ELSEVIER

Best Practice & Research Clinical Endocrinology & Metabolism
Vol. 20, No. 1, pp. 15–33, 2006
doi:10.1016/j.beem.2005.09.001
available online at http://www.sciencedirect.com

BEST PRACTICE & RESEARCH

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Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity

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The endocrine and reproductive effects of xenobiotics are believed to be due to (1) their *mimicking* the effects of endogenous hormones; (2) their *antagonizing* the effects of endogenous hormones; (3) their *altering* the pattern of synthesis and metabolism of natural hormones; and (4) their *modifying* hormone receptor levels. It has been suggested that endocrine disruptors may play a role in the decrease in human semen quantity and quality, an increase in the anomalies of male genital tract, and an increase in the testicular and breast cancer incidence during the last 50 years. Testing these hypotheses will require: (1) identifying estrogen and androgen agonists and antagonists among the chemicals present in the environment; (2) assessing the interactions among the endocrine disruptors to which humans are exposed; and (3) finding markers of estrogen (and androgen) exposure. The development of fast and sensitive bioassays is central to the achievement of these three goals.

Key words: xenoestrogens; antiandrogens; cell proliferation; endosulfan; phthalates; antioxidants; alkylphenols; endocrine disruptors; E-SCREEN; A-SCREEN.

THE WINGSPREAD STATEMENT

The term 'endocrine disruptor' was coined at the Wingspread Conference held in Racine, WI, in 1991. The participants proposed that the developmental alterations observed in a diversity of wildlife species was due to exposure to multiple chemicals

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that, through different modes of action, disrupted the endocrine system of developing metazoan organisms. They noticed that hormonally active chemicals were being introduced into the environment and human food. The antioxidant nonylphenol, which had just been shown to leach from laboratory plastic ware[1], was one such example, and headed a growing list of chemicals that were subsequently identified as being hormonally active agents: plasticizers, agrochemicals, disinfectants, dental materials, and sunscreens among them.[2–5]

The Wingspread participants made the observation that some of the effects documented in the genital tract in wildlife were comparable to those seen in the daughters and sons of women who had been exposed during pregnancy to the synthetic estrogen diethylstilbestrol (DES), which was administered therapeutically to prevent spontaneous abortion between the years 1948 and 1971.[6,7] It was postulated that the DES syndrome was an extreme expression of the plasticity of the fetus to environmental cues. The participants of the Wingspread Conference concluded that, as in the metaphorical canaries in the mines, the developmental abnormalities observed predominantly in birds might foretell what was happening in mammals, including humans.[8]

One year later, a meta-analysis concluded that the quantity and quality of human sperm had decreased during the last half-century, coinciding with the introduction of chemicals into the environment.[9] Further, a variety of reports confirmed an increased incidence of male genital tract defects, such as cryptorchidism, hypospadias and testicular cancer.[10] It was postulated that these diverse outcomes might be the result of extemporaneous exposure to man-made estrogens during fetal development.[11] These studies motivated the formation of government-sponsored committees in the USA, the European Union and Japan that were charged with the task of evaluating the evidence. Research programs were established to assess whether the reported effects were reproducible, and to test the hypothesis that environmental hormonally active agents were causing these effects. In the USA, the 1996 amendments to the Safe Drinking Water Act and the 1996 Food Quality Protection Act required that the Environmental Protection Agency (EPA) develops a program to screen and test chemicals used in large volumes that may contaminate water and food in order to assess their potential activity as endocrine disruptors. In order to perform these law-mandated screenings, in vitro and in vivo assays have been developed and validated.

FROM WINGSPREAD TO THE PRESENT

Due to the large number of chemicals introduced into the environment during the last 60 years, a major effort was devoted to the development of methodology for the identification of endocrine disruptors, particularly of estrogen and androgen agonists and antagonists. Two main approaches were pursued: (1) the development of methodology to study quantitative structure–activity relationships (QSARs) (reviewed in Chapter 5), and (2) the design of in vitro assays. The development of these methodologies is enlarging the list of potential endocrine disruptors. However, for the most part these chemicals seem to be far less potent than the natural hormones in these in vitro assays. Moreover, when the xenoestrogens identified through these methods were tested using the uterotropic assay, their low potency relative to that of estradiol (E_2) was confirmed. However, significant effects were observed when these compounds were administered perinatally at doses that were several orders of

magnitude lower than those producing an uterotropic effect. This lack of correlation between the potency revealed by these simple assays and the developmental effect suggests that the relative potency revealed by the conventional assays should not be used to infer whether a chemical will or will not produce deleterious effects at current exposure levels. Despite the limitations of these in vitro assays with regard to in vivo effects, the assays are necessary and useful for the initial screening of chemicals, for monitoring exposures, and for the analysis of mixtures found in the environment.[12]

ESTROGEN AGONISTS AND ANTAGONISTS

How do natural and synthetic estrogens act on their target tissues?

Estrogens regulate the expression of specific genes and the secretion of certain polypeptidic and steroidal hormones, and coordinate diverse processes such as cell proliferation, cell differentiation and tissue organization.[13] Once estrogens reach the bloodstream, they remain either free or bound to serum estrogen-binding proteins such as α-fetoprotein (AFP) in rodents[14] or sex hormone binding globulin (SHBG) and albumin in humans.[15] There is a consensus regarding the notion that only the free (unbound) hormone is able to diffuse inside the target cell and reach the cell nucleus, where it binds to the estrogen receptors (ER) to form a hormone–receptor complex. The prevailing model suggests that this complex then dimerizes and interacts with the estrogen-response element (ERE) of a target gene and with the transcriptional machinery. There are at least two ERs: the 'classical' ERα and ERβ.[16] ERα is present in the uterus and is thought to drive the uterotropic response, since the uterus of the ERα 'knock-out' mouse does not respond to estrogen administration.[17] ERβ, on the other hand, is present in organs such as the prostate, hypothalamic nuclei and pituitary gland.[18] It is also present, together with ERα, in some human breast cancer cell lines. It is unknown at present whether there are effects that are exclusively mediated by ERβ.

