

**Current Status of Test Methods
for Detecting Endocrine Disruptors:
In Vitro Androgen Receptor Binding 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The objectives of this BRD are to: (1) provide comprehensive summaries of the published and publicly available unpublished data on the scientific basis and performance of *in vitro* assays used to test substances for their ability to bind to the androgen receptor (AR); (2) assess the *in vitro* AR binding assays considered for their effectiveness in identifying endocrine-active substances; (3) identify and prioritize *in vitro* AR binding 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 binding 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 was conducted to retrieve records on publications reporting on the testing of substances for their endocrine disrupting effects *in vitro*. Of the 459 records obtained from the initial search, 108 contained information on AR binding. Additional citations were located while reviewing these publications. Ultimately, data from 23 publications were extracted for consideration during the preparation of this BRD. Some of the peer-reviewed publications that contained AR binding 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.

Data were abstracted on 108 substances tested in 11 different AR-binding assays. These assays used:

- cytosol prepared from animal tissues containing the AR (rat prostate [RPC], rat epididymis, calf uteri), human cell lines (MCF-7, LnCaP) with an endogenous AR, and a mammalian cell line (COS-1) transfected with human (h) AR;
- primary human genital fibroblasts (HGF) with an endogenous AR;
- mammalian cell lines (COS-1) transfected with either hAR or rainbow trout (rt) AR ; and
- recombinant hAR Sf 9 insect cells.

The assays measured the competitive displacement of one of four androgens, two naturally occurring (testosterone, 5 α -dihydrotestosterone [DHT]) and two synthetic (mibolerone, 17 α -hydroxy-estra-4,9,11-trien-3-one [methyltrienolone or R1881]) radiolabeled with tritium (^3H) from the AR. Seventy-four substances were evaluated in competitive AR binding experiments that used DHT as the reference androgen; 47 were tested with R1881, 24 were tested with testosterone, and 16 were tested with mibolerone.

The chemical classes that have been tested most extensively in *in vitro* AR binding assays have been nonphenolic steroids, organochlorines, and phenolic steroids, while the most common product classes have been pharmaceuticals and pesticides. Not all substances could be assigned to a product class.

Of the 108 substances tested in the 11 different *in vitro* AR binding assays, only 34 (31.4%) had been tested in two or more assays, irrespective of the reference androgen used. No substance had been tested in all 11 assays. The assays for which the most substances had been tested are the HGF assay (38 substances, 35.2%), the RPC assay (33 substances, 30.6%), and the COS-1+hAR assay (19 substances, 17.6%). A majority of the substances (66; 61.1%) were tested in only one test.

The majority of the publications reported the data as IC_{50} values (the concentration that reduces the binding of the reference androgen by 50%) or as relative binding affinity (RBA) values, that is, the ratio of the IC_{50} of the reference androgen, divided by the IC_{50} of the test substance $\times 100$.

As so few substances have been tested more than once in the same *in vitro* AR binding assay or in multiple assays using the same reference androgen, no quantitative or qualitative analyses of the comparative performance or the reliability of these assays were possible. However, based on general principles, recommendations were made in regard to the use of *in vitro* AR binding assays as a component of a Tier 1 endocrine disruptor screening battery:

- After consideration of such factors as a desire to eliminate animal use when feasible, and a possible advantage associated with the use of hAR transfected into a cell line free of other endocrine receptors (to avoid possible cross-reactivity) or the use of a recombinant hAR

assay, the COS-1+hAR and hAR assays are recommended as the *in vitro* AR binding assays with the greatest priority for validation. If an assay chosen for validation requires the use of animals, efforts should be made to minimize the number of animals used, and animal pain and distress.

- In conducting future validation studies with these assays, the RPC assay, which is currently undergoing validation efforts sponsored by the U.S. EPA, should be used as the reference test method.
- Formal validation studies should be conducted using appropriate substances covering the range of expected RBA values to adequately demonstrate the performance characteristics of the *in vitro* AR-binding assays recommended as possible screening assays.
- There is little information about the AR binding activity of metabolites of xenobiotics and it is not clear whether metabolic activation needs to be included in *in vitro* AR binding test methods used as screening assay. This issue should be considered prior to the implementation of future validation studies.

An important step towards acceptance of an *in vitro* AR binding 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* AR binding 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. In conducting these validation studies, all of the original data and documentation supporting the validation of a test method must be carefully documented, and include detailed protocols under which the data were produced.

The facilities needed to conduct *in vitro* AR binding 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 all of the animal cytosol assays are roughly equivalent, as would be the costs for the cell culture assays and assays using semi-purified AR.

Since only one guidelines for conducting an *in vitro* AR binding assay has been published, and no formal validation studies have been performed to assess the reliability or performance of *in vitro* AR binding assays, the U.S. EPA requested that minimum procedural standards based on a comparative evaluation of *in vitro* AR binding assays be provided. In addition it was requested that a list of recommended test substances be provided for use in validation studies,

The minimum procedural standards include selection of the reference androgen, methods for determining the K_d of the reference androgen, methods for test substance preparation, the concentration range of the test substance (including the limit dose), the use of negative and positive controls, the number of replicates per test substance concentration, dose spacing, assay acceptance criteria, data analysis, evaluation and interpretation of results, minimal information to include in the test report, and the need for replicate studies.

Four *in vitro* AR binding assay protocols, including the RPC assay protocol being used in the U.S. EPA-sponsored validation study for androgen receptor competitive binding, were provided for consideration (**Appendix B**). Inspection of these protocols provides a perspective on how various assays are conducted by different investigators and for developing a more general protocol, one that takes into account the recommended minimum procedural standards.

A number of factors were considered in developing a list of recommended substances to be used in validation efforts, including the number of times the substance had been tested in various assays, the median RBA value of the substance across assays and its extent of concordance. The selected substances were sorted according to their median RBA values, over six orders of magnitude, ranging from 100 to 0.0001. Weakly-binding substances (RBA values <0.001) were difficult to identify because they were not always consistently positive in tests within an assay or using different assays. Also included were substances classified as "negative" for AR binding based on the lack of a positive response in multiple assays when tested at doses of at least 1 mM. Where possible, five substances were selected for each RBA category and three for the negative category group. To ensure that each RBA category contained a representative sampling of chemical classes, selection was based on the chemical class to which the substance belongs, whether it was representative of a chemical class used in commerce or found in the environment,

and whether the substance is commercially available. The latter criterion was based on whether the substance could be located in a chemical supply catalogue.

The resulting list of 31 substances was compared with an U.S. EPA list of 19 substances that has been proposed for testing in an RPC assay procedure by Battelle Memorial Institute. The U.S. EPA has fewer substances in the organochlorine chemical class. Two of the substances on the U.S. EPA list were not included in the list of recommended substances because of the absence of published data on their AR-binding activity.

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