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Award Number: W81XWH-05-1-0243

TITLE: Estrogen-Related Receptor alpha (ERR (alpha))-Coactivator Interactions
as Targets for Discovery of New Anti-Breast Cancer Therapeutics

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REPORT DATE: March 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY) 01-03-2007		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 Feb 06 – 14 Feb 07	
4. TITLE AND SUBTITLE Estrogen-Related Receptor alpha (ERR (alpha))-Coactivator Interactions as Targets for Discovery of New Anti-Breast Cancer Therapeutics			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-05-1-0243		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Richard R. Burgess, Ph.D. E-Mail: rburgess@wisc.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin Madison, WI 53706-1490			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES - Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT: The steroid nuclear receptor estrogen receptor alpha (ERα) is the primary target of current breast cancer therapies, which block ERα activation or estrogen synthesis. Estrogen-related receptor alpha (ERRα), a protein with high sequence similarity to ERα, has functional similarity to ERα in certain breast cancer cell types; though unlike ERα, ERRα acts independently of steroid ligand. We hypothesize that its activity may be due in part to its interaction with coactivator proteins, and in some instances, ERRα may be functionally substituting for ERα and playing an important role in the progression of a subset of breast cancers. We found that coactivator recruitment is necessary for full ERRα activation of transcription; furthermore, GRIP1 (SRC-2, TIF-2) is cell type- and promoter- specific in its coactivation of transcription with a constitutively active form of ERRα. We used this information to develop a cell-based, high-throughput screen for small molecules that inhibit the transcription of an estrogen response element (ERE)-containing luciferase reporter gene in the presence of overexpressed GRIP1 and "activated" ERRα. This screen, performed in MCF-7 cells in 96-well plates, used a 4,160 "known bioactives" compound library. Recently, we were able to improve the cell-based screen by converting to batch transfection of cells and to a 384-well plate format, which allowed us to use 75% less cells and chemical per well, thereby making the assay much more efficient, reproducible, and adaptable to larger chemical libraries. We also have developed four cell-based, high-throughput counter screens to validate "hits" from primary screens by ruling out compounds that are toxic to cells or that generally inhibit transcription or translation. Lastly, we are generating a targeted, high-throughput screen to determine the effects of remaining "hits" on inhibition of the GRIP1-ERRα interaction in vitro. We will characterize the in vivo and in vitro "hits" by dose response curves and by the activity of closely-related compounds. We believe that the GRIP1-ERRα interaction is a promising target for new breast cancer therapies.					
15. SUBJECT TERMS Estrogen-related receptor alpha (ERRα), glucocorticoid receptor interacting protein (GRIP1), protein-protein interactions, Luminescence Resonance Energy Transfer (LRET), cell-based high-throughput screening					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Breast cancer is the second leading cause of death among women in the United States. Estrogen receptors (ER α , ER β) are a primary focus of the etiology and therapy of breast cancers. ER α -positive breast cancers have a better prognosis and have traditionally been treated with tamoxifen, a selective estrogen receptor modulator (SERM), or inhibitors of aromatase, an enzyme involved in estrogen synthesis. ER α -negative breast cancers do not respond to hormone therapy, are more aggressive, and function through much less well understood mechanisms. Estrogen-related receptor alpha (ERR α), a protein with high sequence similarity to ER α , has functional similarity to ER α in certain breast cancer cell types (Ariazi *et al.*, 2007; Kraus *et al.*, 2002). Unlike ER α , ERR α acts independently of steroid ligand; bulky amino acid side chains in ERR α 's putative ligand-binding pocket, including Phe329, recapitulate interactions analogous to ones classically provided by ligands in other nuclear receptors, thereby promoting binding of coactivator proteins (Chen *et al.*, 2001). In cell types in which ERR α is present but ER α is either absent or inactive, ERR α may functionally substitute for ER α and play an important role in the progression of a subset of breast cancers. Because ERR α 's activity is due in part to its direct interaction with coactivator proteins, we believe that this protein-protein interaction is a promising target for new chemotherapeutic drug development.

BODY (RESEARCH ACCOMPLISHMENTS)

The three tasks of our approved Statement of Work were the following:

Task 1 - Determine ERR α -associated proteins (purify ERR α -associated proteins using gentle immunoaffinity chromatography and identify the proteins using MALDI-TOF mass spectrometry);

Task 2 - Study the binding properties of ERR α and a coactivator; and

Task 3 - Develop a high-throughput screen (HTS) for molecules that interfere with the binding of ERR α and a coactivator.

