SCREENING ASSAY FOR SELECTIVE ESTROGEN RECEPTOR MODULATORS

by

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Estrogen influences the development and progression of breast cancer and of other types of cancer, such as ovarian and lung cancer. The best strategy for prevention and treatment of estrogen dependent cancers is to selectively block estrogen activity in the affected estrogen dependent tissues. The beneficial role of estrogen in the other tissues should be preserved. One of the most common methods to prevent the harmful effects of estrogen is to block the estrogen receptor signaling. The intense research in the breast cancer treatment and prevention field produced a number of estrogen related compounds. The existing screening assays to test the selectivity and potency of these compounds have major limitations. I propose here the development and validation of a rapid screening assay for selective estrogen receptor modulators. This assay is based on the use of an ERE (estrogen response elements) to drive expression of a fluorescent protein that can be visualized directly in living cells. I presented here the first step in developing the screening assay, the generation and evaluation of two fluorescent clones, ERE-GFP and ERE-DsRed. The clones were introduced in CV-1 cells, together with ER, using transient transfection in order to test whether they are under tight estrogenic control. The cells were further treated with know ER ligands. These results predict that the clones function as expected. A robust signal resulted in the presence of estradiol, while with a pure antiestrogen such as ICI 182,780 resulted in very little red/green fluorescence. The vehicle control (ethanol) also elicited very little response (fluorescence). Further, these clones can be stably integrated in CV-1 cells together with either ER alpha or ER beta in order to develop a high content screening assay for SERMs. The new SERMs identified using this assay can be used eventually in therapy of breast or lung cancers or as hormone replacement. In addition, compounds that differentiate ERα and ERβ will be valuable tools to further dissect ER signaling pathways. It is important to know more about coactivator recruitment, gene expression profile or about the response with ER mutations. This will lead to a better understanding of estrogen related cancers and will help designing new therapeutic approaches.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS:

AIB1 - amplified in breast cancer 1

CV-1A cells - CV-1 cells + ERE-GFP + ERα

CV-1B cells - CV-1 cells + ERE-RFP +ERβ

DTAC - 1, 1-dichloro-2,2,3-triarylcyclopropane

ER – estrogen receptor

ERE - estrogen responsive elements

GFP – green fluorescence protein

SERM - selective estrogen receptor modulator

ZOHT – 4-hydroxy tamoxifen

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1.0 BACKGROUND

1.1 ESTROGEN

Estrogen is a steroid hormone and it has an essential role in the development and maintenance of female secondary sex characteristics. This hormone is produced mainly by the ovaries and mediates a broad spectrum of physiologic functions.

1.1.1 Importance

Estrogen can have both beneficial and harmful effects. Some of the beneficial effects of estrogen are: programs the breast and uterus for sexual reproduction, controls cholesterol production protecting the coronary arteries from forming plagues, maintains the proper balance between bone buildup and breakdown helping to preserve bone strength. The harmful effects of estrogen are mainly a consequence of its ability to promote the cell proliferation in the breast and uterus. Breast cancer, endometrial cancer, endometriosis, adenomyosis and leiomyomas are diseases that develop and progress in an estrogen-dependent fashion. Also, estrogen status is a recognized factor in lung cancer risk in women (1).

1.1.2 Estrogen Dependent Diseases

Breast cancer is the second leading cause of cancer deaths in women today and the most common cancer among women. Breast cancer also occurs in men. Although this is much less common, breast cancer in men is usually very aggressive. Estrogen influences breast cancer development and progression. Also, estrogen seems to be involved in lung carcinogenesis, which is the leading cause of death from neoplasia in the United States. Recent studies show that estrogens have growth-promoting effects in lung tumors and that estrogen receptor is expressed in this tissue (2). Many types of cancer are estrogen dependent. Consequently, the best strategy for prevention and treatment is blocking estrogen activity in certain estrogen dependent tissues. There is an intensive search for compounds that block estrogen receptor function or inhibit estrogen production. Such compounds should inhibit the growth of estrogen responsive cancers or even prevent their development. Selective estrogen receptor modulators (SERMs) are a class of drugs which have great therapeutic utility in treating ER-positive breast cancers (3). Also, SERMs may have therapeutic benefit in non–small cell lung cancer (4).

1.2 ESTROGEN RECEPTORS

1.2.1 Estrogen Receptor

Estrogens act on target tissues by binding to estrogen receptors (ER). ER is a steroid receptor and belongs to the family of nuclear hormone receptors. These proteins function as transcription factors when they are bound to their ligands.

Classically, estrogen receptor proteins function as transcription factors in the nucleus controlling estrogen-regulated genes (5). Besides the nuclear signaling cascade, ER can also signal via second messengers, such as receptor-mediated protein kinases, at the membrane or in the cytoplasm (6).

1.2.2 Mechanism of Action

The estrogen receptor has at least two major mechanisms of action. ER can use a genomic pathway and a non-genomic pathway.

Genomic action:

The genomic ER action occurs in the nucleus, when the receptor binds specific DNA sequences directly (classical pathway) or indirectly (non-classical pathway).

Classical (ERE) pathway:

In this pathway ER protein functions as a transcription factor in the nucleus. Upon ligand binding, estrogen receptor (ER) is activated through phosphorylation and dissociates from the heat-shock proteins. Then, the ligand-bound activated ER changes its conformation, dimerizes, and binds to specific DNA sequences called estrogen responsive elements (ERE) in order to regulate transcription (7, 8) **(Fig. 1)**. In the presence of the natural ligand estradiol, ER induces chromatin remodeling and increased transcription of estrogen regulated genes.

Non-classical pathway:

AP-1 (9) and SP-1 (10) are alternative regulatory DNA sequences used by both isoforms of the receptor, ERα and ERβ, to modulate gene expression. In this case, ER does not interact directly with DNA but interacts with other DNA-bound transcription factors such as c-jun or c-

fos, or with other coactivator proteins (9). Some data suggest that these alternative ER signaling pathways, mainly at AP-1 sites, may be involved in Tamoxifen therapy resistance in breast cancer (11).

The same ligand can have different effects on AP-1 (fos/jun)-dependent transcription depending on the receptor subtype. For example, E2 is an agonist for ERα but not for ERβ at AP-1 elements (12). ERα and ERβ also show differential affinity for p160 coactivators and transcriptional responses. As a result of these differences, the overall response of a cell expressing ERα or ERβ to a particular ligand cannot be simply predicted.

Non-genomic pathway:

There are few mechanisms proposed for the non-genomic ER action or membrane-initiated steroid signaling. One mechanism involves activation of insulin-like growth factor receptor 1, cellular tyrosine kinases, mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3 kinase (13). This action occurs within minutes of the addition of estrogen. SERMs such as Tamoxifen may also activate membrane ER (6). Estrogen activates ER in or close to the membrane and then ER binds to growth factor signaling elements such as IGFR-1. Growth factor signaling is activated and further activates key molecules such as Akt or MAPK (14). Estrogen initiates powerful signals for cell survival and proliferation using these pathways. Furthermore, ER and its coregulators can be phosphorylated by these kinases. This causes an increase in ER nuclear signaling and also in estrogen agonist-like activity of tamoxifen and other SERMs (15).

