

In Vitro Models in Endocrine Disruptor Screening

Grantley D. Charles

Abstract

The public and scientific concern that chemicals present in the human diet and the environment and their ability to disrupt the normal hormonal milieu in humans and wildlife have become a high-profile international issue. In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) convened by the Environmental Protection Agency (EPA) recommended a tiered testing approach for the evaluation of estrogen, androgen, and thyroid-related effects of some 87,000 commercial chemicals and environmental contaminants. The function of this committee concluded with its final report, and the further implementation of the recommended testing strategy has now been carried forward with the assistance of the Endocrine Disruptor Methods Validation Subcommittee. The function of this body is to provide advice to the EPA on scientific and technical issues related specifically to the conduct of studies required for the validation of assays proposed by the EDSTAC as part of the tiered screening program. The EDSTAC recommended and alternative screening batteries encompass four *in vitro* mammalian assays. The current methodologies and validation status of the proposed *in vitro* EDSTAC assays are discussed and consist of estrogen/androgen receptor binding, estrogen/androgen gene transactivation, and minced testis, and one alternate (placental aromatase) *in vitro* screening assay.

Key Words: androgen; aromatase; EDSTAC; endocrine disruption; estrogen; *in vitro*; receptor binding; steroidogenesis

Introduction

In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC¹) (<http://www.epa.gov/scipoly/oscpendo/edspoverview/edstac.htm>) convened by the Environmental Protection Agency (EPA¹) recommended a tiered testing approach for the evaluation of estrogen, androgen, and thyroid-related effects of commercial chemicals and environmental contaminants. The further implementation of the recommended testing strategy has

now been carried forward with the aid of the Endocrine Disruptor Methods Validation Subcommittee (EDMVS¹) (<http://www.epa.gov/scipoly/oscpendo/assayvalidation/edmvs.htm>), whose function is to provide advice to the EPA on scientific and technical issues related specifically to the conduct of studies required for the validation of assays proposed by the EDSTAC as part of the tiered screening program. This conceptual, tiered testing framework has since become part of a larger global initiative with the establishment of the Organisation for Economic Cooperation and Development (OECD¹) Working Group on Endocrine Disrupter Testing and Assessment.

The tiered testing approach proposed by the EDSTAC allows for a rapid initial prioritization and sorting of chemicals for further evaluation in mechanistically based screens at the Tier I level. This Tier I battery was designed to be quick and inexpensive, and more sensitive than specific. The battery results were designed to clarify and assess multiple modes of endocrine action and so potentially inform priority setting and further testing at the more definitive Tier II *in vivo* stage, which is designed to verify whether potential adverse effects of an endocrine-active substance exist. The EDSTAC Tier I battery consisted of a recommended battery and two alternate screening batteries (deemed worthy of further evaluation by the EDSTAC) as possible substitutes for the recommended battery based on the fulfillment of the battery's requirements as outlined above.

The EDSTAC-recommended batteries (including the alternate) encompass four *in vitro* and five *in vivo* mammalian assays, with the EPA and OECD currently confronting the challenges of protocol selection, standardization, and assay validation (Schmidt 1999). The proposed batteries should be capable of detecting endocrine-active test materials with differing sensitivities and specificities, with the challenge of the final selection based on factors such as battery predictivity, sensitivity, animal usage, and cost. Many *in vitro* and *in vivo* assay methodologies have been

¹Abbreviations used in this article: AR, androgen receptor; CAT, chloramphenicol acetyl transferase; DHT, dihydroxytestosterone; EDMVS, Endocrine Disruptor Methods Validation Subcommittee; EDSTAC, Endocrine Disruptor Screening and Testing Advisory Committee; EPA, Environmental Protection Agency; ER, estrogen receptor; IC₅₀, concentration of test chemical required to displace half of the specific binding of the labeled native hormone; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; OECD, Organisation for Economic Cooperation and Development.

Grantley D. Charles, Ph.D., D.A.B.T., is a Research Specialist in the Molecular, Cellular and Biochemical Toxicology Group within Toxicology & Environmental Research & Consulting, The Dow Chemical Company, Midland, Michigan.

utilized in assessing potential endocrine activity (reviewed in Holmes et al. 2001; Reel et al. 1996). This article focuses on a discussion of the current *in vitro* screening assays that are part of the Tier I EDSTAC-recommended test batteries consisting of the following: (1) estrogen/androgen receptor binding, estrogen/androgen gene transactivation, and minced testis; and (2) the alternate (placental aromatase) *in vitro* screening assays.

