Efficacy of Highthroughput Pre-Screening Procedure Based on Reporter Gene Assay

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Thank you, Dr. Kanno for your kind remarks and giving me this opportunity to make a presentation in this symposium.

I would like to talk about the efficacy of HTPS procedure based on reporter gene assay. Most of our works are conducted as joint development project with the Japanese Ministry of Economy, Trade and Industry, METI, and Ministry of Health, Labor and Welfare, MHLW, so I introduced the joint project at first.

The joint development project of HTPS system for endocrine modulating chemicals with METI and MHLW started in 1998. The aim of this project is to establish a high throughput pre-screening procedure for prioritizing chemicals to be tested in higher screening stages. Validation works of this assay system was mainly supported by MHLW, and development work and DATA collection work of this system were mainly supported by METI, Japan.

The current status of HTPS systems are shown in this slide. A human ER α cell line was established, and examinations with more than 500 chemicals were completed within last fiscal year, and data analysis is in progress now.

Transient transfection system with rat ER α, human AR, medaka ER α, mamichog ER systems are already established. Totally, more than 1,000 chemicals will be examined with human ER α, human AR, and human TR α systems. Now we show the results of comparative studies of ER binding assay and reporter gene assay with 500 chemicals.

Pilot chemicals were consisted of several categories listed here: aliphatic compounds, steroids, benzene derivatives, polycyclic aromatic compounds, condensed polycyclic compounds, and other types of chemicals. Numbers in parentheses show the number of chemicals belonging to each category.

Competitive receptor binding assay using radioisotope labeled 17 β-estradiol was conducted using human ER α ligand binding domain, expressed in E. coli. Reporter gene assay was conducted with stable transfected cell line bearing Viterogenin ERE upstream of luciferase gene and human estrogen receptor α expression construct. We confirmed this cell line was stable at least 4 months with over 30 passages.

In our reporter gene assay system, PC values were employed to express the hormonal activity of chemicals. This slide shows the description of PC50 and PC10 values. The left figure shows an example of chemical induced dose responses. The right figure shows the positive and negative control responses in each assay.

These PC values were defined as the test chemical concentrations estimated to cause 50% or 10% activity of positive control response. We routinely use 100 pM or 1 nM E2 as positive control. These PC values can be calculated using simple linear regression between two variable data points.

This figure shows the distribution of chemicals having PC50 values and binding affinity to ER. The y axis indicates the reciprocal number of PC50 in reporter gene assay, and the x axis indicates the relative binding affinity to ER.

In the overall aspect, there is a J shaped correlation between these two parameters, and some exceptions belonging to Area 1 and Area 2 exist. Chemicals in Area 1 are defined as the chemicals, which

cause stronger hormonal activity than the expected activity by binding affinity. Area 2 is contrary to Area 1.

This slide shows the structure of chemicals belonging to Area 1. These chemicals have particular structures; namely, these are consisted of ester derivatives of hydroxyl group of carbon-3 or carbon-17 of estradiol. So we think these chemicals were hydrolyzed in the medium or in the cell to be the potent estrogenic compounds.

This slide shows the results of ER binding assay with several types of butyl phenols. Among these types of butyl phenols, para isomers possess binding affinity to ER. However, ortho and meta isomers lack of binding affinity to ER.

As shown in this figure, a similar tendency was noted in the reporter gene assay. Moreover, these results suggest that the branch type of alkyl chains contribute to the estrogenic potency of chemicals.

In our next trial, rat ER mediated reporter gene assay results were compared with that of an *in vivo* screening test, uterotrophic assay using immature female rats. This slide shows the relationship between the results obtained from these two assays.

Twenty-five test chemicals were consisted with typical steroids, alkyl phenols and the other types of chemicals. PC50 values can be calculated in 8 chemicals out of 25 chemicals, and PC10 values can be calculated in 16 chemicals. 15 chemicals caused a positive reaction in uterotrophic assay. These results indicated that the positive chemicals based on the PC10 values were almost completely corresponding to the positive chemicals in the uterotrophic assay.

