

APPENDIX F

Public Comments in Response to the *Federal Register* Request for Comments

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December 19, 2002

TO: Dr. William S. Stokes, Director of NICEATM
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FROM: Dr. Charles B. Breckenridge
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SUBJECT: COMMENTS ON THE LISTING OF ATRAZINE ON THE ICCVAM EDWG
PROPOSED LIST OF SUBSTANCES FOR VALIDATION OF *IN VITRO*
ENDOCRINE DISRUPTOR METHODS.

Atrazine was selected as one of 9 pesticides on the ICCVAM EDWG proposed substance for validation of ER and AR binding and transcriptional activation assays. In that regard, atrazine is listed in Appendix A (ICCVAM EDWG Proposed Substance for Validation of ER and AR Binding and Transcriptional Activation Assays) as a chemical with an anticipated *in vitro* response in the ERTA and ARTA and/or binding as positive. The basis for these conclusions can be purported found in a summary file of *in vitro* data for NICEATM (http://iccvam.niehs.nih.gov/methods/endodocs/ed_brd.htm). However, when one examines the basis for these assumptions, the weight of evidence would support that atrazine does not bind to the estrogen receptor either in ERTA or ER cytosol. In fact, atrazine did not bind to the human ER α transfected to CHO-K1 cell (Otsuka Pharmaceutical, 2001), human ER transfected to HeLa cell (Balaguer et al., 1996), human ER α transfected to MCF-7 cells (Connor et al., 1996; Soto et al., 1995), and human ER transfected to yeast (Graumann et al., 1999). The only positive response was observed in rat ER transfected to yeast (Petit et al., 1997). Besides, the work by Graumann et al. (1999) with human ER transfected with yeast, Connor et al. (1996) also used an estrogen-dependent recombinant yeast strain PL3; these authors found estrogen-dependent PL3 yeast strain was not capable of growth on minimal media supplemented with atrazine in place of E2. Therefore, it would appear more appropriate to list atrazine as negative in the ERTA and /or binding assays and unknown in the ARTA and /or binding assays. In addition, atrazine under *in vitro* data (NICEATM) in Appendix A, binding; atrazine is identified as weakly ER+/AR+; there not basis for this supposition as atrazine was found not to bind to ER isolated from rat uterus (Tennant et al., 1994).

Also in Appendix A, under studies proposed by the U.S. EPA, atrazine was slotted for an AR binding assay, pubertal male assay and potentially for the *in utero* through lactation assay. The AR binding assay, although anticipated to be negative, may add value if completed, the pubertal male has been completed (Stoker et al., 1999), and the *in utero* through lactation assay as a screen

is far from being validated, is not needed as a test, and should not be used for evaluating the substance on the ICCVAM EDWG proposed substances list.

Thank you for your consideration of these comments.

Sincerely Yours,

Charles B. Breckenridge, Ph.D.
Head, Global Risk Assessment Methodology
Syngenta Crop Protection, Inc.
Greensboro, NC 2741

References:

Balaguer P, Joyeux A, Denison MS, Vincent R, Gillesby BE, Zacharewski T. 1996. Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using *in vitro* recombinant receptor-reporter gene assays. *Can J Physiol Pharmacol* 74(2):216-22

Connor K, Howell J, Chen I, Liu H, Berhane K, Sciarretta C, Safe S, Zacharewski T. 1996. Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses in vivo and *in vitro*. *Fundam Appl Toxicol* 30(1):93-101.

Graumann, K., Breithofer, A., & Jungbauer, A. Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds. *Sci. Total Environ*, 1999, 12, 225, 69-79

Otsuka Pharmaceutical, 2001

Petit et al., 1997

Soto et al., 1995

Stoker, TE, Laws, SC, Guidici, DL, and Cooper, RL. 2000. The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function. *Toxicological Sciences* 58: 50-59.

Tennant MK, Hill DS, Eldridge JC, Wetzel LT, Breckenridge CB, Stevens JT. 1994. Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. *J Toxicol Environ Health* 43(2):197-211.

