

# Chapter 11: Endocrine Disruption in Humans

## Introduction

### Concerns about possible wildlife and human health effects

Many *in vivo* and *in vitro* tests have been proposed for screening chemicals for endocrine-disrupting activity, and several regulatory agencies, including the US Environmental Protection Agency (EPA), have recommended strategies involving tiered testing schemes, in response to concerns about the possible adverse effects of such chemicals on the hormonal systems of wildlife and humans. Such chemicals have been called endocrine disruptors (EDs), and are alleged to mimic (agonise) or block (antagonise) the effects of endogenous steroid sex hormones. Reported adverse human health effects associated with exposure to them include deterioration in semen quality, various defects on gonadal development, and testicular, prostate, uterine and breast cancers, as well as hypospadias, endometriosis and effects on the thyroid gland (1, 2).

### US legislation

Legislation in the USA requires the testing of chemicals for endocrine activity (the *Food Quality Protection Act* of 1996, Public Law 104–170, and the *Safe Drinking Water Act Amendments* of 1996, Public Law 104–182; 3). Other regions of the world, particularly Japan and Europe, are also introducing legislation for testing for EDs (for example, 4).

A major problem with investigating the possible modulation of the hormonal system is that normal development is controlled by the endogenous sex steroid hormones (such as oestrogen, testosterone and progesterone), which are characterised by the presence of a 4-ringed cyclopenta[ $\alpha$ ]phenanthrene nucleus, and are highly soluble in lipids.

### Definition of endocrine disruptor

Various definitions of EDs have been proposed. For example, the International Programme on Chemical Safety (IPCS) and the World Health Organisation (WHO) define an endocrine disrupting chemical as “an exogenous substance or mixture that alters function(s) of the endocrine system and causes adverse health effects in an intact organism, or its progeny, or (sub)populations”. This is the definition that is used in this chapter, although the reader is referred to Harvey *et al.* (5) for further discussion.

## Types of endocrine disruptors

A wide diversity of chemicals can exert deleterious effects on the endocrine system, including synthetic chemicals (such as polychlorinated biphenyls, phthalates, organochlorines, polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and a range of synthetic steroids) and naturally occurring chemicals (such as flavonoids, lignans, sterols, aromatic amines and indole-3-carbinol).

## Scientific Background

### The endocrine system

The endocrine system comprises many organs which produce a number of different hormones that are part of a complex feedback regulatory system (see 6 for a review).

Endogenous hormones act primarily by binding specifically to an extensive family of nuclear receptors (steroid hormone receptors [SHRs]; 7). SHRs have structural features in common, which include a central, highly conserved, DNA-binding domain that targets the receptor to specific DNA sequences (hormone-response elements [HREs]). The terminal portion of the receptor includes a ligand-binding domain that interacts directly with the hormone. This domain contains a hormone-dependent transcriptional activation region. Binding of the steroid hormone to the receptor protein causes it to undergo a conformational change, resulting in binding of the DNA to the HRE to form a complex that triggers or suppresses the transcription of a specific set of genes (2, 8).

### The activity of endocrine disruptors

EDs act through a diversity of mechanisms, including receptor binding, altered post-receptor activation, steroidogenesis (modulation of hormone synthesis), hormone storage and clearance, and perturbation of homeostasis. The immediate effects of this activity, depending on the nature of the ED, are: a) oestrogen agonism or antagonism; b) androgen agonism or antagonism; c) progesterone agonism or antagonism; d) suppression or induction of levels of endogenous hormones; and e) other effects, such as the release of follicle-stimulating hormone (FSH), luteinising hormone (LH) or prolactin.

The oestrogen receptor (ER) exists in at least 2 subtypes,  $\alpha$  and  $\beta$ , each encoded by a separate gene.

These ER subtypes differ in their tissue distribution and in their relative ligand-binding affinities for the same hormones (a fact that could account for differential hormonal effects, according to the responding tissue).

