Appendix B3

Development of new reporter gene assay systems for screening Endocrine Disrupters

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EcoScreen assay ™ (high throughput transfection assay)

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INTRODUCTION

There is a great need for effective in vivo screening methods for detecting (anti)estrogenic and (anti)androgenic chemicals. We have developed rapid and sensitive reporter gene assays for detection of the chemicals that have agonist and antagonist activity against the estrogen, androgen, and thyroid hormone receptors. We believe that these methods have the potential to become powerful tools for identifying endocrine disrupters.

MATERIALS and METHODS

Chemicals

17-beta-estradiol, 5-alpha-dehydroxy testosterone, T3, T4, dimethylsulfoxide (DMSO) and rat S-9/Cofactor A Set were from Wako (Osaka, Japan). MTT was from Dojin (Osaka Japan) ALAMABLUE™ from Serotec (Oxford UK). The test solutions were prepared from stock solutions in DMSO and then 10 times serial dilutions were made with DMSO and finally diluted 100 times in the culture media with no supplement (the final DMSO concentration in the media was 1.0%). The test samples were adjusted to the concentrations ranging from 10^{-11} M to 10^{-5} M.

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

The estrogen receptor agonist assay and androgen receptor agonist assay were carried out on 61 chemical compounds (See Appendix1 CHEMICAL LIST) designated by the Japanese Ministry of Economy and Industry for studies on the feasibility of screening for endocrine disrupters. All measurements were done in quadruplicate. We repeated this assay 2 times, and the results were in very good agreement. The configuration of the samples on a 96 well plate is shown in Figure 1-a and 1-b. The wells in row H contained positive and negative controls. Results are shown in figures in the appendix. Samples were identified as HTS “No”.

Samples for Estrogen and Androgen receptor antagonist activity detection assay

The samples listed in Table 1a, and 1b were examined for activity as antagonists for ER and AR as described below.

Plasmids

For estrogen receptor reporter gene assay

pGL3ERE-7: an estrogen responsive reporter plasmid harboring the TATA box from herpes simplex virus thymidine kinase (tk) promoter (1) and four copies of estrogen response element (2), linked to the luciferase gene.

pcDNA ER-alpha: mammalian expression vector for estrogen receptor-alpha with Zeocin resistant gene.

For androgen receptor reporter gene assay

pIND ARE B10: contains the hygromycin resistant gene and 4 copies of the androgen response element: (AGTACG nnn TGTTCT) from the C3 gene (3), linked to the luciferase gene.

pZeoSV2AR: An expression plasmid with the androgen receptor driven by the SV40 promoter, and the Zeocin resistance gene.

For thyroid hormone receptor reporter gene assay

PINDTRE: contains 4 copies of the thyroid response element TRE pal: GGTCATGACC)(5) linked to the luciferase gene.

pZeoSV2TR-beta: expression plasmid containing the thyroid hormone receptor-beta driven by SV40 promoter and the Zeocin resistance gene

For cell viability/non specific inhibition assay

pGL3 control: luciferase expression vector driven by CMV promoter

pcDNA-EGFP: mammalian expression vector containing the green fluorescence protein cDNA.

Estrogen Receptor Agonist Activity detection assay.

1st day: Chinese Hamster Ovary cells (CHO- K1) were maintained in DMEM/Fl2 supplemented with 100 U/ml penicillin, 100ug/ml streptomycin, and 10% fetal bovine serum. The cells were trypsinized and suspended at 1x 10^5/ml. They were seeded with 84 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.

2nd day:

Preparation of Plasmid cocktail: For one 96 well plate assay, 6 ug of pGL3ERE-7 and 60 ng of pcDNA ER-alpha (100:1) was added to a 1.5 ml eppendorf tube. The total volume of DNA solution was kept below 50 ul.

Preparation of transfection mix per one 96 well plate: 18 ug of nonliposamal transfection regent Fugene™ (Roche Diagnostic Corp. IN USA) were added to 660 ul of DMEM/F12 (with no supplement) in a small sterile tube. Then the plasmid cocktail (see above) was added to the tube and incubated for 25 min at room temperature.

Transfection: 6 ul of the transfection mix were added into each well of the seeded 96 well plate by multi channel pipet, and then incubated 3 hr. After incubation, 10 ul of

each chemical diluted with the culture media (see Chemicals) were added, and the cells incubated for 16-24 hr.
3rd day: Followed incubation, 100 ul of the luciferase substrate with cell lysis reagent Steady-GloTM (Promega) was added to all assay wells. After shaking at room temperature for 5 min. the luminescence was measured in an ARVO multi-label counter (Perkin-Elmer).
(see Appendix 2 for the scheme of the high throughput transfection assay)

Note 1
We found that the most stable and reproducible data were obtained when Nunclon™ plates (NalgeNunc, Denmark) were used. Plates from other manufacturers often gave high backgrounds, perhaps because ingredients in the plastic were stimulatory.

