Identification of Selective Estrogen Receptor Modulators by Their Gene Expression Fingerprints*

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Clinical studies have shown that estrogen replacement therapy (ERT) reduces the incidence and severity of osteoporosis and cardiovascular disease in postmenopausal women. However, long term estrogen treatment also increases the risk of endometrial and breast cancer. The selective estrogen receptor (ER) modulators (SERMs) tamoxifen and raloxifene, cause antagonistic and agonistic responses when bound to the ER. Their predominantly antagonistic actions in the mammary gland form the rationale for their therapeutic utility in estrogen-responsive breast cancer, while their agonistic estrogen-like effects in bone and the cardiovascular system make them candidates for ERT regimens. Of these two SERMs, raloxifene is preferred because it has markedly less uterine-stimulatory activity than either estrogen or tamoxifen. To identify additional SERMs, a method to classify compounds based on differential gene expression modulation was developed. By analysis of 24 different combinations of genes and cells, a selected set of assays that permitted discrimination between estrogen, tamoxifen, raloxifene, and the pure ER antagonist ICI164384 was generated. This assay panel was employed to measure the activity of 38 compounds, and the gene expression fingerprints (GEFs) obtained for each compound were used to classify all compounds into eight groups. The compound’s GEF predicted its uterine-stimulatory activity. One group of compounds was evaluated for activity in attenuating bone loss in ovariectomized rats. Most compounds with similar GEFs had similar in vivo activities, thereby suggesting that GEF-based screens could be useful in predicting a compound’s in vivo pharmacological profile.

Determining the function of proteins encoded by all human genes in normal and pathological states is a current challenge faced by the scientific community now that the cloning and sequencing of a large percentage of the expressed human genomic sequences has been accomplished. This effort to determine function will be facilitated by recent advances in the methodology used to simultaneously monitor the expression of hundreds to thousands of genes (1, 2) in various phenotypic states (e.g., normal versus neoplastic, diseased, or activated cells). Such studies will lead to the discovery of specific genes that are regulated under pharmacological or pathological conditions and may suggest strategies for the development of novel therapeutics. A major advance in reducing time and expense in drug discovery would be achieved if the gene expression changes elicited by drug treatment of cultured cells could be correlated with in vivo pharmacological or therapeutic activity. This is a major challenge considering the many factors that influence in vivo pharmacology (e.g., cell- and tissue-specific therapeutic and toxicologic effects as well as pharmacokinetics).

To test the feasibility of using in vitro gene expression profiles to identify and characterize compounds, we chose a drug-induced biological response that is known to be dependent on gene expression modulatory events: the estrogen receptor (ER).1

Clinical studies have shown that estrogen replacement therapy (ERT) reduces the incidence and severity of osteoporosis and cardiovascular disease in postmenopausal women (for reviews see Refs. 3 and 4). However, long term estrogen treatment also increases the risk of endometrial and breast cancers (5). Therefore, our goal was to find a new method for identifying selective estrogen receptor modulators (SERMs) (6) that could be used to reduce the severity of osteoporosis in postmenopausal women without causing endometrial hyperplasia (and the concomitantly increased risk of uterine cancer). We sought an in vitro gene expression profile that would enable us to find compounds with selective in vivo activities. We did not require that the genes whose expression was monitored would necessarily encode proteins that are critical mediators of the desired or measured in vivo effects of the drug. Rather, the genes only needed to serve as reporters of the drug’s activity. Unlike other approaches to SERM identification that are based on transactivation assays using mutated or chimeric ER and estrogen-responsive promoters driving reporter genes (7), our studies asked whether compounds could be distinguished by their diff-

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1 The abbreviations used are: ER, estrogen receptor; SERM, selective estrogen receptor modulators; Tam, tamoxifen; Ral, raloxifene; GEF, gene expression fingerprint; MEM, minimal essential medium; DCC, dextran-coated charcoal; PR, progesterone receptor; HRP, horseradish peroxidase; E2, 17β-estradiol; EE, 17β-estradiol; HT, 4-OH-tamoxifen; IC1, IC1164384; PRL, prolactin; TGFA, transforming growth factor α; IGFBP-1, insulin-like growth factor binding protein-1; TRHR, thyrotropin-releasing hormone receptor; RU, RU39411; Cen, centchroman; bp, base pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction.
different abilities to modulate the mRNA levels of multiple endogenous genes.

The anti-estrogen tamoxifen (Tam) has ‘estrogenic’ activity in the bone and the cardiovascular system (for review see Ref. 8) even though it is an antagonist of estrogen action in the breast. More recent data has demonstrated that another anti-
estrogen, raloxifene (Ral), has ER agonist effects in reducing the severity of postmenopausal osteoporosis (9, 10). Ral has less pronounced stimulatory effects on the endometrium (11) than Tam or estrogen, thereby suggesting its potential advantage over Tam as ERT for postmenopausal women. Because they have different in vivo effects, we expected that Tam and Ral would be distinguishable from each other and from estrogen by differences in the gene expression changes they elicit. Indeed, studies have shown that both Tam and Ral have cell- and tissue-specific effects on transcriptional activation mediated by the ER. For Tam, there is a large body of evidence supporting its estrogen agonist activity in regulating endogenous gene expression (12), whereas there is less known for Ral (13). To be useful in distinguishing SERMs, the cells and genes monitored in response to compound treatment should enable the generation of a different “gene expression fingerprint” (GEF) for estrogen, Tam, and Ral. Ideally, the resultant GEF should also be able to predict the compound’s in vivo biological effects.

