S phases are plotted in the figure. Data are representative of three independent experiments.

gene expression analysis in cell populations at equilibrium. Where indicated, cells were exposed to antiestrogens for the last 24 h of treatment, to measure the ability of each drug to interfere with this equilibrium. This approach was selected to allow assessment of antiestrogen effects on the expression of direct (primary) and indirect (secondary) estrogen-responsive genes under conditions that mimic in vivo exposure of BC cells to the drug. cDNA microarrays encompassing 8,286 unique EST/gene clusters were used and 395 genes were found significantly regulated by at least one of the three antiestrogens in the presence of E2, 159 showed significant expression changes in response to one or more drug in steroid-free

environment and 589 genes were responsive to E2 after 72 h treatment (Supplemental Information: Table S1).

Pharmacologic Classification of Antiestrogen-Responsive Genes

Regulated genes were then classified relative to their pattern of response to the different antiestrogens in the presence and/or absence of E2. To complement the data obtained with 72 h stimulation of the cells with E2, estrogen responsiveness of the same gene set analyzed here was assessed also from the gene expression data we obtained previously by kinetic analysis of E2-stimulated ZR-75.1 cells [Cicatiello et al., 2004b]. This allowed us to include among the E2-responsive genes also those showing significant changes in expression following 20 to 28 h of hormonal stimulation.

Four clusters of genes were thereby identified among the antiestrogen responsive ones, to include those showing hormone-agonist and -antagonist expression patterns, as well as antiestrogen-specific and -independent gene sets (Fig. 2).

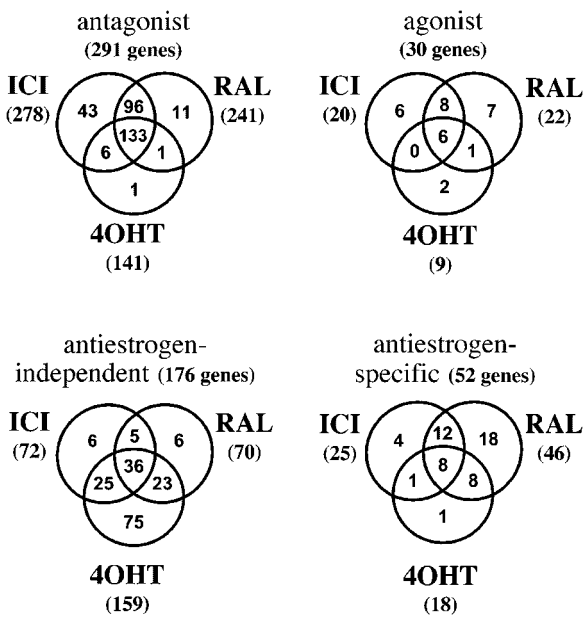


Fig. 2. Analysis of estrogen- and antiestrogen-responsive gene expression profiles in ZR-75.1 breast cancer cells. ZR-75.1 cells were treated with E2 for 24 and 72 h. After 48 h E2 treatment, antiestrogens were added as reported in Figure 2. Cells were collected and the RNA prepared as described in Materials and Methods. Venn diagrams were used to visualize the number of the antiestrogen-regulated genes belonging to each of the pharmacologic classes identified as described in Materials and Methods.

The agonist cluster comprises 30 genes regulated in the same way by antiestrogens and E2. It is worth noting that the agonist behavior was observed only in the absence of E2, since it is well known that estrogen per se is sufficient to induce a maximal gene response in these cases [MacGregor and Jordan, 1998].

The antagonist cluster, on the other hand, includes 291 genes regulated by antiestrogens in an opposite sense, respect to E2. Two distinct response patterns can be distinguished among these genes: a "competitive antagonist" one, observed when the antihormone prevents only estrogen effects on gene expression, without influencing basal activity, and an "inverse agonist," or "negative antagonist" pattern, when the antiestrogen affects gene expression also in the absence of hormone, suggesting that the drug may target in these cases one or more processes controlling basal gene activity. Finally, we defined a cluster of 52 antiestrogen-specific genes, regulated by antiestrogens but not by E2 and an additional one, comprising 176 antiestrogen-independent genes responding to E2 but not to any of the antiestrogens tested.

Based on these measurements, the main mechanism of action of antiestrogens in BC cells is represented by competitive antagonism for the estrogen receptor (ER), respect to estrogen. The different intrinsic power of the three antiestrogens (ICI > RAL > OHT), observed in our growth inhibition assay (Fig. 1C) and elsewhere [Wakeling, 1989; Wijayaratne et al., 1999], is clearly reflected by the number of genes regulated by each compound (278 by ICI, 241 by RAL, 141 by OHT). Furthermore, 133 such genes (46%) are regulated by all the three antiestrogens. It is worth noting that for many genes classified as selectively regulated by one of the drugs, the other antiestrogens exerted a similar trend but lower, not significant, fold changes. The expression data (Tables 1 and S1) suggest, thus, that many of the observed differences among antiestrogens are quantitative rather than qualitative. Distinguishable pharmacologic properties might thus rely onmore on different efficacy/strength of transcriptional regulation than on targeting different gene sets. Interestingly, the response of a number of cell growth-related genes, including *MCM2*, *CD28*, *BUB3*, *MAD4*, and *PIG11*, appears directly related to

the ability of E2 to promote cell-cycle progression and of the antiestrogens to hamper estrogen-induced proliferation of ZR-75.1 cells.

Only 52 genes (14%) belong to the "antiestrogen-specific" class, with the highest number responsive to Raloxifene. Seven genes (15%) are RAL-inhibited zinc finger proteins, implying the possibility that transcriptional effects of this drug are not only due to the inhibition of ER activity but also to the down-modulation of transcription factors.

The "agonist" class contains only a minority of antiestrogen-regulated genes (5.9%), as both 4OHT and RAL have weak estrogen-like activity in BC cells. Nonetheless, ICI, whose antiestrogen action in thought to depend primarily on intracytoplasmatic degradation of ER [Dauvois et al., 1993], shows clear agonist action on 20 genes. This could depend by the fact that estrogen regulation of these genes depends on hormone-induced receptor degradation, which is mimicked by ICI, or, alternatively, of the existence of ER-independent pathways activated by ICI [Levenson et al., 2002b].

Finally, 176 out of 497 estrogen-responsive genes (35%) are "antiestrogen-independent." Among them, we found 12 transcription factors, like for example, *BATF* and *NFIC*, and 8 transcriptional regulators, including *RXRA* and *HDAC5*. We can exclude the possibility that the concentration of antiestrogen used was insufficient to block E2 effects on gene transcription, as under those conditions these compounds were all effective even at lower concentrations [Fig. 1A; Wakeling et al., 1989, 1991; Levenson et al., 2002b]. It is possible, on the other hand, that these E-responsive transcripts are very stable and thus remain in the cell well after the block of their synthesis exerted by antiestrogens. The fact, however, remains that these genes are fully or partially unresponsive to antiestrogens, suggesting the existence of E2-dependent cellular pathways which are not readily blocked by antiestrogens and could represent a factor conditioning BC cell responsiveness to antiestrogens and a molecular mechanism for development of hormone-resistance in tumors treated with these drugs.