Dear Sir / Madam,

Please let me introduce myself. I am the Product Manager for Amersham Biosciences' range of Biotrak Assays.

You may be aware of Amersham Biosciences' active presence in the immunoassays market. Amersham's Biotrak range of assays are targeted towards a range of important therapeutic targets, many using novel patented detection technology.

I have been very interested to read about your proposed list of current and new endocrine disruptors. Unfortunately, I am not sufficiently qualified to comment on such an area. My major interest is however in the assay detection technologies.

As far as I can understand, the current NIEHS endocrine disruptor, receptor binding assays use a radiolabelled ligand in a filter binding assay format. Conscious of the fact that such heterogeneous assays involve a considerable amount of 'hands-on' washing time, I would like to introduce you to Amersham's patented Scintillation Proximity Assay (SPA) format.

SPA's are homogeneous assays following exactly the same reaction kinetics as conventional receptor binding assays, but without any washing steps. The assays use glass beads (5 to 10µM diameter), impregnated with a highly efficient scintillant. The beads are directly coated with the specific receptor of interest and form one of the components of a typical receptor binding assay format. Tritium or [125] iodine ligands are used in the assays. After an appropriate incubation period, those radiolabelled ligands bound to the beads result in a detectable scintillation event. Any unbound ligand will not be in close enough proximity to the bead to generate a scintillation event. SPA's are true homogeneous assays and due to the absence of washing steps, are fully amenable to automation.

Amersham Biosciences SPA technology has already been used by a number of pharmaceutical companies for receptor binding assays. The following publications illustrate these specific receptor binding assays:

P. Coward et al., PNAS, Vol. 98, No 15., pp. 8880-8884 (2001). (Estrogen-related receptor) J. Osmond et al., Biology of Reproduction, 63, pp. 196-205, (2000). L. Moore et al., PNAS, Vol. 97, No 13., pp. 7500-7502 (2000). L. Moore et al., Journal of Biological Chemistry., Vol. 275., No 20., pp. 15122-15127., (2000)

Amersham are currently developing an estrogen receptor SPA for general availability. Given a common interest in this type of assay format, we would be very interested in hearing your views on this application of the SPA format. We would also be very happy to discuss any potential collaborative development projects, or reagent supply, that would be beneficial to both organisations.

I look forward to receiving any comments or ideas on potential collaborative projects that you may have in this area.

Yours faithfully,

Mike Sully

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December 6, 2002

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Re: APERC Comments on Proposed List of Substances for Validation of *In Vitro* Endocrine Disruptor Screening Methods (67 FR 64902; October 22, 2002)

Dear Dr. Stokes:

The Alkylphenols & Ethoxylates Research Council (APERC) appreciates the opportunity to submit comments on the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Endocrine Disruptor Working Group's "Proposed Substances for Validation of Estrogen Receptor (ER) and Androgen Receptor (AR) Binding and Transcriptional Activation (TA) Assays," October 16, 2002 (67 FR 64902). APERC represents the major manufacturers of alkylphenols and alkylphenol derivatives in North America. APERC members include: Dover Chemical Corporation; GE Plastics; Great Lake Chemical Corporation; Mitsubishi Chemical Corporation; Rhodia Inc.; Rohm and Haas Company; Schenectady International, Inc.; Stepan Canada; Sunoco, Inc.; and, The Dow Chemical Company. Information on APERC and its activities can be found at www.aperc.org.

Based on the recommendations of the ICCVAM Expert Panel and in consultation with the Endocrine Disruptor Working Group (EDWG), a combined list of proposed substances was developed to facilitate future validation of *in vitro* endocrine disruptor screening methods, which included *n*-nonylphenol, CAS number 104-40-5. Nonylphenol (NP) is produced by the reaction of phenol with branched nonene. The nonyl group is positioned predominantly in the *para* position on the phenol ring. Commercial synthesis results in a mixture of various branched nonylphenol isomers rather than one discrete chemical structure and is usually represented by CAS number 84852-15-3. Normal or *n*-NP represents a phenol group with a linear nonyl group. The ICCVAM and EDWG should be aware that this compound is difficult to produce and is therefore not likely to be commercially relevant. APERC considers CAS number 84852-15-3 to

Dr. William S. Stokes
 December 6, 2002
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be most descriptive of commercially available NP. Other CAS numbers are less descriptive with respect to the branching and position of the nonyl group on the phenol ring. The following table summarizes the CAS numbers that are commonly associated with NP.

