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Dr. William S. Stokes
National Toxicology Program (NTP) Interagency Center for the
Evaluation of Alternative Toxicological Methods (NICEATM)
P.O. Box 12233, MD EC-17
Research Triangle Park, NC 27709

Dear Dr. Stokes**:**

Subject: Otsuka's Comments on the ICCVAM Endocrine Disruptor Expert Panel Report

We would like to respond to the list of recommendations and prioritizations issued by the ICCVAM panel. We believe that our assay systems satisfy most of the committee's concerns. In addition to our comments listed here we have included FIVE figures which illustrate our assays and support of the following discussion:

The Panel stated that the ideal cell line should have:

Little metabolic activity

Cytochrome P450 levels in CHO cells are too low to be detected spectrophotometrically. These cells are commonly used as hosts for the expression of genes encoding drug-metabolizing enzymes.

An endogenous wild type hAR, with little or no PR protein. The panel noted that some low level of GR was unavoidable.

The Otsuka AR-EcoScreen cells (the stably transfected cells) use an ARE for which the AR has high affinity. Thus there is a strong response to DHT. In contrast, activation by the GR is relatively low. This is shown in the comparison of induction by DHT and dexamethasone (Fig 1). We believe this compares quite favorably with

the assays developed at NIEHS. In both those systems the ARE is from MMTV, which is quite responsive to GR. As a result induction by dexamethasone is much greater than by DHT.

The expression system should be introduced by adenovirus infection or be stably expressed (by construction of stable transfected cell lines).

We have described cells lines that stably express the reporter system with properties are entirely consistent with the goals of the Panel. The preference by the Panel for a transient transfection system utilizing adenovirus is, we suggest, based on a misunderstanding about current technology for transfection of plasmids. Plasmid preparation and purification is simple and rapid, and large stocks can be produced. Our assay procedure involves addition of plasmid and transfection reagent directly to the cells in the medium in which they were plated. No manipulation of the cells is necessary. State of the art reagents support highly efficient and reproducible transfection. We see a transfection efficiency CV of only 5% between the wells of a 96 well plate**.** In contrast, the viral infection method requires a series of washes prior to addition of virus. These can remove cells (a source of uncontrolled variation from well to well), and necessitates complete removal of the wash solutions (to avoid dilution of test samples and virus). Furthermore, the viral stock must be prepared from plaque purified isolates (to eliminate defective variants which accumulate during serial passage), followed by purification and determination of the titer of each preparation.

At least 20 fold induction with 0.1-1nM R1881/DHT

Our AR-EcoScreen system shows a 9-fold induction with 1nM DHT, and 5-fold induction with 0.1nM DHT. We believe that with some minor adjustments to the system the induction level will be doubled. At the same time we would argue that the crucial issue is the stability and reproducibility of the assay. Detection of compounds with weak activity is feasible if the assay is reliable and highly reproducible (see below).

Activity with estrogens and glucocorticoids

See above and Fig 1.

Large scale screening capability

Our assay has been established in a multi-well format, appropriate for automation. At this time we can screen 10,000 samples/assay/year. However this can be increased with automation. The list of receptor systems for which we have developed assays is shown in Fig. 2.

Patent restrictions

The AR patent does not claim the use of the AR cDNA for transcription assays. Instead the patent claims focus on the production of the AR protein. Consequently our patent counsel believes that the Otsuka technology does not infringe the AR patent.

Monitor of cytotoxicity

We use the GFP expression system to monitor toxicity as shown in Fig 3. Our comparison of different methods for this determination shows comparability between GFP and luciferase assays, which are superior to MTT and ALAMAR.

A 20 % inter- and intra- assay coefficient of variation.

The Otsuka transient assay system shows an intra-assay CV of 5.9%, and an inter-assay CV of 16-22%. Our stable transfected cell line has an intra-assay CV of 3.2% and an inter-assay CV of 8-14% (Fig. 4, 5). This compares favorably with the NIEHS systems in which the adenoviral transduction assay has an intra-assay CV of 34% and an inter-assay CV of 85%. The NIEHS stable cell line has an intra-assay CV of 28% and an inter-assay CV of 53%. The high CV values require very high induction/background ratios if the measurements are to be useful.

Weak agonists should increase induction by 2-3 fold, antagonists should decrease induction by 25%.

This was covered in our initial submission, but an example of measurement of antagonist activity is shown in Fig. 3. We have detected both weak and strong agonists and antagonists. The weak antagonists include Linuron with an IC40 (40% decreased induction) of 9.3 x 10-6 M, while 2,24,4-tetrahydroxybenzophenone had an IC 40 of 8.2 x 10-6 M.

It should be noted that the NIEHS stable transfected cell line has been transferred to the Tokyo Metropolitan Institute of Hygiene in Japan. At the recent meeting of the Japan Society of Endocrine Disrupters Research (Hiroshima, November 26, 2002) this laboratory reported that the Otsuka system was 10 fold more sensitive than the NIEHS cell assay.