We describe here the development of a method to classify compounds based on differential gene expression. Thirty-eight compounds were tested and grouped into classes based on their GEF. The endometrial-stimulatory activities and anti-osteoporotic efficacies of some of the compounds were evaluated to determine the predictability of the in vitro “fingerprint” for in vivo activity. The results demonstrate that the majority of compounds with similar GEFs also have similar in vivo activities.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Compound Treatment**—The human breast cancer cell lines MCF7, MDA361, and ZR75–1, the human hepatoma cell line HepG2 (The Wistar Institute; American Type Culture Collection (ATCC) HB8065), and the rat pituitary cell line GH3 were obtained from the ATCC (Manassas, VA). The Fe33 (14) hepatoma ER-transfected FTO-2B cell line was provided by Dr. Hilgenfeld (Heidelberg, Germany), and the BG-1 human ovarian carcinoma cell line (15) was obtained from Dr. J. Boyd (University of Pennsylvania).

MDA-231 ER transfectant E-28 human breast cancer cells (16) were routinely cultured in phenol red-free alpha-modified minimum essential medium (MEM) supplemented with 1× DMEM, 2× HEPES, 2× insulin, 0.1× MEM nonessential amino acids, 1× sodium pyruvate, 50 mg/ml gentamycin (all from Life Technologies, Inc.), 1× insulin (Sigma), and 5× dextran-coated charcoal (DCC) (16)-treated fetal bovine serum (FBS) (Intergen). Cells were plated at approximately 40% confluence (1.5 × 10^6/plate) in 150-mm culture dishes. Following an overnight cell attachment, the medium was changed to include 0.2% ethanol or the test compounds and cultured for an additional 48 h before harvest.

GH3 rat pituitary cells were routinely cultured in Dulbecco’s modified Eagles’ medium (DMEM):F10 (1:1) medium containing 12.5% horse serum, 2.5% FBS, 25× MEM HEPES, 2× t-glutamine, and 50 μg/ml gentamycin sulfate at 37 °C, 5% CO2. Under these conditions, the cells were partially adherent, and both adherent and nonadherent cells were maintained during the passage of the cells. For the measurement of mRNA expression, cells were seeded (10^6 per 100-mm dish) in culture medium without phenol red and containing DCC-treated serum. After 3 days, the medium was changed to one containing 0.2% ethanol or the test compounds, and the cells were further incubated for 2 days before harvest.

BG-1 ovarian carcinoma cells were cultured in DMEM:F12 (1:1) medium containing 10% FBS, 2× t-glutamine, and 50 μg/ml gentamycin sulfate. For measurement of mRNA expression levels, cells were cultured for 24 h in phenol red-free medium containing 5% DCC-treated FBS prior to plating in the same medium at a density of 2 × 10^6/150-mm plate. The following day, the medium was changed to include 0.2% ethanol or the test compounds and cultured for an additional 72 h before harvest.

**Determination of Relative ER Binding Affinities**— Competition experiments were performed as described (19). Briefly, cytosol prepared from rat uterus was incubated for 2 h at room temperature with 50 μM \(^{3}H\)-labeled estradiol either in the presence or absence of unlabeled estradiol for the standard curve, or with test compounds at appropriate concentrations. Displacement curves were calculated and the relative binding affinity (RBA) was determined as the percentage of estradiol binding as described (19).

**Northern Blot Analyses**—At the end of the compound treatment time, cell monolayers were harvested into Ultra spec (Biotecx Laboratories, Houston, TX) or RNeasy (Qiagen Inc., Santa Clarita, CA) RNA isolation reagent (Biotexc Laboratories) or TRIzol (Biotecx Laboratories, Houston, TX) and reverse-transcribed to cDNA using a random priming enzyme (Vivantis, Clarita, CA) RNA isolation reagent (Biotecx Laboratories) and selected in poly(A)+ cDNA (Promega). The resulting cDNA was used for subsequent RT-PCR experiments. For each gene of interest, a specific primer set and probe were designed based on the known sequence. The PCR products were separated by electrophoresis and visualized under UV light. The intensity of the bands was quantitated using a PhosphorImager (Fuji Medical Systems, Inc.). Any variation in sample loading was normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels determined following hybridization of membranes that were treated with a boiling solution of 0.1× SSC/0.5% SDS to remove the original probe. The ratio of the signal intensities in compound-treated samples relative to controls provided the value for fold change used in the assessment of the compound activity for each assay. Changes in mRNA levels greater than or equal to 1.5-fold were scored as positive (interexperimental standard deviations were typically 10–15%).