CAS NUMBER	DESCRIPTION
25154-52-3	Phenol, nonyl- (Historically viewed as not descriptive regarding branching. EPA now assumes that CAS numbers that do not specify branching on alkyl groups represent linear structures. Not viewed as descriptive of commercial NP)
104-40-5	Phenol, 4-nonyl- (Assumes linear alkyl, not viewed as descriptive of commercial NP)
84852-15-3	Phenol, 4-nonyl-, branched (Viewed as descriptive of commercial NP)

The ICCVAM and EDWG should be aware that most of the *in vivo* endocrine research conducted on NP has used commercially available, branched NP when deciding which substances should be included in future validation studies of *in vitro* endocrine disruptor screening methods.

Please contact me at 732-557-5524 or blosey@regnet.com if you have questions or would like additional information about NP nomenclature, chemistry or sources.

Sincerely,

Barbara S. Losey
 Deputy Director



December 6, 2002

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Re: Federal Register / Vol. 67, No. 204 / Tuesday, October 22, 2002 /
Expert Panel Report on the Current Validation Status of *In vitro* Endocrine Disruptor
Screening Methods and a Proposed List of Substances for Validation of *In vitro*
Endocrine Disruptor Screening Methods

Dear Dr. Stokes,

The American Chemistry Council (ACC or the "Council") has played an active role in the development and implementation of the EPA's endocrine disruptor screening and testing program (EDSP) for several years¹. The Council strongly supports EPA's efforts to seek technical advice and recommendations from expert scientists and the public concerning matters related to the validation of endocrine disruptor screening and testing methods. ACC encourages the timely development and implementation of a scientifically robust EDSP.

The Council submits the attached comments on the Expert Panel Report on the Current Validation Status of *In Vitro* Endocrine Disruptor Screening Methods and a Proposed List of Substances for Validation of *In Vitro* Endocrine Disruptor Screening Methods.

With respect to the binding and transcriptional activation assays, we make three main points:

1. In accordance with The Food Quality Protection Act of 1996 (21 U.S.C. Section 346

¹ The Council represents more than 90 percent of the productive capacity for basic industrial chemicals within the United States and its members are the leading companies engaged in the business of chemistry. EPA's endocrine disruptor screening and testing program (EDSP) may significantly affect the Council and its members. For that reason, the Council and its members have attempted to assist the Agency in developing and implementing its EDSP. In that regard, ACC and its members actively participated in EDSTAC and are actively participating in EPA's EDMVS.



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(p)) and the ICCVAM Authorization Act of 2000 (42 U.S.C. 2851), EPA is obligated to validate a binding assay and a transcription activation assay for estrogen receptor ligands and for androgen receptor ligands if it intends to require submission of data from such assays as part of its EDSP.

2. There is an urgent need for EPA to validate a single technique for each assay. As was noted in the expert panel review, currently there exists significant variability of techniques and results, and to date, the inter-laboratory variability, sensitivity, reproducibility and precision of these techniques have not been sufficiently evaluated.
3. EPA needs to address patent restriction issues. It is essential that the assays required for regulatory programs are widely available and that they will not put the regulated community in jeopardy of patent violations in order to comply with screening and testing requirements.