## Tests for Endocrine Disruptors

Testing for EDs is a new area of toxicology, and various testing strategies are now being developed. At least 56 tests have been identified for detecting EDs (3, 9; summarised in Tables 11.1 and 11.2). These methods have been combined into tiered testing strategies comprising *in vitro* and *in vivo* assays.

### *In vivo* assays

The rodent uterotrophic assay, one of the original methods developed for studying oestrogenicity, is still regarded as one of the most useful indicators of endocrine disruption. The test directly measures stimulation of mitotic activity caused by binding to the oestrogen receptor, in tissues of the female reproductive tract. Another animal test was devised by Hershberger and colleagues, to detect the ability of a chemical to elicit agonistic or antagonistic effects by binding to the androgen receptor in male rodents (10). It is necessary to eliminate any interfering effects from endogenous sources of oestrogen and androgen, respectively, in these tests, either by using ovariectomised and castrated animals, or by using immature animals.

Many other *in vivo* tests have been, or are being, developed (11), and some of these are summarised in Table 11.1.

### Drawbacks of *in vivo* tests

There are four major problems with the available animal tests for EDs: a) lack of reproducibility; b) insufficient validation; c) difficulties with applying standard validation criteria; and d) concerns about interpreting the resulting hazard information for risk-assessment purposes. EDs can exert contrasting activities according to the dose administered and the nature of the target cells. Many EDs exert very weak effects, and there are problems with intralaboratory and interlaboratory reproducibility. Also, the interpretation of data can be confounded by a number of factors, including: a) the oestrogenic activity of components of the normal animal diet; b) the use of different species and strains; and c) differences in animal housing and husbandry.

The above problems, together with the experimental procedures required, contribute to severe animal welfare concerns arising from the use of animal procedures. Some of the assays require the use

of considerable numbers of animals in time-consuming experiments, which can involve complicated procedures, possibly including surgery, implantation and repeated injections. Also, considerable animal wastage could occur, when assays are conducted that involve animals of only one sex.

### *In vitro* assays

Several *in vitro* methods are available as screens for detecting EDs, which are designed to be sensitive and rapid, facilitating the testing of large numbers of chemicals (see 2 and 12 for reviews). The main tests involve subcellular hormone receptor ligand-binding and the induction of proliferation (mitogenesis) in hormone-responsive mammalian cell lines, as well as transactivation systems in yeast and mammalian cell lines (Table 11.2).

#### *(Subcellular) hormone receptor ligand-binding assays*

These assays involve assessing the molecular binding of the hormone to isolated and purified receptor proteins. Competitive ligand-binding assays, whether alone or combined with reporter gene expression, are designed to detect chemicals that interact directly with the endocrine receptor. Similar cell-free binding assays involving the androgen receptor (AR) have been developed (13).

#### *Hormone-responsive mammalian cell proliferation*

The most widely used test is based on the induction of mitogenesis in oestrogen-responding cells, particularly in the human breast cancer cell line, MCF-7. MCF-7 cells express the ER and the AR, as well as receptors for progesterone, glucocorticoid, vitamin D and retinoic acid. The induction of proliferation in these cells is believed to result from the binding of oestrogenic substances to the ER within the cells. Several other *in vitro* assays have also been developed that involve the use of diverse human and animal tissues and primary cell cultures.

#### *In vitro assays for steroidogenesis*

These include the use of primary cultures of Leydig cells of the testis (see also Chapter 10). These cells are primarily responsible for steroidogenesis, and are susceptible to various androgen-binding chemicals, such as flutamide. Ovarian steroidogenesis can be measured *in vitro*; for example, Johannsson *et al.* (14) have described a method based on measuring glucocorticoid steroidogenesis in a human adrenocortical carcinoma cell line (H295R).