Note 2
Another source of variability is in the accuracy of dispensing the transfection mixture into the wells, if the distribution is done manually. The use of devices for automated delivery can reduce this source of error. However, if this is not feasible we have found that another seeding and transfection protocol is useful.

Alternative method for transfection
1st day: CHO-K1 cells were trypsinized and prepared at a density of 1x 10^5/ml. 11ml of cell suspension were placed in a sterile 50 ml conical tube (for one plate). The transfection mix was added (see original protocol) to the 50 ml conical tube, mixed gently, and incubated for 15 minutes. Then each well was seeded with 90 ul of cell suspension and incubated for 16-24 hr.
2nd day: 10 ul of test sample (see original protocol) were added to the wells and incubated for 16-24 hr.
3rd day: Same as original protocol.
(see Appendix 3 for the scheme of the alternative transfection method)
This method is easy to perform. Although the signal intensity may decrease because the transfection efficiency decreases, there is still sufficient intensity for measurement.

Estrogen Receptor Antagonist Activity detection assay

The protocol for antagonist activity detection assay is the same as agonist detection assay except that cell viability is evaluated by measuring the fluorescence of EGFP prior to the luminescence measurement. Only the differences between the protocols are described here.

1. Plasmid cocktail: 6 ug of pGL3ERE-7 and 60 ng of pcDNA ER-alpha + 480 ng of pcDNA-EGFP.
2. The test solutions were prepared using “Spiked Media” that contains 5x10^{-11} M of 17-beta-estradiol.
3. After the final incubation period of 24 hr, green fluorescence was measured (excitation: 485 nm, emission: 535 nm) prior to the luminescence measurement by the ARVO multi-label counter (Berthold).

Androgen Receptor and Thyroid Hormone Receptor Agonist / Antagonist Activity detection assay

They are the same as described above except for the use of different plasmids for expression of each receptor and the luciferase-reporter containing the corresponding hormone response element.

For Androgen Agonist detection assay

Plasmid cocktail: 6 ug of pIND ARE B10 and 240 ng of pZeoSV2AR.
5-alpha dehydroxy-testosterone (DHT) was used as a positive control.

For Androgen Antagonist detection assay

Plasmid cocktail: 6 ug of pIND ARE B10 and 240 ng of pZeoSV2AR + 480 ng of pcDNA-EGFP. The test solutions were prepared using "Spiked Media" that contains $5x10^{-9}$ M of 5-alpha-DHT

For Thyroid Hormone Receptor Agonist detection assay

Plasmid cocktail: 6 ug of pINDTRE and 120 ng of pZeoSV2TR-beta.
T3 was used as a positive control.

For Thyroid Hormone Receptor Agonist detection assay

Plasmid cocktail: 6 ug of pIND TRE and 120 ng of pZeoSV2TR-beta and 480 ng of pcDNA-EGFP
The test solutions were prepared using "Spiked Media" which contains $5x10^{-8}$ M of T3.

Cell proliferation assay for evaluation of cell viability in antagonist activity assay

CHO-K1 cells were transfected with 6 ug of pGL3 control plasmid and 480 ng of pcDNA-EGFP by the same method as the above-mentioned protocol with Fugene™, and cultured with different concentrations of DMSO, from 0% to 10%, for 24 hr. DMSO inhibits cell growth, and thus serves as a model for a non specific expression and growth inhibitor. Then the luciferase activity and EGFP fluorescence were measured. The MTT assay and ALAMARBLUE™ cell proliferation assay were also done in order to determine the reliability of the GFP assay as an indicator of nonspecific inhibition/cytotoxicity in the actual antagonist detection assays.