With respect to the Proposed List of Substances for Use in Validation Studies, we comment that:

1. The first step towards evaluating substances to be used in standardizing and validating specific Tier 1 screening methods for the EPA's EDSP should be the development of criteria to select substances for the standardization and validation studies.
2. In compiling substances for standardization and validation, NIEHS and EPA must appropriately qualify and characterize any and all such lists. EDSTAC spent a great deal of time and effort addressing communications issues, and EPA should implement the EDSTAC recommendations to ensure proper understanding by the public of such a list of substances. The Council supports NIEHS' use of disclaimer language, but requests that such language be included in bold face, larger type as an integral part of the table, and not as a footnote.
3. Each entry in which reference is made to a particular hormonal mechanism of action or to potency or activity must be referenced. This is necessary for transparency and accuracy. Appendix A (ICCVAM EDWG Proposed Substances for Validation of ER and AR Binding and Transcriptional Activation Assays October 16, 2002) needs to be reviewed, citations added and any errors or omissions need to be corrected.

We urge NIEHS and EPA to carefully consider the following comments and recommendations. Please contact me directly if you have additional questions at (703) 741-5210 or Rick_Beckers@AmericanChemistry.com.

Sincerely,

Original Signed By

Richard A. Becker, Ph.D., DABT
Senior Director

Attachments

ACC Comments on:

**Expert Panel Report on the Current Validation Status of
In Vitro Endocrine Disruptor Screening Methods**

1. The Food Quality Protection Act of 1996 (21 U.S.C. Section 346 (p)) requires EPA to develop a screening program “using appropriate validated test systems” to determine whether certain substances have endocrine effects. In addition, the ICCVAM Authorization Act of 2000 (42 U.S.C. 2851) dictates that any new or revised acute or chronic toxicity test method, including animal test methods and alternatives, must be determined to be valid for proposed use prior to an Agency requiring, recommending, or encouraging the application of such test method. Thus, EPA is obligated to validate a binding assay and a transcription activation assay for estrogen receptor ligands and for androgen receptor ligands if it intends to require submission of data from such assays as part of its endocrine screening and testing program.

Before an assay can be used for regulatory purposes, its performance characteristics should be documented through a formal validation and standardization process. The goals and requirements of validation for regulatory use are different from and not fulfilled by the goals and requirements of validation for basic academic research. This is not to say that regulatory validation requires a higher standard of performance. Rather, the differences reflect the fact that assays for regulatory use must be reasonably resilient to small deviations in protocol and be amenable to standardized interpretation within narrowly defined limits. It is critical that EPA recognize that extensive use of any particular assay in basic academic research does not *de facto* validate its use for regulatory toxicity testing.

The requirement for regulatory assays to be amenable to a standardized interpretation within narrowly defined limits argues strongly for EPA to validate a single protocol for ER / AR binding and transcription activation assays. Merely adopting performance criteria for these four types of assays will not ensure that a standardized interpretation can be made. Without a standardized interpretation, confusion and controversy will abound and regulatory decision-making will be more contentious than ever. As was pointed out by an EDMVS panel member during the July 23rd 2002 meeting, only *after* a single, standardized, validated protocol has been in regulatory use for some time will meaningful performance criteria become clear, which can then be applied to potential alternative assays for ER / AR binding and transcription activation.

A definite set of pass-fail criteria should be elaborated for each *in vitro* test system/ methodology so as to minimize the potential confusion that may result from individual laboratory determinations. These would include criteria such as acceptable coefficients of variation (CVs), techniques for assessing cytotoxicity and definition of acceptable levels of cytotoxicity, required numbers of replicate data points per experiment, as well as cutoffs for designating a positive/negative response relative to defined controls.

2. There are at present several different methodologies for the performance of estrogen and

androgen receptor binding (Nikov et al., 2000; Blair et al., 2000; Nagel et al., 1997) and reporter gene transactivation assays (Pons et al., 1990; Zacharewski et al., 1994; Kelce et al., 1995; Gaido et al., 1997; Maness et al., 1998; Vinggaard et al., 1999). Although it has been demonstrated that alterations in specific assay parameters leads to significant variability (Beresford et al., 2000; Charles et al., 2000), to date, the inter-laboratory variability, sensitivity, reproducibility and precision of these techniques have not been sufficiently evaluated. This argues strongly for the need to validate a single technique for each assay.