Recent discoveries made as part of a collaboration between our laboratory and the Mertz laboratory have identified a constitutively-activated form of ERR α and a clearly relevant coactivator (glucocorticoid receptor interacting protein 1, GRIP1). As a result we have chosen to bypass Task 1 and to focus heavily on Tasks 3 and 2. We have made major progress in developing a cell-based, *in vivo* high-throughput screen, carrying out our first screening of a chemical library, and generating even more efficient large-scale screens and counter-screens. Below we describe this progress and our plans to carry out counter-screens, further cell-based screens, *in vitro* Luminescence Resonance Energy Transfer (LRET) target-based high-throughput screens, and characterization of resulting chemical lead compounds.

Task 1- Determine ERR α -associated proteins

Because of our recent discoveries and progress, we have chosen not to actively pursue the original Task 1, which was to identify relevant coactivators of activated ERR α for use in Tasks 2 and 3. Since we have identified such a coactivator, we felt justified in

moving directly to high-throughput screen development and use. During the first part of this last year, however, we made progress in further characterizing the useful monoclonal antibodies we previously prepared against ERR α .

➤ Production and characterization of nine murine monoclonal antibodies against ERR α .

Nine murine monoclonal antibodies against ERR α that our laboratory generated (described in the previous annual report) were further characterized and subsequently used in many assays by other members in the laboratory. Chromatin Immunoprecipitation (ChIP)—chip assays are being performed to determine novel biomarkers under the transcriptional control of ERR α , but not ER α . Immunoprecipitation of ERR α , using the monoclonal antibody 1ERR87, assisted in determining novel phosphorylation and sumoylation sites at the N-terminus of ERR α in MCF-7 mammary carcinoma cells (Vu *et al.*, manuscript submitted).

➤ Localization of endogenous ERR α in HeLa cervical carcinoma cells.

Because ERR α -specific antibodies were not available until only very recently, questions of distribution and relative abundance of endogenous ERR α in various cell lines could not easily be answered. Now, however, the nine monoclonal antibodies can be used to perform immunofluorescence (IF) and nuclear/cytoplasmic fractionation experiments in various cell lines. We chose the HeLa cervical carcinoma cell line to study first because of its ease of growth, transfection, and handling. It was observed by IF that endogenous ERR α is located primarily in the nucleus of HeLa cells (see **Figure 1**). This observation was confirmed using nuclear/cytoplasmic fractionation (data not shown); we used heat shock protein 90, Hsp90, as the cytoplasmic marker protein and a transcription factor, Oct-1, as the nuclear marker protein to ensure complete separation of fractions.

IF analyses of MCF-7 mammary carcinoma and BT-474 mammary carcinoma cells are currently being performed; preliminary nuclear/cytoplasmic fractionation of these cell lines gives similar conclusions as that of HeLa cells, that endogenous ERR α is located predominantly in the nucleus.

Task 2 - Study the binding properties of ERR α and coactivator

While we have focused much of this last year on Task 3 using a cell-based high-throughput screen, we have also worked to create a complementary *in vitro* high-throughput screen, which involves the purification and fluorescence-labeling of the two main proteins in our cell-based screen, activated ERR α and the p160 family member coactivator, GRIP1. The *in vivo* cell-based high-throughput screen relies on the inhibition of ERR α -GRIP1-dependent luciferase expression (see below in **Task 3**); consequently, there is a significant risk of false-positive chemical hits, such as those that generally inhibit the transcription or translation process of the cell. Even though it is relatively easy to identify and discard hits that are generally toxic to the cell, we nevertheless need to create an assay that will determine if the hits identified in the cell-based high-throughput screen inhibit the ERR α -GRIP1 interaction specifically.

➤ Development of an *in vitro*, target-based high-throughput screen for small molecule inhibitors of the GRIP1-ERR α protein-protein interaction.

Our laboratory has previously published on the development of an *in vitro* Luminescence Resonance Energy Transfer (LRET)-based assay to determine small molecule inhibitors of a critical protein-protein interaction in bacterial cells (Bergendahl *et al.*, 2003). We believe this *in vitro* assay can be adapted to the current task, to determine small molecule inhibitors of the important GRIP1-ERR α interaction in mammary carcinoma cells. Therefore, we have spent the latter part of the year obtaining plasmids, overproducing, and purifying GRIP1 from *Escherichia coli* and cloning activated ERR α into bacterial expression plasmids.

