

Appendix B7

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

**(Provided by Dr. Grantley Charles, Toxicology and Environmental
Research and Consulting, The Dow Chemical Company, Midland,
MI, USA, and Dr. William Kelce, Pharmacia Corporation,
Kalamazoo, MI, USA)**

[This page intentionally left blank]

Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program:

In Vitro EDSTAC Guideline Protocols¹

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

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.

Dependence on specific receptor or response element interactions not mimicked *in vivo*

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 steroidogenesis 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 Transcriptional Activation Assays

III. A. Purpose & Background

The purpose of this procedure is to screen chemicals for the capacity to activate or inhibit ligand-induced transcription mediated by the mammalian estrogen and androgen nuclear receptors. The general premise is that nuclear hormone receptors bind ligand, which leads to alteration of their conformation, and subsequent binding to specific response element sequences on DNA and the initiation of transcription of the downstream gene. For convenience, the downstream gene codes for a protein (e.g., luciferase) that can be easily and accurately measured (i.e., a reporter gene) and therefore signals the potency of various ligands/chemicals to bind the receptor and either initiate or inhibit receptor-induced transcription of the reporter. Reporter gene assays then assess both agonist (test chemical alone) and antagonist (test chemical in the presence of stimulating ligand) activity.

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 transcriptional activation assays.

Reporter genes can be transiently introduced into cells and used over the course of several days or stably integrated into the cells genomic DNA and used indefinitely, provided their responses to known ligands are stable and verified on a periodic basis. The response variability of transient expression systems is, however, an issue for routine use. Few stable cells lines for nuclear (estrogen and androgen) hormone receptor reporter gene assays are currently available, therefore the protocol recommended here uses accepted methods for transient reporter gene transfections.

III. B. General Assay Design

In brief, cells should be seeded into tissue culture plates, transiently transfected with the reporter gene, fed media containing treatment compounds with and without stimulating ligand. Following a defined treatment period, cell lysates are harvested and assessed for reporter (e.g. luciferase, -galactosidase) activity. Concurrent with the reporter assay, an identically transfected and treated, satellite assays should be run and evaluated for cytotoxicity. For the screening of test chemicals, a dose-response assay is recommended in order to discriminate between highly potent ligands that may be cytotoxic at high concentrations from weak non-cytotoxic ligands that exhibit agonist activity at higher concentrations.

III. C. Recommended Design Features

The dose range should encompass the low pM range to the chemical solubility limit as the upper concentration to be evaluated. Alternatively, the upper limit should also be defined as that below which no cytotoxicity is observed.

Cells should be cultured aseptically in appropriate media using standard cell culture techniques. The optimal number of cells seeded into each dish or well should be determined empirically by each laboratory and is directly dependent on the transfection efficiency of the reporter gene. It is critical that seeding density is uniform, as alterations in cell number per well will introduce unnecessary variability in the assay.

The use of charcoal stripped serum is important to remove endogenous steroids from the serum which can activate transcription of the reporter gene and confound the experiment. A steroid free environment is especially important for estrogen receptor mediated transactivation experiments as many general laboratory procedures and supplies have been shown to artificially induce estrogen receptor mediated responses. In this context, laboratories should strive for an estrogen free environment.

Higher transfection efficiencies using lipofectin, FUGene or electroporation mean that fewer cells are necessary to induce an easily measured response and the assay can be completed using an efficient 96-well format. These transfection methods are recommended over more traditional calcium-phosphate precipitation and DEAE-Dextran that generally give much reduced transfection efficiencies.

Since the assays are generally performed in large multi-well formats, each plate should have its own positive and negative controls and should be considered a single experiment for data

analysis purposes. A concentration of the inhibitor control should be selected that reduces transcriptional activation by at least 90% in the presence of stimulatory ligand. Duplicate evaluations of each test chemical concentration should be assessed per experiment. Experiments should be replicated at least three times on different days.