The most significant ($FC \geq \pm 2$) antiestrogen-responsive genes are listed in Table I, while the complete data relative to the pharmacologic classification described above are reported as Supplemental Information in Table S1. We validated our data by comparing the expression changes revealed by our experiments with previously published lists of estrogen- and antiestrogen-regulated genes detected by gene expression profiling with cDNA or oligonucleotide microarrays [Finlin et al., 2001; Soulez and Parker, 2001; Inoue et al., 2002; Cunliffe et al., 2003; Hodges et al., 2003; Omoto et al., 2003; Frasor et al., 2004; Wang et al., 2004; Cicatiello et al., 2004b; Table S1]. The full data sets of from these experiments, including raw fluorescence data will be made available upon publication through MGED (www.mged.org) and GEO [Edgar et al., 2002; www.ncbi.nlm.nih.gov/geo] public data repositories.

Functional Classification of Estrogen and Antiestrogen-Responsive Genes

In order to gain insight in the cellular pathways differentially regulated by antiestrogens in BC cells, we performed functional classification of the gene sets identified here according to "Gene Ontology" (GO) [Ashburner et al., 2000; www.geneontology.org] (Fig. 3). This was accomplished by searching biological processes associated with the regulated genes in the GO annotation databases from the European Bioinformatics Institute (EBI, www.ebi.ac.uk) and The Institute for Genomic Research (TIGR, www.tigr.org) web sites.

Among genes of the antagonist cluster, a significant number is involved in regulation of protein homeostasis (biosynthesis and catabolism), cell communication/signal transduction, transport, transcriptionand RNA processing, cell cycle, and proliferation. On the other hand, the "agonist" cluster includes genes involved in signal transduction (i.e., *EphB1*, member of the ephrin receptor family, and *akt*), which are significantly regulated by antiestrogens. Regulation of transcription and development showed the highest representation amoung genes of the "estrogen-independent" cluster (Fig. 3).

A statistical analysis of these results was carried out with Onto-Express [Khatri et al., 2002; http://vortex.cs.wayne.edu:8080]. The software associates UniGene clusters to GeneOntology terms relative to biochemical and molecular functions, biological processes, cellular components and cellular roles, and then computes for each function the probability that the regulated genes differs from a reference gene population (the total number of genes

TABLE I. List of the Most Representative Estrogen and/or Antiestrogen-Regulated Genes, Classified According to Their Pattern of Response[a]

GB Acc[b]	Description[c]	Locus[d]	Function[e]	Regulation[f] −E2 E2	I	R	T	Validation of microarray data[g] E2	I	R	T
A. Agonist pattern											
AB020718	Calsyntenin 1	1p36.22	Homophilic cell adhesion	−3.4	−2.9	−3.4	−2.0	*g*			
AF052106	Chromosome 6 open reading frame 106	6p21.31	Unknown	−1.7	−1.9	−2.0	−1.7				
AF146692	Filamin C. gamma (actin binding protein 280)	7q32-q35	Cytoskeleton organization and biogenesis	−2.3	−2.8	−2.6	−2.1				
AC004493	Transcription elongation factor B (SIII), polypeptide 2 (18 kDa, elongin B)	16p12.3	Unknown	−2.1	−2.9	−3.6	−2.0	*g*			
B. Antagonist pattern											
NM_001657	Amphiregulin (schwannoma-derived growth factor)	4q13-q21	Cell proliferation	**3.6**	**−3.2**	**−3.3**	**−2.8**	*a,g*	*i*	*a,i*	*a,i*
AC005326	Asparagine synthetase	7q21.3	Amino acid metabolism	2.8	−2.7	−2.4	−2.5	*e,g*	*d*		*d*
D38553	Barren homolog (*Drosophila*)	2q11.2	Unknown	1.7	−2.1	−2.1	−2.2	*c,d,g*	*d*		*d,j*
U14518	Centromere protein A, 17 kDa	2p24-p21	Chromosome organization and biogenesis	1.6	−2.8	−2.9	−2.3	*c,g*			
U41387	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	10q21	Regulation of transcription, DNA-dependent	2.1	−2.3	−2.4	−2.0	*d,g,h*	*d*		*d*
L13848	DEAH (Asp-Glu-Ala-His) box polypeptide 9	1q25	Unknown	2.3	−3.5	−3.1	−2.5				
L08069	DnaJ (Hsp40) homolog, subfamily A, member 1	9p13-p12	Protein folding	2.4	−3.7	−3.6	−2.9	*c*			
AI079954	Ecotropic viral integration site 1	3q24-q28	Unknown	1.6	−2.6	−2.3	−2.0				
AL034374	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	6p21.1-p12.1	Unknown	3.1	−3.2	−2.8	−2.0	*c,g*	*d*		*j*
AW249010	Heat shock 70 kDa protein 8	11q24.1	Protein folding	1.5	−2.4	−2.2	−2.3	*b,c,d,g*	*d*		
BE795553	Heat shock 90 kDa protein 1, beta	6p12	Protein folding	1.8	−2.5	−2.2	−2.2	*c*			
BE266776	High-mobility group box 1	13q12	Unknown	2.1	−4.0	−3.8	−3.1	*c,f,g,h*			
Y10313	Interferon-related developmental regulator 1	7q22-q31	Myoblast determination	2.0	−2.1	−2.2	−2.0	*g*			
BE254681	Karyopherin (importin) beta 1	17q21.32	Protein-nucleus import	1.8	−2.7	−2.7	−2.1	*c,g*			
NM_002266	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	17q23.1-q23.3	DNA metabolism	2.3	−3.8	−3.4	−2.4	*c,g*			
NM_014736	KIAA0101	15q22.1	Unknown	2.4	−3.3	−3.1	−2.5	*c,d,g,j*	*d*		*d,j*
AW975835	Kinesin family member 20A	5q31	Vesicle-mediated transport	2.4	−4.3	−4.2	−2.9	*c,g*			
BF679062	Matrix Gla protein	12p13.1-p12.3	Oncogenesis	3.9	−3.2	−2.5	−2.4	*d,g*	*d*		
M23613	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	5q35	Cell growth and/or maintenance	2.5	−3.4	−3.1	−2.6	*c*			
D14663	Proteasome regulatory particle subunit p44S10	3p21.1	Unknown	1.8	−2.7	−2.4	−2.2				
AB040911	Rac GTPase activating protein 1	12q13.12	Signal transduction	2.1	−3.4	−2.9	−2.6	*g*			
AL049557	RAP1A, member of RAS oncogene family	1p13.3	Signal transduction	2.8	−3.6	−2.8	−2.0	*c,g,j*			
BE378321	Retinoblastoma binding protein 7	Xp22.22	Cell proliferation	1.6	−2.2	−2.1	−2.1				