Though discovered over 10 years ago, the role of ERβ remains to be fully determined; it has been postulated that it may be to curtail or oppose the effects mediated by ERα.[19] Although selective ligands are being developed, it can be confidently stated that as of now all the xenoestrogens tested bind to both ERs, albeit with different affinities.

In addition to this mechanism of action on gene expression, other estrogenic effects—such as the secretion of prolactin—are thought to be mediated by a subset of ERα bound to the plasma membrane through extranuclear mechanisms that do not involve transcription.[20]

In a related context, our research has highlighted the fact that estrogens affect the proliferation of their target cells by mechanisms involving the plasma membrane-bound ERα.[21] Evidence accumulated during the last 20 years suggests that the default state of all cells is *proliferation*, and the proliferation of estrogen target cells is regulated by a complex interaction between a plasma membrane-bound ER, a plasma-borne inhibitor of the proliferation of estrogen target cells (i.e. albumin), and perhaps additional factors.[21–23] Xenoestrogens also interact with the membrane-bound ER. Interestingly, some of these xenoestrogens that behave as weak agonists in the uterotropic assay are strong agonists regarding the effects mediated by the membrane-bound receptor.[24,25]

What are the uses for in vitro assays?

Reliable 'in culture' assays are needed for several purposes. From a preventive viewpoint, chemicals that will be entering the food supply (such as those used in food packaging) or those that would be released massively into the environment (such as pesticides) should be tested for hormonal activity while still in the development stage of manufacturing. This would provide the means to avoid producing chemicals with unintended hormonal activity. A second reason would be to identify the chemicals that are currently found in the environment for further testing. A third use of bioassays is to discover markers of estrogen exposure: for example, testing water courses to find out whether they are contaminated with estrogenic substances. This will allow the detection of bioactivity without having to know a priori the chemical nature of all estrogens involved. This is important because only a very small list of chemicals has been tested for hormonal activity. This decision precludes the use of chemical analysis to determine whether or not xenoestrogens are present in environmental samples. Hence, there is a need to use bioassays as markers of exposure.

Provided that mixtures act additively, the measurement of estrogenic activity may be used for comparative purposes: for example, to identify a focal source such as sewage[26], to monitor the efficiency of a water treatment plant[12], or to assess water contamination downstream from animal feedlots.[27] Moreover, a comparison with the predicted activity from chemical contaminants identified in these water samples often reveals an excess estrogenic activity that cannot be accounted for in the contaminants already identified. In this case, performing the bioassay after fractionation by chromatography could be used to guide the identification of xenoestrogens.[26]

In addition to testing water, a marker of exposure to hormone mixtures based upon these bioassays may be used to assess human exposure.[28] This would be accomplished by testing the estrogenic activity due to environmental agents found in the blood and fat of humans.[29] For example, the E-SCREEN assay has been successfully used to determine the total xenoestrogenic activity ('total xenoestrogen burden') in adipose tissue in an epidemiological case–control study of breast cancer.[30]

End points measured by in vitro assays

In order to develop a bioassay, it is necessary to choose which effect—in this case that of an estrogen—will be used as an end point. There are at least three definitions of estrogens based on their effects. The one we prefer for the purpose of characterizing a reliable bioassay is the narrow definition that was proposed by Hertz, i.e. 'estrogens are substances, which elicit the proliferative activity of the organs of the female genital tract'. According to Hertz, nothing but estrogens induces the proliferation of these cells.[31] The E-SCREEN assay is based on this property.

A second definition centers on the estrogen receptors as mediators of estrogen action. This biochemically oriented definition states that estrogens are substances that elicit the expression of genes that are controlled by estrogen-responsive elements. Hence, depending on which type of promoter is chosen for the reporter gene, different results can be obtained. And finally, the third definition unifies the biological and biochemical aspects of estrogen action and states that 'estrogens are substances that elicit the proliferative activity and the control of specific gene expression in tissues of the female genital tract'. Each definition implies different specific end points.

Receptor binding assays

The receptor binding assays may use extracts from the uteri of different animal species (bovine and rat), extracts from human cell lines that contain estrogen receptor (MCF7, T47D cells), or recombinant receptor protein as starting materials. The parameter measured is the relative binding affinity (RBA) for E_2. Originally, tissue extracts were used to measure the ability of the tested chemical to compete with radiolabeled E_2 for binding to the estrogen receptor. The concentration point in which the tested chemical results in a 50% decrease of the binding of labeled E_2 to the receptor is denoted as the inhibitory concentration (IC)50. Results are expressed as IC50 or as relative binding affinity, which is the ratio between the IC50 of the test compound and that of unlabeled E_2. For example, if the IC50 of the test compound is 1 nM and the IC50 of E_2 is 1 nM, the RBA is 1. Modern, high-throughput versions of this assay have been developed in recent years.[32] These assays are easy to perform. However, they fall short in distinguishing agonists from antagonists, and partial agonists from full agonists. Moreover, they are quite insensitive when compared to other bioassays (reporter gene and cell proliferation assays).

A typical competitive binding assay is depicted in Figure 1. The curve for E_2 reveals that the IC50 in receptor binding assays is 1 nM, whereas the half-maximal effect for E_2 in the E-SCREEN assay, which measures cell proliferation of the estrogen-target MCF7 cells, is 7–12 pM. This shows one example of the insensitivity of the competitive binding assay. In addition, compounds that are much less potent than 17-β E_2 may give false negatives because of their low solubility. In some cases, it is not possible to obtain total displacement of labeled E_2 and hence to measure an IC50.