Receptor Binding and Transactivation Assays

The receptor binding and transactivation assays are based on classical mechanistic receptor models. The receptors under consideration include the estrogen receptor (ER¹), which has ER α (Green et al. 1986) and ER β (Kuiper et al. 1996) subtypes, and the single isoform androgen receptor (AR¹) (Lubahn et al. 1988). The receptors are transcriptional regulatory proteins and members of the nuclear receptor superfamily (Evans 1988). Classically, interaction of native steroid hormones with their cytosolic/nuclear receptors results in conformational changes, receptor dimerization, and binding to DNA regulatory sequences, which in turn result in the recruitment of coactivator proteins. These complexes can then initiate or inhibit the transcription of hormonally regulated genes and subsequent cellular functions (Brzozowski et al. 1997; Evans 1988).

It should be noted, however, that so-called nongenomic (non-gene transcription-mediated) mechanisms have been demonstrated for these receptors via cell membrane expression and signaling through G-proteins, with subsequent protein kinase activation resulting in pleiotropic cellular effects (Razandi et al. 1999; Revelli et al. 1998; Song et al. 2002; Sun et al. 2003). In the proposed endocrine disruptor screening paradigm, the ER and AR binding and transactivation assays are therefore based on the more classical mechanistic model whereby they evaluate the potential of exogenous agents to mimic or block the action of the natural ER (17 β -estradiol; E2) and AR (testosterone and dihydroxytestosterone; [DHT¹]) ligands with their receptors and so alter hormonal regulation through altered gene expression and not via nongenomic mechanisms.

Binding Assays

Receptor binding assays comprise a well-established methodology within the literature that have found great utility in the investigation of receptor-ligand interactions. In the context of this article, such an assay measures the ability of various concentrations of a test compound to compete for binding with a single concentration of the labeled (usually ³H) native hormone that has been incubated with a preparation containing the receptor of interest. The competitive binding curves are usually generated by plotting bound reference (³H-E2 or DHT) against the log concentration of the

test compound. This activity of the test material is most often reported as the concentration of test chemical required to displace half of the specific binding of the labeled native hormone (IC₅₀) or the relative binding affinity, which is the ratio of the IC₅₀s of the reference or native ligand to that of the test chemical.

Binding assays are generally conducted utilizing cell-free receptor preparations generated from hormonally responsive tissues—typically uterus for ER (Blair et al. 2000) and prostate for AR (Kelce et al. 1995). Aside from tissue receptor preparations, alternative sources are available, such as cytosolic extracts from cultured cells (Schoonen et al. 1995) and transformed yeast and baculovirus expression systems that have been used to produce recombinant receptor preparations after transfection with the appropriate DNA (Bauer et al. 2002; Matthews et al. 2002). In addition, although traditional receptor binding assays have used radio-labeled ligands, more recent assay methodologies have incorporated the use of fluorescence (Bolger et al. 1998; Mueller et al. 2003; Ohno et al. 2003; Saito et al. 2000).

Although simple to conduct and well established, competitive ligand binding assays have certain limitations that restrict their effectiveness in screening for potential endocrine-active chemical agents (Zacharewski 1997). First, the original binding assays proposed by the EDSTAC used rat cytosolic tissue preparations. The fact that, for example, the two ER isoforms α and β display tissue-specific expression profiles (Weihua et al. 2003) could potentially lead to binding data that depend on tissue selection. In this context, the use of recombinantly expressed receptor protein isoforms should allow for enhanced specificities of the assays and minimize the use of animals, both desirable outcomes.

Second, it is notable that although assessing the ability to bind the receptor, these assays, in contrast to gene transactivation assays (described below), cannot distinguish between agonists and antagonists. In this context, agonism and antagonism have been shown in many cases to be a tissue-specific and dose-dependent phenomenon for a given “estrogenic” agent like raloxifene (Evans et al. 1996) or the AR ligand S-40503 (Hanada et al. 2003), and such compounds are now better characterized as selective estrogen or androgen receptor modulators. Such subtle modulation in activity cannot be recognized by a simple evaluation of binding that consequently offers less information for the clarification of potential mode of action, which could subsequently inform priority setting and later *in vivo* Tier I and II studies.