We believe that our assay systems satisfy the requirements for simplicity, reproducibility, high throughput potential, and with monitors for toxicity. We continue to improve the assays but we suggest that they can be productively and reliably applied at this time.

Thank you for your consideration.

Sincerely,

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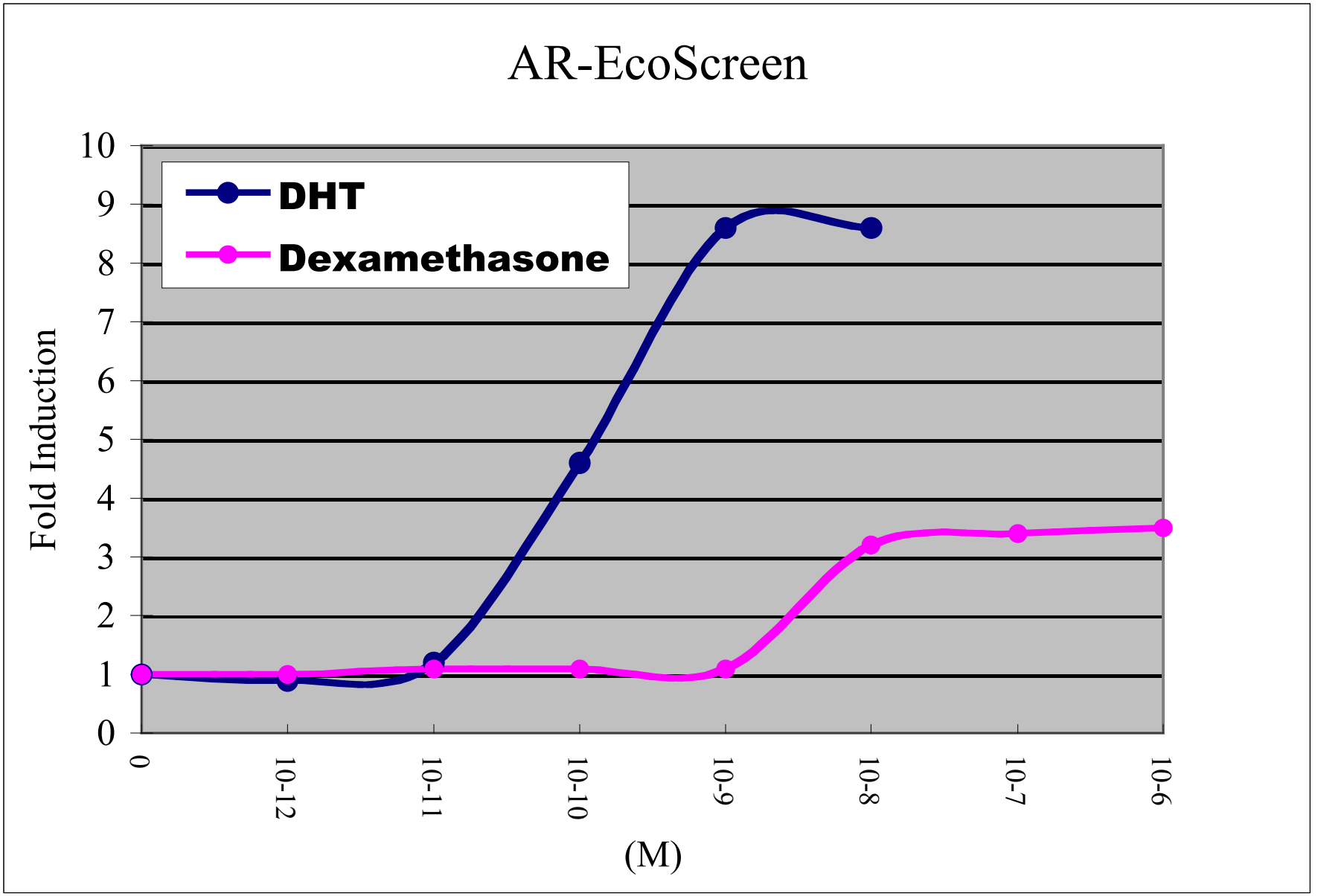

AR-EcoScreen
Fold Induction
10
9
8
7
6
5
4
3
2
1
0
DHT
Dexamethasone
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
(M)