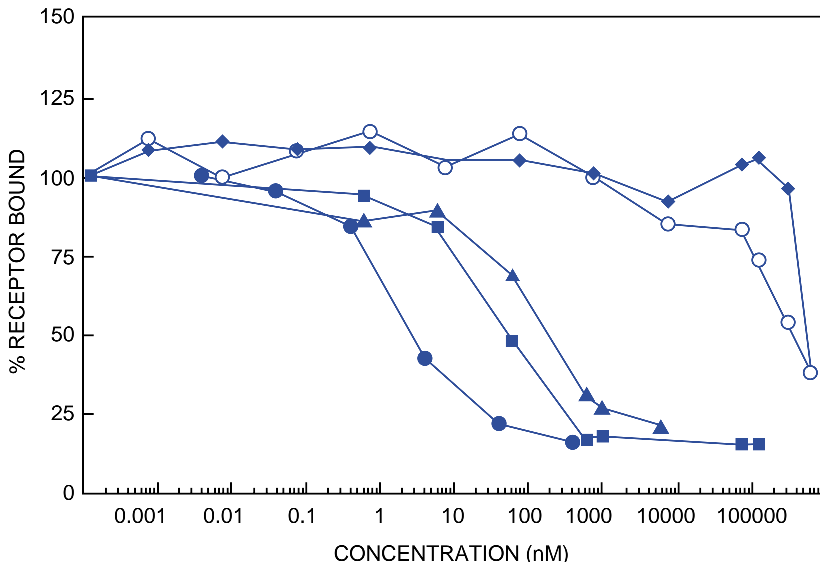

Figure 1. Competition between ^{3}H-estradiol and unlabelled chemicals for binding to estrogen receptors from MCF7 cell extracts. The ordinate represents percentage of ^{3}H-estradiol bound to the receptor. One hundred percentage bound is computed as specific ^{3}H-estradiol binding in the absence of competitor. Competitors are estradiol (●), bisphenol-A (▲), *p*-nonylphenol (■), *o,p*′-DDT (○), and endosulfan-B (◆). Method described in Soto et al[3].

Summing up, measuring relative binding affinities is a fast way to screen chemicals. However, we believe that there are better approaches that can be done for the same money.

Cell culture assays: induction of progesterone receptor and PS2

Established cell lines that express the estrogen receptor provide two end points to assess estrogen activity: cell proliferation and induction of estrogen-regulated gene expression. The breast cancer MCF7 cell line responds to estrogens by increasing its proliferation rate and the expression of endogenous genes like progesterone receptor and PS2.[33–35] We have used both end points to confirm the estrogenicity of the pesticides endosulfan, toxaphene, and dieldrin, which were found to be estrogenic using cell proliferation as the end point (E-SCREEN assay).[2] Like the E-SCREEN assay, the expression of progesterone receptor and PS2 assay discriminates between agonists and antagonists[3]; however, they are less sensitive than cell proliferation assays. Unlike the reporter gene and the E-SCREEN assays, neither PS2 nor progesterone receptor induction have been used for screening purposes.

Cell culture assays: induction of cell proliferation

A cell proliferation assay using the estrogen target human breast cancer MCF7 cells (E-SCREEN assay) was developed about 20 years ago. As alluded above, it measures what is considered to be the biological hallmark of estrogen action, i.e. induction of cell proliferation. Probably because it was available earlier than the other in vitro assays, most of the xenoestrogens were identified using this assay. The E-SCREEN assay discriminates between agonists and antagonists, and full and partial agonists and antagonists.

The E-SCREEN is a simple assay in which similar numbers of cells are seeded into the wells of a tissue culture multiplate; the cells in each well are allowed to attach for 24 hours, and then the medium is changed. Cells are allowed to proliferate for 5 days in the presence of medium containing estrogenless serum (prepared using charcoal–dextran adsorption), along with a range of concentrations of the chemical being tested. The 'negative' control is provided by cells cultured in the absence of estrogen; the 'positive' controls are provided by cells cultured with a range of concentrations of E_2. A typical E_2 dose–response is depicted in Figure 2 together with the dose–response of two xenoestrogens, bisphenol-A and nonylphenol. The E_2 concentration resulting in half-maximal proliferation is about 7–12 pM. It is the most sensitive assay published so far, and could be made more sensitive using a micro-method. The assay also discriminates between partial and full agonists (Figure 3). In the experimental conditions described above, only estrogens elicited cell proliferation.

This assay reveals whether the compound is a partial or a full agonist by comparing the maximal cell number obtained with the test compound with that obtained with E_2. The proliferative effect (PE) measures efficacy; it is measured as the ratio between the highest cell yield obtained with the test chemical and the hormone-free control. Under these experimental conditions, cell yield represents a reliable estimate of the relative proliferation rate achieved by similar cell inocula exposed to different proliferation regulators. This parameter is called the *relative proliferative effect* (RPE); this is $100\times$ the ratio between the highest cell yield obtained with the chemical and with E_2. RPE is calculated as $100\times$ (PE-1) of the test compound/(PE-1) of E_2. Thus, the RPE indicates whether or not the compound being tested induces (1) a proliferative response