Third, the ability to detect compounds metabolized to estrogens and androgens (proestrogens or proandrogens), such as methoxychlor and benzo(a)pyrene for the ER (Bulger et al. 1978; Fertuck et al. 2001) and vinclozolin for the AR (Kelce et al. 1994), is also limited due to a lack of appropriate metabolic capacity. Receptor-ligand interaction also may be nonspecifically disrupted (e.g., by denaturation of receptor protein) and misinterpreted as a reduction in ligand binding, potentially resulting in false-positive characterization of a test material. Such misinterpretation is a likely possibility when the universe of industrial chemistries

that include materials such as solvents, chelants, and surfactants are screened. This issue has been examined for the AR using various detergents to produce apparently distinctive displacement curves relative to true competitive ligands (Freyberger and Ahr 2004). More recently, the use of fluorescence polarization for competitive ligand binding raises the issue of the assessment of test materials that themselves fluoresce or interfere with light emission, potentially resulting in flawed assessment of test material interaction with the receptor. Therefore a formally standardized assay that would define the operational limits of the methodology is necessary.

Transactivation Assays

The isolation of the genes for the human ER α , ER β , and AR, together with the emergence of recombinant DNA technology, allowed for their application to the development of what are now termed reporter gene or gene transactivation assays. The general principle is that ligand-bound nuclear hormone receptors alter their conformation, dimerize and bind specific response element sequences on DNA, and initiate transcription of a downstream gene. For convenience, the downstream gene in these assays is part of a recombinant genetic construct that codes for a protein like luciferase (Legler et al. 1999), β -galactosidase (Gaido et al. 1997), chloramphenicol acetyl transferase [CAT¹] (Tully et al. 2000), and green fluorescent protein (Bovee et al. 2004) whose activity, usually enzymatic, can be measured easily and accurately after treatment of the cell cultures with a chemical agent—hence the term “reporter gene.” This activity measurement thus signals the potency of various ligands/chemicals to bind the receptor and either initiate or inhibit receptor-induced transcription of the reporter. Consequently, reporter gene assays can assess both agonist (test chemical alone) and antagonist (test chemical in the presence of stimulating ligand) activity.

As currently performed for the investigation of the hormone-like transcriptional activity of xenobiotic agents, these transactivation or reporter assays exhibit a great deal of variability in their methodology, and are far less standardized than receptor binding assays (Charles et al. 2000a; Gaido et al. 1997; Kuiper et al. 1998; Saito et al. 2000; Shelby et al. 1996; Vinggaard et al. 1999). This variability stems from a number of sources, including the culture system (e.g., yeast vs. mammalian cells), the nature of the receptor and/or reporter gene constructs, and the methods of introduction or incorporation (transfection) of the constructs into their host cell systems. Each methodology has its own unique advantages and disadvantages in the investigation of potential ligand activity as a consequence of receptor binding and activation.

In terms of culture systems, both yeast (Elsby et al. 2001; Gaido et al. 1997; O'Connor et al. 2000; Routledge and Sumpter 1997; Sugawara et al. 2002) and mammalian cells (Charles et al. 2000a,b; Hartig et al. 2002; Maness et

al. 1998; Terouanne et al. 2000; Vinggaard et al. 1999) have been used in transactivation assays to characterize the activity of potential endocrine-active agents. Yeast requires introduction of both a receptor and a reporter construct (e.g., β -galactosidase) into the cells, whereas mammalian cell lines, which in some cases naturally express the receptor of interest (e.g., MDA-MB-453 [AR] [Hartig et al. 2002]), require only the insertion of a reporter gene construct (e.g., CAT, luciferase). However, dual receptor-reporter transfections are common for mammalian endocrine screening assays, especially in the context of the evaluation of activity against the AR and the requirement for selectivity against ER α versus ER β (Gaido et al. 1999). For large-scale screening purposes like that proposed by EDSTAC, this dual introduction of gene constructs raises legal issues as a consequence of the patenting of this methodology along with those held on the AR for commercial uses (ICCVAM 2003b; O'Connor et al. 2002).