**Progesterone Receptor and \(pS2\) Reverse Transcriptase-Polymerase Chain Reaction Assays**—The measurement of progesterone receptor (PR) in all cell lines and of \(pS2\) in the MCF-7 and ERA-MDRA cell lines was performed using reverse transcriptase polymerase chain reaction (RT-PCR). All RNA samples were diluted to 20 ng/μl in diethylpyrocarbonate-treated water. RT-PCR was performed using 100 ng of total RNA. The reaction mixtures contained 5 units of T7th DNA Polymerase (Perkin-Elmer; Foster City, CA), 1× E buffer (Perkin-Elmer), 2.5 mM Mn(OAc)2, 300 μM dNTPs (mixture from Amersham Pharmacia Biotech), and 10 pmol of each biotinylated primer in a final volume of 50 μl.
PCR primers, synthesized by Synthetic Genetics (San Diego, CA), were PRβ1 (5′-GCT AGT GGG CAT ATG CTT TAT TT) and PRβ2 (5′-AAC TTC AGA CAT CAT TTC TGG AAA TTC) to give a 426-base pair (bp) PCR product; FS241 (5′-CGT GAA AGA CAG AAT TGT GGT TT) and pS2β2 (5′-TCA GAG CAG TCA ATC TGT GGT TT) generated a 299-bp product. An agarose gel was run for 30 min at 60 °C immediately followed by 33 cycles of a two-step PCR reaction (95 °C for 15 s, 60 °C for 45 s) and a final 7-min extension at 60 °C in a Perkin-Elmer Thermocycler 9600. Following PCR, 1/20 reaction volume was analyzed using streptavidin-coated 96-well microplates (Xenogene, Gaithersburg, MD) and oligonucleotide probes specific for the PCR target (i.e. PR: 5′-horseradish peroxidase (HRP). The probe was coupled to either HRP or alkaline phosphatase and addition of either colorimetric (tetramethylbenzidine for HRP; KPL, Gaithersburg, MD.) or chemiluminescent (chloro-5-substituted adamantyl-1,2-dioxetone phenyl phosphate for alkaline phosphatase; Tropix, Bedford, MA) substrates permit quantitation of 300–500 initial copies of specific RNA template in a 20- to 100-ng total RNA sample. RNA samples were tested in triplicate, and the level of mRNA in each sample was extrapolated from a standard curve generated using purified mRNA transcribed in vitro. Changes in mRNA levels were scored as positive if they were greater than or equal to 3-fold. The typical coefficient of variation for the PR/BG-1 assay was 5–10%.

**Uterine Histomorphometric Analysis—**For determination of uterine stimulatory activity, immature, 19- to 21-day-old female Wistar rats (Schering AG, Berlin, Germany), weighing 45–50 g, were given daily subcutaneous injections of compounds or vehicle for 3 days. The compounds were dissolved in a vehicle consisting of 10% ethanol in arachis oil or a mixture of benzylbenzoate/castor oil (1:4). On day 4, the animals were weighed and euthanized by carbon dioxide asphyxiation. The uteri were then embedded in paraffin, cut into 4-μm transverse sections, and stained with hematoxylin and eosin, and the sections were evaluated for luminal epithelial cell height as described (21, 22). The difference in epithelial cell height between the estrogen (0.3 μg of E2 per animal) and vehicle-treated groups was calculated and expressed as 100%. The activity of the compound of interest as a percentage of E2 was calculated as:

\[
\%_E2 = \frac{100 \times [\text{Height (test compound)} - \text{height (vehicle)}]}{\text{height (E2)} - \text{height (vehicle)}} \quad (\text{Eq. 1})
\]

The average luminal epithelial cell height in vehicle-treated rats was 21.1 ± 8.2 μm and 68.5 ± 15.4 μm in E2-treated rats.

**Bone Protection Assays—**For determination of efficacy in preventing bone loss, 3-month-old female rats (Harlan Sprague-Dawley/Schering AG breeding facilities) were ovarioctomized under ether anesthesia and treated immediately after surgery. Compounds were dissolved in either ethanol or a mixture of benzylbenzoate/castor oil (1:4) and administered subcutaneously to the test groups. The control group was treated with vehicle only. The animals were sacrificed 4 weeks after surgery. Left and right tibiae were excised and placed in neutral buffered 3.7% formaldehyde for a minimum of 24 h. The uteri were then embedded in paraffin, cut into 6-μm transverse sections, and stained with hematoxylin and eosin, and the sections were evaluated by a blinded observer using in vivo measurement of bone mineral density measurements as described (23). Bone mineral density was measured in the secondary spongiosa of the proximal tibia 5 mm distal from the joint using peripheral quantitative computed tomography. The activity of the compound of interest as a percentage of E2 was calculated according as:

\[
\%_E2 = \frac{100 \times [\text{BMD (test compound)} - \text{BMD (vehicle)}]}{\text{BMD (E2)} - \text{BMD (vehicle)}} \quad (\text{Eq. 2})
\]

The average bone mineral density, BMD, of ovarioctomized animals was 294.7 ± 19.9 mg of calcium/cm² and 388.5 ± 29.3 mg of calcium/cm² for E2-treated animals. Standard deviations were less than 10% for the compound treatments.

