Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 341–351

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Development of an image analysis screen for estrogen receptor alpha (ERα) ligands through measurement of nuclear translocation dynamics

Angie Dull[a,*], Ekaterina Goncharova[b], Gordon Hager[c], James B. McMahon[d]

[a] Molecular Targets Laboratory, SAIC-Frederick, Inc., National Cancer Institute-Frederick, United States
[b] Data Management Services, Inc., National Cancer Institute-Frederick, United States
[c] Laboratory of Receptor Biology and Gene Expression, Center for Cancer Research, National Cancer Institute, United States
[d] Molecular Targets Laboratory, Center for Cancer Research, National Cancer Institute-Frederick, United States

ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 23 July 2010
Accepted 27 August 2010

Keywords:
Estrogen receptor
High-content screening
Cell-based assay
Nuclear translocation
Cytotoxicity

ABSTRACT

We have developed a robust high-content assay to screen for novel estrogen receptor alpha (ERα) agonists and antagonists by quantitation of cytoplasmic to nuclear translocation of an estrogen receptor chimera in 384-well plates. The screen utilizes a green fluorescent protein tagged-glucocorticoid/estrogen receptor (GFP-GRER) chimera which consisted of the N-terminus of the glucocorticoid receptor fused to the human ER ligand binding domain. The GFP-GRER exhibited cytoplasmic localization in the absence of ERα ligands, and translocated to the nucleus in response to stimulation with ERα agonists or antagonists. The BD Pathway 435 imaging system was used for image acquisition, analysis of translocation dynamics, and cytotoxicity measurements. The assay was validated with known ERα agonists and antagonists, and the Library of Pharmacologically Active Compounds (LOPAC 1280). Additionally, screening of crude natural product extracts demonstrated the robustness of the assay, and the ability to quantitate the effects of toxicity on nuclear translocation dynamics. The GFP-GRER nuclear translocation assay was very robust, with z' values >0.7, CVs <5%, and has been validated with known ER ligands, and inclusion of cytotoxicity filters will facilitate screening of natural product extracts. This assay has been developed for future primary screening of synthetic, pure natural products, and natural product extracts libraries available at the National Cancer Institute at Frederick.

1. Introduction

The estrogen receptor (ER) is a ligand-activated transcription factor, which is a member of the steroid/nuclear receptor super family. In women, ERα is expressed in the brain, cardiovascular system, uterus, bone and liver, and is the predominate form expressed in breast cancer. ER-mediated signal transduction is a complex pathway, which regulates cellular proliferation, differentiation and reproductive physiology. Elevated estrogen levels can lead to initiation, promotion and progression of breast tumors by several pathways in postmenopausal women. Estrogen production from the ovaries ceases following menopause, and the source of estrogen in postmenopausal women is conversion of androgens to estrogens in peripheral tissues, including the breast [1]. ER signaling through the nucleus, mitochondria, and non-genomic signaling at the plasma membrane lead to rapid cell proliferation that may lead to elevated mutation rates, altered cell-cycle control, and inhibition of apoptosis which perpetuate the growth and survival of the cancer cell [2,3]. Additionally, metabolic conversion of estrogen to genotoxic or mutagenic metabolites by the phase I detoxification pathway may result in DNA adduct formation or oxidative DNA damage [4,5]. These mechanisms of carcinogenesis mediated through estrogen signaling illustrate the importance of targeting ERα for therapeutic intervention.

Many of the drugs used for breast cancer therapeutics and hormone replacement therapy present adverse side effects, therefore, our goal was to identify compounds which target the estrogen receptor, which may exhibit reduced adverse side effects. These side effects are often related to the mixed agonistic/antagonist activity of a given drug, which is dependent on tissue, cell, promoter, co-activator or co-repressor expression profiles. While hormone replacement therapy (HRT) reduces menopausal symptoms, maintains bone mineral density and decreases the risk of colon cancer, these drugs also elevate the risk for the development of breast cancer, coronary heart disease, stroke, Alzheimer's disease and blood clots [6–8]. Selective estrogen receptor modulators (SERMs) are drugs that elicit agonism or antagonism depending on tissue, cell, promoter, co-regulator expression. Tamoxifen is a

* Corresponding author at: Molecular Targets Laboratory, SAIC-Frederick, Inc., NCI-Frederick, Mailstop 538-01, Frederick, Maryland 21702, United States.
Tel.: +1 301 846 1830; fax: +1 301 846 7522.
E-mail address: dulla@mail.nih.gov (A. Dull).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.08.009

SERM that is used for the treatment of hormone-responsive breast cancer, which is typically used as an adjuvant therapy after surgery and radiation. While tamoxifen exhibits ER antagonistic activity in the mammary tissue, this drug also exhibits partial agonistic activity in the uterine tissue, which increases the risk for development of endometrial cancer and uterine sarcoma [9]. Nearly half of patients do not respond to tamoxifen treatment, and patients with metastatic disease are certain to develop tamoxifen resistance, while 30–50% of patients with early stage ER-positive breast cancer that are administered tamoxifen relapse with resistant disease [10]. Tamoxifen has been demonstrated to induce non-alcoholic steatohepatitis in humans, which is a fatty acid disease that can develop into hepatocarcinoma or cirrhosis of the liver [11,12]. Furthermore, several studies in rats have demonstrated that tamoxifen is hepatocarcinogenic [13], and this carcinogenesis is based on the ability of tamoxifen to be both a tumor initiator and tumor promoter in the liver [14,15].