(Continued)

TABLE I. *(Continued)*

GB Acc[b]	Description[c]	Locus[d]	Function[e]	Regulation[f] −E2 E2	I	R	T	Validation of microarray data[g] E2	I	R	T
AV727364	Ribosomal protein L6	12q24.1	Protein biosynthesis	2.1	−2.7	−2.6	−2.3	*g*			
AF011468	Serine/threonine kinase 6	20q13.2-q13.3	Mitosis	1.8	−2.6	−2.5	−2.2	*a,c,d,f,g,h*	*a,d*	*a*	*a,d*
AK025078	Solute carrier family 26 (sulfate transporter), member 2	5q31-q34	Unknown	3.7	−3.7	−3.5	−2.6	*c*			
NM_012319	Solute carrier family 39 (zinc transporter), member 6	18q12.2	Unknown	2.0	−2.8	−3.0	−2.7	*a*			
X70944	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	1p34.3	mRNA splicing	1.6	−2.7	−2.5	−2.1	*c*			
AU130109	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	17q21.3-q22	mRNA splice site selection	1.9	−3.2	−2.8	−2.5	*c,g*			
X94912	Stathmin 1/oncoprotein 18	1p36.1-p35	Cell growth and/or maintenance	2.0	−2.9	−2.6	−2.6	*d,g,j*	*a,d*	*a*	*a,d,j*
M81339	Thrombospondin 2	6q27	Cell adhesion	1.7	−2.5	−2.4	−2.2	*g*			
NM_003362	Uracil-DNA glycosylase	12q23-q24.1	Base-excision repair	1.4	−2.1	−2.0	−2.0	*c*			*j*
J04977	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80 kDa)	2q35	Double-strand break repair	1.9	−2.9	−2.6	−2.3				
AB011121	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	2q33	Neurotransmitter transport	1.7	−2.2	−2.5	−2.0				
AI458786	Asparaginyl-tRNA synthetase	18q21.2-q21.3	Protein biosynthesis	2.2	−2.8	−2.8	−2.3				
AL046741	Chromobox homolog 1 (HP1 beta homolog Drosophila)	17q	Chromosome organization and biogenesis	1.6	−3.1	−2.7	−2.5				
AW582251	Chromosome 6 open reading frame 68	6q22.31	Unknown	1.4	−2.2	−2.0	−2.0				
X70476	Coatomer protein complex, subunit beta 2 (beta prime)	3q23	Exocytosis	1.9	−2.8	−2.8	−2.3				
AF000984	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Yq11	Unknown	2.2	−3.4	−2.7	−2.1				
AA306546	Eukaryotic translation initiation factor 2, subunit 1 alpha, 35 kDa	14q24.1	Regulation of protein biosynthesis	2.3	−3.8	−2.9	−2.1	*c,g*			
NM_001969	Eukaryotic translation initiation factor 5	14q32.33	Regulation of translational initiation	2.3	−3.6	−3.2	−2.7	*c*			
D89729	Exportin 1 (CRM1 homolog, yeast)	2p16	Nucleocytoplasmic transport	2.4	−3.6	−3.0	−2.4				
NM_005113	Golgi autoantigen, golgin subfamily a, 5	14q32.12-q32.13	Unknown	1.7	−2.1	−2.2	−2.1	*g*			
M22382	Heat shock 60 kDa protein 1 (chaperonin)	2q33.1	Protein folding	3.1	−5.5	−4.5	−3.2	*b,c,g*	*d*		

(Continued)