Another potential mechanism for membrane-initiated ER signaling indirect activation of the epidermal growth factor receptor (EGFR) (16). SERMs have differential effects on membrane–initiated ER signaling. Tamoxifen, like estrogen, activates membrane ER, while the

pure antiestrogen fulvestrant does not activate membrane ER in this way (15). Tamoxifen resistance may be explained by this stimulation of ER through non-genomic pathways (15).

1.2.2.1 ER Signal Transduction using ERE sites:

When ER is activated by agonist ligand binding, conformational changes are induced and intracytoplasmic chaperones, such as heat-shock proteins 70 and 90, dissociate from the receptor molecule (17). The receptor then interacts with DNA and the transcriptional response is modulated by the recruitment of co-regulatory proteins (18). The conformation of the receptor is altered by different ligands and by ERE structure. When ER is bound to antiestrogens, a part of the receptor (helix 12) is displaced and blocks co-activator access. As seen in crystallographic studies, ER alpha reacts to a specific single nucleotide alteration within the ERE by changing its DBD conformation by means of a side-chain rearrangement (19).

The ligand structure and the specific ERE sequence influence ER conformation resulting in differential recruitment of co-regulatory proteins to the ER–ERE complex. This can be a mechanism for modulation of gene transcription at different EREs. Coregulators recruitment is primarily dependent on the ERE sequence when the ERs are bound to 17b-estradiol. By contrast, the ERE sequence loses its influence on AF-2-dependent cofactor recruitment when the ERs are occupied by anti-estrogens (20).

The ability of the DNA-bound receptor to modulate transcription is dependent not only on cofactors recruitment but also on coactivator/corepressor expression as well as on promoter context and ligand structure (3, 21).

The ERE pathway **(Fig. 1)** is mainly important in reproductive tissues. Selective estrogen receptor modulators bind to the ER causing a conformational change which modifies receptor

capacity to dimerize, to form transcription complexes with coactivators and/or to bind to ERE-dependent genes. Therefore, SERM-bound receptor is unable to activate ERE pathway but the new conformation allows ER to activate alternate response domains. These alternate pathways are operative in other estrogen responsive tissues, such as bone and vascular tissue. The ligand-induced conformation of the receptor and the variable availability of some coregulators in different tissues make these compounds selective. This might explain why SERMs can be agonists as well as antagonists depending on their structure and the cell context.

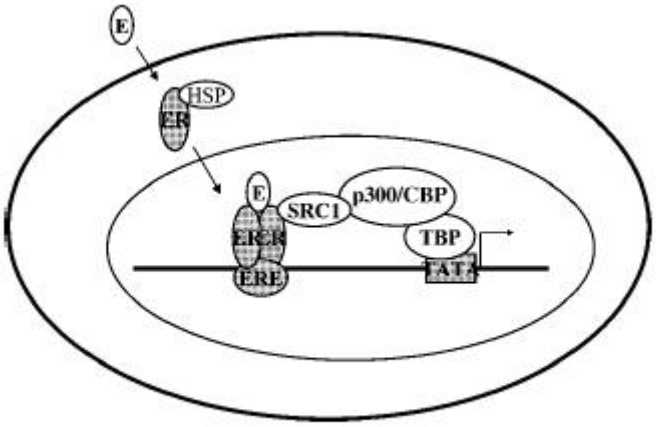


Figure 1 - *Model for ER-mediated signal transduction ERE pathway (7).*

1.2.2.2 Importance of Coactivators and Corepressors

ER coregulators are proteins that are recruited by the receptor to form the transcription complexes. The ER needs these factors to mediate cellular responses to endocrine signals. As cellular environments change, ERs can associate with distinct subsets of cofactors depending on

binding affinities and availability of these factors as well as on the ligand bound to the receptor. The coregulators are generally divisible into coactivators and corepressors. The coactivators turn on target gene transcription and the corepressors inhibit gene activation and possibly turn off activated target genes. These proteins are part of the transcription complexes and create a bridge between ER, chromatin components (histones) and components of the basal transcription machinery. Each class of ligand induces a different ER-ligand binding domanin conformation. The coregulatory proteins bind to the exposed surface of the receptor, so different ligands induce recruitment of different coregulators.

The coactivators can be categorized in several subgroups: the p160/SRC coactivator family (SRC-1, GRIP-1, AIB-1), CBP/p300 coactivators and SRC-associated acetyltransferases, the TRAP/DRIP coactivator complex. The coactivators associate with ER in the presence of agonistic ligands.

The corepressors, like N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors), are recruited to ERs in the presence of antagonistic ligands.

Coregulators influence transcription depending on ER-subtype, the structure of the receptor–ligand complex, and the tissue specificity.

Affinity of the ER alpha interaction with AIB-1 is much higher than that observed for the ER beta (22). In contrast to ER alpha, ER beta apparently interacts in a ligand independent manner with SRC-1 in vitro.

Compounds selective for ER alpha or ER beta have different preferences in recruiting coactivators (23). The SRC family members have been shown to interact in vivo with both isoforms of the receptor (24). In the presence of E2, SRC-1 or GRIP1 are recruited with similar

affinity to both ER subtypes. In contrast, both coactivators are more strongly recruited to ER beta than to ER alpha in the presence of genistein (25). The capacity of ER ligands to selectively modulate the recruitment of coregulators would be useful in the development of pharmaceuticals with superior tissue selectivity.

Altered expression of ER cofactors has been reported during human breast tumorigenesis (26). Hormonally regulated cancers such as breast, uterine, and prostate cancers overexpress AIB-1 coactivator (27). Overexpression of AIB-1 is suspected to contribute to tamoxifen resistant breast tumors (26). The variation in transcriptional response mediated by ER alpha and ER beta might be due to variation in expression levels and differential recruitment of coactivators.

1.2.3 Receptor Domains

ERs are composed of 6 domains; A to F. Domain A/B (N-terminal domain) encodes a ligand-independent activation function (AF1). This region was shown to be a target of the MAP-kinase regulatory pathway, indicating a cross-talk between signals initiated by growth factors and steroid hormones at the level of the receptor (28). The C domain contains a two-zinc finger structure which is important in dimerization and DNA binding. The C-terminal domain (E/F domain) mediates ligand binding, nuclear translocation and transactivation of target gene expression. The ligand binding domain (LBD) also contains a ligand dependent activating function (AF-2) and it spans from helix 3 to helix 12. The ligand-binding pocket is composed of helixes 3, 4, 5, and 12.The position of helix 12 is altered by ligand binding and influences the recruitment of cofactors (29).

1.2.4 Receptor Isoforms

The biological actions of estrogens are mediated mainly by two receptor isoforms, ER alpha (30) and ER beta. The two isoforms are products of distinct genes on different chromosomes. Although the two isoforms share mechanistic similarities, they are genetically and functionally distinct. ER alpha has biological roles that are different from those of ER beta. Knock-out mice deficient in ER beta show a quite distinct phenotype then ER alpha knock-outs. The two ER subtypes have different tissue distribution: the ERα subtype is predominant in breast, uterus and ovaries, whereas ERβ predominates in lung, bone, endothelial cells (31) and prostate (32). These two isoforms have an intranuclear localization. Few membrane-associated subtypes of the receptor have been reported lately. An intracellular transmembrane estrogen receptor, GPR30, was reported to mediate rapid non-genomic signaling events (33). Also, ER-X is expressed in brain and mediates the influences of estrogen on neuronal differentiation, survival, and plasticity using MAPK pathway (34).

