

## 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The objectives of this BRD are to: (1) provide comprehensive summaries of the published and publicly available unpublished data on the scientific basis and performance of *in vitro* assays used to test substances for their ability to initiate transcriptional activation of the androgen receptor (*in vitro* AR TA assays); (2) assess the *in vitro* AR TA assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* AR 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* AR 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 105 records related to androgen binding and TA assays with 26 publications containing relevant data on *in vitro* AR TA assays for inclusion in this BRD. Some of the peer-reviewed publications that contained *in vitro* AR 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 In addition to the published data, the BRD includes *in vitro* AR TA data from one submitted report containing unpublished information.

In *in vitro* AR TA assays, the cell lines used include those that have been transfected with foreign DNA consisting of an AR and a reporter gene (luciferase, chloramphenicol acetyltransferase, or -galactosidase) that is transcribed when an androgen or test substance binds to the AR, and mammalian cells harboring an endogenous AR in which a reporter gene is added. In these tests, one of four reference androgens (5 -dihydrotestosterone, methyltrienolone, testosterone, or mibolerone) was used. Studies that evaluated the potential AR agonism of a test substance used enzyme activity as an indirect measure of AR-induced transcriptional activation, while AR antagonism studies measured the ability of the test substance to inhibit reporter gene enzyme activation induced by the reference androgen.

Data were abstracted for 18 different *in vitro* AR TA assays. These assays used either CHO (Chinese hamster ovary cells), CV-1 (monkey kidney cell line), HeLa (human cervical cancer cell line), HepG2 (human liver tumor cell line), MDA-MB-453 (human breast carcinoma cell line), PC-3 (human prostate tumor cell line), and EPC (carp skin tumor cell line) cells, or yeast (*S. cerevisiae*) transfected predominantly with the human (h) AR, although mouse (m) and rainbow trout (rt) AR have been used also. Some cell lines were manipulated so that the foreign DNA was incorporated permanently into cellular DNA. However, many of the assays described in the BRD used cells transiently transfected with the AR and the reporter gene. Under these conditions, the transfected DNA remains intact in the cell for a few days.

*In vitro* AR TA assay data were collected for a total of 145 substances, of which 68 were tested for both agonism and antagonism activity, 51 for agonism activity only, and 20 for antagonism activity only. The chemical classes tested most extensively have been nonphenolic steroids, organochlorines, phenolic steroids, and polycyclic aromatic hydrocarbons, while the most common product classes tested have been pharmaceuticals and pesticides.

More substances (65; 44.5%) were tested in the CHO-K1 hAR(T)+Luc(T)+EGFP(T) than in any other assay. The next most frequently used assay was the PALM hAR(S)+Luc(S) (43 or 29.5% of substances tested). Thirty-two substances (21.9%) were tested in the Yeast (*S.cer*) AR + gal assay and 27 (18.5%) were tested in the CV-1+hAR(T)+Luc(T) assay.

The quantitative results of the *in vitro* AR 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 androgen, or as the concentration of the test substance that produced a certain percent response relative to the reference androgen. An EC<sub>50</sub> value (the half-maximal concentration) was provided infrequently. For antagonism studies, the inhibition of reference androgen-induced enzyme activity by the test substance was measured and sometimes expressed as an IC<sub>50</sub> value (i.e., the concentration that inhibited the reference androgen-induced AR transcriptional activation by 50%).

Relatively few of the substances had been tested by more than one investigator in the same *in vitro* AR 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 AR-induced transcriptional activation and not at identifying substances with AR 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* AR 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 AR 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. Patent issues are another disadvantage of the transiently transfected cell lines. Taking these factors into consideration, it would seem that a cell line that contains endogenous AR and is stably transfected with a luciferase reporter plasmid (e.g., MDA-MB-453-kb2) would offer the greatest utility by eliminating the need to continuously prepare multiple batches of transiently transfected cells. This cell line would also eliminate concerns regarding patents on the transient transfection of cell lines with the AR.

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* AR TA assay recommended as a possible screening test method for AR agonists and antagonists. The role of metabolic systems in activating some substances to an AR agonist or antagonist needs to be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* AR TA assay into a regulatory screening program is production of high quality data. To achieve this goal, it is recommended that any

future prevalidation and validation studies on *in vitro* AR 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* AR TA assays are widely available, as is the necessary equipment from major suppliers. Although information on 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* AR 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* AR TA assays be developed. The minimum procedural standards provided include methods for determining the ability of the reference androgen 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* AR 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* AR 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* AR TA test methods recommended as screening assays. A number of factors were considered in developing this list of substances, including the EC<sub>50</sub> and IC<sub>50</sub> value of the substance in all the assays in which it had been tested. Because the number of substances with replicate quantitative agonist or antagonist data was insufficient to generate the desired number of substances for consideration, selection of most substances was based on results obtained in a single assay by a single investigator. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* AR TA assay.

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

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