Appendix B6

Development of stably transfected cell lines to screen Endocrine Disrupters

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ER-EcoScreen assay™ and AR-EcoScreen assay™ (Stable CHO clones containing luciferase based reporter gene and expressing hormone receptors)

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INTRODUCTION

We have developed genetically engineered stable transfected cell lines, expressing hormone receptor and luciferase based reporter genes, for screening compounds and compound mixtures for endocrine disrupter activity. We have named the lines "**ER-EcoScreen™** " (expressing estrogen receptor) and "**AR-EcoScreen™** " (expressing androgen receptor). To establish these cell lines we introduced the plasmids used in our transient transfection Eco-Screen Assay™ systems. We have demonstrated that the cells have the same reactivity to the samples tested in the Eco-Screen Assay™ system. We also have confirmed that these cell lines do not lose reporter activity during continuous cell passage.

METHODS

Stable transfection of Hormone Receptor and Reporter Gene in CHO-K1 cell

About 16 hr prior to transfection, CHO-K1 cell were seeded at 50% confluence in a 6-well plate in 2 ml culture medium per well. Transfections were carried out with Fugene™ according to the Instruction Manual. For the estrogen receptor (ER) reporter assay, 12 ug of pINDERE-15 (containing the luciferase gene, under the control of the minimal heat shock promoter with 4 copies of the estrogen response element, as well as the hygromycin resistant gene) and 120 ng of pcDNA ER-alpha (estrogen receptor expression plasmid). For the androgen receptor (AR) reporter assay, 12 ug of pIND ARE B10 (4 copies of the androgen response element linked to luciferase, and the hygromycin resistance gene) and 480 ng of pZeoSV2AR (androgen receptor expression plasmid) were transfected per well. After 24 hr the cells were trypsinized and the cells from each well plated in two 100-mm petri dishes. The culture medium was replaced every three days with medium containing 200 ug/ml of Zeocin and 200 ug/ml of Hygromycin until colonies were large enough to isolate (about 10 days). Luciferase-positive clones were isolated using a photon detecting CCD camera (Night OWL, Perkin-Elmer). Briefly, clones were exposed to 0.2 nM luciferin and 1 nM E2 for the ER assay, 10 nM testosterone for the AR assay, for 24 hr, and then introduced into the CCD camera. Luminescence intensity was monitored for 10 min per dish and the

luminescence image from cell was superimposed on to the light field image of the cell clones in the dish. Positive clones were isolated using cloning rings and further cultured in 24 well plates. After growth each clone was trypsinized and seeded into 2 wells in two 96 well plate (Nunclon™ NalgeNunc Denmark) and further cultured. After 24 hr culture, cells in one plate were exposed to 0.1% DMSO as a control, while the cells in the other plate were incubated with 1nM of E2 for ER assay and 1nM of 5-alpha-dehydrotestosterone for AR assay, respectively. Followed 24 hr culture, 100 ul of the luciferase substrate with cell lysis reagent Steady-Glo ™ (Promega) were added to all assay wells. After shaking at room temperature for 5 min the chemiluminescence was measured in the ARVO multi-label counter (Perkin-Elmer). The most responsive clone was selected.

Procedure for ER/AR-Eco screen assay™

1st day: The most responsive CHO-K1 stable clone was maintained in DMEM/Fl2 supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, and 10% fetal bovine serum. The cell were trypsinized and prepared at a density of $1x10^5$/ml, and then seeded with 90 ul of culture medium in 96 well microtiter plates (Nunclon™ #137101, NalgeNunc Denmark) in DMEM/F12 containing 5% charcoal-treated fetal bovine serum (Hyclone, Logan, UT) and incubated for 24 hr at 37 °C in a humidified atmosphere of 5% CO_2 / air.

2nd day: After 24 hr culture 10 ul of sample solution from serial dilutions of each chemical with the culture media (see Chemicals on protocol 1) were added to the plates and cultured for 16-24 hr.

3rd day: Followed 24 hr culture, 100 ul of the luciferase substrate with cell lysis reagent Steady-Glo™ (Promega) were added to all assay wells. After shaking at room temperature for 5 min the chemiluminescence was measured by ARVO multi-label counter (Perkin-Elmer).

Chemicals for Estrogen and Androgen reporter assay (agonist activity detection)

The estrogen receptor agonist assay and androgen receptor agonist assay were carried out with 12 chemicals for ER-EcoScreen™ and AR-EcoScreen™**,** respectively (Table 1 and 2).