17 β -estradiol and 5 α -dihydrotestosterone are recommended stimulatory ligands for the estrogen and androgen receptor assays, respectively. The concentration of stimulatory ligand used in test article antagonism studies should induce transcriptional activity to levels approximately 80-90% of maximum; use of submaximal levels insures that the receptor is not saturated with agonist ligand and incapable of responding to inhibitory compounds. ICI-182,780 and hydroxyflutamide (Wakeling *et al.*, 1991; Clark *et al.*, 1981; Kelce *et al.*, 1995) are the respective recommended antagonism controls and should be used at concentrations that inhibit transcriptional activation by 90% or more. Other stimulatory and antagonist controls are acceptable provided that they are appropriately validated against the standard controls.

Control and test chemicals should be solubilized in ethanol or DMSO and added to the media in each well to a final concentration determined empirically as part of the initial standardization and validation effects for that cell line. Particular attention should be given to the solubility of test chemicals especially at the high doses. Any precipitate, discoloration, or persistent light refractive changes on the media surface should be noted and included in the final report indicating potential solubility problems. Other vehicles may be used provided appropriate determination of its effects on the cell line and reporter activity are properly standardized and validated.

III. D. Data Presentation and Pass-Fail Criteria

The percent coefficient of variation (%CV) of replicate samples at each concentration of test or control chemical cannot exceed 20% in any assay. 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 antagonist control must reduce transactivation by at least 90% within a 20%CV or the assay will be considered unacceptable.

Data from transactivation experiments should be replicated at least three times each on different days. Data should be tabulated and graphed as reporter activity (relative light units) on the ordinate versus log dose of test chemical on the abscissa. For convenience, reporter data can be presented as %-control (%-maximal activity induced by stimulatory ligand) provided actual control values are clearly indicated.

The EC₅₀ (agonist experiments) is calculated as the concentration of test chemical that activates transcription by 50% relative to the maximal activity induced by stimulatory ligand.

The IC₅₀ (antagonist experiments) is calculated as the concentration of test chemical that inhibits transcription by 50% relative to the maximal activity induced by stimulatory ligand. EC₅₀ and IC₅₀ values for each test chemical and the positive and antagonist controls, respectively, 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.

An efficacy of 25% of the positive control (or the negative control in the case of antagonist activity) should be considered a positive response for that test chemical in that assay.

III. E. Limitations

The following limitations of transcriptional activation studies should be recognized:

Differences in sensitivity exist among clones of a given cell line (Villalobos *et al.*, 1995) in terms of their endocrine responses. Hence adequate characterization of cell lines are necessary and the testing methodology should address factors such as drift in responsiveness, sensitivity and specificity to minimize variability in response across laboratories.

Test end points are dependent upon interactions with a given receptor structure or engineered response element. Therefore, results from any single gene transactivation system for a given chemical may vary significantly from that of another.

Reproducibility of results will always be a potential concern, consequently, test systems should be widely available to enable confirmatory findings by other laboratories. It is therefore essential to perform an ICCVAM-type validation on a specific estrogen and androgen transactivation systems to act as gold standards to which other assay systems could be compared.

It should be noted that transiently transfected cell lines exhibit some degree of variability across experiments in terms of their responses making stable cell lines a potentially more appealing alternative for validation purposes. In the event that new, stable cell lines are developed and are generally available, it is recommended that they be used with the caveat that they are properly validated in accordance with the ICCVAM principles already outlined. Their sensitivity, accuracy, precision and specificity should also be reviewed on a periodic basis to protect against genetic drift and cellular mutations that may compromise the integrity of the assay system.