Expression plasmids containing GST-tagged GRIP1 (amino acids 563-1121, a generous gift from M. Stallcup, University of Southern California) have been transformed into Rosetta2(DE3)pLysS cells (Novagen, Madison, WI), allowing overproduction of soluble protein that may be partially purified from cell lysates using glutathione immobilized onto agarose (**Figure 2**). We are now working hard to purify GST-tagged GRIP1 to homogeneity by using size-exclusion and ion-exchange chromatography.

We have cloned the DNA sequence for activated ERR α (amino acids 77-423) into a pET vector (Novagen, Madison, WI) that provides an N-terminal (His)₆-tag for rapid purification of the protein. We are currently sequencing this plasmid. We will transform the plasmid into Rosetta2(DE3)pLysS for overproduction of tagged activated ERR α . We plan to purify this protein using Ni²⁺-resin, but we are aware that we may have to purify activated ERR α to homogeneity using additional chromatographic steps, as we are currently doing with GST-tagged GRIP1.

Once GRIP1 and activated ERR α are purified to homogeneity, we will fluorescently tag them, as stated in Task 2 of our original Statement of Work: ERR α will be labeled with the donor fluorophore and GRIP1 will be labeled with the acceptor fluorophore. We will then develop and use a LRET-based high-throughput screen following protocols we have previously published (Bergendahl *et al.*, 2003).

➤ Generation of murine monoclonal antibodies against GRIP1.

Over this past year, we have noticed a great need, not just in our laboratory but in the nuclear receptor field, for a good monoclonal antibody against GRIP1. Because we now have relatively pure GRIP1 protein to use as an immunogen, and because our laboratory has years of experience in producing and characterizing monoclonal antibodies to bacterial and mammalian proteins, we have decided to start the production of murine monoclonal antibodies against GRIP1. We have performed the third immunization and, in approximately three weeks, we will perform a fusion of the spleen from the antigen-stimulated mouse and plasmacytoma cells. We will screen the resulting hybridoma cells for clones that produce monoclonal antibody against GRIP1. The resulting monoclonal antibodies will be made widely available.

Task 3 - Develop a high-throughput screen (HTS) for molecules that interfere with the binding of ERR α and a coactivator

➤ GRIP1 is a cell type- and promoter-specific coactivator of ERR α .

During our collaborations with the Mertz laboratory this past year, we made some important discoveries that shifted the focus of our research. The Mertz laboratory was

the first to report that ERR α regulates ERE-dependent transcription in a cell type-dependent manner, repressing in MCF-7 cells while activating in HeLa cells (Kraus *et al.*, 2002). In trying to answer why this observation was so, the Mertz laboratory discovered that if the N-terminus of ERR α is removed (deleting amino acids 1-76), it becomes a constitutively activated form of ERR α , no matter the cell type. We felt this piece of information was highly relevant for Task 1 of our approved Statement of Work: to purify ERR α -associated proteins. We could use this constitutively activated form of ERR α as “bait”, thereby pulling out proteins associated only with the activated form of ERR α from mammary carcinoma cell lysates. To be sure this form of ERR α was functionally active, we performed small scale transfections of full-length or activated ERR α in numerous mammary carcinoma cell lines, and by transfecting in known coactivators of ERR α (such as the p160 family members, and PGC-1 α), we found that recruitment of coactivators was, indeed, important for full ERR α activation of transcription in breast cancer cells lines, just as we had hypothesized. But to our surprise, we stumbled upon something most interesting: of the three members of the p160 family of transcriptional coactivators, only GRIP1 (SRC-2, TIF-2) was cell type- and promoter- specific in its coactivation of transcription with the constitutively active form of ERR α (**Figure 3**). We had a solution to Task 1, which is that GRIP1 is an important coactivator when ERR α is involved in transcription of estrogen-dependent gene transcription (even in the absence of ER α) in mammary carcinoma cells.

➤ Cell-based high-throughput screen for small molecule inhibitors of the GRIP1-ERR α protein-protein interaction.