Data Analysis

We used the criteria of PC50 for data analysis. Refer to the “Definition of PC50” on a report 1 ”Development of new reporter gene assay systems for screening Endocrine Disrupters.” The data were analyzed with software by applying Cubic Spline Curve Fitting Method. EC50 is also shown for reference.

RESULTS

Clone stability for ER-Eco Screen™

The cloned line (ER-Eco Screen™) that was stably transfected with pINDERE-15 and pcDNA ER-alpha showed stable expression over at least 15 passages during more than two months of culture (fig.1-a).

In over 10 assays, this clone reported about 3.5 fold induction with 100 pM E2 compared to solvent treatment (0.1%DMSO), with a detection limit of 0.5 pM. The PC50 was 9.2 pM.

Clone stability for AR-Eco Screen™

Our cloned AR-Eco Screen™ was stably transfected with pIND ARE B10 and pZeoSV2AR. It was responsive to DHT for 30 passages over three months culture (fig.1-b), as observed in over 10 assays. This clone showed about a 5 fold induction with 1nM DHT compared to solvent (0.1%DMSO), with a detection limit of 15 pM. The PC50 was 153 pM.

Estrogen receptor agonist activity

Table 1 shows the rank of the compounds that had ER agonist activity on the basis of the PC50. Although there were slight differences, the ranking on the basis of PC50 was almost the same as that of the high throughput transfection assay. (Refer to Table 1 on the report of "high throughput transfection assay") The reaction curves for all measurements are shown in appendix 1. Although DDT (HST0099) and DEE (HST0100) had detectable ER agonist activity, they did not reach the PC50 (see appendix). As noted before, with weakly active compounds, standards such as PC40 (40% of positive reaction) or PC30 (30% of positive reaction) are more useful for ranking purposes.

Androgen receptor agonist activity

Table 2 shows the rank of the compounds, which have AR agonist activity, on the basis of the PC50. The reaction curves for all measurements are shown in appendix 2. Most of listed compounds that showed high agonistic activity were natural ligands or synthetic steroid hormones. Progesterone (HST0008) and Aldosterone (HST0009) showed only slight activity. RU486 (HST0087) and Cortisol (HST0099) at the highest concentration (10^{-6} M) examined were about 40% of the reaction of the positive control. The results were almost same as that of high throughput transfection assay. (Refer to Table 2 on the report of "high throughput transfection assay").

DISCUSSION

In our presentation of the high throughput assay we discussed the problem of ranking weakly active compounds, those whose reaction curves failed to reach one half of the plateau level of the positive control, and for which a PC50 could not be calculated. In some cases, although a PC50 could not be determined, the reaction curves did plateau (Estrone (THS00022), RU486 (HTS00087) and Cortisol (HTS00088), in the androgen agonist assay), and so an EC50 could be calculated (see table 2 and HTS00022, HTS00087, HTS00088 on Appendix 7). Comparing chemicals with weak and strong activity on the basis of EC50 determinations can be controversial and cause some with genuine activity to be disregarded. The PC50 ranking is a practical approach and weaker compounds can be qualified in terms of PC40 (40% of positive reaction) or PC30 (30% of positive reaction). The results presented above show that

both cell lines can distinguish compounds with strong activity and with weak activity, and the results can be used to rank the compounds.

As pointed out in our report on high throughput screening, others have developed cell lines with stable transfected reporter genes. These include MCF-7 (4), Hela (5), T47D (6), and PC-3 (7) cells. These lines all express multiple steroid receptors. For example, T47D cells express ER-alpha and ER-beta, androgen, progesterone and retinoic acid receptors. Therefore, in the assays, cross- reaction may be observed, and it is impossible to distinguish whether ER-alpha or ER-beta has bound ligand. PC-3 cells actively metabolize steroids, and so natural ligands like testosterone and 5alpha-DHT cannot be used as standards. In contrast the CHO-K1 cells do not metabolize steroid hormones and do not express endogenous steroid receptors. Thus it is possible to measure the signal from only the transfected receptor.

CONCLUSION

This method is suitable for high throughput screening applications, and generates reliable data.

POSTSCRIPT

We continue to improve our system. Recently we have derived clones that give stronger signals on receptor activation, and thus are more sensitive. We are now preparing cell lines that express both EGFP and the reporter system simultaneously, and our preliminary results are promising. These will be developed for receptor antagonist activity assays, similar to those described in the transfection assay system.

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