**RESULTS**

**Selection of Estrogen-responsive Genes and Cells—**Cell lines were chosen based on prior knowledge of their responsiveness to estrogen treatment (e.g. growth stimulation/inhibition, gene expression modulation, and the presence of a receptor protein that binds estrogen specifically and with high affinity). Included were cell lines that express endogenous ER (GH3 pituitary adenoma (24); BG-1 ovarian carcinoma (15); MCF7, ZR75–1, and MDA361 breast carcinomas (25)) and cells stably transfected with ERα (breast carcinoma MDA231-E28 (called MDA-ER) (16), human mammary epithelial cell line 184B5-E1 (called B5-ER) (16), human heptoma HepG2-ER1 and ER2, and rat hepatoma Fe33 (14)). Known estrogen-responsive genes, including the 52-kDa cathepsin D (52kd (26)), growth hormone (GH) (27), prolactin (PRL) (28), progesterone receptor (PR) (29), pS2 (16), transforming growth factor alpha (TGFα) (30), insulin-like growth factor binding protein 1 (IGFBP-1) (31), corticosteroid-binding globulin (CBG), amphiregulin (32), and thyrotropin-releasing hormone receptor (TRHR) (33) were also chosen for our studies.

To determine which of the genes and cell lines showed measurable responses to estrogen treatment, cells were grown in estrogen-free culture medium and treated with the natural ligand, E2, or the nonmetabolizable estrogen EE for varying lengths of time from 3 to 72 h. Analysis of the levels of mRNA for the genes of interest gave an estimate of the kinetics of the response to E2 or EE treatment and an indication of the optimal conditions to measure the responsiveness of each gene. An example is shown for regulation of CBG mRNA levels in HepG2-ER2 cells (Fig. 1). Maximal stimulatory effects were observed by 48 h of treatment.

Gene/cell combinations (i.e. assays) that responded to E2 or EE treatment with at least 3-fold changes in mRNA level relative to vehicle-treated controls are shown in Table I. The degree of estrogen-responsiveness in this panel ranged from 3- to 450-fold, but most of the assays showed less than a 15-fold response to estrogen treatment (for fold change plus estrogen, see Table I).

**Identification of Informative Gene/Cell Combinations for Compound Discrimination—**Based on the assumption that there would be a limit to the number of distinguishable mechanisms by which a ligand-bound ER could modulate gene expression, we performed studies to determine whether a subset of these gene/cell combinations would be sufficient to differentiate known pharmacologically distinguishable compounds. We tested compounds with known ER-binding properties and activities in animal models for endometrial hyperplasia and bone protection. Two key compounds were the partial agonists, Tam (the 4-OH-tamoxifen (HT) derivative was used in the initial studies) and Ral, because they have distinctly different in vivo activities compared with each other and with estrogen. One full agonist (2-hydroxy-estradiol (2HE)), three putative partial agonists (RU39411 (RU) (34), centchroman (Cen) (35), ZK119010 (119) (36), and a pure antagonist,ICI164384 (ICD) (37) were also included in these initial studies.

Since 1.0 μM or lower concentrations of compounds elicited maximal responses in most of the assays (data not shown), all compounds were tested at 1.0 μM. The response of each gene/cell combination to compound treatment was scored by measuring steady-state levels of mRNA by Northern, slot blot, or RT-PCR analysis (Table I). The degree of stimulation of gene expression by each of these compounds differed as a function of the gene and cell type. In some cases, the partial agonists HT, RU, and Cen had the same effectiveness as estrogen in stimulating gene expression (i.e. in TRHR/GH3, TGFα/B5-ER, TGFα/MDA-ER). But, in most instances, the full agonists (i.e. E2, EE, 2HE) were significantly more active in enhancing gene expression than the other compounds. Analysis of 24 different gene/cell combinations with these nine compounds revealed that most of the assays provided redundant information (seen as the same pattern of activity across the series of compounds in Table I). Five major activity patterns across this set of compounds were discernable among all of these assays when two activity states (i.e. active and inactive) were considered (patterns I–V on the right side of Table I). Response pattern I was found in most of the genes and cell types tested, whereas the other patterns were seen less frequently. There were only
four assays that registered responses to Ral treatment (i.e., those in patterns II and V), whereas HT was active in nine assays (i.e., those in patterns II–V). Interestingly, some compounds exhibit cell type-dependent abilities to activate the same gene, as illustrated by the differential response of 52kD gene expression to treatment with the partial agonists HT, RU, and Cen in ZR75–1 mammary carcinoma compared with BG-1 ovarian carcinoma cells. Furthermore, the same compounds can have different effects on different genes within the same cell (e.g., compare 52kD and TGF-α in the B5-ER cells).

![FIG. 1. Time course of EE-dependent CBG gene expression in HepG2-ER2 cells. Total RNA was isolated from HepG2-ER2 cells at the indicated time points following treatment with EE or vehicle (VC). Northern blots were prepared with 20 μg of RNA and analyzed for mRNA levels of CBG (top gel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, bottom gel). The CBG signal intensities (averages of duplicates) were normalized for glyceraldehyde-3-phosphate dehydrogenase levels and are represented in the graph as fold change in gene expression relative to the vehicle control at the corresponding time point.