Data Analysis

Definition of PC50 (50% of Positive Reaction)

Chemicals that can be used to determine an EC50 (half maximal activity of a particular compound) are limited to a small number that have a similar activity/toxicity profile as E2. This is because the activity curves of many compounds do not reach a plateau before the maximum tolerated dose is reached. In order to compare the activity of chemicals whose activity does not reach a plateau at the maximum tolerated dose we have defined the PC50 as the concentration of compound that corresponds to ½ the value of the transcriptional activity of the positive control (10^{-9} M of 17-beta-estradiol). Thus the PC50 can be used to rank compounds when true half maximal values cannot be determined because of toxicity (Refer to fig. 2). This PC50 concept is based on the guideline of the Japanese Ministry of Economy and Industry. The use of the PC50 removes the requirement for a standard curve at every measurement. (Only a solvent control and positive control at plateau level are needed). We have found that there is

very little variance in the PC50 value from experiment to experiment, even with discernable variation in luciferase activity due to differences in culture condition and transfection efficiency.

To determine the PC 50, one concentration of the standard at the maximal activity level was included in each assay as a positive control (for ER assay: 10^{-9} M of 17-beta estradiol; for AR assay: 10^{-8} M of 5alpa-DHT). In each assay the reaction curve of the sample (ranging from 10^{-12} M to 10^{-6} M), and the ½ maximal point was determined by analysis of the data by a Cubic Spline Curve Fitting Method using soft ware designed by us.

RESULTS AND DISCUSSION

Estrogen receptor agonist activity

Table 2 shows the rank order of compounds that have ER agonist activity on the basis of the PC50 determination. DDT (HST0099) and DEE (HST0100) have detectable ER agonist activity, but do not reach the PC50 level (see Appendix 4: results of ER agonist assay). Assessment of compounds with weak activity requires the use of comparison standards adjusted to lower activity such as PC40 (40% of maximal positive reaction) or PC30 (30% of maximal positive reaction).

Androgen receptor agonist activity

Table 3 shows the rank of the compounds that have AR agonist activity on the basis of the PC50. Most of the listed compounds were natural ligands or synthetic steroid hormones. The results are shown by the graph in appendix 3. Progesterone (HST0008) and Aldosterone (HST0009) showed only slight reaction, and RU486 (HST0087) and Cortisol (HST0099) were about 40% of the reaction of positive control at the highest concentration (10^{-6} M).

Thyroid hormone receptor agonist activity

Four sub types of the thyroid hormone receptor (TR) are known: alpha1, alpha2, alpha3, and beta. We have performed assays with reporter plasmids for TR-alpha1and beta. Figure 3 shows the result of agonist assay of TR-beta receptor. There was about a 20 fold induction relative to the solvent control (0.1%DMSO) with 100nM T3, with a detection limit of 500 pM and PC50 of 2 nM. We have not carried out large-scale screening for TR receptor. However, after testing about100 compounds, we found that only T3 and T4 had clear agonist activity.

Determination of non-specific inhibition/cell toxicity in the antagonist activity assay

In the antagonist activity assay a constant amount of the standard ligand was added to the test sample containing the unknown. The antagonist activity was observed as a decline in the luciferase activity. It is essential to distinguish a decline in luciferase activity due to true receptor antagonism from the non-specific inhibition of expression or cell toxicity that some compounds display.

Fig.4 shows how the reporter activity was affected by the nonspecific inhibitory activity of a test sample, using DMSO as an example. CHO-K1 cells were transfected with pcDNA-EGFP (green fluorescence protein expression plasmid) and pGL3 control (luciferase expression plasmid), and were cultured in various concentrations of DMSO. Expression of the markers in both plasmids is constitutive. In 4% DMSO the activity of EGFP and Luciferase fell to 5% or less of control. However, the ALAMABLUE™ assay and MTT assay reported 56% and 88%, respectively, of the 0% of DMSO control. This experiment showed that expression of genes on the plasmids was more sensitive to DMSO than the other assays. The MTT assay, which measures the reduction activity of the intracellular dehydrogenases, is widely used as an index of the cell proliferation or cell number. The ALAMABLUE™ assay is a simple method suitable for measuring large number of samples, and is said to be well correlated with the MTT assay. The ALAMABLUE™ method measures change of the reduction/oxidation state of the culture environment as a result of cell proliferation. However, our results indicate that these assays are not reliable indicators of nonspecific inhibition of plasmid gene expression. The pattern of decline in expression of EGFP was in good agreement with that of luciferase. Consequently we monitor nonspecific inhibitory/cell toxicity effects of the samples by measuring the expression of EGFP in the receptor activity assays. This is straightforward, and can be performed on living cells in a 96 well plate format. Usually this measurement is taken just before measuring the activity of luciferase. The advantage of this strategy is that both EGFP and luciferase assays can be performed on the same cells.