A further lack of standardization is shown, for example, in mammalian systems in which a variety of receptor response elements (ARE, ERE) have been used in the reporter constructs. These elements have included incorporation of the promoter regions of hormone-responsive genes (Klotz et al. 1996; Tarumi et al. 2000), the specific response element DNA sequences themselves recognized by the ER or AR (Legler et al. 1999; Rogers and Denison, 2000; Seinen et al. 1999; Yoon et al. 2001), and chimeric receptor reporter systems (Fertuck et al. 2001; Matthews et al. 2002). The use of hormone-responsive promoter regions, and to a lesser extent response element constructs, has been shown to exhibit reduced responsiveness of the reporter gene, in part because of activation by non-AR- or ER-mediated processes, and the cross-reactivity or interaction with other signaling pathways (Keller et al. 1995; Wilson et al. 2002). It should be noted that the activity of mammalian screening systems is also cell line specific (Cho and Katzenellenbogen 1993).

The recent introduction of chimeric receptor reporter systems in endocrine screening has attempted to minimize the issues described above by utilizing hybrid receptor and reporter constructs. The receptor construct is created by a fusion of the ligand binding domain of the ER with the DNA binding domain of a yeast-specific protein (GAL4). This “chimeric” construct is then able to recognize and activate the reporter gene construct containing the GAL4 DNA response element, which is not known to be bound by other mammalian factors. Such systems have been used in the investigation of the estrogenic activity of synthetic chemicals (Fertuck et al. 2001) and extracts of air particulates (Clemons et al. 1998). The methodology has also allowed for incorporation into the screening evaluation of species-specific endocrine responses by application to ERs from varying organisms (Matthews et al. 2002), although similar cross-species comparisons have not utilized this approach exclusively (Sumida et al. 2003).

Yeast assays have been used in endocrine screening and have the advantage of easy genetic manipulation, allowing

the incorporation of exogenous receptor subtypes and reporter constructs for the ER and AR to produce stable reporter cell lines (Bovee et al. 2004; Routledge and Sumpter 1997). However, yeast-based systems have two disadvantages: They are unable to discriminate agonists from antagonists in terms of the classical responses to compounds like the antiestrogenic ligand ICI 182,780. In addition, they have altered rank order potency differences, potentially due to issues of cell wall permeability, multidrug resistance transport proteins (which mediate chemical extrusion or uptake), and metabolism and cell signaling interactions in terms of the presence of accessory proteins and other signaling molecules (Gaido et al. 1997; Pham et al. 1992; Zysk et al. 1995).

The scope of the receptor binding assays discussed above does not incorporate evaluation of the activity of test materials requiring metabolism. Yeast and mammalian transactivation systems exhibit limited metabolic competency, as evidenced by their ability to detect activity attributed to putative estrogenic and antiandrogenic metabolites of some parent molecules like methoxychlor and vinclozolin, and consistent with observations of *in vivo* activity of these materials (Charles et al. 2000b; Kelce et al. 1994; Shelby et al. 1996; Wong et al. 1995). This restricted metabolic competency has led to attempts to incorporate exogenous metabolizing systems like liver microsomal preparations to allow for greater potential metabolism to a broader range of chemistries in both yeast (Elsby et al. 2001; Shiraishi et al. 2003) and mammalian assays (Charles et al. 2000b; Sumida et al. 2001; Yoshihara et al. 2001). However, these attempts have had limited success.

The reduced degree of standardization with transcriptional activation assays compared with their receptor binding counterparts results in significant interlaboratory variability in terms of sensitivity, reproducibility, and precision. Furthermore, alterations in assay-specific parameters such as pH and solvent effects can also lead to significant variability (Beresford et al. 2000; Charles et al. 2000c). This observation raises significant issues regarding the need for the EPA to validate these assay methodologies for use as screening tools in the context of the EDSTAC-proposed screening-testing battery. A recent expert panel convened under the auspices of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM¹) to assess the present readiness of the receptor binding and receptor transactivation methodologies for validation was unable to recommend any single assay method. However, the panel recommended minimum procedural standards that should be incorporated in future standardized assays for validation studies (ICCVAM 2003a).

Minced Testis and Aromatase Assays

The minced testis and placental aromatase assays are intended to assess the potential of chemicals to interfere with

steroid biosynthesis or more directly alter steroid hormone levels. However, it is important to note that these assays do not cover the plethora of possible ways by which the synthesis, metabolism, and feedback interaction of steroid hormones can be disrupted (Ashby 2000). Instead, the complexity of the steroid hormone biosynthetic pathways was reduced expeditiously for purposes of deriving simplified assay models more amenable to large-scale chemical screening (Miyamoto and Burger 2003).