1.2.4.1 Tissue Distribution

The tissue distribution of ER alpha and ER beta transcripts is fairly different, suggesting that the two receptor subtypes have distinct functions in different tissues. ER alpha is the predominant subtype expressed in the breast, uterus, cervix, and vagina. ER beta is important in

other tissues such as the central nervous system, bone, lung, cardiovascular system, ovary, testis, kidney and colon. There are synthetic ER ligands that can act in a tissue-specific manner. These compounds interact differently with the two isoforms of the receptor and consequently induce recruitment of a particular set coregulators as well as binding to different DNA response elements. Some tissues express both subtypes of the receptor. When these tissues are exposed to a particular ER ligand, the effect of the ligand depends on the ER alpha to ER beta ratio (35).

1.2.4.2 Genes Regulated by ER alpha and ER beta

Estrogen regulates an important number of genes involved in cell proliferation, apoptosis, stimulation of invasion and metastasis, and angiogenesis (13).

It was demonstrated that most genes regulated by ER alpha are distinct from those regulated by ER beta in response to E_2 and SERMs. For example, E2 stimulated ER alpha regulates genes involved in cell proliferation and signal transduction, while ER beta regulates genes that function as tumor suppressors. These results indicate that estrogens and SERMs exert tissue-specific effects by regulating unique sets of targets genes through ER alpha as well as through ER beta (36).

In order to develop more tissue-selective and safer drugs for menopausal symptoms and estrogen dependent cancers it is important to understand how ER alpha and ER beta regulate different genes in response to various ligands.

1.2.4.3 Selective Ligands for ER alpha or ER beta

Although the two ER subtypes are both activated by binding 17β-estradiol, the ligand binding domains (LBD) have only 56% amino acid identity (37). If the ligand has a 1000- fold excess affinity for one receptor over another, this may provide therapeutic selectivity. Agonist ligands at ER alpha and ER beta may have selectivity of action based on the perturbation of their respective complexes. ER beta has an impaired AF-1 domain compared with ER alpha and the necessary synergy with AF-2 is dramatically reduced. These differences suggest that it is possible to develop ligands with different affinities, potencies, and agonist vs antagonist behavior for the two ER subtypes. In fact there are some known ligands with subtype-selective affinities and a degree of subtype selective agonist/antagonist character. In most cases where this selectivity is high, it favors ER alpha.

ER alpha selective ligands:

Katzenellenbogen and co-workers (38) have synthesized ER subtype-specific ligands: triaryl-substituted pyrazoles. The most ER alpha selective ligand, pyrazole, had 120-fold higher agonist potency for ER alpha than for ER beta. Another selective ligand, a cis-diethyl-substituted tetrahydrochrysene and shows full ER alpha agonism but pure ER beta antagonism (39). This compound differs from antiestrogenic compounds such as Tamoxifen and Raloxifene, which are full antagonists on ER beta but have negligible agonist activity on ER alpha.

Tetrasubstituted propylpyrazole (PPT) has a 410-fold binding selectivity for ER alpha and is an ER alpha-specific agonist, as it activates gene transcription only through ER alpha (40).

HPTE (2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane) behaves as an ER alpha agonist and an ER beta antagonist (41) in human hepatoma cells (HepG2) and HeLa cells.

ER beta selective ligands:

Ligands that are ERß selective are also known. Some phytoestrogens such as genistein and coumestrol activate both ER isoforms but they preferentially bind ER beta (32). DPN (2,3-bis(4-hydroxyphenyl) propionitrile) acts as an agonist on both ER subtypes, but has a 70-fold higher relative binding affinity and 170-fold higher relative potency in transcription assays with ER beta than with ER alpha (42).

1.3 DRUGS THAT TARGET THE ESTROGEN RECEPTOR

There are two sources of estrogen in estrogen-dependent diseases. One is secretion by the ovaries and the other one is biosynthesis in local tissue catalyzed by enzymes such as aromatase. This enzyme catalyzes the conversion of testosterone to estradiol. Aromatase is located in estrogen-producing cells in the adrenal glands, ovaries, placenta, testicles, adipose tissue, and brain. A strategy to block estrogen signaling involves systemic inhibition of estrogen biosynthesis through the use of aromatase inhibitors. These compounds have limitations also, the most important being the long-term impact of estrogen deprivation (osteoporosis, hypersensitivity to low levels of estrogen) (43).

Another strategy to treat estrogen-dependent cancers is to block the activity of the estrogen receptor using specific ligands. Endocrine therapy uses selective estrogen receptor modulators (SERMs), such as Tamoxifen, for treatment and prevention of breast cancer (44). Although Tamoxifen is widely used and highly effective, a considerable percent of the patients

fail to respond to treatment or acquire resistance in time. Tamoxifen has adverse side effects, mainly due to its partial agonist action in endometrium.

Besides Tamoxifen, second, third and fourth generations of SERMs are now available (**Fig. 2**). Second-generation SERM, Raloxifene is an antiestrogen in breast, but unlike tamoxifen, it has significantly reduced uterotropic activity and prevents hip fractures (45). Yet Raloxifen has several undesirable effects, including hot flushes and venous thrombosis (46). The risk benefit ratio has yet to be clinically determined for a third generation of SERMs (lasofoxifene and basofoxifene). Arzoxifene (third generation SERM) and Acolbifene (fourth generation SERM) are both more potent than Tamoxifen and they are active in metastatic breast cancer (47). Acolbifene, but not Arzoxifene, inhibited growth of Tamoxifen resistant breast cancer cell lines. Nevertheless, these drugs induce hot flashes, fatigue and muscle or bone pain.

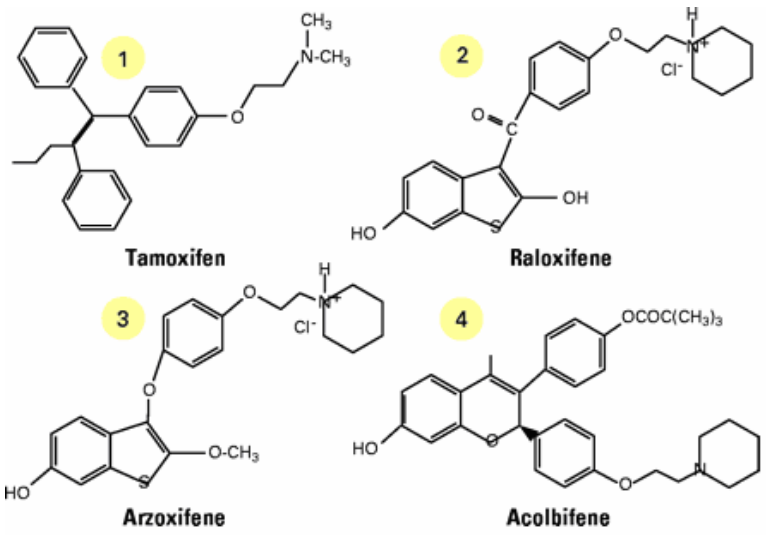


Figure 2 – *Representative chemical structures of the fourth classes of selective estrogen receptor modulators (SERMs)(3)*

Although the current classes of drugs have had a positive impact on the morbidity and mortality associated with breast cancer, they have important disadvantages. Considering that SERMs are to be used in healthy women for breast cancer prevention or to treat menopausal symptoms, it is crucial to develop more favorable drugs.