The observations that when GRIP1 is co-transfected with “activated” ERR α , there is a robust activation of transcription of an ERE-containing reporter gene, provided a strong foundation on which to develop a cell-based assay to search for small molecule inhibitors of the ERR α -GRIP1 interaction.

We used this novel cell-based assay to scale-up and develop a high-throughput screen, performed in 96-well plates in MCF-7 cells treated with 10⁻⁸ M ICI 182,780 (a compound that inhibits ER α ; Tocris, Ellisville, MO). We used a 4,160 “known bioactives” (KBA) compound library (**Figure 4**) in our pilot screen. Eighteen hours after MCF-7 cells were plated in the presence of ICI 182,780 compound, specific plasmids carrying GRIP1, activated ERR α , and a (5x)ERE-containing luciferase reporter vector were co-transfected using *TransIT*-LT1 transfection reagent (Mirus, Madison, WI). Six hours post-transfection, one KBA compound was added per well, and twenty-four hours post-transfection, cells were harvested using a mixture of 1:2 ratio of Bright-Glo luciferase reagent to 1.5x Passive Lysis Buffer (Promega, Madison, WI). A well in which a 4-fold (75%) or more decrease in luminescence occurred compared to control was considered a “hit”.

➤ Cell-based counter screens for validating small molecule inhibitors of the GRIP1-ERR α protein-protein interaction.

A pilot cell-based high-throughput screen of the KBA library produced approximately 200 unique “hits” that were not generally cytotoxic in a number of different cell-based screens. Secondary screens were developed and optimized to rule out compounds that generally inhibit transcription or translation (affected activity of a

constitutively active luciferase reporter gene; pGL3-promoter vector, Promega, Madison, WI), and those that cross-react with other nuclear receptor or co-activator family members (see examples of secondary screens, **Figure 5**). For example, we will discard compounds that can decrease PGC-1 α —ERR α dependent transcription of an ERE-containing luciferase reporter gene. PGC-1 α is an important coactivator of ERR α , but in metabolic cells and tissues; in fact, PGC-1 α is not present at detectable levels in mammary carcinoma cells. We are ready to perform these secondary screens.

➤ Technical improvements to increase ease and reproducibility of cell-based high-throughput screen

After our pilot cell-based high-throughput screen of 4,160 chemical compounds, we noticed that a high-throughput screen can really highlight the strengths and weaknesses of an assay. We needed to work on transfection efficiency and reproducibility, as well as well-to-well cell number reproducibility. We spent a good portion of the end of last year optimizing our cell-based assay, and we are excited to report that we are now able to perform batch transfection and plating of cells in one step, which greatly decreases variability across wells and plates. It also greatly reduces handling time of the plates and changing of pipette tips. We also have been able to transfer our assay into a 384-well plate format. This move allows us to decrease the amount of cells and chemicals needed per well by 75%. These two improvements—batch transfection and a 384-well format—allows us greater efficiency, ease and reproducibility of our assay, and facilitates cell-based screening of larger (16,000 compound) libraries.

KEY RESEARCH ACCOMPLISHMENTS

- Further characterization of nine murine monoclonal antibodies against ERR α including localization of endogenous ERR α in the nucleus of HeLa cervical carcinoma cells.
- GRIP1 is a cell type- and promoter-specific coactivator of a constitutively-activated mutant ERR α .
- Purification of key proteins for development of an *in vitro*, target-based high-throughput screen for small molecule inhibitors of the GRIP1-ERR α protein-protein interaction.
- Cell-based high-throughput screen for small molecule inhibitors of the GRIP1-ERR α protein-protein interaction.
- Technical improvements to increase ease and reproducibility of cell-based high-throughput screens.

REPORTABLE OUTCOMES

➤ Abstracts and presentations

- Presentation: *Estrogen-related Receptor alpha—Coregulator Interactions as Possible Targets for Novel Breast Cancer Therapies*. Molecular and Cellular Pharmacology Student Seminar Series; January 29, 2007.
- Abstract and poster: *Generation and characterization of murine monoclonal antibodies for use in studying the biology of the estrogen-related receptor alpha (ERR α)*. The Keystone Symposium, Nuclear Receptors: Orphan Brothers; Banff, Alberta, Canada; March 18-23, 2006.

➤ Patents and licenses applied for and/or issued

- *Development of a cell-based assay for use in a high-throughput screening of small molecules that interfere with a transcription factor (“activated ERR α ”) and a coactivator to decrease transcription of a reporter construct*. Disclosed to the University of Wisconsin-Madison patenting and licensing group, WARF. Provisional patent submitted December 2006.

