Appendix B4

Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program: *In Vitro* EDSTAC Guideline Protocols

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U.S. EPA Endocrine Disruptor Screening Program:

In Vitro EDSTAC Guideline Protocols[1]

I.   Introduction

The Food Quality Protection Act of 1996, amending the Federal Food, Drug and Cosmetic Act, directed the Environmental Protection Agency (EPA) to develop a screening program to evaluate whether or not certain chemical agents could potentially have hormone-like effects in humans. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) convened by the EPA recommended a tiered testing approach for the evaluation of endocrine, androgen and thyroid related effects of commercial chemicals and environmental contaminants (EDSTAC, 1998).

Under this testing paradigm, Tier I screening would identify chemicals with a potential to affect the estrogen, androgen and thyroid systems. The recommendations of the EDSTAC for a Tier I screening battery encompassed the utilization of *in vitro* test system methodologies that recognize known mechanisms by which chemicals can interact directly with the estrogen, androgen and thyroid hormone systems. These *in vitro* assays included evaluations of direct binding to the hormone receptors as well the ability of test compounds to activate marker response genes (reporters), linked to hormone responsive genetic elements. The Tier I assays are intended for use in rapid initial screening and prioritization of chemicals for further definitive *in vivo* Tier II testing to determine any potential adverse effects of an endocrine-active substance.

Tier I *in vitro* assays are used as screening tools to provide mechanistic data. These data should not be used as the sole element in a risk assessment regulatory context for test compounds. The *in vitro* screening assays are intended to be used in a hierarchical system which includes, as appropriate, *in vivo* Tier I screening assays and *in vivo* Tier II tests. In this hierarchical system a negative Tier II outcome would supercede a positive Tier I finding (EPA, 2000).

There are limitations inherent in the recommended *in vitro* assays that restrict their effectiveness as large scale, precise, valid, screening tools (Holmes *et al.,* 1998; Zacharewski, 1998). These include but are not limited to:

> Inability to distinguish agonists from antagonists (receptor binding)
> Issues of limited metabolic capacity and bioaccumulation
> Limited/variable chemical uptake
> Dependence on specific receptor or response element interactions not mimicked *in vivo*

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[1] This technical perspective was prepared by experienced scientists engaged in *in vitro* and *in vivo* toxicological research and testing of industrial chemicals/ pesticides/pharmaceuticals.   The primary authors of this commentary are listed under acknowledgements.

Lack of 'gold standard' protocols/methodologies for evaluation of assay results across laboratories

Issues of proprietary and/or restricted use under US patent law regarding the use of human cDNA sequences coding for human nuclear hormone receptors (and/or simultaneous co-transfection of receptor and reporter constructs; cis-trans technology) for use in reporter gene transactivation assays

These limitations need to be addressed in order to maximize the potential use of these assays/methodologies in a properly functional, tiered, screening paradigm required for the assessment of adverse chemical effects on the endocrine system. This paper seeks to aid in moving forward the process of producing sensitive, specific, accurate and properly validated Tier I *in vitro* methods that could be used as screening assays for hormonal activity.

II. Major Elements To Be Considered for Standardization and Validation of *In Vitro* Assays

The following factors need to be taken into consideration in developing, validating and implementing *in vitro* assays for hormonal activity:

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). To date, the inter-laboratory variability, sensitivity, reproducibility and precision of these techniques have not been sufficiently evaluated. Furthermore, alterations in specific assay parameters can also lead to significant variability (Beresford *et al.,* 2000; Charles *et al.,* 2000). A single methodology therefore needs to be properly standardized and validated as the 'gold standard' by which other alternative protocols can be reliably compared.

This gold standard *in vitro* protocol/methodology should be validated under an Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) type process in which several laboratories utilize identical protocols to assess the robustness of the assay in terms of reproducibility and accuracy. An agreed upon set of reference chemicals should be used to assist in the validation especially with regard to specificity and sensitivity.

