Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Estrogen Receptor Transcriptional Activation Assays

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EXECUTIVE SUMMARY

The objectives of this BRD are to: (1) provide comprehensive summaries of the published and publicly available unpublished data on the scientific basis and performance of *in vitro* assays used to test substances for their ability to initiate transcriptional activation of the estrogen receptor (*in vitro* ER TA assays); (2) assess the *in vitro* ER TA assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* ER TA assays that might be considered for incorporation into future testing programs for validation; 4) develop minimum performance criteria by which to judge the effectiveness of proposed *in vitro* ER TA assays; and (5) generate a list of recommended substances to be used in validation efforts.

The data summarized in this BRD are based primarily on information obtained from the peer-reviewed scientific literature. An online literature search identified 258 records related to *in vitro* ER TA assays with 86 publications containing relevant data on ER-induced transcriptional activation for inclusion in this BRD. Data from two submitted non-peer reviewed documents were included also. Some of the peer-reviewed publications that contained *in vitro* ER TA assay data were not abstracted for inclusion in this BRD because the studies lacked the appropriate details or contained data from unique procedures or substances that were not clearly identified.

In *in vitro* ER TA assays, the cell lines used have been transfected with foreign DNA consisting of an ER from human or mouse and a reporter gene that is transcribed as a consequence of a substance binding with the ER. Studies that evaluated the potential ER agonism of a test substance used enzyme activity as an indirect measure of ER-induced transcriptional activation, while ER antagonism studies measured the ability of the test substance to inhibit reporter gene (luciferase and chloramphenicol acetyltransferase in mammalian cell assays, β-galactosidase in yeast assays) or as cell proliferation in mammalian cells harboring an endogenous ER. The agonistic potency of a substance in *in vitro* ER TA assays has been determined by comparing the enzyme activity induced by the test substance with that produced by the reference estrogen. Cell proliferation studies reported results as cell number, foci/cm^2, EC_{50} values, cell growth relative to hormone free control, increase in protein or DNA content, and fold increase in cell proliferation

relative to vehicle control. Data from each study was assigned a qualitative response of "positive" or "negative" for the particular assay system.

A variety of yeast and mammalian cell reporter gene systems were used in the studies. The yeast assays used different strains of yeast and sources of ER (e.g., human, mouse, rainbow trout) that were usually stably integrated into the yeast genome. The mammalian cell reporter gene assays used a variety of cell types, including human breast cancer cell lines (MCF-7, MDA-MB-231, T47D, ZR-75); human ovarian carcinoma cells (BG-1); Chinese hamster ovary cells (CHO-K1); monkey kidney cells (COS-1); rat uterine leiomyoma cells (ELT-3); human endometrial cancer cells, (HEC-1 and Ishikawa); human embryonal kidney cells (HEK293); human cervical tumor cells (HeLa); and human liver tumor cells (HepG2). The mammalian cell reporter gene systems used various permutations of stable and transient transfections of ER, ERα and ERβ isoforms (human, mouse). Different methods were used to assess cell toxicity (e.g., β-galactosidase expression and cell counts and staining). Data and methods were collected also for *in vitro* ER cell proliferation assays.

In vitro ER TA assay data were collected for a total of 703 substances of which 362 substances were tested in reporter gene agonism assays and 185 in reporter gene antagonism assays. A total of 272 substances were tested for agonism and 70 for antagonism in the cell proliferation assays. Only 36 substances were tested in five or more reporter gene agonism assays. The chemical classes that have been tested most extensively are polychlorinated biphenyls, organochlorines, polycyclic aromatic hydrocarbons, phenolic steroids, nonphenolic steroids, phthalates, phenols, and alkylphenols. The most common product classes tested in *in vitro* ER TA assays have been pesticides (including their metabolites and degradation products), pharmaceuticals, chemical intermediates, dielectric fluid components, natural products (including several phytoestrogens), and plasticizers.

Data was generated from 22 *in vitro* yeast ER reporter assays involving 13 different strains and from 63 *in vitro* mammalian cell ER reporter assays using 12 different mammalian cell lines. Three cell limes using different transfected ER and reporter plasmids were used to measure cell proliferation.