**Table 11.1: An overview of animal assays for endocrine disruptors**

| Test name  | Endpoint  | Mechanism of effect  | Proposed role  | Validation status                                | Comments   |
|--|---|--|--|--|--|
| 3-day uterotrophic assay                                   | Change in uterine weight  | Binding to the oestrogen receptor, resulting in oestrogen agonism and antagonism   | Provide <i>in vivo</i> screening data for preliminary hazard identification (in EDSTAC Tier 1)                         | OECD validation study completed; outcome awaited | Phases I and II of validation study completed and show that immature and ovariectomised animals give similar responses; few studies have included anti-oestrogens  |
| 5-day or 7-day Hershberger assay                           | Change in weights of seminal vesicles and ventral prostate  | Binding to the androgen receptor, resulting in androgen agonism and antagonism   | Provide <i>in vivo</i> screening data for preliminary hazard identification (in EDSTAC Tier 1)                         | OECD validation study in progress                | Validation study still in progress; early data suggest that there is no need to use castrated animals  |
| 30-day pubertal male assay with thyroid endpoints (see 25) | Intact 23-day-old weanling male rats are exposed to the test substance and pubertal indices are measured. Reproductive and thyroid tissues are weighed and evaluated histologically, and serum is taken for hormone analysis  | Binding to various receptors associated with thyroid-related effects, steroid synthesis and 5-reductase inhibition, to detect alterations of pubertal development, thyroid function, and hypothalamic-pituitary-gonadal system peripubertal maturation | Provide <i>in vivo</i> screening data for preliminary hazard identification (in EDSTAC Tier 1)                         | None   | EPA about to start a validation study. Puberty in mammalian species is a period of rapid interactive endocrine and morphological changes, and a variety of chemicals can dramatically alter pubertal development   |
| Enhanced OECD TG 407 Repeat-dose 28-day study              | A wide range of pathological and clinical biochemical effects, focusing on neuro-toxicity, as in the standard protocol, but with extra endpoints for endocrine-sensitive effects  | All possible mechanisms of ED action   | None, intended to be an <i>in vivo</i> screening method to detect EDs acting by mechanisms other than by ER/AR binding | OECD validation study in progress                | The relevance and reliability of the extra endpoints are not yet established, but these will be evaluated on the basis of the OECD study; use of the assay would not increase the overall number of animals, as the protocol is part of standard testing anyway. |
| <i>In utero</i> developmental (lactation) assay            | A wide range of reproductive effects in the embryo and/or offspring, following <i>in utero</i> exposure, for example, neurobehavioural endpoints, hormone levels, motor activity, malformations, anogenital distance, nipple retention, time to puberty, organ weights, sperm number and motility | Mechanisms based on placental transfer followed by effects in the embryo and/or offspring, including the receptor-mediated effects of oestrogen, androgen and thyroid hormone  | Provide <i>in vivo</i> screening data for preliminary hazard identification (in EDSTAC Tier 1)                         |  | EPA about to start a validation study  |

ED = endocrine disruptor; EDSTAC = Endocrine Disruptors Screening and Testing Advisory Committee.

**Table 11.1: continued**

| Test name  | Endpoint  | Mechanism of effect  | Proposed role   | Validation status | Comments  |
|--|---|--|---|-------------------|---|
| Enhanced 2-generation mammalian reproductive toxicity study with extra endocrine endpoints | A variety of effects on all parameters concerned with reproduction (including fecundity, litter size, birth defects), and some endocrine organ function tests | Agonism and antagonism to oestrogen and androgen receptors, thyroid and neurodevelopmental effects | Provide <i>in vivo</i> data for hazard identification and risk-assessment purposes (in EDSTAC Tier 2) |                   | Modification of existing OECD TG 416 to include extra endpoints |

*ED* = endocrine disruptors; *EDSTAC* = Endocrine Disruptors Screening and Testing Advisory Committee; *EPA* = US Environmental Protection Agency.