In vitro assays performed as part of the Tier I screening methodology should be performed in compliance with Good Laboratory Practice (GLP) provisions of the USEPA, OECD and/or MAFF so as to ensure the quality of the data derived from the studies. This includes the proper characterization of the test material for potential purity and/or contamination prior to assay utilization.

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.

In light of the desire to minimize the number of animals that will be used in the implementation of any new toxicological testing procedures, the utilization of methodologies which make limited use of animals (e.g. recombinant receptor proteins for binding assays) should be promoted.

The following discussion provides technical perspectives and recommendations on the design, methodology, and evaluation criteria of nuclear hormone receptor binding assays and nuclear hormone transcriptional activation assays. In addition, the limitations of the testicular steroidogenisis assay are described. These perspectives and recommendations have been developed to promote technical discussions among the scientists engaged in the development, standardization and validation of *in vitro* methods for use as Tier I screening assays for hormonal activity.

III.   Nuclear Hormone Receptor Binding Assays

III. A. Purpose & General Design
The purpose of this procedure is to screen chemicals for the capacity to compete for binding to mammalian nuclear hormone receptors. This technique has been used in the mechanistic evaluation of chemical-receptor interactions. It is assumed that if a test material binds to a receptor with some degree of affinity, then some biological activity on the part of the chemical is usually inferred.

The binding of ligand to the receptor (i.e., specific binding) is a saturable process. Unsaturable binding of ligand is called nonspecific binding and is due to ligand binding to non-receptor elements in a preparation. Total binding is defined as the sum of specific (saturable) and non-specific (unsaturable binding):

Total binding = Saturable binding + Unsaturable binding.

Total and nonspecific binding are determined empirically, while specific binding is calculated as their difference. Total ligand binding is determined by incubating the receptor preparations with increasing concentrations of radiolabelled ligand (^{3}H, ^{125}I etc) for sufficient time to reach equilibrium. The total bound ligand (i.e., saturable + unsaturable binding) is separated from free ligand and quantified using liquid scintillation spectrometry. Nonspecific binding is determined exactly as above except that a 100-fold molar excess of radioinert ligand is included in all

incubations, together with the increasing concentrations of radiolabelled ligand (i.e., binding of radiolabelled ligand in the presence of a 100-fold molar excess of radioinert ligand represents nonsaturable binding). Specific binding is defined and calculated as the difference of total binding and non-specific binding:

Specific binding = Total binding - Nonspecific binding

Specific binding is analyzed graphically via Scatchard analysis to determine the K_d and B_{max}. Radiolabelled 17 -estradiol and methyltrienolone (R1881) are generally recommended for use as ligands for the estrogen and androgen receptors, respectively.

The general protocol followed herein is based on the use of isolated mammalian receptor preparations as currently being pursued by the National Center for Toxicology Research (NCTR) as part of their Quantitative Structure Activity Relationship (QSAR) modeling effort (Blair *et al.,* 2000). This methodology is recommended as the standard that would be validated under an ICCVAM process. The use of recombinant or purified receptors is not precluded once proper validation exercises are performed against the standard procedure so as to ensure equivalency of the technique in terms of precision, reproducibility and sensitivity.

III. B. General Methodology

The receptor protein to be used in the receptor binding assays should be initially characterized by determining the apparent K_d for endogenous ligand binding (i.e., androgen or estrogen) and the maximum number of binding sites/tube (B_{max}) in the receptor preparation. These objectives are normally accomplished by completing an initial Scatchard analysis on each receptor preparation. Once the receptor preparation has been characterized it can be used to assess the ability of test chemicals to displace endogenous ligand from the receptor in binding assays. Appropriate performance criteria will need to be established, for example receptor Kd's in the 0.1-1.0 nM range.