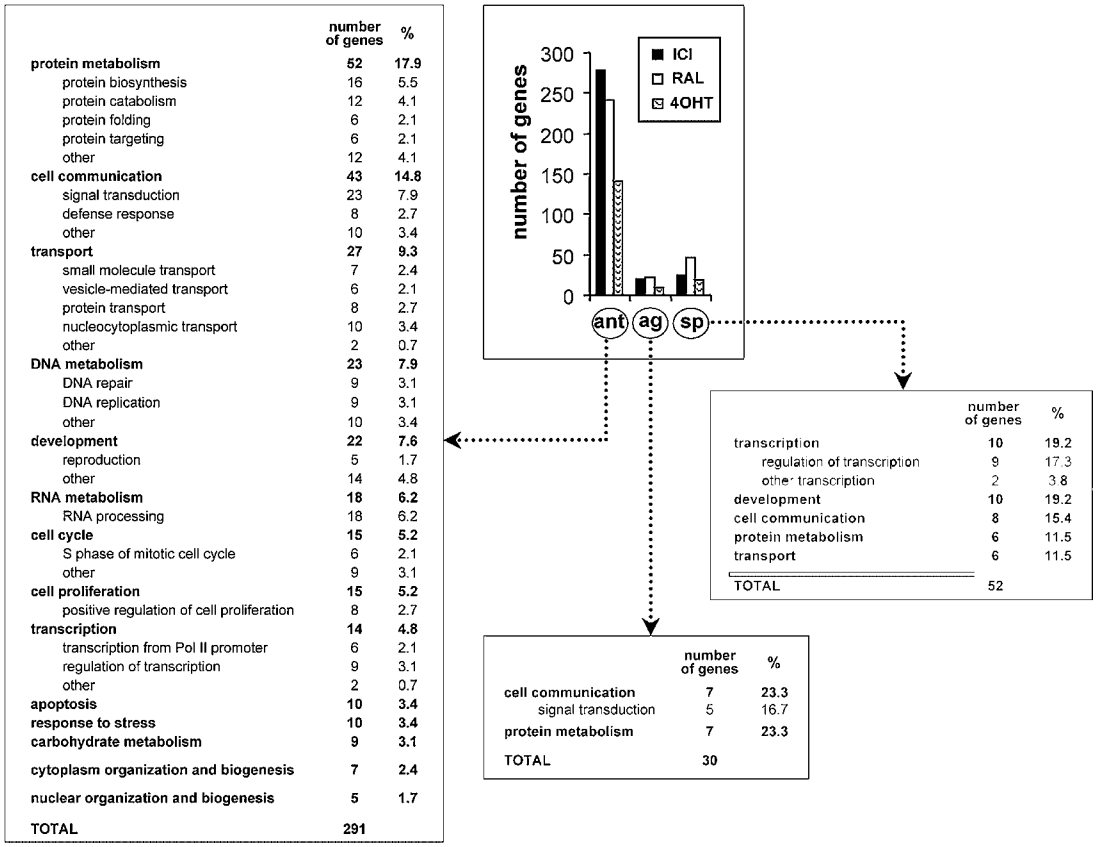
BE742483	Heat shock 70 kDa protein 4	5q31.1-q31.2	Protein folding	2.5	−3.2	−2.8	−2.1				
D28877	Heterogeneous nuclear ribonucleoprotein A2/B1	7p15	RNA processing	1.8	−2.6	−2.4	−2.0	*b,c*			*j*
NM_005520	Heterogeneous nuclear ribonucleoprotein H1 (H)	5q35.3	RNA processing	1.6	−2.8	−2.5	−2.5	*g*			
X65488	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	1q44	RNA processing	1.7	−3.0	−2.8	−2.6				
BE706412	Hypothetical protein DKFZp761B1514	3q29	Unknown	1.8	−2.7	−2.6	−2.1				
NM_003870	IQ motif containing GTPase activating protein 1	15q26.1	Cell shape and cell size control	1.9	−2.6	−2.5	−2.2				
AF117236	Matrin 3	5q31.3	Cell shape and cell size control	2.4	−3.2	−3.1	−2.4				
AB040057	Mst3 and SOK1-related kinase	Xq26.2	Protein amino acid phosphorylation	2.7	−3.5	−3.4	−2.6	*c,g*			
AK000250	Nucleolin	2q12-qter	Ribosome biogenesis and assembly	2.4	−5.7	−3.6	−2.4	*c,g*			
NM_000938	Polymerase (RNA) II (DNA directed) polypeptide B, 140 kDa	4q12	Transcription from Pol II promoter	2.3	−3.1	−2.9	−2.5				
U86782	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	2q24.3	Ubiquitin-dependent protein catabolism	2.5	−4.0	−3.3	−2.3	*g*			
AK025517	Proteasome (prosome, macropain) activator subunit 4	2p16.3	Proteolysis and peptidolysis	2.2	−2.5	−2.7	−2.3				
U34994	Protein kinase, DNA-activated, catalytic polypeptide	8q11	Double-strand break repair	1.7	−2.2	−2.0	−2.1				
NM_003620	Protein phosphatase 1D magnesium-dependent, delta isoform	17q23.3	Negative regulation of cell proliferation	1.4	−2.4	−2.5	−2.0				
NM_001033	Ribonucleotide reductase M1 polypeptide	11p15.5	DNA replication	1.9	−2.6	−3.3	−2.7	*c,d,g*	*a,d*	*a*	*a,d*
M97935	Signal transducer and activator of transcription 1, 91 kDa	2q32.2	Signal transduction	1.9	−2.3	−2.3	−2.0	*g*			
X63071	SON DNA binding protein	21q22.1-q22.2	Anti-apoptosis	1.6	−2.6	−2.5	−2.5				
U21858	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32 kDa	5q11.2-q13.1	Transcription from Pol II promoter	2.0	−2.8	−2.6	−2.3	*c,g*			
AF077367	Thioredoxin reductase 1	12q23-q24.1	Signal transduction	2.3	−3.6	−3.2	−2.3				
NM_003191	Threonyl-tRNA synthetase	5p13.2	Protein biosynthesis	3.0	−4.0	−3.3	−2.7	*g*			
U09086	Thymopoietin	12q22	Unknown	2.0	−2.5	−2.8	−2.2	*c,g*			
U07806	Topoisomerase (DNA) I	20q12-q13.1	DNA metabolism	1.5	−3.0	−2.9	−2.7				
X68060	Topoisomerase (DNA) II beta 180 kDa	3p24	DNA metabolism	1.8	−2.6	−2.5	−2.0				
U72069	Transportin 1	5q13.2	Protein-nucleus import, translocation	1.5	−2.3	−2.5	−2.2	*c*			
BE818373	Tumor rejection antigen (gp96) 1	12q24.2–q24.3	Response to stress	3.1	−4.7	−4.0	−3.2	*c,g,h,j*	*d*		*d*
AA022783	Ubiquitin specific protease 32	17q23.3	Ubiquitin-dependent protein catabolism	2.8	−3.3	−3.2	−2.4				
C. Antiestrogen-specific pattern											
M80899	Hypothetical protein MGC5395	11q12.2	Neurogenesis	1.2	−2.2	−2.1	−2.1				

(Continued)

TABLE I. *(Continued)*

GB Acc[b]	Description[c]	Locus[d]	Function[e]	Regulation[f] −E2 E2	I	R	T	Validation of microarray data[g] E2	I	R	T
D. Antiestrogen-independent pattern											
AB038162	Trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	21q22.3	Carbohydrate metabolism	2.0	−1.3	−1.2	−1.3	*a,b,c,d,e, g,j*			
L13740	Nuclear receptor subfamily 4, group A, member 1	12q13	Signal transduction	−2.0	1.3	1.2	1.1				
J02611	Apolipoprotein D	3q26.2-qter	Lipid metabolism	−2.7	1.4	1.5	1.3	*c,d*			
AB002305	Aryl-hydrocarbon receptor nuclear translocator 2	15q24	Signal transduction	−2.4	1.6	1.3	1.2	*a,g*			
NM_004390	Cathepsin H	15q24-q25	Proteolysis and peptidolysis	−2.1	1.5	1.6	1.2	*a,g*			
AU126499	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	6q21	Humoral immune response	−2.0	1.4	1.3	1.1	*a,c,g*			

[a]Only the genes that responded significantly (fold-change $\geq \pm 2.0$) to all the three compounds tested are reported, grouped according to the pharmacological classification described in the text.
[b]GB Acc: GenBank Accession number is the unique identification number of the sequence spotted on the array, according to the GenBank database.
[c]Descriptions are those reported in the UniGene database (last update: July 2004).
[d]Chromosomal localization was assigned according to the LocusLink database (last update: July 2004).
[e]Functions were assigned by matching the UniGene clusters to Gene Ontology biological processes, according to the EBI and TIGR Gene Ontologies Annotations (last update: February 2003).
[f]Gene induction or repression, expressed as fold-changes, in response to each of the compounds tested (I: ICI; R: RAL; T: 4OH-tamoxifen; E2: cells stimulated with 17β-estradiol for 72 h), measured as described in the text; *±E2*: the response to antiestrogens was measured in the absence (*−E2*) or presence (*+E2*) of 17β-estradiol.
[g]Genes that showed a response to estrogen (E2) or one of the antiestrogens (ICI, RAL, TAM, respectively, for ICI, 4OH-tamoxifen, RAL) in the following independent microarray analyses: *a*, Frasor et al. [2004]; *b*, Wang et al. [2004]; *c*, Cicatiello et al. [2004b]; *d*, Cunliffe et al. [2003]; *e*, Omoto et al. [2003]; *f*, Hodges et al. [2003]; *g*, Inoue et al. [2002]; *h*, Lobenhofer et al. [2002]; *i*, Soulez and Parker [2001]; *j*, Finlin et al. [2001].
For further details see Table S1 in Supplementary materials.