**Table 11.2: An overview of non-animal assays for endocrine disruptors**

| Test name                                       | Endpoint  | Mechanism of effect   | Proposed role  | Validation status                        | Comments   |
|---|---|---|--|--|--|
| (Q)SAR  | <i>In silico</i> modelling of receptor binding  | Mathematical relationship between physicochemical properties of ligand and receptor                   | Compound prioritisation (HTPS)                       | None                                     | Being developed by EPA laboratories, especially for pesticides; extensively studied in Japan; some modelling work elsewhere  |
| Receptor binding                                | Radioactive binding to hormone receptor   | Specific binding of ligand to receptor  | Compound prioritisation (HTPS)                       | None, but about to be reviewed by ICCVAM | Concerns about readiness for validation; much activity in USA by EPA laboratories, involving the rat uterine cytosolic and rat prostate cytosolic methods for ER and AR binding, respectively  |
| Cell proliferation                              | Induction of cell division in ED-sensitive cell lines   | Specific binding to receptor, resulting in induction of expression of genes controlling cell division | Compound prioritisation (HTPS)<br>EDSTAC Tier 1 test | None                                     | Issues of specificity for EDs, repeatability, and serum binding can confound data interpretation; Phase I and II metabolism need to be incorporated, to improve the predictivity of the assays |
| Reporter gene assays                            | Induction of transcriptional activation of regulatory element linked to a gene whose expression is easily monitored | Specific binding to receptor, resulting in induction of gene expression                               | Compound prioritisation (HTPS)<br>EDSTAC Tier 1 test | None, but being reviewed by ICCVAM       | The EPA has had problems with reproducibility of assays, but some systems routinely used in-house by industry  |
| Steroidogenesis in Leydig cells (minced testis) | Measurement of testosterone   | Susceptibility of Leydig cells to androgen-binding chemicals  | EDSTAC Tier 1 alternative/extra test                 | None                                     | A detailed review paper has been prepared for EPA  |
| Aromatase assay (26)                            | Aromatase inhibition  | Inhibition of oestrogen biosynthesis by aromatase inhibitors  | EDSTAC Tier 2 alternative test                       | None                                     | A detailed review paper has been prepared for EPA  |

ED = endocrine disruptor; EDSTAC = Endocrine Disruptors Screening and Testing Advisory Committee; HTPS = high-throughput screening; (Q)SAR = (quantitative) structure-activity relationship.

### *Hormone-sensitive transcription of reporter genes*

These tests involve genetically engineered mammalian cells or yeast strains (*Saccharomyces cerevisiae*) expressing human or mammalian ER nuclear receptors, in conjunction with the respective response elements linked to promoter regions for a reporter gene, together with the reporter gene itself. Receptor activation is detected as a stimulation of reporter gene expression. Several different receptor–response, element–reporter gene combinations have been used in the yeast (for example, ERE–gal1–*lacZ* for the detection of oestrogenicity through  $\beta$ -galactosidase production), and in mammalian cell transactivation systems (for example, ERE–luciferase or ERE–CAT [chloramphenicol transferase]). Strains of mammalian cells have also been constructed for the detection of chemicals with prostatic and androgenic activities through interaction with the appropriate receptors.

Genomic analysis is also beginning to be applied to the screening of chemicals for ED activity, by developing microarrays containing DNA sequences for ER and HREs (Jun Kanno, personal communication).

### **Drawbacks of *in vitro* assays**

Apart from the limitations inherent in using all *in vitro* systems, several EDs are known to bind strongly to serum proteins. Such binding can occur with components of culture media, which results in variable *in vitro* data, as well as in differences between the responses obtained in animal and non-animal assays. This phenomenon has been alleviated by removing serum from the medium, but this raises the problem that the *in vitro* model does not then account for serum binding *in vivo*.

*In vitro* assays also require the addition of an exogenous metabolising system. However, for ED testing, this has been considered to be confounded by the binding of the test chemical and its metabolites to components of the enzyme fractions used. However, Hashimoto *et al.* (15) have shown that it is feasible to detect the effects of exogenous metabolism on ED activity *in vitro*.