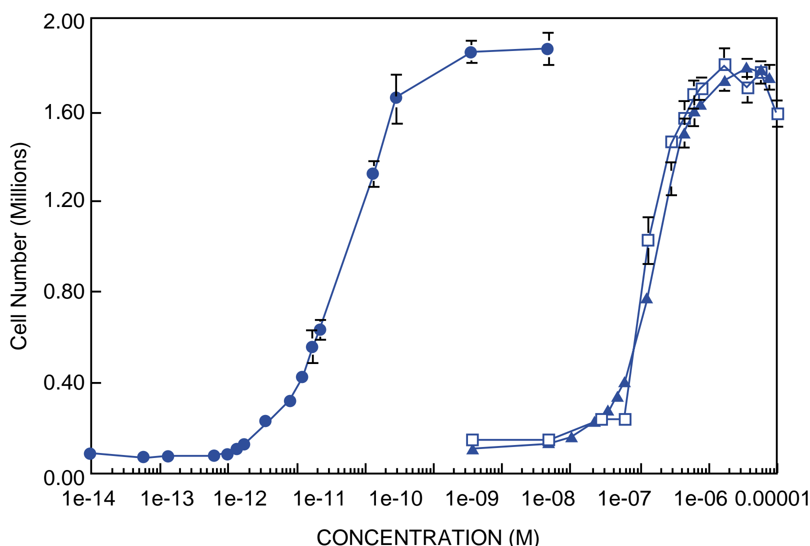

Figure 2. Dose–response curve to estradiol (●), bisphenol-A (▲) and nonylphenol (□) by the E-SCREEN assay. Cells were harvested after 5 days of exposure to 5% estrogenless serum plus the indicated concentration of the estrogenic chemical. Method as described by Soto et al[27,29].

quantitatively similar to the one obtained with E_2, i.e. a full agonist (RPE = 100), or (2) a proliferative yield significantly lower than the one obtained with E_2, i.e. a partial agonist.[36] The potency of xenobiotics is assessed by determining their relative proliferative potency to E_2 (RPP); it measures the ratio between the minimal concentration of E_2 needed for maximal cell yield and the minimal dose of the test compound needed to achieve a similar effect. Alternatively, relative potency may be measured by comparing the concentrations at which proliferation is half-maximal; this parameter is akin to the IC50 used in the competitive binding assays. Figure 4 displays a schematic representation of these concepts. For screening purposes, the range of xenobiotic concentrations was from 1 nM to 10 μM, and for E_2 from 0.1 pM to 1 nM.

Antagonists are detected in a two-step test by a modification of the E-SCREEN assay. In the first step, the ability of the chemical to inhibit estrogen action is tested. A range of concentrations of the presumptive antagonist is added to the medium containing the minimal dose of E_2 that induces maximal proliferation. Once it is found that a compound inhibits estrogen action in step 1 (Figure 4), it becomes imperative to verify that this is a receptor-mediated phenomenon, i.e. it can be reversed by increasing the concentration of estrogen. This reversal by estrogen, called ‘estrogen rescue’, is the hallmark of a true antagonist. In this second step, the minimal dose of the antagonist needed for maximal inhibition is tested in the presence of a range of doses of E_2 (Figure 4).

How are these novel assays validated? Although the uterotrophic assay is the in vivo assay considered to be the ‘gold standard’ of estrogenicity, we and others have found that this end point is quite insensitive.[37,38] Therefore, we use the mitotic index of the uterine lining as an assay to verify our E-SCREEN results.

Using the E-SCREEN assay, many xenoestrogens have been identified. When we started our research on the subject, the only insecticides known to be estrogenic were

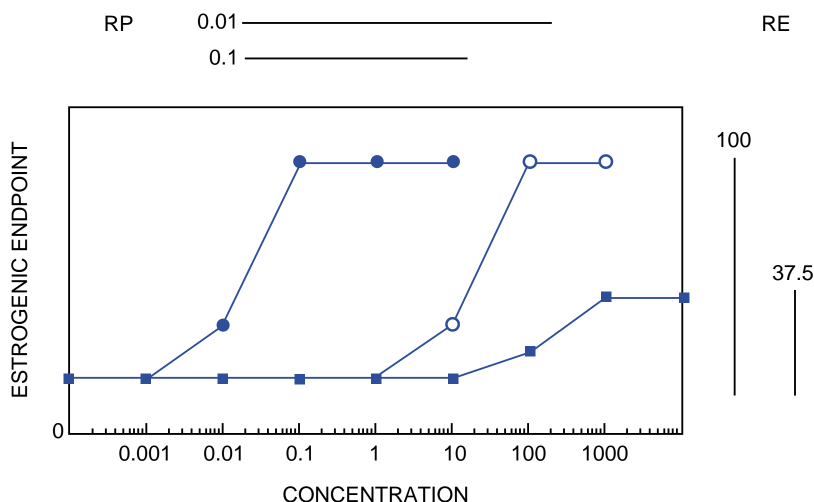

Figure 3. Schematic representation of the dose–response curve to estradiol (●), a full agonist (○), and a partial agonist (■). Relative potency (RP) is the ratio between estradiol and xenobiotic doses needed to produce half-maximal effects $\times$ 100. The horizontal bars indicate that RP is a comparison between effective *concentrations* of the agonist and estradiol. Efficacy (*E*) is the ratio between the maximal effect obtained with the compound tested and that of the untreated control. Relative efficacy (RE) compares the maximal effect achieved by the xenobiotic with that obtained with estradiol. RE is calculated as $100 \times (E - 1)$ of the test compound/$(E - 1)$ of estradiol; a value of 100 indicates that the compound tested is a full agonist, a value of 0 indicates that the compound lacks estrogenicity at the doses tested, and intermediate values suggest that the xenobiotic is a partial agonist. The vertical bars at the right of the graph box illustrate that RE compares the ability of estradiol and of agonists to increase the cell yield (E-SCREEN) or product (reporter gene assays) over the values obtained in untreated controls. Method described in Soto et al[3].