### TABLE I

Activity of selected compounds in modulating gene expression

The indicated cell lines were treated with compounds (1.0 μM), total RNA was isolated, and mRNA levels corresponding to the indicated genes were quantitated by Northern (n), RT-PCR (p), or slot blot (s) analyses as described under “Experimental Procedures.” The gene expression response of each cell line following E2 or EE treatment (average of at least two independent experiments) is provided in the third column (i.e., Fold change 1 estrogen).

| Gene | Cell line | Fold change + estrogen | E2 | EE | 2HE | HT | RU | Cen | Ral | 119 | ICI | Pattern |
|------|-----------|------------------------|----|----|-----|----|----|-----|-----|-----|-----|-------|
| PR (p) | GH3 | 10 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| PR (p) | MCF7 | 15 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| PR (p) | B5-ER | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| PR (p) | ZR75–1 | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| GH (n) | GH3 | 20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 52kD (n) | MCF7 | 25 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 52kD (n) | B5-ER | 30 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| IGFBP-1 (n) | HepG2-ER1 | 35 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| pS2 (n) | MCF7 | 40 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| pS2 (n) | B5-ER | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Amphireg (n) | MDA361 | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| pS2 (n) | ZR75–1 | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| TRHR (n) | GH3 | 30 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| PR (s) | GH3 | 35 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| pS2 (p) | MDA-ER | 450 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| TGFα (s) | MDA-ER | 20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| pS2 (n) | B5-ER | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| TGFα (n) | B5-ER | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 52kD (n) | ZR75–1 | >20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| CBG (n) | HepG2-ER2 | 30 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| IGFBP-1 (s) | Fe33 | 20 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

* ND, not determined.

* The + in this assay is <50-fold. Assays were grouped according to the compound activity pattern shown at the right (i.e., I–V).
Selection of Nonredundant Gene/Cell Combinations—Addi-
tional studies were performed to determine which of the “re-
dundant” assays were most amenable to screening strategies
(e.g. highest reproducibility and extent of change relative to
table). Based on these considerations, PR/BG-1 (pattern I),
PRL/GH3 (pattern II), TGFα/MDA-ER (pattern III), and
IGFBP-1/Fe33 (pattern V) were selected to use in screens of
additional compounds. We did not include the assay for pattern
IV, because the estrogen response was only 4- to 6-fold.

The response of each compound in the four assays generated
a GEF characteristic of each compound (seen as + and –
patterns of activity in the row corresponding to each compound
in Table II). The GEFs of the full agonists, E2 and EE, are
identical to each other but different from those of HT, RU, and
Cen, which all have the same GEF. Importantly, the different
GEFs of E2, Ral, and HT demonstrate that these assays dis-

**TABLE II**

**Compound classification by selected GEF assays**

Data in Table I are re-arranged and simplified here to emphasize the
grouping of compounds based on their activities in each of the four
selected assays. See legend of Table I for details.

| Gene cell line | PR/BG1 | PRL/GH3 | TGFα/MDA-ER | IGFBP-1/Fe33 |
|---------------|--------|---------|-------------|-------------|
| E2            | ++     | ++      | ++          | ++          |
| EE            | ++     | ++      | ++          | ++          |
| 2HE           | ++     | ++      | ++          | +           |
| HT            | –      | +       | ++          | +           |
| RU            | –      | +       | ++          | +           |
| Cen           | –      | +       | ++          | +           |
| Ral           | –      | +       | –           | +           |
| 119           | –      | –       | +           | –           |
| ICI           | –      | –       | –           | –           |

The concentration dependence of the gene expression re-
sponse for E2, EE, HT, Ral, and ICI in each of the four assays
is shown in Fig. 2. EC_{50} values for EE and E2 were 0.01–0.1 nM
in the PR/BG-1 (Fig. 2A) and PRL/GH3 (Fig. 2C) assays and
were significantly greater in the TGFα/MDA-ER (i.e. 1 and 10
nM, respectively; Fig. 2B) and the IGFBP-1/Fe33 assay (i.e. 0.5
and 50 nM, respectively; Fig. 2D). The large difference in the

**FIG. 2.** Concentration dependence of gene expression changes in selected GEF assays. Cells were treated with vehicle, E2 (open squares), EE (open circles), HT (diamonds), Ral (solid squares), or ICI (triangle) at the indicated concentrations. The fold change in mRNA levels relative to vehicle control is graphically depicted for each gene/cell combination. A, PR mRNA levels were measured using RT-PCR analysis following treatment of BG-1 ovarian carcinoma cells for 72 h. B, TGFα mRNA levels were quantitated by slot blot analysis using 10 μg of total RNA following treatment of MDA231-ER cells for 48 h. C, PRL mRNA levels were measured by slot blot analysis of total RNA (1.0 μg) prepared from 48-h compound-treated GH3 pituitary cells. D, IGFBP-1 mRNA levels were quantitated in total RNA (0.5 μg) isolated from Fe33 liver hepatoma cells treated for 48 h with the indicated compounds.
potency of E2 and EE in the Fe33 liver cells has previously been described (38) and is most likely due to metabolism of E2 in the liver cells. The maximal response obtained with HT and Ral in the Fe33 liver cell assay. The other groups contain compounds that were inactive in the PR/BG-1 assay but showed differing activities in the other three assays.