### **(Q)SAR studies**

Several studies have been conducted to identify the structural features of steroid molecules necessary for efficient binding to nuclear hormone receptors, such as the ER (see 16). Many oestrogens contain one or more phenolic OH groups on a small lipophilic molecule of about 200–300Da. Removal of these moieties normally markedly reduces binding efficiency. One obvious reason why many chemicals

seem to possess oestrogenicity is that the phenolic OH group is a commonly used substituent in synthetic chemistry. It should also be remembered that this chemical moiety is also found widely in the body, arising from the first-pass metabolism of endogenous and xenobiotic chemicals by way of mixed-function oxidase activity.

Several computer-generated models of ligand–ER interactions have been developed to investigate the mechanisms of binding and to predict binding to the ER. This work is limited by the availability of crystal structures of the various SHRs (see Jacobs & Lewis [17] for more information).

Several pharmacophore and QSAR models have been generated for predicting the binding affinities of chemicals to the ER (2, 18). However, most commercially available expert systems lack extensive rulebases for detecting EDs. The MULTICASE system identifies a 6Å unit spacer biophore as being associated with ED activity (18).

Hong *et al.* (19) developed a decision tree-based model to evaluate 58,000 chemicals for binding to the ER, by using physicochemical information and SARs derived from a training set of chemicals with known binding affinities for the rat ER. The model also involves rejection (exclusion) filters, the first of which is a molecular mass range of < 94 or > 1000. The second (inclusion) filter is the need for a ring structure and three structural alerts: a) the steroid ring; b) the phenolic ring; and c) the double-ring structure of the diethylstilboestrol skeleton. Filtering is also based on log P (where P is the octanol–water partition coefficient), positive surface charge area (incorporating molecular shape and electronic distribution), and the breadth of the molecule. This computational approach resulted in a low false-negative rate, and predicted that 80% of the 58,000 chemicals (i.e. 46,000) would show negligible binding to the ER.

## **Existing Testing Strategies for Endocrine Disruptors**

### **Activities of the US Environmental Protection Agency (EPA)**

The EPA has been charged by the US Congress with the task of developing a screening strategy involving the use of validated tests or other scientifically valid information. The EPA therefore established an Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) to consider possible testing strategies for EDs, and to propose tiered testing schemes. EDSTAC proposed a testing strategy based on the combined use of various tests (20, 21), involving an initial priority-setting stage, followed by a Tier I screening phase, to be succeeded by a Tier II testing battery for

detecting and characterising EDs (Figure 11.1). Tier I tests are intended to identify substances with endocrine-disrupting potential for further testing, and Tier II tests (all *in vivo* tests) are designed to identify adverse effects and establish dose–response relationships for hazard and risk assessment.

Priority setting ranks the most important chemicals for more resource-intensive and more-costly Tier I evaluations. This entails the organisation of data on chemicals according to exposure, biological activity and statutory criteria. All the information obtained is being entered into a database (the Endocrine Disruptor Priority Setting Database [EDPSD]). After prioritisation, the chemicals with the highest priority will be tested first. However, developing the EDPSD has been hindered by a general lack of information, and by delays in developing and using receptor-binding tests to provide some of this information. More than 87,000 chemicals were initially selected for evaluation, many of which were polymers or

were otherwise considered unlikely to be active, leaving about 58,000 chemicals to be tested in Tier I.