More selective drugs will potentially have improved therapeutic utility. Since the two isoforms of the ER have different tissue distribution, ER antagonists that selectively inhibit only one isoform of the receptor would have fewer side effects. An ideal SERM would block the estrogen effects in reproductive tissues while retaining the positive estrogenic character in non-reproductive tissues such as bone, liver, and the cardiovascular system.

The benefits of finding selective compounds are numerous. One would be finding drugs that target only a specific pathway involved in disease progression. The ability of the DNA-bound receptor to modulate transcription is dependent on coactivator/corepressor expression. The nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is overexpressed in breast cancer (27). Since ER alpha is the predominant isoform of the receptor in breast tissue, a compound that will specifically inhibit ER alpha – AIB1 modulated transcription may be used to selectively target this pathway in breast cancer while leaving the other ER pathways operative. Another possibility would be to combine two selective drugs. It was shown that when ERβ is coexpressed with ERα in breast cancer, the tumor sensitivity to 4-hydroxytamoxifen is increased and the overall prognostic of the disease is better than in patients with low or no expression of ERβ (48). Also, the two isoforms of the receptor play different roles in mammary epithelial cells. ERα induces proliferation while ERβ has a role in apoptosis (49). So, a combination of two compounds (a selective ERα antagonist and a selective ERβ agonist) will be more potent in breast cancer treatment.

2.0 NEW SCREENING ASSAY

2.1 RATIONALE FOR THE PROJECT

The intense research in the breast cancer treatment and prevention field produced a number of estrogen related compounds that have shown great therapeutic promise. There has been much effort put into the design and synthesis of libraries of compounds that target the estrogen receptor (50, 51). The goal is to find compounds that block the effects of estrogen in some tissues and conserve the positive estrogenic character in others. So far, there is no rapid screening assay to test these compounds. The screening assays used to date to test compounds that bind ER have serious limitations.

I propose here the development and validation of a rapid screening assay to follow ER function in vivo. This assay is based on use of ERE (estrogen response elements) to drive fluorescence that can be visualized directly in living cells. Additionally, no exogenous substance is necessary to detect fluorescence, so the determinations will have less variability. Reporter gene and traditional biological assays require cell lysis to measure transcription. A benefit of this system is that the cells do not have to be lysed to measure fluorescence, so they can be studied easily at different time points. Using this system, I will be able to very quickly screen a large number of compounds under the same conditions and with a functional readout. The screening can be done simultaneously for ER alpha and for ER beta.

2.2 MATERIALS AND METHODS

Materials:

17β-Estradiol (E_2) was purchased from Sigma-Aldrich (St. Louis, MO). They were prepared as 1 x 10^{-2} M stocks in 100% ethanol and stored at -20°C.

CMV-ERα contains the full-length coding sequence of human ERα (595 amino acids). ERE-tk-luciferase contains a single ERE cloned upstream of the thymidine kinase promoter and the luciferase gene (gift of Don DeFranco, Dept. of Pharmacology, University of Pittsburgh).

The pcDNA5/FRT encoding the Hygromycin resistant gene was purchased from Invitrogen.

The pEGFP-N1 and pDsRed2-N1 plasmids are commercially available (Clontech).

DMEM (Dulbecco's modified Eagle's medium with 4.5 g/L-glucose) was purchased from Mediatech, Herndon, VA and supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA).

Construction of ERE-GFP reporter gene:

The Luciferase reporter gene was excised from the plasmid ERE-tk-Luc by restriction digest with the enzymes StyI and XhoI. An AflII-XhoI fragment containing the coding region of EGFP gene was excised from pEGFP-N1 (Clontech) and ligated into digested ERE plasmid to generate ERE-GFP. A 1500 bp PvuII-AseI fragment was containing ERE-GFP clone was then inserted into the pcDNA5/FRT (Invitrogen) at the multiple cloning site. This makes the ERE-

GFP clone suitable for stable transfections, since pcDNA5/FRT contains a Hygromicin resistant gene. The resulting ERE-GFP plasmid contains a copy of ERE sequence a TATA box and a tk promoter upstream from the GFP gene.

Construction of ERE-DsRed reporter gene:

The Luciferase reporter gene was excised from the plasmid ERE-tk-Luc by restriction digest with the enzymes StyI and XhoI. An AflII-XhoI fragment containing the coding region of DsRed gene was excised from pDsRed2 (Clontech) and ligated into digested ERE plasmid to generate ERE-DsRed. The resulting ERE-DsRed plasmid contains a copy of ERE sequence a TATA box and a tk promoter upstream from the DsRed gene.

Construction of ER alpha/Neomycin gene:

An EcoRI-EcoRV fragment containing the coding region of ER alpha gene was excised from CMV-ERalpha plasmid and ligated into pcDNA3.1 plasmid to generate ER alpha/Neomycin plasmid. This makes the plasmid suitable for stable transfections, since pcDNA3.1 contains a Neomycin resistant gene.

Cell culture and transfections:

CV-1 cells (African green monkey kidney cells) were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L-glucose (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 atmosphere. All cultures were in a passaged every 3-4 days. For transient transfection assays, cells were plated in 10mm dishes at a density of $1X10^6$ cells per well in phenol red-free Dulbecco's modified Eagle's medium (HyClone) containing 10% charcoal-dextran-stripped fetal bovine serum (HyClone). CV-1 cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the

manufacturer's protocol. Transfections contained 2 μg of reporter plasmid and 2 μg of ERα expression vector. The transfection complexes were incubated with cells for 5 h and then DMEM media added to achieve a final concentration of 10% CSS. After 16 h, the medium was replaced, and the cells were treated with vehicle or ligand for an additional 24 h. (CSS). Transfection efficiency has been estimated to be 10-20%. The amount of DNA per sample was kept constant in all experiments. The transfection complexes were incubated with cells for 5 h and then DMEM media added to achieve a final concentration of 10% CSS.

Generate ER alpha/Neomycin stable cell line:

For stable transfections, 70% confluent CV-I cells were transfected by Lipofectamine following the protocol provided by the supplier (Life Technologies, Rockville, MD). For transfection, 5×10^6 cells were suspended in 3 ml of Opti-MEM I (Life Technologies) containing 40 μg of ER alpha/Neomycin plasmid in a 100 mm tissue culture dish. After transfection, cells were plated in complete medium (DMEM supplemented with 10% FBS) for 48 hr, and then selected in media containing 400 μ/ml G418. Several individual clones were isolated and expanded.

2.3 RESULTS

2.3.1 Principle of the Screening Assay

The new screening assay will follow the ER-mediated transcription in living cells. An ERE promoter element in front of a fluorescent protein reporter gene will be used to evaluate estrogenic transcriptional activity, driven by test compounds. The consensus ERE

(AGGTCAnnnTGACCT) in front of the tk promoter will control the expression of the fluorescent protein. The amount fluorescence visible in living cells should be proportional to the amount of transcription through ER bound to the ERE enhancer element.