To further elucidate the mechanisms responsible for the different gene expression modulatory activities of compounds in groups 2–7, most of the compounds were tested for their abilities to antagonize estrogen-stimulated gene expression changes in the assays where they had no activity. None of the compounds in group 2 could antagonize the stimulatory effect of E2 at 10 M in the IFGBP-1/Fe33 assay at 100-fold molar excess, whereas all of the tested compounds in groups 3–8 inhibited E2-induced responses in the IFGBP-1/Fe33 cell assay. The results suggest that compounds in groups 3–7 are classical partial agonist-antagonists of the ER, whereas the compounds in group 2 are either weak agonists or are metabolized in the Fe33 liver cells.

The two “standard” SERMs in our experiments were Tam and Ral. Tam and the other compounds in its group (i.e. group 3) have distinctly different GEF from Ral and other members of group 5, all of which have no activity in the TGFα/MDA-ER assay. Interestingly, Tam and its more active metabolite HT were separated from each other by the inactivity of Tam in the Fe33 liver cell assay and by Tam’s weaker response in the TGFα/MDA-ER assay at 100-fold molar excess, whereas all of the tested compounds in groups 3–8 inhibited E2-induced responses in the PR/BG-1 assay (data not shown). The results suggest that compounds in groups 3–7 are classical partial agonist-antagonists of the ER, whereas the compounds in group 2 are either weak agonists or are metabolized in the Fe33 liver cells.

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sponse observed at the maximally effective dose of each compound is shown in Fig. 3A. All of the compounds in groups 2 and 3 elicited endometrial stimulatory effects that were equal to or better than E2 treatment. Most of the compounds in groups 4 and 5 were similar to or less potent than Ral in increasing uterine epithelial cell height. There was no apparent distinction for this in vivo parameter between members of these two groups, which were active in two of the four GEF assays. The two exceptions were Tam and ZK183955, both of which co-classified with poorly active compounds by their in vitro GEFs but were more stimulatory than E2 in the in vivo evaluation.

The single members of groups 6 and 7 were very different in their in vivo effects on endometrial cell height; ZK183659 was highly effective in stimulating the endometrium and ZK186217 was weakly effective. All but one of the compounds in the "inactive" class (i.e., group 8) that includes the pure ICI antagonists were very weak or inhibitory in the endometrial stimulation assay. The only active compound, ZK183659, produced a moderate stimulatory response only at the highest dose tested (i.e., 10 mg/kg/day).

To determine whether compounds that grouped with Ral (group 5) would have anti-osteoporotic activities, the four com-

**TABLE IV**

*Compound activities in GEF assays*

Data represent the average maximal response (fold change in mRNA level relative to vehicle control) of at least three individual experiments with duplicate determinations. Each compound response is also provided as percentage of E2 response in parentheses. Shading indicates active; blank indicates inactive. The minimum differential expression changes that were significant for each assay are listed at the bottom of each column. ER binding affinities of each compound relative to E2 are shown in the column labeled RBA. nd, not determined.

| Compound | PR/BOG-1 | PRL/GH3 | TGF/MDA-ER | IGFBP-1/Fc33 |
|----------|----------|---------|------------|--------------|
| E2       | 21       | 37.9    | 12.6       | 100          |
| EE       | 167      | 42.9    | 12.1       | 106          |
| E1       | 13       | 32.5    | 12.1       | 111          |
| E3       | 8.3      | 32.5    | 12.1       | 91           |
| ZK167502 | 0.7      | 32.4    | 4.1        | 33           |
| 2HE      | 25       | 8.8     | 1.7        | 33           |
| Dihydrogesterol | 6.3 | 32.1 | 5.3 | 42 |
| Dihydrogesterolinin | 1.8 | 33 | 2.9 | 23 |
| ZK183491 | 0.2 | 3.4 | 4 | 32 |
| ZK158543 | 2.1 | 3.4 | 4 | 32 |
| ZK166780 | 44 | 13 | 4.4 | 35 |
| ZK166781 | 14 | 10 | 4.1 | 33 |
| Coumestrol | 4.2 | 40.8 | 7.8 | 72 |
| ZK22496 | 12 | 12.1 | 9.2 | 86 |
| 17c-E2 | 2.2 | 11.7 | 1.9 | 18 |
| HT | 50 | 11.9 | 3.2 | 25 |
| RU39411 | 42 | 13.3 | 3.1 | 25 |
| Dostaxol | 15 | 6.3 | 1.7 | 14 |
| Centchroman | 2 | 2.6 | 1.8 | 14 |
| ZK183819 | 5.3 | 2.8 | 1.7 | 18 |
| Tamoxifen | 1 | 4.3 | 3.5 | 28 |
| ZK182254 | 24 | 4.3 | 3.5 | 33 |
| ZK185704 | 4 | 1.6 | 1.6 | 15 |
| ZK185010 | 7.7 | 1.6 | 1.6 | 15 |
| ZK183955 | 33 | 3 | 3.5 | 28 |
| Raloxifene | 77 | 1.6 | 2.3 | 18 |
| ZK167566 | 71 | 1.7 | 1.6 | 13 |
| ZK180686 | 17 | 1.6 | 2.1 | 17 |
| ZK184104 | 17 | 1.5 | 1.6 | 13 |
| ZK186217 | 20 | 1.6 | 1.4 | 15 |
| ZK185427 | nd | 0.9 | 0.7 | 1.2 |
| ICI164384 | 18 | 0.7 | 0.9 | 1.2 |
| ICI182780 | 67 | 0.5 | 1.3 | 0.8 |
| ZK167957 | 44 | 0.5 | 1.4 | 1.4 |
| ZK185157 | 18 | 0.9 | 1.0 | 8 |
| ZK185956 | 2.9 | 0.8 | 1.0 | 8 |
| ZK183565 | 4.2 | 1.2 | 1.0 | 0.8 |
| RU56688 | 25 | 1.1 | 1.0 | 0.8 |