EPA should be commended for making good progress toward validating and standardizing single rat estrogen receptor and androgen receptor binding assays. The use of recombinant receptor proteins for these assays should be encouraged in order to reduce use of animals and to more fully standardize components of the assay.

3. EPA needs to address patent restriction issues. It is essential that the assays required for regulatory programs are widely available and not put the regulated community in jeopardy of patent violations in order to comply with screening and testing requirements. In order to avoid potential US patent restrictions regarding the use of human cDNA sequence coding for human nuclear hormone receptors (and/or simultaneous co-transfection of receptor and reporter constructs; cis-trans technology), cell lines known to express endogenous human nuclear receptors are recommended. Cells expressing the human nuclear receptor of interest need only have the reporter gene introduced into them in order to be used for transcription activation assays. EPA and the EDMVS should focus on standardizing and validating these types of transcription activation assays for ER and AR as they are the most likely to be usable by the regulated community.

**ACC Comments on:
Proposed List of Substances for Use In Assay Validation Studies**

The American Chemistry Council believes the first step towards evaluating substances to be used in standardizing and validating specific Tier 1 screening methods for the EPA's EDSP should be to develop criteria to select substances for the standardization and validation studies. At this stage of early protocol development, the emphasis should be on using relatively well-characterized substances. Such substances should allow the EPA, the EDMVS and others to assess two essential aspects of the data to be generated: 1) the early performance and long-range promise of a particular protocol and 2) the commonality or differences of the protocols. ACC recommends the following selection criteria for consideration by the Agency. (Note – these criteria are for Tier 1 assay standardization & validation studies. Evaluation of Tier 2 tests may need dramatically different criteria and substances.)

1. The hormonal activity and mechanism of hormonal effect of a substance should already be known from both *in vitro* and *in vivo* research methods. There must be sufficient and robust information and data from scientific reports on each substance with respect to the hormonal mode of action, the hormonal potency and specificity and ADME2 characteristics. These data enable a prediction of results for the screening method and a reasonable assessment of protocol performance.
2. Substances selected must be readily available through commercial vendors. These substances are likely to be used over a number of years, in several protocols and by a number of laboratories as part of the standardization and validation program. Further, other labs will have an interest to establish and demonstrate their proficiency with these screening methods. Therefore, it is necessary to select substances which will be readily available through commercial sources presently and in the future.
3. The Agency must focus on substances with known estrogen, androgen and thyroid (EAT) activity, consistent with the Agency's EDSP Statement of Policy. The priority for the EDSP should be estrogen, androgen and thyroid hormonal activities or modes of action. The focus should be on direct modes of EAT actions and should include receptor agonists/antagonists and, if applicable, hormone synthesis inhibitors. Importantly, the Agency should avoid use of substances that exert endocrine effects via indirect modes or mechanisms (except to establish specificity, as described in point 7 below).
4. Substances with high specificity (either as agonists or antagonists) are preferred and should be used to the maximum extent practicable. In cases where the use of a mixed agonist/antagonist is necessary or where there are other overlapping specificities, EPA must select the concentrations and doses carefully, keeping in mind the effects such mixed activities may have upon the type, magnitude and nature of the response(s).
5. Substances with particular EAT activity should be evaluated in the appropriate screening method. While there may be some overlap, it is not necessary to use exactly the same set of substances in the validation of each screening method. For example, substances with

estrogenic activity should be used for validation of the uterotrophic assay, but it would make no sense to use the same complete set of substances in the Hershberger assay for androgens.

