Toxicology in Vitro 28 (2014) 916–925

Contents lists available at ScienceDirect

Toxicology in Vitro

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

A robotic BG1Luc reporter assay to detect estrogen receptor agonists

Matthew A. Stoner [a], Chun Z. Yang [a], George D. Bittner [a,b,*]

[a] CertiChem, Inc., Austin, TX, USA
[b] Neuroscience Department, University of Texas, Austin, TX, USA

ARTICLE INFO

Article history:
Received 23 January 2014
Accepted 21 March 2014
Available online 18 April 2014

Keywords:
BG1Luc
Bisphenol A
Estrogenic activity
Estrogen
Endocrine disruptor
Tritan™

ABSTRACT

Endocrine disrupting chemicals with estrogenic activity (EA) have been associated with various adverse health effects. US agencies (ICCVAM/NICEATM) tasked to assess *in vitro* transcription activation assays to detect estrogenic receptor (ER) agonists for EA have recently validated a BG1Luc assay in manual format, but prefer robotic formats. We have developed a robotic BG1Luc EA assay to detect EA that demonstrated 100% concordance with ICCVAM meta-analyses and ICCVAM BG1Luc results in manual format for 27 ICCVAM test substances, i.e. no false negatives or false positives. This robotic assay also consistently assessed other, more problematic ICCVAM test substances such as clomiphene citrate, L-thyroxin, and tamoxifen. Agonist responses using this robotic BG1Luc assay were consistently inhibited by the ER antagonist ICI 182,780, confirming that agonist responses were due to binding to ERs rather than to a non-specific agonist response. This robotic assay also detected EA in complex mixtures of substances such as extracts of personal care products, plastic resins or plastic consumer products. This robotic BG1Luc assay had at least as high accuracy and greater sensitivity and repeatability when compared to its manual version or to the other ICCVAM/OECD validated assays for EA (manual BG1Luc and CERI).

1. Introduction

Endocrine disrupting chemicals (EDCs) mimic or otherwise alter the activities of hormones. Estrogenic activity (EA) is by far the most common type of hormonal activity for known or suspected EDCs (National Research Council, 1999; ICCVAM, 2003, 2006, 2010, 2011; vom Saal et al., 2005; Vandenberg et al., 2012). Many studies (National Research Council, 1999; ICCVAM, 2003; Calafat et al., 2005; Swan et al., 2005; vom Saal et al., 2005; Heindel and vom Saal, 2009; Talsness et al., 2009; Thompson et al., 2009; Gray, 2010; Adewale et al., 2011; de Cock et al., 2012; Hall and Korach, 2012; Vandenberg et al., 2012) have reported that xenobiotic chemicals with EA *in vitro* can produce adverse effects *in vivo* in laboratory animals and humans. These effects include decreased sperm counts, ovarian and uterine disorders, abnormalities in male reproductive organs, obesity, abnormal brain maturation, learning disabilities, attention disorders, increases in immune and autoimmune disease and increased incidence of some cancers. Fetal, infant, and juvenile mammals are especially sensitive to low dosages [nanomolar (nM) to <picomolar (pM) concentrations, or ppb to <ppt levels] of chemicals with EA (vom Saal et al., 2005; Gray, 2010; Vandenberg et al., 2012).

Many scientists and consumers are concerned about the potential public health effects of chemicals with EA that are released from commonly used products such as plastics and cosmetics (Gray, 2010). In the US, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are tasked to co-ordinate the development, validation, and acceptance of *in vitro* toxicological tests. [These combined agencies are hereafter referred to as ICCVAM.] Acceptable *in vitro* toxicological tests to assess whether chemicals have EA include estrogen receptor (ER)-dependent transactivation assays such as BG1Luc and CERI, and cell proliferation assays such as those using MCF-7 cell lines

Abbreviations: CCi, CertiChem, Inc.; CS, calf serum; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EA, estrogenic activity; EC50, half-maximal response of a test substance in its dose-response curve; EDC, endocrine disrupting chemical; EFM, EA-free medium; ER, estrogen receptor; EtOH, ethanol; FBS, fetal bovine serum; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; ICI, ICI 182,780, an ER antagonist; NICEATM, National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods; NTP, National Toxicology Program; OECD, Organization for Economic Cooperation and Development; RLU, relative light unit [a measure of luciferase bioluminescence]; RPMI, Roswell Park Memorial Institute; SD, standard deviation; Sham Control, control solvent that went through all the steps that an extract did; VC, vehicle control.

