December 6, 2002

Dr. William S. Stokes
Director of NICEATM,
NICEATM, NIEHS,
PO Box 12233, MD EC–17,
Research Triangle Park, NC, 27709,
(phone) (919) 541–3398, (fax) (919) 541–0947,
(email) *niceatm@niehs.nih.gov.*

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 years[1]. 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] 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.

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