For the purposes of screening test chemicals, an initial three point assay at zero (vehicle) and two concentrations, at the upper solubility limit and 2 log concentrations below is recommended. Chemicals that inhibit receptor binding by 50% (IC_{50}) or more at either of these concentrations in at least two of three replicate assays should be considered positive (i.e., able to bind the respective nuclear hormone receptor and displace endogenous ligand). In these three point binding assays the concentration of radiolabelled ligand is held constant at a value equal to its K_d (determined above) and competing test chemical is added with and without a 100-fold molar excess of radioinert ligand (nonspecific binding).

Alterations in nonspecific binding by test chemical reflect possible direct interference of the test chemical with the assay (i.e., protein denaturation, precipitation, etc) and should preclude an assessment of the test chemical on nuclear hormone receptor binding. Triplicate analyses using a positive control test chemical (concentrations of a chemical known to inhibit receptor binding by 90% or more) should be included in every screening assay for quality control.

Positives should be further assessed using relative binding affinity experiments to more precisely define the dose-response relationship between test chemical concentration and inhibition of receptor binding. Relative binding affinity (RBA) assays determine in a quantitative manner the relative ability of test chemicals to compete with radiolabelled ligand. The ligand is held constant at concentrations equal to its K_d for binding to the nuclear hormone receptor and competing test chemical is added with and without a 100-fold molar excess of radioinert ligand (nonspecific binding). Concentrations of test chemicals used in RBA assays should be deliberately broad ranging from 10 pM up to 25 uM (or the upper limit of solubility of the chemical in the receptor preparation) in 10-fold concentration increments.

Specific binding is then calculated by subtracting nonspecific from total binding at each concentration and the data are plotted in a line graph. Specific binding (% total binding that occurs in the absence of added chemical) is plotted on the ordinate vs log dose of test chemical on the abscissa. The IC_{50} value is calculated as the concentration of test chemical that displaces 50% of the radiolabelled ligand from the receptor.

$$RBA = IC_{50} \text{ Test chemical}/ IC_{50} \text{ Radioinert ligand x 100.}$$

RBA values of test chemicals can be compared to determine relative potency. RBA values should be compared only when the slopes of the RBA data curves between 20 and 80 percent of the maximal response are parallel. Non-parallel slopes suggest atypical interactions of ligand and receptor; binding by these compounds should be evaluated separately for the presence of different interfering mechanisms, which may preclude the use of receptor binding assays.

III. C. Data Evaluation & Assay Pass-Fail Criteria

IC_{50} and RBA values for each test chemical and the positive controls should be tabulated for each assay and the means together with a measure of the variability (e.g., standard deviation) from all assays clearly indicated.

Chemicals that inhibit receptor binding by 50% (IC_{50}) or more in at least two of three replicate assays will be considered positives.

The percent coefficient of variation (%CV) of replicate samples at each concentration of test or control chemical cannot exceed 20% in any assay as per GLP. Data which exceeds the 20% CV at any concentration of test or control chemical within an assay will fail these criteria and all data for that concentration of test or control chemical for that particular assay must be excluded from the data analysis. All data failing these criteria should be so indicated in the data tables.

The positive control test chemical must reduce radiolabelled ligand binding by at least 90% within a 20% CV or the assay will be considered unacceptable.

Scatchard analyses for each receptor preparation should be completed and the calculated K_d and B_{max} clearly indicated and within prescribed limits.

III. D. Limitations

Several limitations of receptor binding assays should be recognized:

Agonist and antagonist activity cannot be discriminated using receptor binding assays.

Positive results may occur *in vitro* at concentrations that far exceed those that are caable of existing *in vivo*.

Only receptor-ligand interactions are assessed.

Furthermore, as part of a Tier I testing scheme if data from a validated *in vitro* gene transcriptional activation assay (discussed below) is already available, (based on that assay's requirement for receptor binding), there should generally be no need for the performance of the hormone receptor binding assays.

IV.   Acknowledgements

The primary authors of this technical perspective are Grantley Charles (Dow), William Kelce (Pharmacia) and Leonard Davis (DuPont).

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