➤ Funding applied for based on the work supported by this grant

- WARF Strategic Technology Enhancement Program Lead Discovery Initiative, 2006-2008. We applied for this program and were approved. This support from WARF (<http://www.warf.org>) will pay for much of the cost associated with upcoming use of the UW-Keck Comprehensive Cancer Center Small Molecule Screening Facility (<http://hts.wisc.edu>) on campus.

CONCLUSIONS

We are excited about our progress this year. We have been able to further characterize our nine anti-ERR α monoclonal antibodies so that others in the laboratory may now begin to answer questions involving the global transcriptional regulation of ERR α , and the post-translational modifications of ERR α , in mammary carcinoma cells. We have established the glucocorticoid receptor interacting protein 1 (GRIP1) as an important coactivator for ERR α on ERE-containing promoter elements in mammary carcinoma cell lines. We used it and a truncated constitutively activated ERR α (amino acids 77-423) to develop an *in vivo* cell-based luciferase reporter assay, *in vivo* cell-based counter assays, and are developing an *in vitro* LRET-based protein-protein interaction assay, that all may be used for high-throughput screening to find small molecule inhibitors of the GRIP1—activated ERR α protein-protein interaction. We look forward to optimizing and utilizing these assays. Hopefully, these assays will allow us to discover a lead compound for the inhibition of the GRIP1—activated ERR α interaction, an interaction we believe may play an important role in the etiology and progression of a subset of presently difficult-to-treat breast cancers.

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SUPPORTING DATA

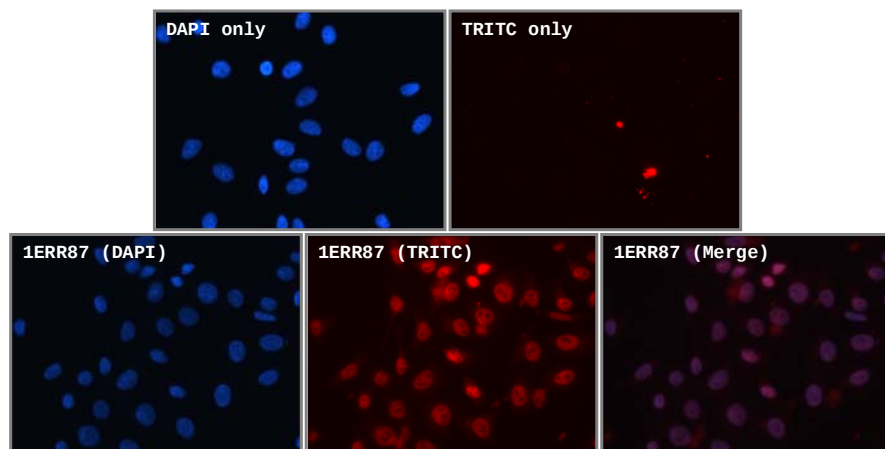


Figure 1. Immunofluorescence detection of ERR α in HeLa cervical carcinoma cells.

Briefly, HeLa cells were plated on glass cover plates in 12-well dishes and incubated for 24 hours at 37°C/5% CO₂. Cells were cross-linked with 3% paraformaldehyde and permeabilized with 0.1% Triton X100. 1ERR87, an anti-ERR α murine monoclonal antibody, was used as the primary antibody, and a TRITC-labeled goat anti-mouse antibody was used as the secondary antibody. DAPI was used to stain the nucleus of cells. The “Merge” panel is the overlay of the DAPI nuclear staining and the presence of ERR α (via TRITC-detection) and shows clear nuclear localization of ERR α .

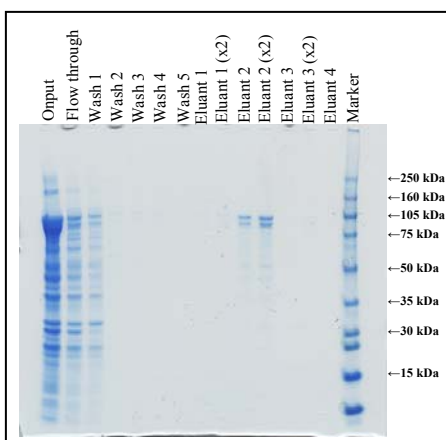


Figure 2. Purification of N-terminal GST-tagged GRIP1 (aa 563-1121) from Rosetta2(DE3)pLysS (~0.2 g wet wt).