Activity cut-offs: 3

* Measured using recombinant human ER protein.
* Data from ref. 46.
Endometrial-stimulatory and bone-protective activities of GEF-sorted compounds. A, compounds from groups 2–8 of Table IV were tested at concentrations ranging from 0.03 to 3.0 mg/kg/day in immature female rats (5 rats per treatment group). Stimulatory effects on uterine epithelial cell height (as percentage of E2) are provided in comparison to a replacement dose of E2 (0.3 μg per animal) for the maximal responsive dose of each compound. The corresponding doses (in milligrams/kg/day) were 0.05 (for RU39411), 0.1 (for HT, ZK167466, 17α-E2, RU58688), 0.3 (for Tam, Ral, ZK183955, ZK180686, and ZK182254), 3.0 (for Droloxifene, Cen, ZK119010, ZK184104, ZK185157, ZK183819, and ZK182956), and 10 (for ZK22496, ZK185704, and ZK183659). B, the compounds from group 5 that were poorly active in the uterine growth assay were evaluated at doses ranging from 0.04 to 1.0 mg/kg/day for bone-preserving efficacy in ovariectomized female rats (8 rats/treatment group). Bone mineral density (BMD) in the proximal tibia following 4 weeks of treatment was measured using peripheral quantitative computed tomography. Compound activity was assessed relative to the effects observed with a bone-protective dose of 1.2 μg/kg/day E2, which was set at 100%. The activity at the maximally effective doses (in milligrams/kg/day: for ZK167466, 0.4; for Ral, ZK180686; and for ZK184104, 1.0) of each compound are shown. The response of RU58688 (at 0.4 mg/kg/day; from group 8) is provided for comparison.

DISCUSSION

Our search for novel SERMs is an example of the use of gene expression profiles in a strategy to discover new drug leads. The results demonstrate that it is possible to group compounds based on differences and similarities in the pattern of gene expression changes that they elicit. Furthermore, the feasibility of employing gene expression profiles to guide selection of compounds for in vivo biological studies is suggested by the separation of compounds with highly stimulatory effects on the endometrium from those that were poorly effective on the basis of their GEFs. The observation that all of the poorly active members of one group of compounds were also effective in ameliorating bone loss in an ovariectomized rat provides additional support that this approach can predict in vivo pharmacological and therapeutic profiles.

In the example described here, we evaluated 24 gene/cell combinations comprising 10 different known estrogen-responsive genes in eight cell lines representing four cell types (i.e., liver, pituitary, mammary, and ovarian). To determine which of the gene/cell combinations would be most useful in discriminating between different compounds, a small group of compounds that had known abilities to bind to the ER and to elicit various estrogenic responses in vivo was used. Included in this group were the two SERMs, Tam and Ral, because they have distinguishable in vivo biological activities in comparison with estrogen and with each other.

We found five different patterns of gene expression activity in the 24 gene/cell combinations in response to the nine initial compounds evaluated (Table I), suggesting that many gene/cell combinations report the same “redundant” information. This may be due to an inherent limitation in the number of molecularly distinct mechanisms by which the ER can be activated by ligand binding (for reviews see Refs. 43 and 44). The majority of the gene/cell combinations were responsive to estrogen and the other full agonists but not to any of the partial agonists tested. There was at least one gene in each of the 10 cell lines that was modulated only by estrogen and the full agonists, whereas genes in only two of the cell lines were regulated by Ral. This may be indicative of a requirement for cell-specific factors for Ral responsiveness. The contribution of cell-specific factors to regulation of gene expression by E, HT, and Ral is also suggested by the observation that the same gene (i.e., pS2) can be differentially modulated by these compounds in different cell types or in distinct cell lines from the same cell type (e.g., breast cancer cell lines ZR75–1 and MDA-ER). In addition, we found that different genes in the same cells responded differentially to these compounds, thereby underscoring the importance of gene-specific factors in determining sensitivity to compound treatment.