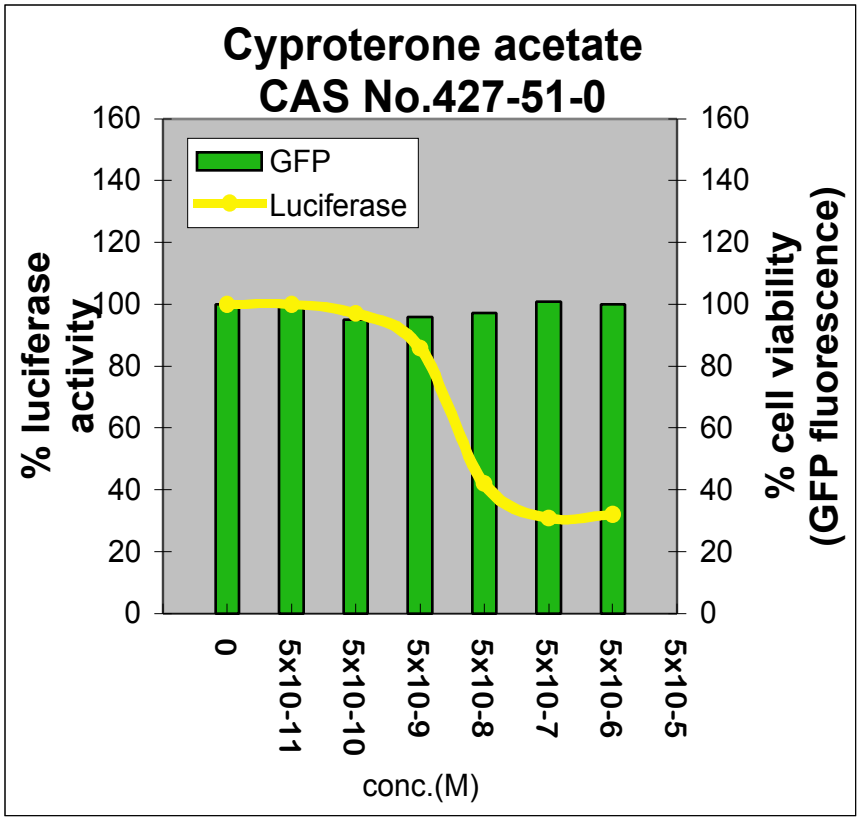
Fig 1

Otsuka has already developed following TA assay for screen EDs

- Estrogen Receptor α
- Estrogen Receptor β
- Androgen Receptor
- Thyroid Hormone Receptor α1
- Thyroid Hormone Receptor β
- TSH Receptor

Fig 2

Examples of AR antagonist assay using EGFP for toxicity monitoring



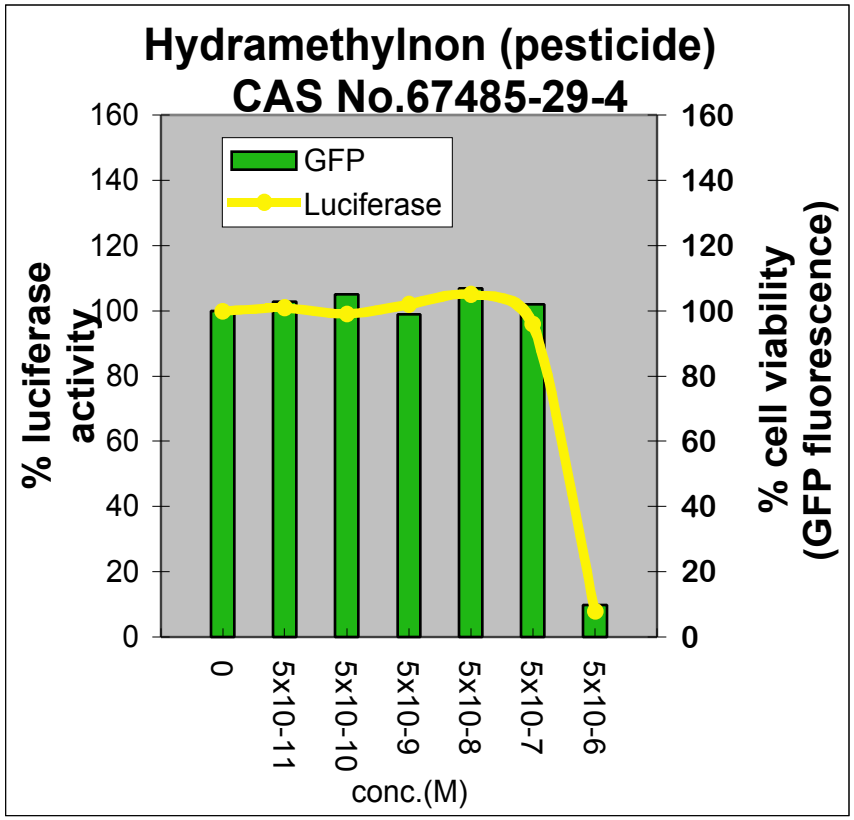


Fig 3

Reproducibility of ER/AR-EcoScreen™

Stably transfected cell lines

Intra-assay

ER assay CV 2.3% (average 30 data in quadruplicated)

AR assay CV 3.2% (average 30 data in quadruplicated)

Inter-assay

EC50 value of E2 and DHT in different day attempt

ER assay CV 14.3% (5 different attempt)

AR assay CV 7.9% (8 different attempt)

Fig 4

Reproducibility of EcoScreen™

high throughput transfection assay

Intra-assay

CV 5.9% (average CV in assays over a hundred compounds in quadruplicated)

Inter-assay

compounds	n-octhylphenol	Dibuthyl phthalate
Day1	1.53	5.86
Day2	2.10	4.03
Day3	1.86	4.11
mean	1.83	4.66
CV(%)	**15.6**	**22.2**

EC50 values are shown ($x10^{-6}$ M)

Fig 5