For these reasons, as well as others, there is a need for discovery of new effective drugs for breast cancer treatment, which can ameliorate the adverse side effects associated with drugs currently available. In addition, new agents are needed for the development of combination therapies that could prevent the onset of endocrine-resistant disease associated with monotherapy.

This study describes a high-content nuclear translocation imaging screen, which used ERα as a therapeutic target for the discovery of new hormone replacement therapies and breast cancer therapeutics.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modification of eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and G418 were purchased from Mediatech (Manassas, VA). The penicillin/streptomycin solution, trypsin–EDTA, staurosporine, and 37% formaldehyde were purchased from Sigma–Aldrich (St. Louis, MO). Sodium butyrate was purchased from Millipore (Billerica, MA). The Nunc glass-bottom 96-well and 384-well plates and Hyclone characterized fetal bovine serum (FBS) and charcoal/dextran-treated FBS were acquired from Thermo Fisher (Pittsburgh, PA). Hoechst 33342 was obtained from Invitrogen (Carlsbad, California). 17-β estradiol (E2), propyl pyrazoletriol (PPT), diethylstilbestrol (DES), 4-hydroxy-tamoxifen (4OHT), diarylpropionitrile (DPN), genistein and the LOPAC 1280 library were acquired from Sigma–Aldrich (St. Louis, MO). The natural product extracts library was obtained from the Natural Products Branch, Developmental Therapeutics Program, at the National Cancer Institute-Frederick (Frederick, MD).

2.2. Cell culture

The 6020 cells were cultured in DMEM, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mg/ml G418 and 10% charcoal/dextran-treated FBS, which will be referred to as complete media. The C127 cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% charcoal/dextran-treated FBS. Cells were cultured in a humidified incubator at a 37 °C, 5% CO_2, 95% air environment.

2.3. Library plate dilutions

Chemical libraries are typically screened at 10 μM final concentrations for pure synthetic compounds and 10 μg/ml for natural product extracts, and dilution plates were made in complete media containing charcoal/dextran-treated FBS. Dilutions were made using the Biomek FX liquid handling instrument (Fullerton, CA). DMSO (0.5% final concentration) served as the negative control, and estradiol (5 μM final concentration) was used for the positive control for the assay. Due to time constraints on the third day of the assay, dilution plates were made the day before and frozen overnight at −20 °C.

2.4. Nuclear translocation assay

The 6020 cells were plated into 96-well or 384-well Nunc glass-bottom black plates at 8000 cells/well or 3500 cells/well, respectively, and cultured in complete media. The 6020 cells were seeded into 384 plates at 50 μl/well (or 100 μl/well for 96-well plates) using a Bio-Tek MicroFill microplate dispenser (Winooski, VT) and incubated at 37 °C, 5% CO_2, 95% air overnight. Twenty-four hours after seeding the cells into assay plates, cells were treated with 70 mM sodium butyrate (25 μl/well for 384-well plates and 50 μl/well for 96-well plates) using a Beckman FX liquid handling device (Fullerton, CA). Cells were treated with sodium butyrate for 24 h to induce expression of the GFP-GRER chimera, which is located in a silent region of the genome. The following morning cells were treated with ER ligand dose–response plates or chemical library plates for 6 h. The Beckman FX was used to add 10 μl of the ER ligand dose–response plates or chemical dilution plates to the assay plates. After a 6-hour treatment with dose responses or chemical library plates, the assay plates were subjected to fixation with 4% formaldehyde for 45 min. After fixation, the assay plates were washed 5 times with 100 μl of DPBS using a Bio-Tek plate washer (Winooski, VT). The plates were stained with 0.3 μg/ml Hoechst 33342 in PBS overnight at 4 °C, which was added (15 μl/well) using a Bio-Tek MicroFill microplate dispenser to the assay plates. The next day, assay plates were washed twice with 100 μl of DPBS using a Bio-Tek plate washer (Winooski, VT). Plates were sealed with aluminum sealing tape and were barcoded with a Velocity-11 VCode Bar Code Label Print and Apply Station (Menlo Park, CA).

2.5. Image acquisition and nuclear translocation analysis

Images were acquired using a BD Pathway 435 imaging system (Rockville, MD) integrated with a Thermo CRS catalyst-5 robotic arm (Waltham, MA) and a Symbol barcode reader (Schaumburg, IL) for unattended imaging. An Olympus 20× 0.75 NA objective was used for image acquisition, four sites (montages) were acquired per well, each well >100 cells, and GFP (180 ms, gain = 10) and Hoechst (30 ms, gain = 0) filters were used. Translocation dynamics were quantitated with BD Attovision software using a ring-based (2 output) algorithm, and a GFP threshold of 300–4095 gray values was used for segmentation of the cytoplasmic area. Nuclear areas were defined by Hoechst staining, and GFP intensity was measured in that defined region of interest. The nuclear/cytoplasmic ratios were measured, and % nuclear translocation was reported. Images were written to a terabyte server during image acquisition. Attovision and Image Data Explorer software (BD Biosciences, Rockville, MD) were used for analysis of image data, and data mining. Image Data Explorer software and SigmaPlot (San Jose, California) software were used to calculate EC_{50} values and generate dose response curves.

2.6. Cytotoxicity assays

The 6020 cells were seeded into Nunc 96-well glass-bottom plates at 10,000 cells/well in complete media (100 μl/well) and incubated for 24 h at 37 °C, 5% CO_2, 95% air. The following morning the cells were treated with 70 mM sodium butyrate (final concentration) to induce GFP-GRER expression for 24 h before staurosporine treatment. Cells were treated with a staurosporine dose–response for 4 h, which was followed by formaldehyde