### Validation of test methods

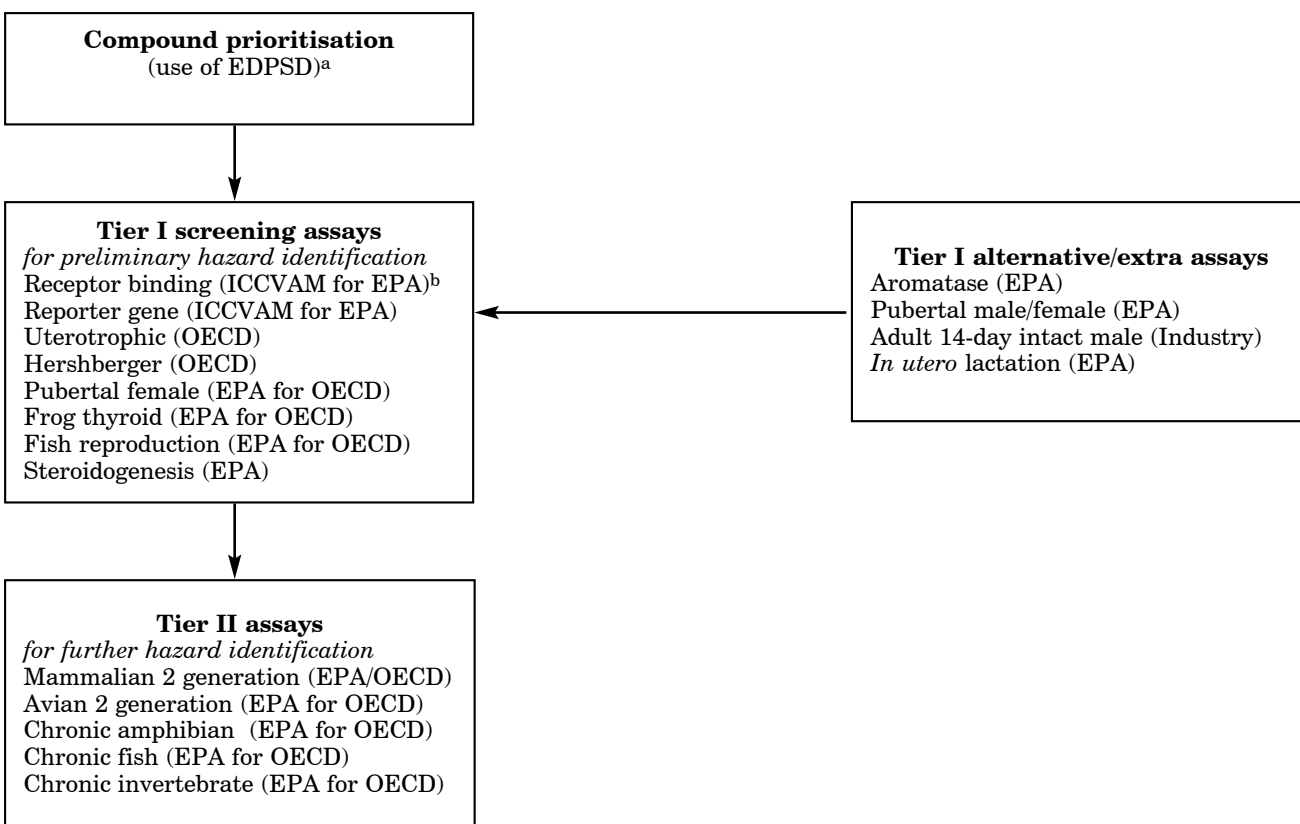
#### *The EDMVS*

A Federal Advisory Committee of the EPA, the Endocrine Disruptor Methods Validation Subcommittee (EDMVS), has been established to advise the EPA on the development and validation of assays for ED testing.

#### *Timetable for implementing the test strategy*

The original EPA timetable for implementing a testing strategy for testing for EDs was: a) priority setting and validation of Tier I tests (2000–2003); b)

**Figure 11.1: A testing strategy for endocrine disruptors as recommended by the Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) of the US Environmental Protection Agency (EPA)**



<sup>a</sup>EDPSD = Endocrine Disruptor Priority Setting Database; <sup>b</sup>indicates the organisation leading the development, validation and assessment of the assay.

validation of Tier II tests (2000–2005); and c) commencement of chemical screening (2003–2005).