6. In general, validation must cover the entire range of activities anticipated from the population of substances that will be selected to be evaluated with the assay. Little or no confidence can be placed upon results of substances whose activities fall outside the activities or modes of action of the set of substances for which the assay has been validated. Further, the set of substances used for development and standardization of an assay should be different from the set of substances used for validation. In the validation series, the substances selected should include materials with a range of potencies; from strong to weak to completely negative for the appropriate EAT mechanisms.
7. It is essential to address the issue of specificity (false positive responses) in the validation studies of each assay. In particular, since the EDSP screening assays and the Tier 1 battery have been selected by EPA to minimize or eliminate false negatives, such characteristics will likely generate false positives. Therefore, in the validation of EDSP screening assays, it is critical to include substances that exert effects (and/or toxicity) by mechanisms that are not primarily hormonal in order to establish the specificity of the assay endpoints (e.g., evaluate potential for false positive responses due to a non-hormonal toxicity). In some cases it may be beneficial to establish specificity by evaluating, for example, a pure estrogen agonist in an assay designed for androgens (and vice versa).
8. EPA must coordinate its activities with the OECD EDTA with respect to study design, selection of substances and dose levels for assay validation. OECD has initiated (and for some assays, largely completed) validation studies using specific chemical substances. EPA's activities with respect to assay validation for the EDSP should demonstrate the Agency's strong support of international harmonization and mutual acceptance of data.
9. The approach EPA adopts for standardization and validation should be sufficiently rigorous to comply with generally recognized scientific principles of study design and conduct. With respect to test articles selected for EDSP validation, this should include knowledge of chemical purity, stability and concentration (particularly the applied or administered dose). In evaluating substances for potential selection for use in particular assays and routes of administration, EPA should consider what degree of analytical chemistry would be necessary to meet these recognized scientific standards.
10. In compiling substances for standardization and validation, NIEHS and EPA must appropriately qualify and characterize any and all such lists. EDSTAC spent a great deal of time and effort addressing communications issues, and both NIEHS and EPA should implement the EDSTAC recommendations to ensure proper understanding by the public of such a list of substances. We support NIEHS' use of the qualifying language, but suggest that such a descriptor be included as an integral part of the table, rather than as a footnote.
11. Each entry in which reference is made to a particular hormonal mechanism of action or to potency or activity must be referenced. This is necessary for transparency and accuracy.

This would permit members of the EDMVS (and the public) to readily access the citation and to review the actual study results (study design, dose levels, endpoints measured and results). This is critical and is necessary for selection of chemicals and dose levels for pre-validation studies – it is also important for constructing the predictive models. Appendix A (ICCVAM EDWG Proposed Substances for Validation of ER and AR Binding and Transcriptional Activation Assays October 16, 2002) should be re-examined, citations added, and any errors and omissions need to be corrected. In the comment sections, at times the terms weak and strong are used, but these are not explained anywhere in the table. Definitions should be added, and such terms should be used in a consistent manner. For example, in a comprehensive study of rat uterine ER receptor binding activity more than 180 compounds, Blair et al (2000) report that “none of the phthalates competed strongly for ER; however benzylbutyl phthalate and bis(2-ethylhexyl) phthalate [diethylhexyl phthalate] showed slight competition for the ER.” In addition, Zacharewski et al. (1998) found that none of eight commercial phthalate esters (including the three in Appendix A) elicited *in vivo* estrogenic responses. Yet in Appendix A, the descriptors for butylbenzyl phthalate and di-n-butyl phthalate do not reflect this minimal (if any) degree of activity.

Blair et al. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicological Sciences* 54:138-153.

Zacharewski T, Meek M, Clemons J, Wu Z, Fielden M, and Matthews J (1998). Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. *Toxicological Sciences* 46:282-293.

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December 5, 2002

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Evaluation of Alternative Toxicological Methods (NICEATM)
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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 9.3×10^{-6} M, while 2,24,4-tetrahydroxybenzophenone had an IC₄₀ of 8.2×10^{-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 Disruptors 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,

Mitsuru Iida, Ph.D.

Eco-Screen R&D Section, EDC Analysis Center.