2.3.2 The Screening Assay Development

In order to develop a new assay, I constructed reporter plasmids that have an ERE element, upstream of thymidine kinase (tk) promoter controlling a GFP or a DsRed reporter gene. Stable CV-1 transfectants can be developed in which a single copy of the ERE-DsRed reporter and ER alpha is integrated. To allow identification of subtype selection, I also constructed a second reporter plasmid that has an ERE element upstream of thymidine kinase (tk) promoter controlling a GFP (green fluorescent protein) reporter gene. This reporter system and ER beta can be stably integrated in CV-1 cells **(CV-1B).** In this case, any compound that binds ERβ and folds it for coactivator interaction will activate the transcription from the ERE enhancer, making GFP and green cells. The key experiments can combine all four of these elements as stably integrated DNA: the ERE-driven GFP reporter, the ERE-driven DsRed2 reporter, the wild-type ERα that binds EREs in **CV-1A** and the wild-type ERβ that binds ERE elements in **CV-1B** cells. ERα and ERβ activity will therefore be assayed simultaneous in living cells using the two cell lines**: CV-1A** and **CV-1B.**

A summary of expected results is presented in **Table 1**.

Table 1- Screening assay for selective ER compounds - Expected results

Cells /Treatment	CV-1B cells (CV-1 cells + ERE-GFP+ERβ)	CV-1A cells (CV-1 cells + ERE-RFP+ERα)
Estradiol	Green	Red
4 OH-Tamoxifen	Some green	Some red
ICI 182,780	No green	No red
Ethanol (control)	No green	No red
Selective ERα antagonist	No green	Some Red
Selective ERβ antagonist	Some Green	No red

2.3.3 Characterization and Optimization of the Assay

Transient transfections in CV-1 cells in the presence of known ER ligands were used to test ERE-DsRed and ERE-GFP plasmids. The best results were attained using ERE-GFP in combination with ER beta and ERE-DsRed in combination with ER alpha. This is probably due to the fact that ER alpha is more potent than ER beta. In addition, GFP is brighter than DsRed in

the same conditions. As expected, the amount of red/green fluorescence, visible in living cells, was dependent on known ligand capacity to modulate transcription **(Fig. 3 and 4)**. A robust signal resulted in the presence of estradiol, while with a pure antiestrogen such as ICI 182,780 (52) resulted in very little red/green fluorescence. The vehicle control (ethanol) also elicited very little response (fluorescence).

A CMV-ERα plasmid has been stably integrated in CV-1 cells using G418 as a selectable marker. Western blots will be used to confirm that ER alpha is indeed express by these cells. To test ER alpha activity in these cells, the G418 resistant colonies will be transiently transfected with ERE-tk-LUC plasmid and treated with different ligands.

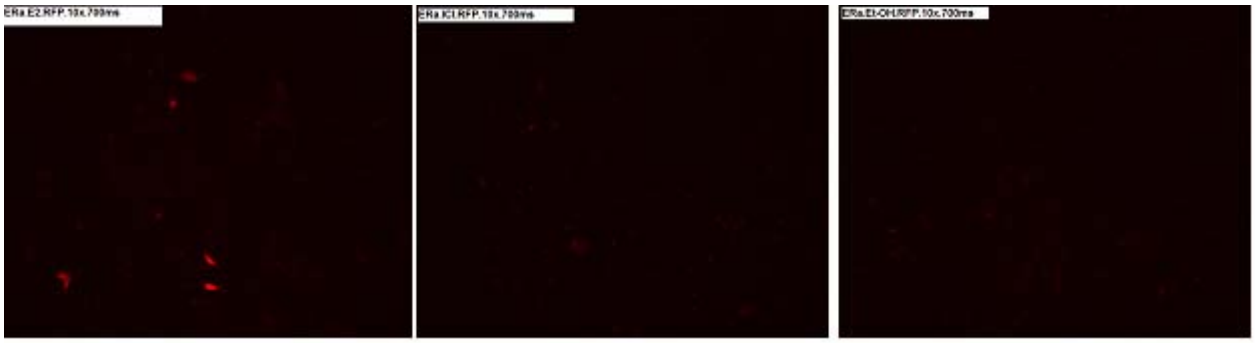


Figure 3 - *The ERE-DsRed plasmid is under tight estrogenic control.*

CV1 cells were co-transfected with CMV-ERα (2µg) and ERE-tk-DsRed (2µg). After 24h, E2 (a) (agonist), ICI 182 (b) (antagonist) or the vehicle control ethanol (c) were added. The final concentrations of each compound were (1×10^{-7}M). The pictures are taken after additional 48h. The exposure time is the same for all pictures (700ms).

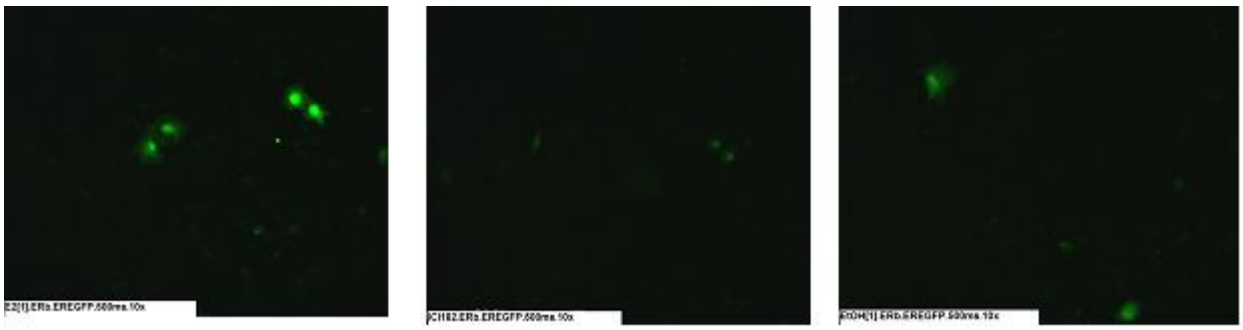


Figure 4 - *The ERE-GFP plasmid is under tight estrogenic control*

CV1 cells were co-transfected with CMV-ERβ (2µg) and ERE-tk-GFP (2µg). After 24h, E2 (a) (agonist), ICI 182 (b) (antagonist) or the vehicle control ethanol (c) were added. The final concentrations of each compound were (1×10^{-7}M). The pictures are taken after additional 48h. The exposure time is the same for all pictures (500ms).

ERE-tk-DsRed reporter plasmid will also be stably integrated in the same cells (CV-1A). Another cell line (CV-1B) should have CMV-ERβ plasmid and ERE-GFP reporter stably integrated. The exact level of transcription induced by compounds that look more promising can be quantified using Luciferase reporter gene construct, such as ERE-tk-Luc.

Once the whole system is set up and validated with known compounds, it can be used to determine the estrogenic character of new SERMs. Some of the compounds that can be tested are DTACs (1,1-dichloro-2,2,3-triarylcycloprpanes) (**Fig. 5**), which have antiestrogenic character. They block uterine growth in mice and also block growth of MCF-7 estrogen-dependent breast cancer cells (51).