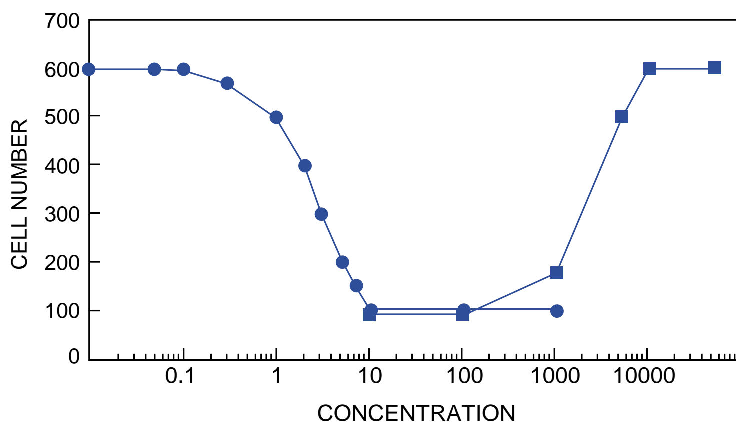

Figure 4. Schematic representation of the two-step method for assessing antagonists by means of the E-SCREEN assay. In step 1 (inhibition), the antagonistic activity is measured in inocula exposed to 0.1 nM estradiol plus the range of concentrations of the putative antagonist indicated in the abscissa (●). In step 2 (rescue), the lowest dose of antagonist that produced maximal inhibition is measured in inocula exposed to a range of concentrations of estradiol as indicated in the abscissa (■). Method described in Soto et al[27].

DDT and its metabolites methoxychlor and kepone. The E-SCREEN assay also revealed that endosulfan, toxaphene, dieldrin, and lindane are also estrogenic. Chemicals found to be estrogenic using this assay include antioxidants such as alkylphenols and butylhydroxyanisole, the disinfectant orthophenylphenol, compounds found in plastics such as benzylbutylphthalate, dibutylphthalate, bisphenol-A and its derivatives, some PCB congeners, sunscreens[5], and preservatives found in cosmetics.[39]

Reporter gene assays

There are two types of reporter gene assay. There are those using cells that already express the estrogen receptor, such as MCF7 cells, and those using cells that do not express endogenous estrogen receptors. In the first case, the cells are transfected with a reporter gene that is inducible by estrogens. Two assays of this type are used, e.g. transient and stable transfectants. The latter are indeed more desirable, because once the cells are engineered they are supposed to remain stable and ready for use. The MVLN, an MCF7 cell line transfected with a reporter gene whereby the vitellogenin promoter regulates the expression of luciferase[40,41], was one of the first to be developed. However, when these cells were briefly exposed to hydroxytamoxifen, their reporter gene no longer responded to estrogens.[42] The mechanism underlying this effect seems to be due to silencing involving heterochromatin protein 1α.[43] In principle, avoiding exposure to hydroxytamoxifen should prevent this from happening. However, this raises the issue of instability due to inadvertent exposure to chemicals during maintenance or propagation of the cells. More recently, the ER-CALUX assay was developed by stable transfection of the estrogen-target T47D cells with an ERE-driven luciferase reporter gene. A comparable assay was developed using MCF7 cells (MELN). The sensitivity and half-maximal activity for E_2 of these two assays is comparable to that obtained with the E-SCREEN assay; however, certain agonists—such as resorcylic acid lactones—result in a significantly higher luciferase activity (overactivation) than that obtained with E_2, making it difficult to define partial and full agonistic activity.

Other assays require a double transfection, with a construct that confers the constitutive expression of the receptor and a construct containing the reporter gene. Most of these assays use yeast cells, taking advantage of the short incubation period needed for a detectable response. However, they do not discriminate effectively between agonists and antagonists.[44] When using yeast cells, problems of membrane permeability and transport have been identified that may spuriously affect the measurement of the relative estrogenic potency.[45] The rate of false negatives is high for these assays.[46]

The recently developed HELM assays use human HELA cells; two variants have been developed, one expressing ERα and the other ERβ. Both activate a luciferase reporter gene. This assay is comparable to the MELN, CALUX and E-SCREEN regarding sensitivity. Like the other reporter gene assays, it shows overinduction by certain ligands.

Comparison among assays

To date, no extensive comparisons of the different methods have been published, with the exception of those by Andersen et al[47] and Fang et al[48]. The former was a blind study comparing the estrogenic activity of 20 compounds using a competitive ligand binding assay, the E-SCREEN assay, and a variety of reporter gene assays using both transient and stable transfectants. Their main recommendation was the use of two

assays: the ligand binding assay using a recombinant human estrogen receptor and the E-SCREEN, 'as these assays have high sensitivity, are easy to perform, and in combination provide information on both receptor binding and a cellular response'. The study by Fang et al compared in vitro estrogen receptor competitive binding assays, yeast-based reporter gene assays, and the E-SCREEN assay to determine their quantitative agreement in identifying structurally diverse estrogens. Data sets for at least 29 chemicals from five laboratories were analyzed pair-wise by *X*–*Y* plots. This comparison showed that the ER binding assay was a good predictor for the other two assay results when the antiestrogens were excluded ($r^2 = 0.78$ for the yeast assays and 0.85 for the E-SCREEN assays). Antiestrogens were identified as outliers in the ER binding/yeast assay, while complete antagonists were identified in the ER binding and E-SCREEN assays. Although these assays involve different hierarchical levels of biological complexity, the major conclusion is that they generally provided consistent information in quantitatively determining estrogenic activity for five data sets examined. Unfortunately, there are no data comparing the mammalian cell-based reporter gene assays with either the E-SCREEN or the yeast-based assays. The overactivation observed when testing resorcylic acid lactones suggests that they may not behave quantitatively when mixtures are assessed. This is a serious limitation, since the behavior of mixtures is of utmost importance in ecotoxicology and in the use of these bioassays to assess total xenoestrogen burden. In summary, it seems that all the assays discussed above are good at identifying xenoestrogens. However, regarding quantitative data, only the yeast reporter gene assay and the E-SCREEN assay have been evaluated and found to provide good quantitative data when evaluating the activity of mixtures. In this regard, Rajapakse et al[49] stated:

'Of all the in vitro assays in use for the screening of endocrine-active chemicals, the E-SCREEN represents the highest level of biological complexity, and we regard this as strength because it affords the opportunity to identify factors that may impact on mixture effect predictability. And here lies the relevance of our observations to ecotoxicology. It will be interesting to see whether apical end points affected by endocrine-active chemicals in fish, such as effects on fertility or fecundity, may also be sensitive to intervening toxicity during the prediction of combination effects. Our findings suggest that this may well be the case and would have to be taken into consideration during experimental planning, design, and assessment of estrogenic combination effects.'

Conclusions

In vitro/'in culture' screens using breast cancer cells are accurate and sensitive for the detection of estrogen agonists and antagonists. Their main limitation is that they cannot detect pro-estrogens, which are compounds that are metabolized in vivo into estrogens. We prefer the cell proliferation assay (E-SCREEN) because it has been extensively studied, and no false positives or false negatives have yet been found. It is also the most sensitive among the published assays. It can discriminate among agonists and antagonists and in addition it lends itself to the study of interactions such as additivity, synergism, and antagonism. MCF7 cells, like all the target cells used in reporter gene assays, do not metabolize many of these chemicals. However, some metabolic activity may exist in these cells. For example, methoxychlor, which is not supposed to be active until metabolized to the free phenolic product, is estrogenic in the E-SCREEN assay. Alkylphenol monoethoxylates and bisphenol-A dimethacrylate

seem to be metabolized to the free phenols, and PCBs are probably hydroxylated, since these compounds are estrogenic in the E-SCREEN assay. But for the most part, in order to make the E-SCREEN or reporter gene assays work reliably for pro-estrogens, an activation step needs to be developed. Yeast assays have the advantage of being fast, but they can produce spurious results due to cell wall permeability. In addition, false-negative results have been reported.[48]

Finally, the E-SCREEN assay has been used in several epidemiological studies to assess total xenoestrogenic exposure.[30,50] In one of these studies, a significant correlation was found between total xenoestrogenic burden and breast cancer.[30]

ANDROGEN AGONISTS AND ANTAGONISTS

Androgen agonists and antagonists

Among the environmental chemicals tested to date, none has been found to be an androgen mimic. A few derivatives of the known non-steroidal antiandrogen bicalutamide (ICI 176,334) have shown androgenic effects in vitro.[51] In contrast, several androgen antagonists have been identified among environmental chemicals, such as the bioaccumulating DDT metabolite *p,p*′-DDE (dichlorodiphenyldichloroethylene), the fungicides vinclozolin, procymidone, and prochloraz, and the herbicide linuron.[52–56] The two vinclozolin metabolites, M1 and M2[57], as well as linuron, but not *p,p*′-DDE, relate structurally to the therapeutic antiandrogen flutamide. Polycyclic aromatic hydrocarbons block androgen receptor (AR) activation in vitro[58] and suppress androgen-dependent growth of accessory sex organs in juvenile male rats.[59] Recently, several phenolic chemicals demonstrated antiandrogenic activity in a reporter cell line.[60]

End points measured by in vitro assays

Binding assays

Androgen receptors were believed to be less ‘promiscuous’ than estrogen receptors; however, Fang et al[61] reported a wide structural diversity among AR ligands in an extensive experiment measuring the AR binding activity of 202 chemicals. In this study, the relative potency based on the ligands’ mean RBAs from higher to lower affinity was as follows: steroids > diethylstilbestrols (DES), diphenylmethanes, PCBs, organochlorines > phytoestrogens, phenols, flutamides, phthalates, and phenol-like chemicals.

Among the steroid hormones, certain progestagens and mineralocorticoids also bind to the androgen receptor. Moreover, the androgen, glucocorticoid, progesterone, and mineralocorticoid receptors have identical consensus response elements and their DNA-binding domains are highly conserved. This also explains in part the overlapping among progestogenic, androgenic and corticoid activities. It is thought that higher specificity is achieved at higher orders of biological organization. This is the reason for developing bioassays addressing higher levels of biological complexity.

Cell proliferation assays

There are few ‘in culture’ models to study androgen action. Those available are mostly based on the human prostate cell line LNCaP-FGC developed in the early 1980s.[62] However, the androgen receptor in this cell line has a point mutation in

the androgen-binding domain that enhances estrogen binding.[63,64] For this reason, this cell line has not been used for screening androgen agonists and antagonists; however, it has been used extensively to study androgen action on the control of cell proliferation and gene expression.[65–69]

We have proposed that the control of the proliferation of androgen target cells occurs by a two-step mechanism.[65,70] In step 1, androgens increase the proliferation of target cells by canceling the inhibition exerted by a specific plasma-borne protein. In step 2, androgens directly trigger the expression of APRIN, an endogenous inhibitor of the proliferation of their target cells.[65,71–73] This latter inhibitory effect of step 2 would be equivalent to a failsafe effect on step 1. To further study step 2, we transfected a full-length, wild-type, human androgen receptor into MCF7 cells. These MCF7–AR1 cells express the proliferative shutoff (step 2).[70] These are the cells used in our A-SCREEN assay, the androgen equivalent to/complement of the E-SCREEN.