Otsuka Life Science Initiative

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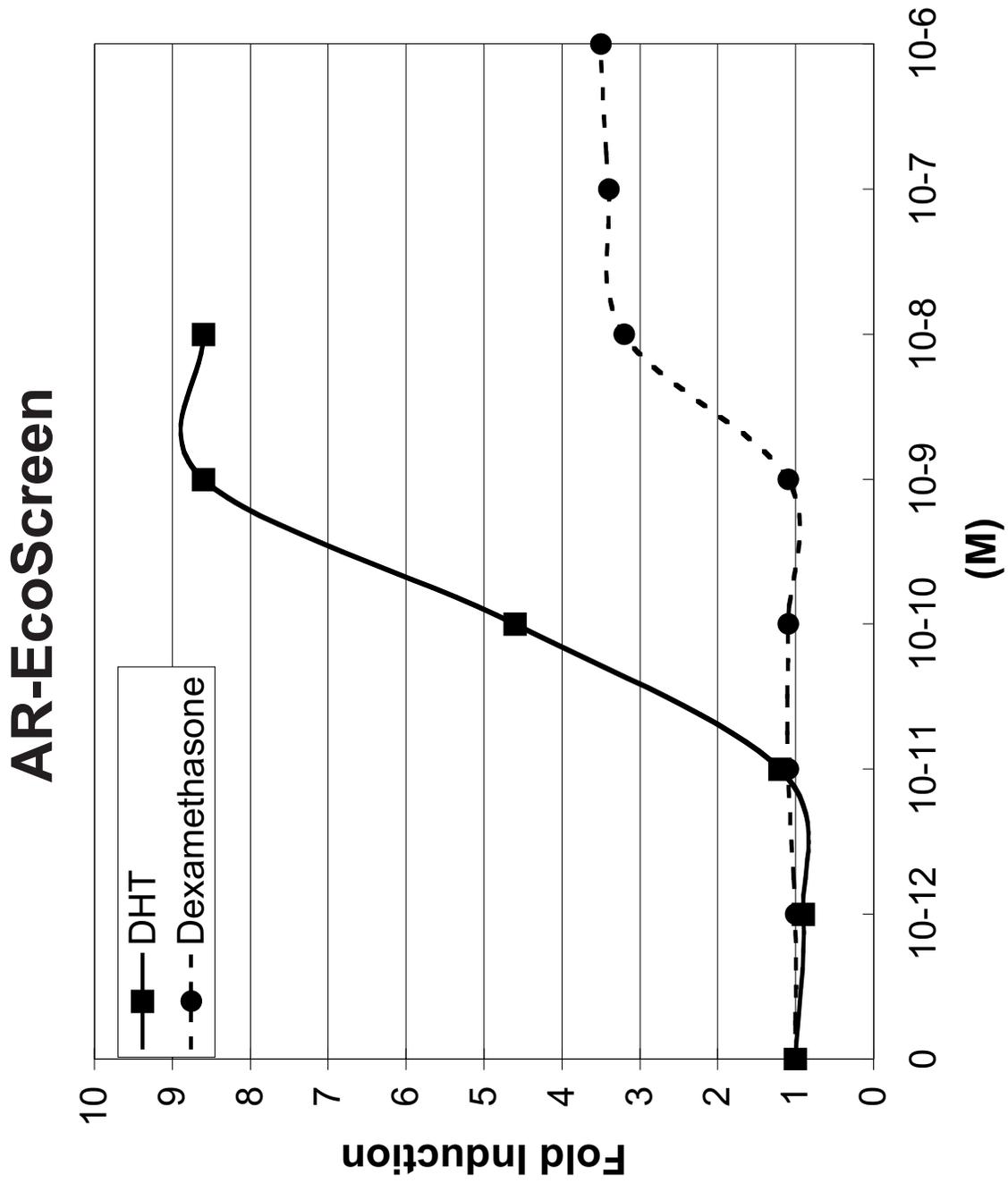


Fig 1

**Otsuka has already developed following
TA assay for screen EDs:**

- **Estrogen Receptor α**
- **Estrogen Receptor β**
- **Androgen Receptor**
- **Thyroid Hormone Receptor $\alpha 1$**
- **Thyroid Hormone Receptor β**
- **TSH Receptor**