	number of genes	%
protein metabolism	**52**	**17.9**
protein biosynthesis	16	5.5
protein catabolism	12	4.1
protein folding	6	2.1
protein targeting	6	2.1
other	12	4.1
cell communication	**43**	**14.8**
signal transduction	23	7.9
defense response	8	2.7
other	10	3.4
transport	**27**	**9.3**
small molecule transport	7	2.4
vesicle-mediated transport	6	2.1
protein transport	8	2.7
nucleocytoplasmic transport	10	3.4
other	2	0.7
DNA metabolism	**23**	**7.9**
DNA repair	9	3.1
DNA replication	9	3.1
other	10	3.4
development	**22**	**7.6**
reproduction	5	1.7
other	14	4.8
RNA metabolism	**18**	**6.2**
RNA processing	18	6.2
cell cycle	**15**	**5.2**
S phase of mitotic cell cycle	6	2.1
other	9	3.1
cell proliferation	**15**	**5.2**
positive regulation of cell proliferation	8	2.7
transcription	**14**	**4.8**
transcription from Pol II promoter	6	2.1
regulation of transcription	9	3.1
other	2	0.7
apoptosis	**10**	**3.4**
response to stress	**10**	**3.4**
carbohydrate metabolism	**9**	**3.1**
cytoplasm organization and biogenesis	**7**	**2.4**
nuclear organization and biogenesis	**5**	**1.7**
TOTAL	291	

	number of genes	%
transcription	10	19.2
regulation of transcription	9	17.3
other transcription	2	3.8
development	10	19.2
cell communication	8	15.4
protein metabolism	6	11.5
transport	6	11.5
TOTAL	52	

	number of genes	%
cell communication	**7**	**23.3**
signal transduction	5	16.7
protein metabolism	**7**	**23.3**
TOTAL	30	

Fig. 3. Functional classification of antiestrogen-responsive genes according to ''Gene Ontology'' nomenclature. The antiestrogen-responsive genes were first classified according to their pattern of regulation by the drugs: antagonist-like (ant), agonist-like (ago), antiestrogen-specific (sp, i.e., regulated by antiestrogen but not by estradiol), and the number of genes of each class was plotted for each of the three compounds. Then for each pharmacologic class a functional classification was performed, according to the ''Gene Ontology Consortium'' (GO) nomenclature, and the biologic processes encompassing the highest number of genes were reported. The ''parent'' terms (i.e., those that hold a higher position in the GO hierarchical classification) are in bold, and the respective ''child'' terms are listed below each ''parent'' definition. Only the processes that comprised at least five genes were enumerated, the remaining were grouped in the ''other'' category.

analyzed, for example) or, alternatively, represent a selection thereof, suggesting in this case that one or more gene pathways were preferentially affected by the compound tested. OntoExpress analysis was performed for all antiestrogen-responsive classes described above, taking the E2-regulated gene list as the reference population.

Since analysis could not be carried out on the "agonist" custer, due to the small number of genes included (only 30), our attention focused on the "antagonist" gene cluster. Results reported in Table II show that among the genes regulated by antiestrogens in an antagonist fashion respect to estrogen only two biological processes result significantly regulated by all three antiestrogens: RNA processing and nuclear-cytoplasmic transport. For what concerns functions specifically influenced by only one of the three drugs studied, ICI affects preferentially cell-cycle regulation by estrogen, represented among other by *cdk7*; *STAT-1*; *BCL-2*; *PCNA*, and *RAN* genes, despite the fact that this compound, among the antihormones tested, affects the highest number of estrogen responsive genes. In addition, together with RAL this compound on specifically interferes with E-regulated genes involved in RNA splicing (*SFPQ*, *SFRS1*, *SNRPB2*, *SNRPG*, etc.), RAL, and 4OHT, in turn, each interferes with hormonal regulation of specific biological processes. In particular, RAL appears to affect preferentially oncogenesis, DNA repair, and signal transduction, while 4OHT targets protein amino acid phosphorylation, nucleocytoplasmic transport, and metabolic pathways.

TABLE II. Biological Processes Significantly Affected in Hormone-Stimulated Human Breast Cancer Cells by the Different Antiestrogens Tested[a]

	ICI		RAL		TAM		E2
Biological process	Number of genes	*P*-value	Number of genes	*P*-value	Number of genes	*P*-value	Number of genes
A: Significant *P*-value for all three antiestrogens tested							
RNA processing/modification	20	0.00928	17	0.00848	11	0.02863	23
Nuclear-cytoplasmic transport	8	0.04659	7	0.03663	7	0.00404	9
B: Significant *P*-value for ICI and RAL							
RNA splicing	8	0.04659	7	0.03663	3	0.24427	9
C: Significant *P*-value for ICI only							
Regulation of cell cycle	11	0.04010	8	0.09686	6	0.07353	13
D: Significant *P*-value for RAL only							
Oncogenesis	13	0.11981	13	0.02976	6	0.28172	19
DNA repair	5	0.23586	7	0.02016	4	0.07483	8
DNA replication	4	0.20163	5	0.04136	3	0.08850	6
Signal transduction	12	0.18396	6	0.02424	8	0.49977	31
E: Significant *P*-value for 4OH-tamoxifen only							
Protein amino acid phosphorylation	4	0.20163	2	0.45746	5	0.00713	6
Nucleocytoplasmic transport	4	0.11981	3	0.15789	4	0.01367	5
Other metabolism	7	0.09835	3	0.48184	7	0.00404	9
DNA synthesis	6	0.18798	6	0.08509	5	0.04160	9

[a]The biological processes involving genes significantly regulated by antiestrogens acting as hormone antagonists were identified, based on Gene Ontology categorization, with Onto-Express analysis software [Khatri et al., 2002; http://vortex.cs.wayne.edu/projects.htm].

When combined, the results of these analyses suggest significant qualitative differences among the three antiestrogens tested with respect to their ability to influence genomic pathways in estrogen-responsive BC cells.

In Silico Promoter Analyses

In order to find the binding sequences for ERs and other known transcription factors in the promoters of estrogen- and antiestrogen-regulated genes, an in silico promoter analysis was performed.

The estrogen response element (ERE) was first searched using three different computational approaches (see Materials and Methods). This analysis yielded 86 genes including 1 or more ERE sequence inside or near their promoter and revealed by at least 2 of the statistical approaches utilized; among them, there are some known E2-regulated genes, *CTSD* and *MUC1*. Noteworthy, we could identify *TFF1* (pS2/trefoil factor-1), another well-known E2 target, only by one computational approach (Table S3). This is due to the fact that in the RRE database [Lazzarato et al., 2004] are annotated only the ERE containing genes characterized by the conservation of the ERE within human and rat/mouse orthologs, and the *TFF1* ERE is not conserved in the rat and mouse othologs. The distribution of ERE-containing genes is apparently independent on the pharmacologic classification and regulation by estradiol: both E2-induced and inhibited, as well as not regulated genes, show a similar proportion of ERE within their promoters. Overall, 13% of the genes identified in this gene expression study resulted to include an ERE. This result is not unexpected, since the long exposure of cells to estrogen (72 h) resulted in both primary and secondary (non-ER mediated) gene expression changes.