The quantitative results of the *in vitro* ER TA studies for agonism were most commonly presented in terms of relative activity expressed as the fold induction of enzyme activity produced by the test substance relative to the activity in the untreated controls, as the ratio of the response of the test substance to that of the reference estrogen, or as the concentration of the test substance that produced a certain percent response relative to the reference estrogen. An EC_{50} value (the half-maximal concentration) was provided infrequently. For antagonism studies, the inhibition of reference estrogen-induced enzyme activity by the test substance was measured and sometimes expressed as an IC_{50} value (i.e., the concentration that inhibited the reference estrogen -induced ER transcriptional activation by 50%).

Relatively few of the substances had been tested more than once in the same *in vitro* ER TA assay or in multiple assays in the same or different laboratories. Furthermore, because the primary focus of many of the studies reviewed in this BRD was on understanding the mechanisms of ER-induced transcriptional activation and not at identifying substances with ER agonist or antagonist activity, much of the published data are of limited value for the analysis of assay performance or reliability.

Based on the limited data available, there is no single *in vitro* ER TA assay that can be concluded to perform better or to be more reliable than any other assay. However, it might be anticipated that mammalian cell assays would be preferred over yeast assays, simply because of the increased ability of test substances to cross the mammalian cell membrane compared to the yeast cell wall. Although the transiently transfected cell lines have some advantages over the stably transfected cell lines in that the level of the ER is higher in the former, the ability to reproducibly transfect the same amount of DNA on a routine basis is difficult. Not all of the laboratories using this technique monitored the transfection efficiency. Taking these factors into consideration, a cell line stably transfected with both the hER expression and the luciferase reporter plasmids (PALM) or one with an endogenous ER and a stably transfected reporter plasmid (e.g., MDA MB-453, BG-1) would offer the greatest utility by eliminating the need to continuously prepare multiple batches of transiently transfected cells.

Formal validation studies should be conducted using appropriate substances covering the range of expected EC_{50}/IC_{50} values to adequately demonstrate the performance characteristics of any *in vitro* ER TA assay recommended as a possible screening test method for ER agonist and antagonist. The role of metabolic systems in activating some substances to ER agonist or antagonist needs to be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* ER TA assay into a regulatory screening program is production of high quality data. To achieve this goal, it is recommended that any future pre-validation and validation studies on *in vitro* ER TA assays be conducted with coded substances and in compliance with GLP guidelines. Ideally, if multiple laboratories are involved in the validation study, the substances should be obtained from a common source and distributed from a central location.

The facilities needed to conduct *in vitro* ER TA assays are widely available, as is the necessary equipment from major suppliers. Although information of the commercial cost of these assays was not available, it can be assumed that the costs for most if not all of the assays are roughly equivalent.

Since there are no published guidelines for conducting *in vitro* ER TA studies, and no formal validation studies have been performed to assess the reliability or performance of such assays, the U.S. EPA requested that minimum procedural standards based on a comparative evaluation of *in vitro* ER TA assays be developed. The minimum procedural standards provided include methods for determining the ability of the reference estrogen to induce transcriptional activation, methods for establishing a stable cell line, the concentration range of the test substance (including the limit dose) to test for agonists and antagonists, the use of negative and positive controls, the number of replicates to use, dose spacing, data analysis, assay acceptance criteria, evaluation and interpretation of results, minimal information to include in the test report, and the potential need for replicate studies. These minimum procedural standards are provided to ensure that *in vitro* AR TA studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

Six *in vitro* ER TA assay protocols developed by experts in the field are provided in **Appendix B**. Inspection of these protocols provides a perspective on how various *in vitro* ER TA assays are conducted by different investigators, and for developing a more general protocol, one that takes into account the recommended minimum procedural standards. Prior to developing that protocol, the submitted protocols need to be evaluated for completeness and adequacy for their intended purpose.

The U.S. EPA requested that a list of recommended test substances be provided for use in validation studies. Testing of substances encompassing a wide range of agonist/antagonist responses are needed to adequately demonstrate the performance characteristics of *in vitro* ER TA test methods recommended as screening assays. A number of factors were considered in developing this list of substances, including the EC_{50} and IC_{50} value of the substance in all the assays in which it had been tested. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* ER TA assay.

It is anticipated that this BRD and the guidance it provides will help to stimulate validation efforts for *in vitro* ER binding assays.