From among these 24 gene/cell combinations, a nonredundant panel of four assays was selected that would enable discrimination between E, Tam, and Ral as well as identify compounds that had similar and different activities from them (Table I). Evaluation of 29 additional compounds using these four assays led to their separation into eight distinct groups (Table IV). Twelve of the 38 compounds tested were grouped with E2 and EE, because they had activity in all four assays.
Nineteen compounds were active in one to three assays, and seven were devoid of activity in any assay. The compounds that were classified into groups 3–7 were of particular interest, because they included the standard SERMs, Tam and Ral. None of the members of these groups could induce PR gene expression in the BG-1 ovarian cells but were distinguished by differing abilities to modulate gene expression in the other three assays. Other studies have shown that the transcriptional responses of Tam and Ral can be distinguished by their activities in trans-activation assays using wild type and mutant ER and the complement C3 promoter driving a reporter gene (45) but did not reveal the existence of other classes of partial agonists. The experiments described here demonstrate the use of multiple endogenous gene expression readouts in different cells to generate a GEF for each compound that ensures the discrimination between Tam- and Ral-like compounds. In addition, our data suggest that endogenous gene expression assays may uncover classes of ER modulators that could not be identified using other methods.

The *in vivo* evaluation of compounds for their maximal uterine stimulatory responses was designed to mirror the approach used in the *in vitro* analyses. The results for compounds in groups 3–8 revealed that there was good correlation between their GEFs and endometrial-stimulatory activity. All of the compounds in group 3, which included HT, a major active metabolite of tamoxifen, were equivalent to or more stimulatory than a replacement dose of E2. The compounds in this group were structurally diverse, had varying degrees of affinity for ERα, and were similar only in their ability to score positively in the same three gene/cell combinations. The compounds in group 8, which includes ICI, were inactive in all GEF assays and all but one of them weakly stimulated or inhibited uterine growth in immature rats. The remaining groups contain compounds, including Ral, with activity in only one or two assays; most of these compounds had considerably less endometrial-stimulatory activity than members of group 3. Thus, compound sorting by GEF analysis can be used to predict compounds that would have a high probability of being very active in increasing endometrial luminal epithelial cell height (i.e. those with activity in three or more assays). However, the number of assays in which a compound is active did not always predict the strength of the uterine stimulatory response for the poor to moderately active compounds. Of the two compounds representative of single assay hits, one of them (i.e. ZK185427, Fig. 3A) was more effective in the endometrial stimulation assay than the compounds that were active in two assays.

An additional point can be made from these data with respect to the importance of metabolism in *in vivo* pharmacology. Tam was placed into group 4 by its *in vitro* gene expression profile even though its *in vivo* effectiveness clearly categorized it with HT and the other potent endometrial stimulators of group 3. These results suggest that Tam was inactive in the IGFBP-1/F33 assay either because the 4-hydroxylated metabolite of Tam is essential for the transcriptional activation of the IGFBP-1 promoter or because HT has 50-fold greater affinity for the ER than Tam. It is possible that the same explanation could apply to the other exception, ZK183956. This compound is a tamoxifen derivative that may undergo similar metabolic conversion in *vivo*. These data also emphasize the limitations of *in vitro* screens that are not able to measure effects of compound metabolites or have insufficient sensitivity to score effects of compounds with low affinities for their biological targets.

To further assess the strength of GEF-based screening for predicting *in vivo* biological activity, we determined whether the compounds that were grouped together with Ral were similarly effective in protecting against bone loss in female rats following estrogen deprivation by ovariectomy. All of the compounds in the Ral group demonstrated significant bone-protective activity. This activity, coupled with the weak to poor endometrial stimulatory activity of these compounds, suggests that they would be potential leading candidates for optimization as novel SERMs.

These results support the concept that gene expression profiles can be employed to classify compounds with respect to a reference standard such as estrogen. However, the compounds tested in this study did not permit statistical evaluation of the correlation between compound classification and *in vivo* effects for the members of all compound groups. Additional studies using a larger number of compounds are required to determine whether the four gene/cell assays employed in this screen are sufficient to adequately categorize compounds for their *in vivo* effects.

The integration of high density cDNA array hybridization methods for the identification of genes and cells to be employed in such screens will greatly facilitate this process. It should be possible to use gene expression profiles in the search for new chemical or biological entities that have similar functions to any compound used as a reference. In addition to small molecules, mimetics of proteins and peptides as well as expressed partial or full length cDNAs, oligonucleotides, or antisense molecules can be identified by screening compounds that cause changes in gene expression that are similar to those elicited by such effectors. In situations where large chemical data bases and corresponding pharmacological and pharmacokinetic data are available, a reverse approach that evaluates *in vivo* characterized compounds in GEF screens similar to those described here can be carried out. The juxtaposition of known *in vivo* efficacy and side effects with the results of GEF analysis can be employed to derive GEFs that are predictive for pharmacology, toxicity, and perhaps even pharmacokinetic effects.

Acknowledgments—We are grateful for gifts of HEGO plasmid (Dr. P. Chambon), F33 rat hepatoma cells (Dr. Hilgenfeld), BG-1 ovarian carcinoma cells (Dr. J. Boyd), and 184B5 cells (Dr. M. Stampfer). We thank J. Simmons, D. Allen, and K. Oliver for technical assistance. Special thanks are extended to Dr. P. Johnson for valuable discussions and comments on the manuscript.

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