To identify the transcription factors (TFs) possibly acting as effectors of secondary responses or co-operating with ER in gene regulation, a computational analysis was performed limited to the −1,000; +200 region of each gene, in the different classes of estrogen- and antiestrogen-regulated genes. To this aim, the PRIMA analysis tool [Elkon et al., 2004] was used. This performs statistical tests aimed at identifying TFs whose binding sites are significantly more abundant in the target set (the different clusters of regulated genes) than in the background set (the total list of genes studied through microarray analysis). Among E2-induced genes, three TF-binding sites resulted over-represented: E2F1, NRF-1, and NF-Y (Table III). The E2F1 factor is known to promote

TABLE III. Results of In Silico Analysis of Estrogen- and Antiestrogen-Responsive Gene Promoters[a]

		+E2/−E2[d]		+E2						−E2	
				+ICI		+RAL		+TAM		+RAL	
Transcription factor[b]	TRANSFAC[c]	Up[d]	Down[d]	Up	Down	Up	Down	Up	Down	*Up*	*Down*
NRF-1	M00652	$4.6 \times 10{-}5$	NS	NS	$3.5 \times 10{-}4$	NS	$1.6 \times 10{-}4$	NS	1.7×-4	NS	NS
NF-Y	M00287	$3.0 \times 10{-}6$	NS	NS	$3.2 \times 10{-}5$	NS	NS	NS	$2.2 \times 10{-}4$	NS	NS
ATF	M00338	NS	NS	NS	$1.3 \times 10{-}4$	NS	$8.8 \times 10{-}6$	NS	$1.4 \times 10{-}4$	NS	NS
E2F-1	M00428/M00940	$1.7 \times 10{-}4$	NS	NS	$1.0 \times 10{-}4$	NS	NS	NS	NS	NS	NS
LF-A1	M00646	NS	$3.1 \times 10{-}4$	$2.8 \times 10{-}4$	NS	$3.6 \times 10{-}5$	NS	NS	NS	NS	NS
CAC-binding protein	M00720	NS	$1.6 \times 10{-}5$	$2.1 \times 10{-}4$	NS	NS	NS	NS	NS	NS	NS
EGR	M00807	NS	NS	$8.1 \times 10{-}5$	NS	$6.0 \times 10{-}5$	NS	NS	NS	NS	NS
Sp1	M00933	NS	NS	$2.1 \times 10{-}6$	NS	$1.6 \times 10{-}5$	NS	NS	NS	NS	NS
ETF-1	M00652	NS	NS	NS	NS	NS	NS	NS	NS	NS	$9.3 \times 10{-}5$

[a]Computational analyses were carried out with the PRIMA tool [Elkon et al., 2004], as decribed in the text.
[b]Transcription factor-binding sites present in responsive gene promoters, within position −1.000 and +200 relative to the putative transcription start site.
[c]Accession number relative to the latest version of the TRANSFAC Transcription Factors database [Matys et al., 2003].
[d]*P*-values calculated for genes positively (*Up*) or negatively (*Down*) regulated by the indicated compound in hormone-stimulated (−*E2*) or -deprived (−*E2*) ZR-75.1 cells. NS: not significant. The columns relative to the tamoxifen and faslodex treatment in the absence of estradiol were omitted because no significant enrichment was observed in these groups.

the G_1 to S transition in estrogen-responsive BC cell lines, as a consequence of cyclin D1/cdk-4/6 activation by the hormone [Cicatiello et al., 2004a], and it is transcriptionally upregulated by E2 [Wang et al., 1999]. *E2F1* gene overexpression in BC cells has been shown to promote hormone-independent proliferation and antiestrogen-resistance [Louie et al., 2004]. The transcriptional activation of *E2F1* gene has been recently shown to be activated by two other TFs that resulted enriched in antiestrogen-responsive genes: NF-Y and Sp-1 [Wang et al., 1999; Ngwenya and Safe, 2003]; this last factor is well known to mediate the tethering of ER to ERE-devoid promoters [Krishnan et al., 1994; Porter et al., 1997]. NRF-1 is a nuclear TF regulating the expression of mitochondrial genes [Kelly and Scarpulla, 2004]; it has been shown to cooperate with other TFs for the induction and maintenance of cytochrome c expression, which is a limiting factor for the G_0 to G_1 transition, as it sustains the higher cell respiration rate required for proliferation [Herzig et al., 2000]. The binding sites for two TFs are enriched in E2-inhibited genes: LF-A1 and CAC-binding protein, whose role in estrogen-responsive BC biology is currently unknown. LF-A1, however, has been shown to cooperate with ER to activate the transcription of apoVLDL II in the liver [Wijnholds et al., 1991]. Interestingly, two TFs, ATF4 and EGR1, whose consensus sites are present in a significant number of antiestrogen-regulated gene promoters (ATF and EGR in Table III), turned out to be themselves regulated by E2 in the microarray experiment (Tables I and S1). ATF4 is a member of the AP-1 network, whose activity is known to be modulated by the ER in BC cells [Webb et al., 1995; Paech et al., 1997]; recent work has suggested a role for ATF4 mammary gland development [Bagheri-Yarmand et al., 2003]. Thus, its upregulation by estradiol, and the presence of its binding site in a large number of genes inhibited by antiestrogens, suggests an important role for this TF as a secondary effector of the hormonal stimulus. EGR1, on the other hand, is an immediate-early gene known to be induced by E2 [Pratt et al., 1998; Cicatiello et al., 2004b] and to mediate the G_1 to S transition [Molnar et al., 1994], its *cis*-acting sequence is enriched in the genes upregulated by ICI and RAL. Finally, the only TF whose recognition site is specifically associated with the genes responsive to RAL alone is ETF-1.

TABLE IV. Correlation Between E2 Regulation and Presence of an ERE in the Promoter of E2 Target Genes[a]

		ERE[d]		non-ERE[d]	
Transcription factor[b]	TRANSFAC[c]	Up[d]	Down[d]	Up	Down
E2 upregulated[d]					
NRF-1	M00652	$2.1 \times 10-5$	NS	NS	NS
NF-Y	M00287	NS	NS	$1.9 \times 10-5$	NS
E2F-1	M00428/M00940	NS	NS	NS	NS
E2 downregulated					
CAC-binding protein	M00720	NS	$1.2 \times 10-8$	NS	NS
LF-A1	M00646	NS	NS	NS	NS

[a]Computational analyses were carried out with the PRIMA tool [Elkon et al., 2004], as decribed in the text.
[b]Transcription factor binding sites present in responsive gene promoters, within position −1,000 and +200 relative to the putative transcription start site.
[c]Accession number relative to the latest version of the TRANSFAC Transcription Factors database [Matys et al., 2003].
[d]*P*-values calculated for genes positively (*E2* upregulated) or negatively (E2 downregulated) regulated by E2 in the groups of genes whose promoter contains (*ERE*) or does not contain (non-*ERE*) an estrogen response element, respectively. NS: not significant.