* Corresponding author at: CertiChem, Inc., 11212 Metric Blvd., Suite 500, Austin, TX, USA. Tel.: +1 512 339 0550×201; fax: +1 512 339 0551.
E-mail address: gbittner@certichem.com (G.D. Bittner).

http://dx.doi.org/10.1016/j.tiv.2014.03.013
0887-2333/

(ICCVAM, 2003, 2006; Yang et al., 2011, 2013). Whenever possible, ICCVAM prefers robotic assays to manual assays (ICCVAM, 2003, 2006).

Only two EA assays are currently validated, or have been undergoing validation, by ICCVAM for regulatory use: the BG1Luc ER transactivation assay in manual format and the MCF-7:WS8 (MCF-7) cell proliferation assay in robotic format, respectively. A third assay (CERI) has been approved in manual format by the EU Organisation for Economic Co-operation and Development (OECD) and this validated assay is accepted by ICCVAM (2011). The validated assays for EA by ICCVAM are also accepted by the US Environmental Protection Agency (EPA).

In order to increase the high through-put and the repeatability, decrease the human errors and assay cost, we have developed a robotic version of the BG1Luc assay subsequently used to evaluate the EA of 44 test substances supplied by ICCVAM and of ICI 182,780 (ICI), a pure strong anti-estrogen. The 44 test substances were used in the ICCVAM validation study of the BG1Luc assay (2011). The half-maximum responses (EC50s) of individual test substances were calculated from concentration–response curves. From these EC50s, the test substances were classified as having strong EA ($EC50 \leqslant 1 \times 10^{-9}$ M, e.g., diethyl-stilbestrol), moderate EA (EC50 between 1.0×10^{-9} M and 1.0×10^{-7} M, e.g., coumestrol), weak EA ($EC50 \geqslant 10^{-7}$ M, e.g., genistein), or no detectable EA (e.g., atrazine). This robotic BG1Luc assay could detect EA in complex mixtures of chemicals. Furthermore, agonist responses detected for a test chemical, or a complex mixture, were suppressed by the ER-antagonist ICI 182,780 (ICI) to confirm that the agonist response is via ER pathway. That is, positive agonist responses classified as exhibiting EA were due to binding of chemicals to ERs, rather than non-specific ER activation, potentially resulting in a false positive classification for EA.

Twenty seven of the 44 ICCVAM test substances used by ICCVAM to assess the accuracy (concordance) of the manual BG1Luc assay with ICCVAM meta-analyses were used to assess the accuracy of this assay. This robotic BG1Luc assay had a 100% concordance with ICCVAM meta-analysis classifications (ICCVAM, 2003, 2006, 2011) for these 27 test substances. Robotic BG1Luc assays of individual test substances are very repeatable (reproducible). The EC50s of individual test substances tested in this robotic assay were usually lower (more sensitive) compared to EC50s previously reported by ICCVAM (2003, 2006, 2011) using other *in vitro* assays, including the validated manual BG1 and CERI assays for EA. We therefore conclude that this robotic BG1Luc assay is at least as accurate, and often more sensitive and reproducible, as the validated test methods accepted by the US ICCVAM/NICEATM, the US EPA and the EU OECD.

2. Materials and methods

2.1. Equipment

A Labconco Class II Biosafety Hood (Kansas City, MO, USA) equipped with a 254 nm fluorescent bulb to enclose EpMotion 5070 robotic workstations (Eppendorf, Hamburg, Germany) was used for all cell seeding, serial dilutions of test substances and for treatments with test substances (Yang et al., 2011, 2013). A Tristar Luminometer (Berthold Technology, Germany) was used to measure luminescence.