Fig 2

Examples of AR antagonist assay using EGFP for toxicity monitoring

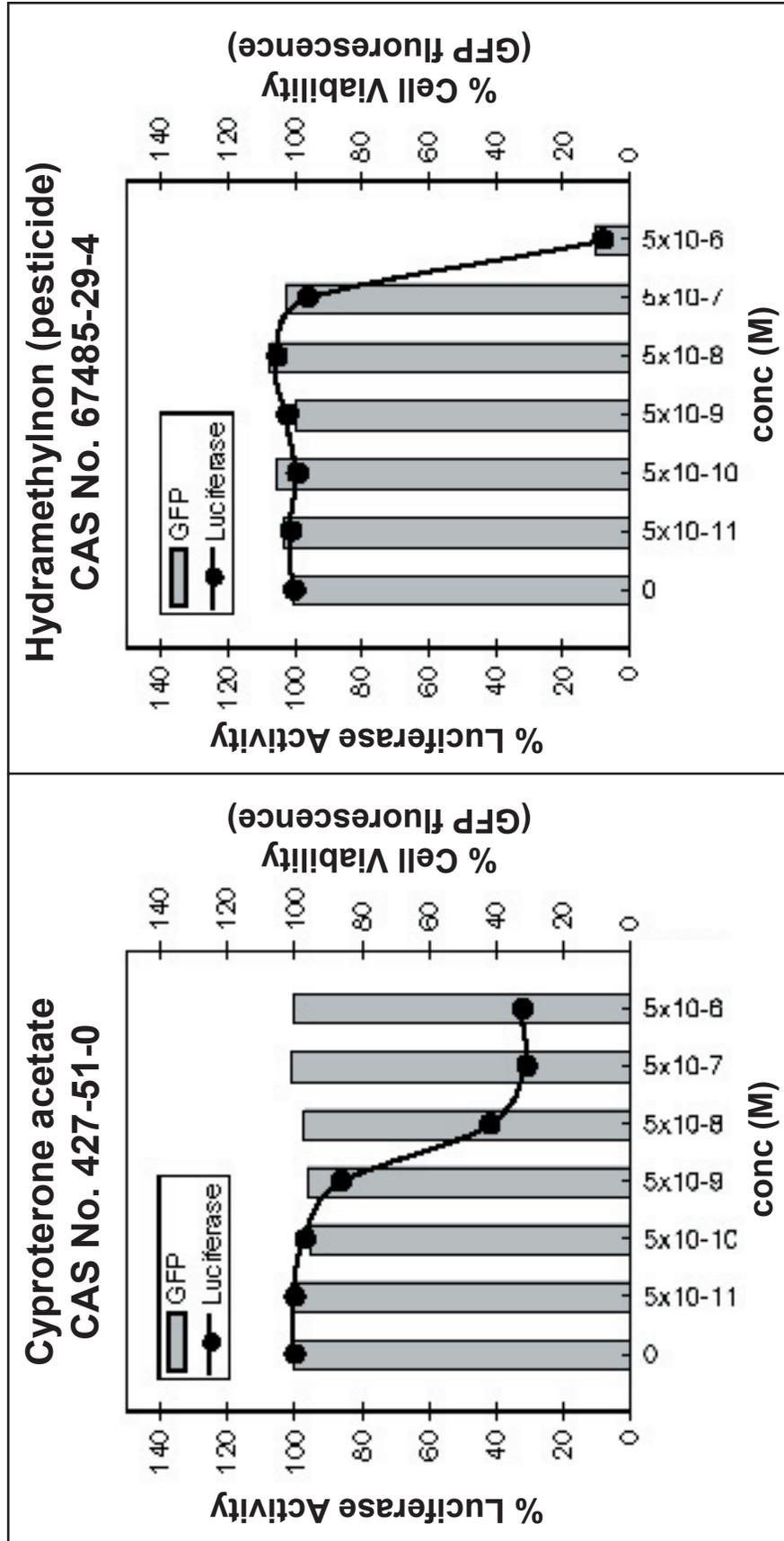


Fig 3

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-Octylphenol	Dibutyl 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

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