Little is known about the role of this TF in BC cell biology and estrogen action, but the PRIMA analysis showed that this TF binding site is significantly enriched in the "antiestrogen-specific" genes downregulated by RAL (*P*-value 3.5×10^{-4}). Indeed, among the 46 "antiestrogen-specific" genes regulated by RAL, 21 bear at least one ETF site in their promoter.

In order to gain some mechanistic hints about the role of different TFs in the regulation of E2-responsive genes, we performed the PRIMA analysis dividing both the genes up- and down-regulated by E2 in two different target sets, according to the presence or the absence of an ERE in their promoter, as identified by the bioinformatic approaches described above (Table IV). The analysis showed that the binding sites for some of the factors are over-represented in the E2-responsive genes whose promoters contain an ERE (NRF-1 for activated genes and CAC-binding protein for inhibited genes), and this suggests that these factors could be co-modulators of estrogen signaling. The NF-Y binding sequence is enriched, instead, in the E2-responsive genes whose promoter does not comprise an ERE; although more data are required for the identification of the precise meaning of this result, two hypotheses can be made: (i) that NF-Y is one of the secondary effectors of E2 action; (ii) that the complex E2-ERE is tethered to the ERE-negative promoters through interaction with NF-Y, analogously to what observed for AP-1 [Ambrosino et al., 1993; Cicatiello et al., 2004a].

Finally, in order to establish a correlation between the response to the estrogenic stimulus and the pattern of regulation by the antiestrogens, the PRIMA analysis was performed dividing the E2 responsive genes according to their pharmacologic class (Table V). The signatures of all the transcription factors which had resulted enriched in the genes activated by E2 are significantly associated also with the genes of the "antagonist" class, regardless of the

TABLE V. Correlation Between E2 Regulation and Pharmacologic Classification of Estrogen- and Antiestrogen-Responsive Genes[a]

Transcription factor[b]	TRANSFAC[c]	Agonist[d]	Antagonist	Antiestrogen-unregulated
E2 upregulated[b]				
NRF-1	M00652	NS	$2.4 \times 10-4$	NS
NF-Y	M00287	NS	$4.0 \times 10-5$	NS
E2F-1	M00428/M00940	NS	$2.3 \times 10-4$	NS
E2 downregulated				
CAC-binding protein	M00720	NS	NS	$3.6 \times 10-6$
LF-A1	M00646	NS	NS	NS

[a]Computational analyses were carried out with the PRIMA tool [Elkon et al., 2004], as decribed in the text.
[b]Transcription factor binding sites present in responsive gene promoters, within position −1,000 and +200 relative to the putative transcription start sit.
[c]Accession number relative to the latest version of the TRANSFAC Transcription Factors database [Matys et al., 2003].
[d]*P*-values calculated for genes positively (*E2* up-regulated) or negatively (E2 down-regulated) regulated by E2 in the groups of genes created according to the pharmacological classification described in the text. NS: not significant.

presence of an ERE in the promoters. The only exception is for CAC-binding protein, which is associated to the genes downregulated by E2 and containing an ERE in their promoter, and the binding site for this factor is enriched in the genes that are not regulated by antiestrogens. The meaning of this pattern of regulation is not clear, but it shows the possibility of identifying some putative targets or secondary effectors of E2 action whose activity is not regulated by the antiestrogen drugs here examined.

As for the analysis of the TF-binding sites associated to the different pharmacologic classes of the genes regulated by each of the three antiestrogens (data not shown), the small number of genes comprised in each class prevents the reaching of significance in the statistical analysis; the only exceptions are the already mentioned TFs associated with genes activated by E2 and inhibited by the antiestrogens ("antagonist" genes, Table V), and the association of ETF with the "antiestrogen-specific" genes downregulated by RAL.

DISCUSSION

Clearing the effects of estrogen and drugs affecting estrogen signaling in BC cells is central to understand the role of these steroid hormones in breast and other neoplastic diseases. Hormonal therapy is one of the key treatments for BC, not only for the palliative management of metastatic disease but also for adjuvant aims and for cancer prevention in high-risk women. One of the strategies for hormonal therapy of BC consists on the use of antiestrogens, receptorial antagonists of estrogens, which blocks the trophic stimulus exerted by these hormones on cancerous cells. There are two main classes of antiestrogens: pure antagonists, such as ICI, and selective ER modulators (SERMs). While compete with the natural ligands for binding to the receptor, the former target ER for degradation while the latter are able to regulate ER-dependent transcription by modulating its interaction with co-regulatory proteins. In this way SERMs can exert estrogen-like activities in certain the cellular contexts, or even estrogenic or antiestrogenic actions in the same cells, depending upon the estrogen-responsive gene context or the presence of specific mutations in ER itself [Levenson and Jordan, 1998; Levenson et al., 1998; Zajchowski et al., 2000; Liu et al., 2002]. The mechanistic differences that underlie these tissue- and gene-specific actions of antiestrogens are not yet fully understood but it is now clear that different drugs, by binding the ER, induce in the protein a spectrum of intermediate conformations between a "fully active" (characteristic of the E2-ER complex) and a "fully inactive" (exemplified by the ICI-ER complex) form. In turn, these alternative conformations of ER are read as more or less estrogenic/antiestrogenic by the cellular environment. Besides the tissue-specific ER modulation, different antiestrogens have shown differences in the antitumoral action, such as lack of cross-resistance between 4OHT and ICI, suggesting the inability of one molecule to affect the very same cellular pathways targeted by the other. The nature of the common and divergent effects of different antiestrogen molecules in BC cells is still unknown.

This study was aimed at evaluating in hormone-responsive BC cells the gene expression profiles of E2 and three drugs—4OHT, RAL, and ICI—which represent standards for classification of antiestrogens. Antiestrogen responsive gene expression analysis was carried out both in the presence and in the absence of E2, in order to relate antiestrogen-dependent transcriptional effects to those exerted by the hormone itself. The result is a pharmacologic classification of antiestrogen-regulated gene expression, which shows a prevalent antagonist action of the drugs respect to E2 in this cell type (78% of the genes). This is not surprising considering what is known on the mechanism of action of these drugs, and the general block of estrogen effects they exert in BC cells.