Output is ~0.9% of total lysate loaded onto an agarose column containing immobilized reduced glutathione. *Wash 1* used buffer containing Tris-HCl pH 7.9, EDTA, NaCl, and NP40, and was effective in removing non-specific proteins. *Wash 2-3* used buffer containing Tris-HCl pH 7.9, EDTA, KCl, and Triton X100. *Wash 4-5* used buffer without detergent. Washes 2-5 caused some leaching of GST-GRIP1 off the column, but also removed non-specific proteins. *Elution* is with buffer containing Tris-HCl pH 7.9, EDTA, KCl, and reduced glutathione. (x2) by eluant fraction indicates double the sample volume loaded compared to the previous lane; this checks for purity of sample. This SDS-polyacrylamide gel was stained for total protein with Coomassie blue stain. A Western blot using an anti-GST monoclonal antibody indicates that the lower band of the eluted doublet is tagged-GRIP. The upper band is unidentified.

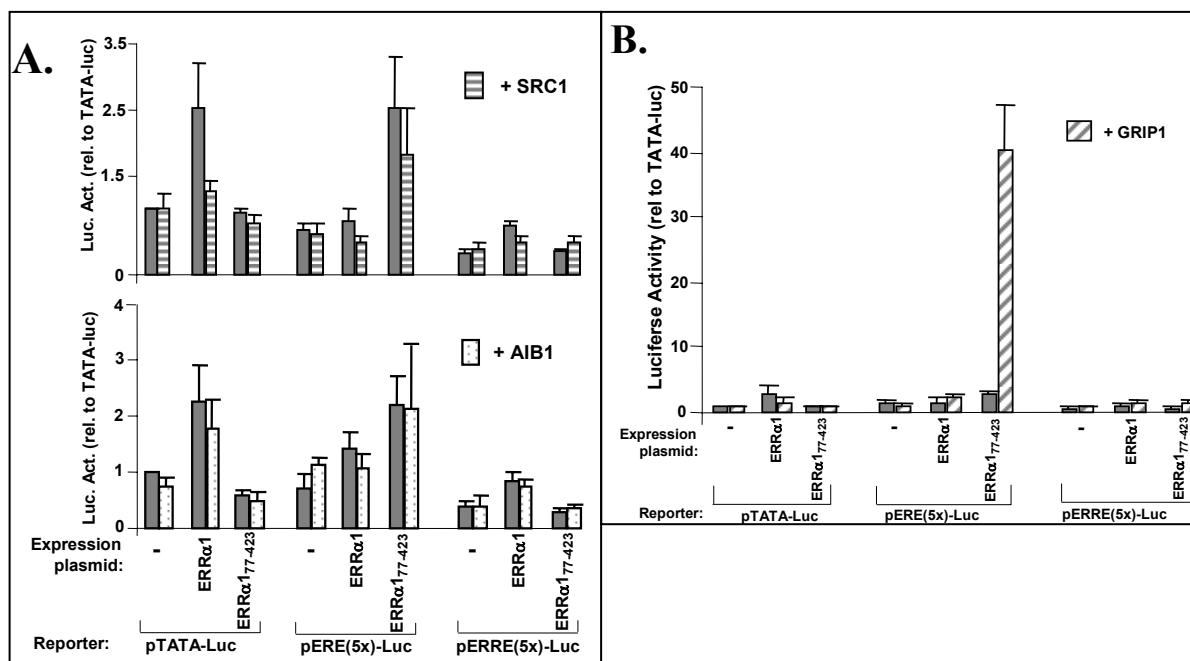


Figure 3. GRIP1 is a specific coactivator of “activated” ERR α on an ERE in MCF-7 cells. MCF-7 cells, in the presence of ICI 182,780, were transiently transfected for 48 hours then harvested for luciferase activity. pSRC1, pAIB1, pGRIP1, and pERR α 1 plasmids allow for expression of full-length protein. pERR α 177-423 plasmid allows for expression of N-terminal truncated (amino acids 1-76) “activated” ERR α . pERE(5x)-Luc contains five copies of the consensus estrogen response element (ERE, 5’-AGGTCACAGTGACCT-3’) upstream of the firefly luciferase reporter gene. pERRE(5x)-Luc contains five copies of the consensus estrogen-related receptor response element (ERRE, 5’-TAAAGGTCA-3’) upstream of the firefly luciferase reporter gene. ERE core half site sequences are underlined. (A) Neither SRC-1 nor AIB1 are coactivators of ERR α in MCF-7 cells. (B) Addition of GRIP1 increases “activated” ERR α transcriptional activity by nearly 20-fold, and only when ERR α is bound to an ERE-containing promoter.