Minced Testis

The minced (sliced) testis assay was designed to identify the potential of compounds to disrupt any of the intracellular pathways involved in the gonadal biosynthesis of testosterone (Gray et al. 1995; Laskey et al. 1995). The assay is based on the steroidogenic activity of testicular tissue, which is almost exclusively performed by the Leydig cells (Huhtaniemi and Toppari 1995). The current assay uses sectioned portions of tissue from the decapsulated testis of naive rats that has been incubated in culture media with the test compound under study in the presence and absence of the steroidogenic stimulant human chorionic gonadotropin. Media collected at various incubation periods are then analyzed for testosterone, usually by radioimmunoassay (Powlin et al. 1998; Sikka et al. 1985), although other analytical measures such as gas chromatography/mass spectrometry are applicable (Smith et al. 1987). The assessment of toxicant effects on steroidogenesis using this method has been limited to testes (Gray et al. 1995; Powlin et al. 1998; Sikka et al. 1985) and ovary (Laskey et al. 1995; Piasek et al. 2002).

Attempted optimization and evaluation of a series of endocrine-active agents in this test system, including known steroidogenesis inhibitors like ketoconazole and finasteride, resulted in poor correlation with their well-characterized *in vivo* activity (Powlin et al. 1998). The sectioned culture has the advantage over isolated Leydig cell cultures that it is easier to prepare than primary cell cultures; however, decreased steroidogenic activity in the assay would be difficult to differentiate from unequal activity across preparations and/or cytotoxicity to Leydig cells. The specific measurement of Leydig cell cytotoxicity is technically challenging in this complex system with multiple cell types (O'Connor et al. 2002). Alternative approaches such as the use of primary Leydig cultures (Biegel et al. 1995; Brun et al. 1991; Klinefelter et al. 1991) or immortalized cell lines (Nishi et al. 2001) may warrant consideration in a validation context as a screening methodology. These systems would allow for the potential incorporation of a cytotoxicity evaluation and also significantly minimize animal usage in the case of immortalized cultures.

Placental Aromatase Assay

Compared with the minced testis assay, the placental aromatase assay seeks to identify specific inhibitors of the aro-

matase enzyme, which catalyzes the conversion of androgens to estrogens (Lephart and Simpson 1991). The aromatase assay was included in the alternate battery to complement the *in vivo* male pubertal Tier I screen that was thought to lack sensitivity to aromatase inhibitors, which act to decrease estrogen levels (EDSTAC 1998). The aromatase enzyme complex is composed of two proteins: a cytochrome P450 (CYP19A1), which converts C19 androgenic steroids into C18 estrogenic steroids, and an NADPH-cytochrome P450 reductase (Simpson et al. 1994).

The assay involves the evaluation of an aromatase enzymatic preparation to convert an androgenic substrate such as testosterone or androstenedione to estradiol/estrone, and the ability of a test material to alter this conversion process quantitatively. The assay is usually carried out by one of two methods: the more rigorous product isolation technique (Kao et al. 2001; Prefontaine et al. 1990) or the radiometric assay method (Lephart and Simpson 1991; Vinggaard et al. 2000). As its name implies, the product isolation method involves incubation with labeled or unlabeled androgen and quantitative isolation of the estrogenic products via chromatographic, mass spectrometric, or immunoassay (Biegel et al. 1995; Kao et al. 2000; Numazawa et al. 2002). By comparison, the radiometric method indirectly evaluates aromatase activity by measuring the amount of $^3\text{H}_2\text{O}$ released from [1β - ^3H]-androstenedione due to stereospecific cleavage of the substrate during conversion to estrogen (Fishman and Raju 1981). The assay has recently been modified by the use of a fluorescent substrate using recombinantly expressed aromatase preparations (Stresser et al. 2000).

Spontaneous release of $^3\text{H}_2\text{O}$ has been observed while evaluating aromatase activity in enzymatic preparations from human endometrium (Prefontaine et al. 1990). In addition, other biochemical pathways of androgen metabolism may result in greater apparent activity than that evaluated by the product isolation method (Lephart and Simpson 1991). The potential for false-positive findings via the radiometric method should require that prevalidation of the assay includes an initial direct comparison of both product isolation and radiometric analysis assessments to facilitate use of either methodology based on laboratory preference. However, the product isolation procedure, although more resource intensive, should remain the gold standard for evaluation.