IV. Acknowledgements

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

V. References

- Beresford N, Routledge EJ, Harris CA, Sumpter JP. (2000). Issues arising when interpreting results from an *in vitro* assay for estrogenic activity. *Toxicol Appl Pharmacol* 162(1):22-33.
- Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol Sci* 54(1):138-153.
- Charles, G. D., Gennings, C., Clemons, J., Zacharewski, T. R., Gollapudi, B. B., and Carney, E.W. (2000). Optimization of an estrogen receptor- transcriptional activation assay for testing a diverse spectrum of chemicals. (*J. Appl. Toxicol*). 20:449-454.
- Clark CR, Merry BJ, Nowell NW. (1981). Effect of alpha, alpha, alpha-trifluoro-2-methyl-4'-nitro-m-lactotoluidide (Sch 16423) on serum testosterone and LH in rats. *J Reprod Fertil*. 61(1):189-191.
- EDSTAC. Endocrine Disruptor Screening and Testing Advisory Committee Final Report, August 1998. Federal Register Notice: 63,(248) 71541-71568. URL: <http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm>.
- Environmental Protection Agency, Endocrine Disruptor Screening Program, Report to Congress (Aug, 2000). <http://www.epa.gov/scipoly/oscpendo/reporttocongresso800.pdf>
- Gaido GW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ and McDonnell DP. (1997). Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol*. 143:205-212.
- Holmes, P., Humfrey, C. and Scullion, M. (1998). Appraisal of test methods for sex-hormone disrupting chemicals capable of affecting the reproductive process. OECD Environmental Directorate, Environmental Health and Safety Division, Paris. <http://www.oecd.org/ehs/test/monos.htm>
- Kelce, W.R., Stone, C.R. Laws, S.C. Gray, Jr, L.E.. and Wilson, E.M.. Persistent DDT Metabolite p,p'-DDE is a Potent Androgen Receptor Antagonist. *Nature* 375: 581-585, 1995.
- Klinefelter, G. and Kelce, W.R. (1996). Leydig cell responsiveness to hormonal and nonhormonal factors in vivo and in vitro. In *The Leydig Cell* (A.H.Payne, M.P. Hardy and L.D.Russell, Eds.) pp 535-553. Cache River Press, Vienna.
- Maness, S.C., McDonnell, D.P., and Gaido, K.W. (1998). Inhibition of androgen receptor-dependent transcription activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. *Toxicol. Appl. Pharmacol*. 151:135-142.

Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect.* 105(1):70-76.

Nikov GN, Hopkins NE, Boue S, Alworth WL. (2000). Interactions of dietary estrogens with human estrogen receptors and the effect on estrogen receptor-estrogen response element complex formation. *Environ Health Perspect* 108(9):867-872.

O'Connor JC, Frame SR, Davis LG, Cook JC. (1999). Detection of the environmental antiandrogen p,p-DDE in CD and long-evans rats using a tier I screening battery and a Hershberger assay. *Toxicol Sci.* 51(1):44-53.

Organisation for Economic Co-Operation and Development (OECD). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997) EVN/MC/CHEM(98)17.

Pons, M., Gagne, D., Nicolas, J.C. and Mehtali, M. (1990). A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. *Biotechniques* 9:450-459.

Powlin, S.S., Cook, J.C., Novak, S. and O'Connor, J.C. (1998). Ex vivo and *in vitro* and ovary explants: utility for identifying steroid biosynthesis inhibitors and comparison to a Tier I screening battery. *Toxicol. Sci.* 46:61-74.

US Environmental Protection Agency - TSCA GLPs. Title 40 CFR, Part 792 - Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards, Final Rule

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V. (1995). The E-screen assay: a comparison of different MCF7 cell stocks. *Environ Health Perspect.* 103(9):844-850.

Vinggaard AM, Joergensen, EC and Larsen, AC. (1999). Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals. *Toxicol Appl Pharmacol.* 155(2):150-160.

Wakeling AE, Dukes M and Bowler J. (1991). A potent specific pure antiestrogen with clinical potential. *Cancer Res.* 51:3867-3873.

Zacharewski T. (1998). Identification and assessment of endocrine disruptors: limitations of *in vivo* and *in vitro* assays. *Environ. Health Perspect.* 106 (Suppl 2):577-582.

Zacharewski, T., Bondy, K., McDonnell, P., and Wu, Z.F. (1994). Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on 17 β -estradiol-induced-pS2 expression. *Cancer Res.* 54:2707-2713.