Our group previously demonstrated that the novel class of compounds 1,1-dichloro-2,2,3-triarylcyclopropanes (DTACs) function as anti-estrogens and block ERα-mediated transcription from an ERE-driven reporter construct and estrogen-mediated stimulation of endogenous target genes c-myc and cyclin D1 in breast cancer cells. CV-1 cells were cotransfected with CMV-ERα, CMV-β-galactosidase, and ERE-tk-luciferase and treated with different ligands.

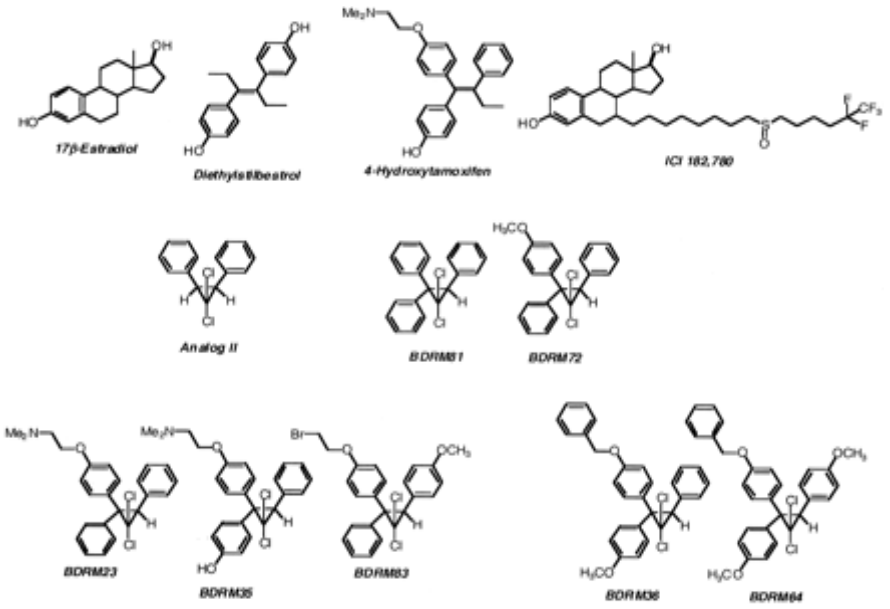
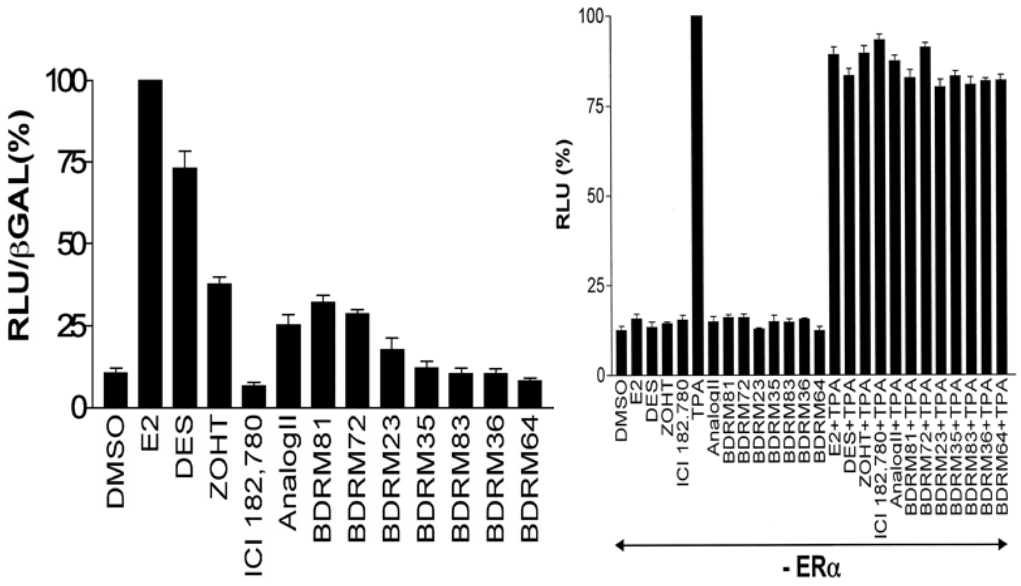


Figure 5 – *DTAC chemical structures.*

First line of this panel shows well characterized ER ligands, such as estradiol (the natural ligand of ER), diethystilbestrol (synthetic agonist), 4-hydroxytamoxifen (the active metabolite of Tamoxifen – a partial agonist) and ICI 182,780, a pure antiestrogen. Second and third lines present various DTACs chemical structures starting with the parent compound, Analog II(53).

Results presented in **Fig. 6a** show that DTACs do not stimulate ER alpha transcription from an ERE site. To show that these compounds are not general transcription inhibitors, their ability to modulate transcription from an AP-1 DNA binding element was analyzed (**Fig. 6b**). TPA (12-O-tetradecanoylphorbol-13-acetate) was used to activate signal transduction in CV-1 cells (ER alpha negative) transfected with AP-1 –Luciferase plasmid. DTAC compounds, as well as other estrogenic or antiestrogenic compounds, had no effect on TPA stimulated transcription. This suggests that DTACs are not general transcription inhibitors (53).



a b

Figure 6 - *DTACs inhibit ER alpha mediated transcription but they are not general transcription inhibitors(53).*

(a)CV-1 cells were cotransfected with CMV-ERα, CMV-β-galactosidase, and ERE-tk-Luciferase and treated with known compounds (1 x 10^{-8} M) or with DTACs (1 x 10^{-5} M).

(b) CV-1 cells were transfected with AP-1-tk-luciferase. The following day, cells were treated as indicated. The activity of Luciferase was measured for both experiments after 24 hours of treatment.

Because of the potential differences in ERα and ERβ activity, a series of studies was conducted to evaluate the behavior of DTAC compounds in the presence of ERβ. The ability of DTACs to modulate ERβ-mediated transcription from estrogen response elements (EREs) (**Fig. 7**) was evaluated.

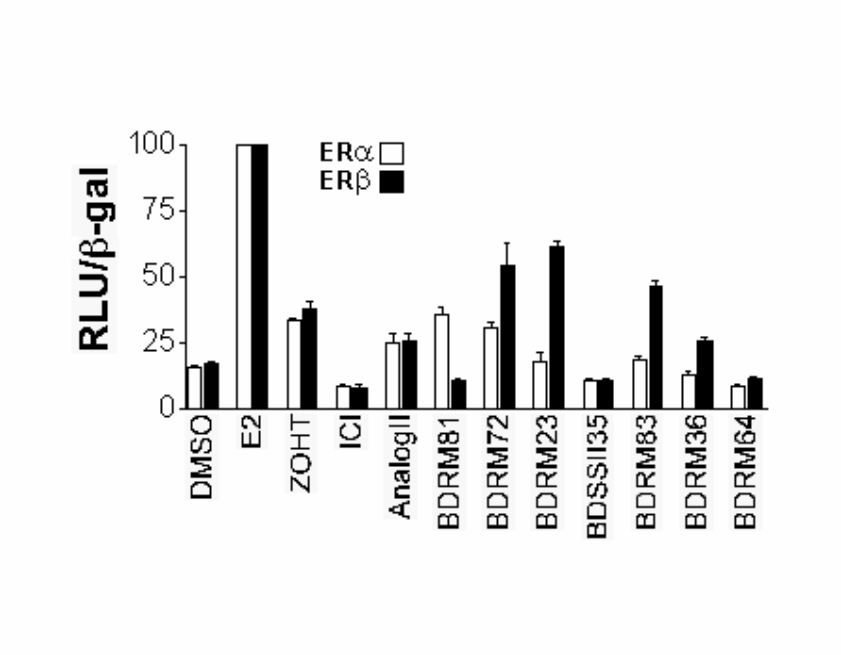


Figure 7 - *The ability of DTACs to modulate ER-mediated transcription from estrogen response elements (EREs)(53)*