The aromatase assay has been used in the assessment of the activity of numerous agents including pesticides like atrazine, diuron, and fenarimol (Andersen et al. 2002; Hennewer et al. 2004; Vinggaard et al. 2000), organochlorines, polychlorinated biphenyls, and TCDD (Drenth et al. 1998; Letcher et al. 1999; Sanderson et al. 2002), organotin (Cooke 2002; Nakanishi et al. 2002), and phytoestrogens (Pelissero et al. 1996). These studies have utilized cell-free enzymatic preparations from both human placenta and recombinant sources (Andersen et al. 2002; Stresser et al. 2000), primary cultures of hepatocytes and Leydig cells (Biegel et al. 1995; Liu et al. 1996), and immortalized JEG-

3, Jar, and H295R chorio- and adrenocortical carcinoma cell culture systems (Letcher et al. 1999; Vinggaard et al. 2000). Although the authors of studies using the cell culture models (Letcher et al. 1999; Sanderson et al. 2002) have remarked as to the negative impact of cytotoxicity on their potential utility in this assay, the ability to discriminate true direct impact of a test material on aromatase activity, rather than reduced activity via impairment of cellular function, is a critical issue in the implementation of a validated screening methodology.

Validation of a cell-free system such as microsomal enzyme preparations from placenta obtained after delivery, or even bovine or porcine sources, raises two issues: heterogeneity in enzyme expression and, in the case of human tissue, availability for commercial screening. Furthermore, the potential use of human tissue raises the question of genetic polymorphisms in the aromatase gene, which may result in differential assay responses by enzymatic preparations from different individuals. Serious consideration should be given to recombinant sources for the enzyme that are now available (Jo Corbin et al. 2003; Stresser et al. 2000) and that allow for a standardized and readily accessible reagent source. As with the receptor binding assays, the need to discriminate direct action (e.g., aromatase inhibition) through binding the enzyme or receptor versus the nonspecific decrement in activity due to issues of denaturation and solvent effects still must be addressed.

Conclusions

The implementation of the *in vitro* component of the tiered testing strategy recommended by EDSTAC awaits acquisition of a set of agreed-upon validated screening assays under established criteria (ICCVAM 2003). Validation requires the establishment of operational characteristics such as sensitivity (proportion of actives correctly identified) and specificity (proportion of inactives correctly identified). Issues that affect such a validation exercise with these *in vitro* test systems have been described and will pose significant challenges. Some stakeholders have expressed concern that the final tests will result in increased animal usage because the assays require receptor and tissue preparations. This concern, coupled with issues of availability and variability, suggest that emphasis on the use of recombinant technologies and cell culture systems in the development of validated methods should be a priority.

The reliable assessment of cytotoxicity is critical for assays such as the transactivation and minced (sliced) testis assays, especially in the context of potential antagonistic responses to test compounds in which the decreased assay response may be a consequence of a cytotoxic manifestation. Furthermore, validation also emphasizes the need for an agreed-upon set of positive and, more importantly, negative control test materials for assay standardization. An agreed-upon test material battery is important in light of the

fact that most of the environmental estrogens characterized to date tend to exhibit weak activity in these assay systems. Hence, an agreed-upon framework for the utility of these assays would require the establishment of a definite set of pass-fail criteria for each in vitro test system/methodology to minimize the potential confusion that may result from individual laboratory determinations of positive or negative test findings. Among the examples of criteria that would be included are acceptable coefficients of variation and required numbers of replicate data points/experiments, as well as cutoffs for designating a positive/negative response relative to controls.

The means by which the biochemical activity of the hormonal milieu may be altered are legion. In vitro assays, which are simplified means of examining specific mechanistic endpoints (an observed activity), provide data that are qualitatively different from in vivo tests (an adverse toxicity) (Ashby 2000; Miyamoto and Burger 2003). The tiered testing strategy proposed by the EDSTAC therefore highlights the scientific consensus for both in vitro (mechanistic activity) and in vivo testing that incorporates absorption, distribution metabolism, and excretion for characterization of potential endocrine activity, although it may oversimplify the complex interactions of some chemicals with the endocrine system in vivo. Despite the potential usefulness of properly standardized and validated in vitro assays in prioritizing substances for definitive Tier II testing, it is important to recognize that Tier I in vitro assays have limitations. They are valuable as screening tools that provide mechanistic data, but they should not be used directly in risk assessment paradigms.

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