A general observation stemming from the data reported here is the absence of a clear-cut distinction between pure antiestrogens and SERMs and even between agonists and antagonists. Indeed, each of the three drugs examined shows both agonist and antagonist actions, despite the reported differences in their respective ability to interfere with ER activity. A minority of genes (8.5%) show an "agonist" pattern of response to antiestrogens, with two thirds of them regulated by ICI despite the fact that the only antiestrogen with a well-known partial agonist action on BC cells is 4OHT [Nicholson et al., 1988; Wakeling et al., 1989]. This finding is in partial agreement with already published gene expression data

obtained on antiestrogen-responsive genes in ER-negative BC cells stably transfected with the *ER* gene [Levenson et al., 2002a,b]. In this case, a surprising set of ICI-activated genes, partially overlapping with RAL-induced genes, was identified and interpreted as the result of an ER-independent action of the antiestrogens. The presence of ER-independent pathways regulated by antiestrogens in BC cells is suggested also by the finding of "antiestrogen-specific" genes, whose transcription is affected by antiestrogens but not by estradiol. While ICI regulates the greatest number of "antagonist" genes as an antagonist, RAL is the antiestrogen with the greatest E2-independent action. The existence of ER-independent pathways is not confirmed by other studies [Inoue et al., 2002; Frasor et al., 2004] of E2- and antiestrogen-responsive gene expression profiling in MCF-7 cells. This discrepancy could be due to the fact that in that study pre-selection of the genes as E2-regulated precluded the possibility to identify "antiestrogen-specific" genes. Also, it is worth mentioning that in the present study we focused on the genomic effects of exposure of BC cells to antiestrogens in to the presence of estrogenic stimulus, to mimic the in vivo effects of administration of the tested drugs to pre-menopausal patients. Indeed, in vivo studies have shown that a short-term administration of antiestrogens is able to influence gene expression in BC [McClelland et al., 1996; Robertson et al., 2001].

Due to the prevailing antagonist action that all three antiestrogens show in the presence of E2, the different gene expression profiles they induce are widely overlapping. Nevertheless only 133 out of 291 (45.7%) genes showing an "antagonist" pattern are regulated by all the three antiestrogens, while 25% of all antiestrogen-responsive genes are specifically regulated by only one of the three molecules. This specificity is even more evident for "agonist" (50% drug-specific genes) and "antiestrogen-specific" (44% drug-specificity) patterns than for the "antagonist" one (only 19% specific genes). These data suggest that there are E2-responsive genes regulated by ICI but unresponsive to TAM, which could be implicated in the lack of cross-resistance between the two drugs observed in clinical studies [Howell et al., 1995]. For all the reasons listed above, we believe that the antiestrogen-specific genes identified here can be used for the classification of new antiestrogens according to similarities with one or the other of the three basic compounds used as standard. Many previous studies underlined the opportunity of classifying antiestrogens according to their expression profiling, and pointed out at a relationship between expression patterns and clinical properties of these drugs [see, for example, Zajchowski et al., 2000]. In particular, it has been shown that similar behavior of novel compounds with respect to RAL in a gene-activation test carried out in different cell lines correlates with a positive effect on bone density of ovariectomized rats. The identification of genes that are differentially regulated by the three reference compounds can be useful for further investigations about tissue- and cell-specific actions of SERMs. To this aim, we provide a comprehensive listing of antiestrogen-regulated genes, that can be exploited for classification of new antiestrogens, based on their effects on the activity of these gene clusters in BC cells (Tables I and S1).

Besides the identification of genes that are specifically regulated by a given antiestrogen, gene-profiling data allows to identify also those genes that are co-regulated during treatment because they exert a common biologic function [Eisen et al., 1998]. A statistical evaluation of estrogen-regulated biological processes that were significantly affected by antiestrogens showed that important cellular functions are differently affected by the three drugs studied. Despite the fact that ICI affects the largest number of genes, this drug does not seem to target any specific hormone-responsive biological process, reflecting the fact that as pure antiestrogen it acts upstream in the estrogenic signaling cascade by blocking most ER functions. On the other hand, 4OHT and RAL clearly exert a more targeted action, with specific antagonist effect toward certain specific biologic functions, such as nucleo-cytoplasmatic transport or DNA repair.

Another puzzling observation stemming from this study is represented by the presence of antiestrogen-unresponsive genes among those hormone-regulated. This finding is not unprecedented, as lack of regulation by antiestrogens of certain estrogen-responsive genes, including *TGF-alpha*, *VEGF*, and *BRCA1*, has been already observed in estrogen-independent BC cells transfected with ER-alpha [Levenson et al., 2002a,b]. In our case it might reveal estrogen-

responsive transcripts with longer half-life, and for this reason less readily responding to antiestrogen blockade of the corresponding gene, and might contribute to explain a certain refractoriness of certain cell pathways to antiestrogen blockade. On the other hand, this result points toward the possibility that currently used antiestrogens only partially interfere with the complex network of estrogen-regulated gene pathways in BC cells. This could explain the partial and temporally limited activity observed for these drugs and suggests that the set of antiestrogen-unresponsive genes identified here represent a useful tool to assess the potency and effectiveness of novel antiestrogenic compounds.

Statistical analysis was performed to identify TF signatures enriched in some antiestrogen-responsive gene clusters, as this can be used as a starting point for reconstruction of transcriptional networks controlled by the hormone in BC cells, as well as of specific effectors of antiestrogen actions. Indeed, the signature for ETF-1 factor is specifically over-represented in "antiestrogen-specific" genes inhibited by RAL, so that it can be assumed that this TF represents one of the effectors of RAL-regulated transcription.

The clustering of genes showing a similar pattern of regulation in high-gene density tracts of the chromosomes suggests involvement of chromosomal domains in the genomic responses to estrogen, as it has been discussed in our previous work [Cicatiello et al., 2004b]. Several cases, within in this study, provide evidences for such regulated gene clustering in BC cells: *KRT7*, *KRTHB1*, *KRT8*, *KRT18*, *EIF4B*, and *RARG* genes, for example, all located within 1 Mb of 12q13 and *ATP2A1*, *CDIPT*, *TAO1*, *MGC10500*, *MAPK3*, *CORO1A*, and *PRSS8* genes that clusters within 2.2 Mbs of 16p12 are all downregulated by E2 and scarcely responsive to antiestrogens; a cluster of genes downregulated by all three antiestrogens (*CSTF2*, *TIMM8A*, *GLA*, *HNRPH2*, *FLJ20811*) is present on Xq22 and spans over 0.8 Mbs, while four genes inhibited by RAL in the absence of E2 (*NSF*, *NPEPPS*, *KPBN1*, *CBX1*) are clustered within 1.5 Mbs on cytoband 17q21 of the long arm of chromosome 17. A full list of the genomic location of all estrogen- and antiestrogen-responsive genes identified in this study is supplied in Table S2 of Supplementary information for further details.

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