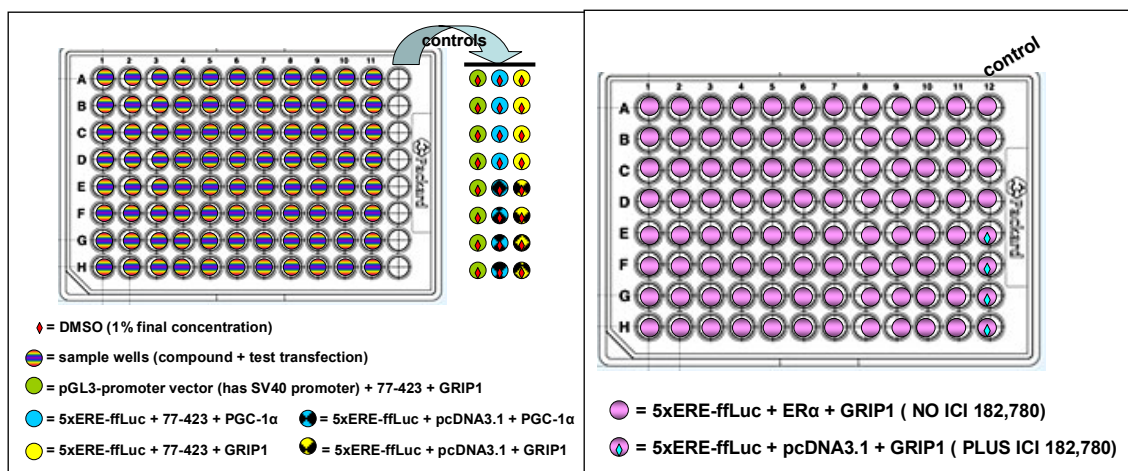
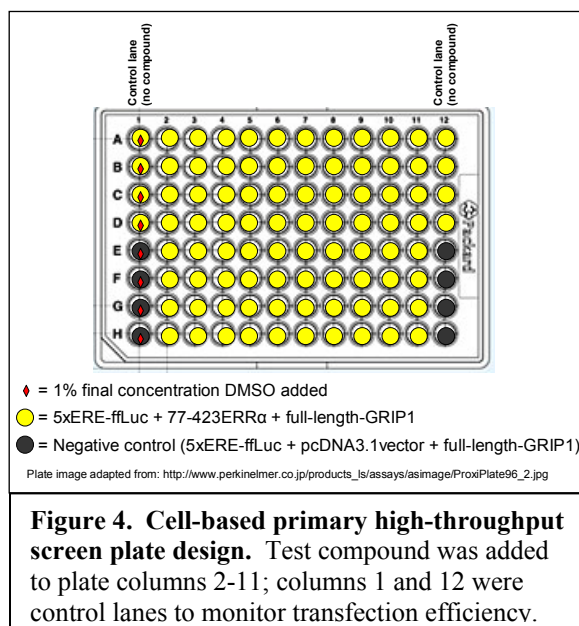


Figure 5. Cell-based secondary high-throughput screening plate design. To remove non-specific inhibitors, the following transient transfections will be performed: [1] a luciferase reporter gene downstream of a constitutive promoter (pGL3-promoter vector) to test for molecules that affect general cellular processes, as well as those molecules that affect luciferase enzyme activity; [2] full-length estrogen receptor alpha (ERα), GRIP1, and a luciferase reporter gene downstream of an ERE-containing promoter, in the absence of ICI compound, to test for molecule specificity (i.e., molecule inhibits ERα- but not ERα-GRIP1 interactions); and, [3] activated ERα, PPAR gamma coactivator 1 alpha (PGC-1α), and a luciferase reporter gene downstream of an ERE-containing promoter, to test for molecule specificity (i.e., molecule inhibits GRIP1- but not PGC-1α-ERα interactions).