2.2. Cell line maintenance, seeding and assay conditions

BG1Luc4E2 (BG1Luc) cells were obtained from Dr. Michael Denison (University of California-Davis). This human ovarian cell line expresses estrogen receptor (ER) alpha and beta receptors and is highly sensitive to 17β-estradiol (E2) at 1 pM (Rogers and Denison, 2000). These cells are modified to stably carry a firefly luciferase reporter vector that expresses luciferase enzyme under the control of multiple estrogen-response elements (ERE) sequences positioned in the gene enhancer/promoter sequence upstream of the luciferase coding sequence. The EA assay examines the ability of a substance to induce expression of luciferase enzyme.

As described in more detail below and by ICCVAM (2011) the BG1Luc assay consists of growing these cells in estrogen-free medium for three days, then exposing the cells to test substances or E2 for 24 h, then measuring any agonist-induced luciferase response against the E2 response (positive control) and the vehicle response (negative control). The enzymatic activity of luciferase is measured in relative light units (RLUs) with respect to the maximum E2 response to E2 (positive control) set at 100% activity obtained by a dose response-curve in each experimental run. Modified cell culture medium serves as the negative vehicle control (VC) and is set at 0% estrogenic activity.

Cell culture medium used to maintain the BG1Luc cells was RPMI (Roswell Park Memorial Institute)-1640 media supplemented with 10 μg/mL phenol red, 4 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 8% fetal bovine serum (FBS) and 1 mM sodium pyruvate. Cells were grown as monolayers in polystyrene tissue culture flasks (T-25 flask, CytoOne, USA Scientific, Ocala, FL; or T-75 flask, BD-Falcon, BD Biosciences, San Jose, CA) in a humidified incubator at 37 °C with 5%CO_2.

The EA-free medium (EFM) was prepared in two ways. ICCVAM EFM (used for ICCVAM validation study) was phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5% dextran-coated charcoal-stripped FBS, 1 mM sodium pyruvate, 4 mM L–glutamine and 100 units/mL penicillin and 100 μg/mL streptomycin solution (Invitrogen, Grand Island, NY). Alternatively, CCi EFM was phenol red-free RPMI-1640 medium supplemented with 1% charcoal-stripped FBS and 4% charcoal-stripped calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B solution, 4 mM L-glutamine, 1/100 (vol/vol) non-essential amino acids (100×: catalog number 11,140,050) purchased from Invitrogen, and 6 ng/mL insulin.

In preparation for experiments and in order to decrease the basal level expression of luciferase enzyme, BG1Luc cells were trypsinized, dispersed with a 22G needle on a 3 mL or 10 mL syringe, counted, re-plated in a T-75 flask and incubated for 1–4 days in EFM that was changed daily. (Three days was subsequently chosen as the standard incubation time.)

After the initial incubation time in EFM, BG1Luc cells were then seeded in 96-well, white-walled, clear bottom cell culture plates (Greiner Bio-One, Monroe, NC) at 10,000–40,000 cells per well in 0.1 mL EFM, followed by a 24 ± 6 h incubation after adding 0.1 mL of serially diluted test substances or extracts in triplicate of each testing concentration (see below). Water was distilled on-site in an all-glass system and collected directly into glass before use in extractions. Extractions were performed in borosilicate glass tubes.

2.3. Visual assessment of cell health/cytotoxicity observations

Some test substances were cytotoxic at high treatment concentrations. Since cytotoxicity can prevent measurement of EA and lead to false-negative interpretations, viability of BG1Luc cells was visually observed under an inverted light microscope immediately before terminating incubation. Cellular cytotoxicity was visually assessed using the following scoring parameters suggested by ICCVAM (NIEHS, 2011): 1 = normal cell morphology, 2 = low cytotoxicity (10–50% of cells with altered morphology), 3 = moderate cytotoxicity (50–90% of cells had altered morphology), and 4 = high