CV1 cells were co-transfected with CMV-ERα or CMV-ER β, CMV-β-galactosidase, and ERE-tk-luciferase. After 16h, E2, DES, ZOHT or the DTAC compounds were added. The final concentrations of each compound were: E2, DES, ZOHT, ICI (1×10^{-8}M), Analog II and DTACs (1×10^{-5}M). After an additional 24h, the cells were harvested and luciferase and β-galactosidase activity measured. To control for transfection efficiency, luciferase activity was normalized to β-galactosidase activity. Data are expressed relative to the activity of E2, which was set at 100%. Bars represent the mean ± SD of at least 3 independent experiments.

In the presence of ERβ at EREs, BDRM72, BDRM23, and BDRM83 displayed agonist activity that was equal to or greater than that observed for the well-characterized partial agonist, ZOHT (**Fig. 7**). These results contrast with those obtained with ERα, wherein BDRM81 and BDRM72 but not BDRM23 or BDRM83 displayed agonist activity. Taken together, these data indicate that the structural determinants of DTAC stimulation of ER-mediated transcription are different for ERα and ERβ (53) .

Additional controls can be added in order to measure the background fluorescence. For this purpose we can use plasmids similar with ERE-GFP and ERE-DsRed but lacking the ERE site. Other controls should include experiments in the presence of SERMs solvents, such as DMSO. Also background fluorescence should be determined by measuring fluorescence in the absence of any ligand.

In order to optimize the assay, a time course experiment can be used to find out when in time the difference between agonists and antagonists is reflected best in the amount of fluorescence visible. The ligand bound receptor takes minutes to bind to DNA and activate gene transcription (54). Fluorescence develops within 24 hours of transfection using pEGFP-N1 or pDsRed2-N1 and increases in intensity in the next 2-3 days. The **CV-1A** and **CV-1B** cells will be treated with estradiol or with ICI 182,780 and the fluorescence will be measured every 12 hours. The last time point will be after 120 hours when the original florescent plasmids were show to have maximum fluorescence intensity (Clontech).

Another optimization for a more uniform fluorescent signal would be to synchronize the cells growth. A regular batch of cultured cell grows randomly and each individual cell is asynchronous with the others. For the purpose of this assay it would be more useful to have the cells going through the cell cycle synchronously, and consequently synthesizing the fluorescent protein in the same time. Synchrony can be induced by hot-cold or light-dark intervals.

2.3.4 Potential Pitfalls and Alternative Approaches

The sequential stable integration of two different plasmids (ER and ERE-fluorescent protein) in the same cells might be a difficult task. Alternatively we could construct a single plasmid that would contain both genes (ER and ERE- fluorescent protein). In this case we will

have to stably integrate only one plasmid (ER alpha/ERE-DsRed) in CV-1 cells to generate CV-1A cells and ER beta/ERE-GFP plasmid to generate CV-1B. One concern is that such a plasmid is very large (approximately 10kb). That size would be very difficult to transfect. Another concern is that ER expression is driven by a CMV promoter, which is a very strong promoter. That can impair expression of DsRed, respectively GFP (driven by ERE-tk promoter which is weaker) if they are both in the same plasmid.

In case the amount of fluorescent protein expression is not enough to generate a detectable fluorescent signal, multiple estrogen-responsive elements can be used to generate (ERE)n – GFP/DsRed clones. Multiple EREs upstream of the promoter will yield a stronger signal that a single ERE copy (55). Also, fluorescent clones that have a nuclear localization signal (NSL) tag might be useful. NLS-tagged ERE-GFP/DsRed clones would generate a stronger nuclear signal and less, if any, cytoplasmic background fluorescence.

3.0 DISCUSSION

Selective estrogen receptor modulators (SERMs) show great promise in treatment of cancer, osteoporosis and endocrine disorders. Tamoxifen, one of the first characterized SERMs, has been successfully used in breast cancer therapy (56, 57). Although many patients benefit from currently available SERMs, these drugs have major limitations. There is a substantial effort put into developing new, more efficient SERMs (50, 51). Many new compounds derived from this intensive search and they need to be analyzed fast but also in a comprehensive manner.

I proposed here a new screening assay that can be used to sort through SERMs. The newly synthesized compounds can be screened for estrogenic/antiestrogenic activity at the level of gene transcription. The principle of the screening assay is the following: an ERE promoter is cloned in front of a fluorescent protein gene and controls the protein expression. So, the fluorescent visible in cells should be proportional to the ER ligand capacity to modulate transcription through ERE. This is a noninvasive assay, unlike the conventional reporter gene assays, that can be used to study the classical estrogen receptor-mediated gene activation. This technique has various applications. Besides compound screening, the assay can be further expanded to analyze promoters and cofactors importance, and to analyze the biological function of estrogen receptor mutants. I have been able to construct an important part of the materials needed for the assay. The results presented here predict that the assay proposed is going to work

as anticipated: the fluorescence visible in living cells is proportional to the ability of the ligands to modulate ER transcription through ERE.

A number of in vitro and in vivo assay were used so far in order to characterize compounds designed to be estrogen receptor ligands. The ligand binding assay measures only the extent of the binding affinity between ligand and receptor. This kind of assay is an in vitro assay and offers limited information about the ligand character. There is no information about the agonist/antagonist activity, the effect on gene transcription or about the toxic effects on a living cell. Other assays, such as the cell proliferation assays or rodent uterotrophic assays (58), are performed in vivo but they lack specificity (ER alpha versus ER beta specific ligands) and do not provide any direct information on transcription. The reporter gene assays, such as the Luciferase assays, require time and they have a lot of variability. Other assay use truncated forms of the receptor. For example the LBD-flanked by the FRET donor fluorophore and the acceptor fluorophore is used to predict the agonist/antagonist character of the ligands, but not the full length of the receptor (59).

The new assay proposed here provides many advantages over conventional reporter genes for the study of transcription factor action. It is an in vivo assay that can screen fast a big number of compounds in the same conditions and with a functional readout. Using this assay we can specifically test the effect of the ligands on the ERE-mediated transcription, it will not test just the ligand-receptor interaction or the proliferation of the cell in the presence of various ligands. This assay offers information about ligand selectivity for ER alpha or ER beta. In addition, fluorescent reporters can measure gene expression of a single living cell in real-time.