Estrogen receptor antagonist activity

Fig.5a shows the result of the estrogen receptor antagonist assay for tamoxifen (CAS No.10540-29-1: anti-cancer drug). The GFP fluorescence is shown by the green line, and the luciferase activity by the yellow line. At the concentration of 10^{-7} M, the luciferase activity was18% of the control, while the GFP activity was about 100%. This shows that tamoxifen is an antagonist of the estrogen receptor. The antagonist activity of 4-hydroxy tamoxifen is about 100 times stronger than that of the tamoxifen (Fig.5b). With triphenyltin chloride (fig.5c), at 10^{-6} M, GFP showed 93% of activity, while the luciferase activity was about 75% of control. In another set of experiments we compared the antagonistic activity of a styrene dimer with, or without, metabolic activation by incubation with a rat liver S9 preparation. We found that without S9 treatment 1-Methly-1-phenylindan (styrene dimer, 10^{-6} M) showed no receptor antagonistic activity, while with S9 treatment the activity of GFP (red triangle) was 91% of control while the luciferase activity (blue triangle) fell to 60%. Although this requires additional study, metabolites of this compound may have weak antagonist activity against the estrogen receptor, while the parent compound does not.

androgen receptor agonist activity

Fig 6a-h shows the results of the androgen receptor antagonist detection assay. Cyproterone acetate (fig.6a) showed the strongest antagonist activity to the androgen receptor of all the compounds we have tested. Consequently we use cyprotenone acetate as a positive control in every measurement. Two pesticides, hydramethylnone (fig.6b) and tralomethrin (fig.6d), were judged to have no true antagonist activity, because the

decline in the luciferase activity was matched by the decline in GFP activity. Two pesticides (CNP: fig.6c, fenitrotion: fig.6e) were clear antagonists. At 10^{-6} M GFP expression was unaffected while luciferase activity was reduced to 23% (CNP) and 13% (fenitrotion). Weaker antagonistic activity was shown by prothiofos (fig.6f) and vinclozolin (fig.6g).

Spironolactone (fig.6h) gave a biphasic activity curve. At low concentrations (10^{-8}–10^{-6} M) in the presence of testosterone it was an antagonist, while at higher concentrations the antagonistic activity was reversed. In the absence of testosterone it was an agonist at high concentrations (10^{-5}–10^{-6} M). These results suggest that for some compounds the definition of antagonist and agonist will have to be qualified by an indication of concentration and the presence of other ligands.

CONCLUSION

Other reporter cell lines that constitutively express steroid receptors, in some cases several receptors, have been developed. For example, T47D expresses ER-alpha and ER-beta, Androgen, Progesterone, and Retinoic acid receptors. There is a Hela cell derivative that expresses the glucocorticoid receptor, while MCF-7 naturally expresses ER-alpha and ER-beta receptors. These lines will report the activity of compounds that stimulate any of the receptors and cannot distinguish which receptor(s) have been stimulated. The strategy we have employed measures the signal from only the receptor introduced by transfection since the CHO cells do not express any endogenous steroid receptor.

Our method can be considered a “ high throughput transfection assay ”. Generally these methods are thought to suffer from variability and lack of reproducibility, due in part to toxicity of the transfection reagent, and uncontrollable variation in cell culture conditions. However we have found that recently developed transfection reagents solve many of these problems. FuGene™ is in one of these reagents. A reporter gene assay using FuGene™ has been reported previously by Vingaard (4). This reagent does not show any toxicity to the cells and if methods for accurate delivery of reagents are established there is little intra-assay variation in measurement. We have found that the average intra-assay coefficient of variation was only 5.9% (CV5.9%) in assays of over a hundred compounds. Our method is simple and affords a significant reduction of lab work and produces reliable data. Actually our method does not have any medium exchange and plate washing step after seeding a cell on 96 well plate until measuring luminescence. If the measurement is carried out on the concentration of 4 doses in duplicate the cost per sample (except for personnel expenses) will be $10 or less. In conclusion, our method is suitable for pre-screening a large number of environmental chemicals and should identify compounds that need further testing.

REFERENCES

[1] Altschmied, J., Duschl, J. (1997) Biotechniques 23, 436-438.
[2] Kumar, V. J. and Chambon, P. (1988) Cell 55, 145-156.

[3] Reese, C. J. and Katzenellenbogen, S. B. (1992) Mol. Cell. Biol. 12, 4531-4538.
[4] Vinggaad, A.M., Joergesen, E.C.B. and Larsen, J.C. (1999) Toxycol. Appl. Pharm. 155, 150-160.
[5] Jeannin, E., Robyr, D., and Desvergne, B. (1998) J. Biol. Chem. 237, 24239-24248.