Contrary to the process for detecting estrogens (measuring the induction of cell proliferation or step 1), we use the *inhibition* of cell proliferation as an end point to screen for androgen agonists, while for androgen antagonism we use the inhibition of this response, which is an *increase* in the cell number. To avoid interference by estrogen on cell proliferation activity, this assay is performed in a serumless medium supplemented with insulin and transferrin.

Figure 5A shows that both DHT and the synthetic androgen R1881 inhibit the proliferation of MCF7–AR1 cells. The cells undergo G_0 arrest within 24 hours of androgen treatment. Maximal activity has been observed at 0.1 nM DHT, 0.1 nM R1881, and 1 nM testosterone. Hydrocortisone is also a partial agonist in this system due to the well-established fact that at supraphysiological doses, it binds to the androgen

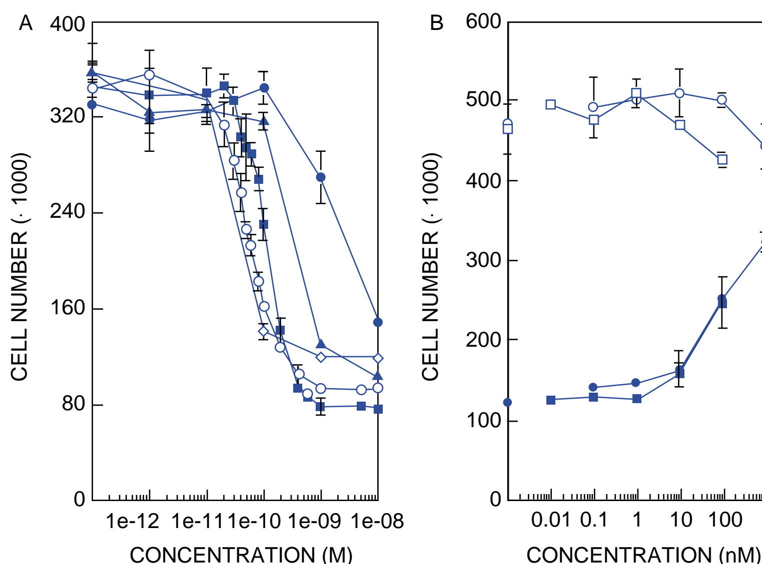

Figure 5. (A) Proliferative response of MCF7-AR1 cells. Dose–response curve to R1881 (○), DHT (■), trenbolone α (●), trenbolone β (◇) and testosterone (▲). (B) Response of MCF7–AR1 cells to antiestrogens. Cells were cultured with bicalutimide (○) or with hydroxyflutamide (□) alone or in the presence of 10 pM R1881 (●),(■), respectively. Methods described in Soto et al[27], Szelei J et al[70], and Körner et al[77].

receptor. Thus, we do not think that discrimination between androgen and hydrocortisone can be further improved.

Most of the environmental chemicals that interact with the androgen receptor are androgen antagonists. Figure 5B shows the detection of antagonistic activity by the antagonists bicalutamide and hydroxyflutamide, in the presence of maximal inhibitory doses of R1881. Both reversed the activity of R1881. The A-SCREEN assay has also been used to measure the additive activity of androgens in feedlot effluents.[27]

Reporter gene assays

Since the development of A-SCREEN assay, several reporter gene assays have been developed, most of them using mammalian cells. The MDA-kb-2 cell line has been developed by scientists at the EPA[74] by stable transfection of AR and a MMTV-driven luciferase reporter gene into the human mammary cancer MDA-MB-453 cell line. This cell line constitutively expresses glucocorticoid receptors. In this model, both androgen and glucocorticoid agonists activate the MMTV luciferase gene.

The PALM cell line was derived from the human prostate adenocarcinoma PC3 cell line by stable transfection of the AR and an MMTV-driven luciferase reporter gene[75]; the same researchers also established a comparable reporter assay based on the CHO cell line.[60]

Most recently, Sonnenveld et al[76] developed the AR CALUX cell line by stable transfection of the human U2-OS osteosarcoma cell line with the androgen receptor and a luciferase reporter gene construct containing three androgen-responsive elements coupled to a minimal TATA promoter. The purpose was to make their reporter more androgen-specific than those in the cell lines mentioned above. Table 1 compares the doses at which the half-maximal effect was achieved in these assays.

Table 1. Comparison of the half-maximal effect of a set of androgens and anti-androgens determined by the A-SCREEN, MDA-kb-2, PALM and CALUX assays.

Compound	A-SCREEN	MDA-kb-2	PALM	CALUX
DHT	7.8×10^{-11} M[a]	21×10^{-11} M[a]	$4–300\times10^{-9}$ M[b]	13×10^{-11} M[c]
R1881	6.8×10^{-11} M[d]	11×10^{-11} M[e]	3.0×10^{-11} M[b]	
Testosterone	29×10^{-11} M[f]		$2-200\times10^{-10}$ M[b]	66×10^{-11} M[c]
Methyltestosterone	43×10^{-11} M[a]	53×10^{-11} M[a]	74×10^{-11} M[a]	
Androstenedione	59×10^{-9} M[a]	140×10^{-9} M[a]	35×10^{-9} M[a]	4.5×10^{-9} M[c]
Bicalutamide	13×10^{-8} M[a]	38×10^{-8} M[a]	17×10^{-8} M[a]	
Vinclozolin	21×10^{-8} M[a]	11×10^{-8} M[a]	47×10^{-8} M[a]	100×10^{-8} M[c]
4,Tris-(4-chlorophenyl) methanol	57×10^{-8} M[a]	110×10^{-8} M[a]	80×10^{-8} M[a]	

Compounds 1–5 are androgen agonists; compounds 6–8 are androgen antagonists. Results are expressed as half-maximal androgenic activity for compounds 1–5 and as the half-maximal inhibitory activity for compounds 6–8. The provenance of the data are indicated by the following symbols.