I presented here the assembly of two clones, ERE-GFP and ERE-DsRed. In order to test the functionality of these clones I performed transient transfections in CV-1 cells. These cells are

one of the most suitable ones for the purpose of this screening assay. They exhibit no endogenous estrogen receptors or any growth responses to estrogens or antiestrogens. Therefore it is possible to generate cell lines that contain only one isoform of the receptor. Additionally, stable cell lines should have similar levels of ER expression in all cells. CV-1 cells grow well in charcoal stripped serum, unlike other type of cells frequently used to study ER ligands (such as MCF-7 cells). These cells have been used for a number of studies of steroid receptors and they respond accurately with exogenously added receptors and receptor constructs. Also, CV-1 cells are easy to transfect, available, inexpensive and suitable for stable transfections.

The clones were introduced in CV-1 cells, together with ER, using transient transfection in order to test whether they are under tight estrogenic control. The cells were further treated with know ER ligands. The results (Fig. 2 and Fig. 3) show that in the presence of full agonist estradiol ER mediated transcription is stimulated through ERE pathway. The amount of fluorescence visible in the cells treated with full antagonist ICI 182 is very low, similar to the one obtained with the vehicle control ethanol. ERE-GFP and ERE-DsRed clones function under tight estrogenic control in the presence of estrogen receptor. These results predict that the clones function as expected and they can be used to develop the SERM screening assay.

One limitation of this assay system is that it investigated only the transcription through ER bound to the ERE enhancer element. ERs have also been shown to modulate gene expression at alternative regulatory DNA sequences such as AP-1 (9), SP-1 (10). Alternative ER signaling through AP-1 sites is of great interest since it was suggested that it is involved in the onset of Tamoxifen therapy resistance in breast cancer (11). An assay that would have stable cell lines that express ER alpha and AP-1-DsRed, respectively ER beta and AP-1-GFP would give

information about this alternative ER signaling pathway. Also, this assay does not address the non-genomic effects of the estrogen receptor (60).

There are other limitations of this assay. One of them derives from the fact that stably transfected cells are going to be used. Transfection results in an overexpression of the gene of interest. Consequently, the transfected cells might have modified phenotype and modified gene function compared to the real gene. Other limitations of the screening assay are limitations common to all cell-based assays. One would be that it takes a long time to develop and optimize the assay. The standardization of the cell culture conditions, the stability of the recorded signal and the sensitivity of detection are other challenges of the cell-based assays. This type of assays has some variability so a big number of cells need to be analyzed to get convincing results. Also, these assays generate a considerable amount of data that needs to be analyzed. The statistical methods used to interpret the data are not trivial and have limitations.

No matter how complex is a cell based assay it can not predict all the effects of a drug on an organism.

I presented here the first step in developing the screening assay, generating and evaluating the fluorescent clones. Further, these clones can be stably integrated in CV-1 cells together with the estrogen receptor. Stable CV-1 transfectants can be developed so that they have a single copy of the ERE-DsRed reporter and ER alpha is integrated (**CV-1A**). The ERE- GFP reporter system and ER beta can be stably integrated in CV-1 cells **(CV-1B)** to allow identification of ER subtype selection**.** ERα and ERβ activity will therefore be assayed simultaneous in living cells using the two cell lines**:** **CV-1A** and **CV-1B. CV-1A** cells will be treated with known ER ligands in order to validate this reporter system. The amount of red fluorescence visible in living cells should be dependent on known ligand capacity to modulate

ER alpha regulated transcription. After the initial screening using DsRed (red fluorescent protein) as a reporter gene, the transcription can be accurately quantified using an ERE-tk-Luc. **CV-1B** cells will be also tested using know compounds as described above. These assays can be performed on a Cellomics ArrayScan multiparameter fluorescence imaging instrument for high-content screening (HCS).

The high content screening generates a huge amount of data in the form of photographic images of cells. The images captured from each well will allow comparison between fluorescence yielded by unknown compounds and known standards. These experiments have variables (plating density, cell number per field, number of fields scanned per well) that can modify fluorescence independent of ligand action. A statistic test, such as Kolmogorov-Smirnoff (KMG) statistic described by Smellie A. and col.(61), will be used measures the difference between fluorescence distributions. The corrected Kolmogorov score (S) will be used for pairwise comparison.

$$S(W,D,P) = \left(\frac{\sum_{i=1}^{N_D} \mathrm{KMG}_p(W,D_i)}{N_D} \right) - B_C$$

A value of 0 for this score means the distributions of the values are identical and a value of 1 means that the distributions are nonoverlapping and distinct. This will compare the distribution of values derived from the images for a row of wells (W) with those of another row of wells (D). In the presented formula S (W,D, P) is the corrected Kolmogorov of the Wth test well, when compared against the set of negative controls wells D using parameter P, W is the

well being compared, KMGp (W,Di) is the Kolomogorov-Smirnoff statistic computed using the distribution of parameter P values in the well test W and the ith negative control well Di, P is the parameter used for computation (in this case average fluorescence inside the nucleus if we consider that there are 4 images generated for each well), Di is the ith negative control well on the plate, N_D is the number of control wells per plate, and BC is the background KMG value defined as the mean KMG distance between all pairs of negative control wells. The background for these experiments will be the fluorescence yielded by the cells without any treatment. This corrected score measures the average KMG distance between a test compound and all negative control wells on the plate, with a correction for the background distance between control wells. The cutoff for the corrected Kolmogorov score is typically between 0.02 and 0.1. S values in this interval indicate that the experiment is reproducible and that the fluorescence varies because of the ligand not because of the way the experiment is setup.

Further we can compare the values of the fluorescence generated by a test compound with the one generated by a control compound. The goal of the screening is to identify compounds that specifically inhibit transcription / fluorescence. To find compounds that inhibit fluorescence similar to compound ICI 182,780 we will calculate confidence interval (95%) around the mean fluorescence of ICI 182,780 using the following formula:

$$\text{Mean} \pm 1.96 \times SD / \sqrt{n}$$

We consider "hits" the compounds that provide a mean fluorescence within the confidence interval.

The new SERMs identified using this assay can be used eventually in therapy of breast or lung cancers or as hormone replacement. In addition, compounds that differentiate ERα and ERβ will be valuable tools to further dissect ER signaling pathways. It is important to know more

about coactivator recruitment, gene expression profile or about the response with ER mutations. This will lead to a better understanding of estrogen related cancers and will help designing new therapeutic approaches.

The steroid receptors are a large class of ligand activated transcription factors. Steroids imbalance or disruption of the pathways involved in hormone action lead to diseases like breast and ovarian cancer, prostate cancer and osteoporosis. The steroid receptors role in many aspects of cell growth and metabolism makes them important targets in drug discovery. A fast and accurate screening assay can optimize the drug discovery process. Many compounds can be screened in the same time reducing the time required to develop new, more selective drugs. The assay proposed here satisfies not only these requirements but, with few modifications, can be used to understand better the estrogen receptor mechanism of action. Once developed and validated this assay can be expanded to study other steroid receptors (such as progesterone receptor, androgen receptor, mineralocorticoid receptor or glucocorticoid receptor) and to screen their ligands. Besides drug screening and mechanistic studies, this assay can be used to test manmade or environmental chemicals found in soil, water and food for their potential effect as endocrine disruptors.

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