Next, we examined the efficacy of endocrine disrupter screening test in detecting anti-estrogenic effect using all *trans* retinoic acid. *Trans* retinoic acid is known to cause anti-estrogenic activity via their receptors or interfere with estrogenic action at estrogen responsive element level.

This slide shows the result of ER binding assay and reporter gene assay of *trans* retinoic acid. The left figure shows the result of binding assay; the open circle indicates the result of *trans* retinoic acid, and the closed circle shows the result of E2. The right figure shows the antagonist assay results for the reporter gene assay. Both x axes are showing log concentration of chemicals.

As you can see, this slide shows a complete lack of binding affinity to ER. *Trans* retinoic acid exhibits clear antagonist activity in reporter gene assay.

This slide shows the results of uterotrophic assay using immature female rats on *trans* retinoic acid. In the agonist assay, *trans* retinoic acid caused no agonist activity. In the antagonist assay, we confirmed the anti-estrogenic activity of this chemical.

Then, we conducted the gel mobility shift analysis to elucidate the mechanism of antagonist activity of *trans* retinoic acid. In this assay, *trans* retinoic acid caused no effect on the binding capacity of ER to ERE.

Results of the series of predictive tests on *trans* retinoic acid such as binding assay, reporter gene assay and uterotrophic assay, suggested that *trans* retinoic acid caused anti-estrogenic activity without ER ligand interaction.

In any case, a screening strategy for endocrine disrupters should be designed to detect various kinds of chemicals possessing endocrine modulating activity, including retinoid-like endocrine modulator. Consequently, the reporter gene assay may be a promising and effective pre-screening procedure because it can be adopted in high throughput pre-screening process with high sensitivity and high selectivity. Moreover, it requires no use of experimental animals.

Finally, I would like to acknowledge the people in our laboratory listed here, especially these technical staffs contributed to the study with their excellent skills. I also express my great appreciation to the Japanese METI and MHLW because most of our work is supported by these government offices. My talk is over. Thank you for your attention.

Q&A

Kanno: Thank you very much. Comments and questions, please.

Sone: Sone from National Institute for Environmental Studies. In the last slide, we heard that the Gelshaft assay might be an effective means of screening. Did you conduct a non-RI Gelshaft assay? Also, I think a non-RI assay should be conducted in order to have a wider range of screening. What do you think of it?

Takeyoshi: The Gelshaft assay was not conducted as a screening test. It was rather a test to look at functions of the anti-estrogenic effect of retinoic acid. As for the other question, the Gelshaft assay was conducted using a fluorescent probe.

Aoki: Aoki from National Institute for Environmental Studies. In the first place, I hate to ask questions that seem argumentative, but am I correct in saying when trying to find 50% effective concentration, you used a 50% concentration for maximum activity of estradiol for all compounds?

Takeyoshi: Yes.

Aoki: If memory serves me correctly, in textbook terms, we are talking about a partial agonist. I think the more accurate value for maximum activity of each respective compound was obtained at a 50% concentration. What is the basis for using 50% as the standard for maximum manifestation of estradiol?

Takeyoshi: We adopted EC50 because it is the most commonly used parameter. The method calls for solving for 50% concentration for the dose response curve using a logistic equation. This method however may not yield values for weak estrogenic chemicals in some cases. We have conducted a study of correlation of EC50 and PC50, and found there is a strong correlation between the two. We can therefore assess a large number of compounds using the concept of PC50.

Aoki: Are you doing this from the standpoint of convenience of assessment.

Takeyoshi: That's correct.

Aoki: Thank you very much.

Q: My question regards the apparent failure of the uterotrophic assay to pick up, as I read the slide, daidzein. Now that is somewhat weaker than genistein. So if you administered SC, did you use doses in excess of 100 mg/kg/day? Because the general literature and some other work would say at those approximate doses and above, that should have been a positive.

Takeyoshi: In our uterotrophic assay in this study, we conducted the uterotrophic assay using a fixed dose method, with highest dose of 200 mg/kg/day. So the negative result of daidzein is dose related phenomenon. So the positive uterotrophic reaction in daidzein is reported at 500 mg/kg/day.