[a] Körner et al[77].
[b] Terouanne et al[78].
[c] Sonneveld et al[76].
[d] Soto et al[27].
[e] Ma et al[80].
[f] Szelei et al[70].

Comparison among assays

The only blind comparative study published thus far involved a transient AR and reporter gene co-transfection, the A-SCREEN and two stable transfectants, the PALM and the MDA-kb-2 cell line.[77] The participating laboratories received four coded compounds and two controls: two steroidal androgens, two antiandrogens, an androgenic control, 5α-dihydrotestosterone (DHT), and an antiandrogenic control, bicalutamide (ICI 176,334), and the solvent used to make the stock solutions. All four test systems produced comparable quantitative results for two androgens and two antiandrogens. The doses resulting in a half-maximal effect and the resulting androgenic potencies relative to the positive control differed by less than a factor of 10 between the assays, with one exception, i.e. the assay consisting of the transient transfection of CHO cells with AR and the reporter gene. The latter also showed an overall greater sensitivity toward detection of androgens. Regarding the same antiandrogenic chemicals tested by the above-mentioned laboratories, differences in relative antiandrogenic potencies and in doses required for a half-maximal antagonistic effect remained well within one order of magnitude. The coefficient of variation obtained for the different half-maximal values stayed generally within the same range. Different metabolic capacities of each cell line for each compound may also play a role. Differences in relative androgenic potency may be due to differences in metabolism of the test compound, the reference compound (DHT), or both.[77] In this regard, the PALM cells were reported to metabolize testosterone, so that its relative potency decreased as the number of cells plated increased.[78] The authors concluded that all four cellular in vitro assays proved to be sensitive screening tools to detect and quantify AR-mediated androgenic and antiandrogenic effects of these chemicals with reasonable accuracy. In addition, the study stressed that in choosing a test system to use, the equipment of the laboratory, specific background and experience of the staff, and cost-effectiveness must also be evaluated.

Conclusions

Androgen screens have not been tested as thoroughly as estrogen screens. The interlaboratory study by Koerner indicates that their results regarding androgens and antiandrogens are quite comparable. As remarked by Sonneveld et al[76], the presence of glucocorticoid receptor in the MDA-k2 cells may make them 'unsuitable as a selective screening tool'; hence, they proposed the use, instead, of AR-CALUX. However, this assay, as well as other reporter gene assays, oftentimes exhibits supramaximal induction for a particular ligand. This phenomenon should be carefully avoided when using the assay to measure the additive effect of mixtures. In this regard, we think that the A-SCREEN is presently the most suitable bioassay available.[27]

GENERAL CONCLUSIONS

Most of the novel xenobiotics with estrogen or androgen agonist or antagonist activities discovered in the last decade have been identified by the use of 'in culture' assays. This screening effort, incomplete by far, reveals that there are hormonally active chemicals that may pose a health risk for humans and wildlife. So far, agrochemicals such as endosulfan, and vinclozolin, plastic monomers such as

bisphenol-A, antioxidants such as BHA and alkyl phenols, and UV filters have been identified. Other sources of exposure include certain phthalates and alkyl phenol polyethoxylates, which are used as dispersant agents for pesticides and in cosmetics and toiletries.

Over 70 000 synthetic chemicals are registered for commercial use in the US EPA's Toxic Substances Control Act Inventory.[79] In vitro bioassays are probably the best tool to screen this large number of chemicals in a timely manner. These assays could also be extremely useful to monitor the presence of endocrine disruptors in the environment as well as to ascertain the efficacy of treatments to decrease the release of these hormone mimics into the environment. Finally, we introduce the concept of measuring the total xenoestrogen burden using the MCF7 estrogenicity assay as a marker of exposure to environmental estrogens. This parameter was used to assess the role of xenoestrogens on the increased incidence of breast cancer, and may be useful to test the role of xenoestrogens on cryptorchidism and lowering of the quality of semen in human populations. Thus, for all practical purposes, bioassays to identify undesirable synthetic sex steroids in our environment are available. One wonders whether the political will to legislate their identification and banning is there to prevent humans and wildlife to be unnecessarily exposed to them.

Research practice points

- all the assays discussed in this review are adequate to determine whether a given chemical, mixture or extract obtained from a biological sample have estrogen or androgen agonist and antagonist activity; however, they are not equally sensitive
- when quantifying the activity of a mixture, the use of assays that display overactivation should be avoided

Research agenda: use of bioassays for assessing xenoestrogen burden

- epidemiological studies should be performed to assess the potential causal role of endocrine disruptors in breast and prostate cancers, undescended testis, hypospadia and lowering age of puberty onset
- there are 80 000 chemicals in the US Federal Registry, and only a few have been tested for hormonal activity. It is clear that using a single estrogenic chemical as a marker of 'xenoestrogens' is not only inappropriate but also dangerous because it may produce false negatives. Bioassays such as the E-SCREEN assay may be used to measure the total xenoestrogen concentration in biological samples. This measurement should provide a useful tool to assess the role of xenoestrogens in health outcomes mentioned above
- the timing of exposure to xenoestrogens is crucial, and hence, exposures should be assessed at the appropriate 'window of vulnerability'

ACKNOWLEDGEMENTS

This work was partially supported by the grants from the W. Alton Jones Foundation, EPA-CR 820301, NIH-ES08314, NIH-AG13807, NIH-CA13410 and NSF-DCB-9105594.

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