### *Involvement of the OECD*

An OECD Working Group on Endocrine Disruptor Testing and Assessment (EDTA) has been established to identify tests for validation, and a separate validation management committee is overseeing the validation of some of the *in vivo* tests identified by EDSTAC, namely the uterotrophic and Hershberger assays, and an enhanced repeat-dose toxicity protocol (OECD Test Guideline [TG] 407).

### *Progress with validation*

The validation of tests for EDs poses a number of problems (2, 22). A series of validation studies have been conducted on the uterotrophic assay, and these have established that it can reliably detect both strongly and weakly oestrogenic substances (23). However, the predictivity cannot be fully assessed, because of a shortage of chemicals lacking activity. As yet, the uterotrophic assay cannot be recommended for screening anti-oestrogenic substances for regulatory purposes, because not enough anti-oestrogens have been involved in the validation studies conducted so far.

The validation studies for the other two assays are at various stages of progress. Phase 1 of the validation of the Hershberger assay was delayed by a need to resolve several basic issues of protocol (such as how to weigh testicular material). However, a protocol has now been agreed, and phase 2 of the study is under way. With regard to the Enhanced TG 407 test, a prevalidation study has been conducted to investigate the interlaboratory transferability of the modified protocol, and to ascertain the usefulness of the additional endpoints in the test. It is intended that the results will be compared for the same set of chemicals tested in the uterotrophic, Hershberger and Enhanced TG 407 assays.

Studies with the uterotrophic and Hershberger assays have shown that there is essentially no need to use ovariectomised or castrated animals (24, 25), and this should be taken into account when OECD test guidelines are being developed for the tests. Also, some investigators have reported low-dose (< 5mg/kg bwt) effects. This is a complex issue, although the current view of the US National Institute of Environmental Health Sciences (NIEHS) Low-Dose Peer Review Group is that the existence of low-dose effects requires confirmation, since there have been many problems in confirming the results. Resolving this issue has been hindered by the technical difficulty of detecting very subtle changes, which can be affected by animal strains,

housing, handling, husbandry and feed. Thus, at the present time, the need for the inclusion of additional dose groups in animal tests for EDs has not been scientifically established.

## **Discussion**

There is considerable controversy concerning the potential adverse effects of EDs on human health. There is an urgent need for careful and realistic assessments of likely exposure, and for appropriate epidemiological analyses of susceptible groups. Such groups include those manufacturing and taking the contraceptive pill before, and unknowingly during, pregnancy (the latter for *in utero* exposure), and those involved in the manufacture and/or use of substances with well-known ED activity.

There has also been debate about the relative uses of non-animal and animal approaches for the detection of EDs. It is widely felt that a fully functional endocrine system is required to investigate whether a substance acts as an endocrine disruptor. Thus, until it is possible for the endocrine system to be modelled outside the body, *in vivo* tests will be required to confirm predictions of ED activity. Thus, a positive result in a non-animal system based on mechanisms of action of EDs can indicate endocrine-disrupting potential. However, activity detected in rodents can only indicate a potential for the substance to act as an endocrine disruptor in humans, because of considerable inter-species differences.

A major problem with developing and validating tests for EDs relates to the absence of general agreement on definitive test data that could be used to provide a high level of certainty that a chemical is not an ED. In view of the diverse and complex effects of ED activity, the definitive test for ED activity is considered by some to be a multi-generation reproductive study. However, such tests are costly and time-consuming, and there is a paucity of data from them. The relevance of other tests, such as the uterotrophic assay, is limited by the difficulty of defining a PM for the assay. In other words, it is not clear how evidence that a chemical binds to the ER in a rodent should be interpreted, when it is known that endogenous hormones bind to their respective receptors as part of developmental processes, including uterine development.

It is becoming clear that the uterotrophic and Hershberger assays can only be used to indicate that a substance is a *potential* ED in humans, and that they should be used in ways analogous to the use of the bone-marrow micronucleus assay in genotoxicity testing (Chapter 9), albeit with respect to a different target organ. The relevance to human health of a positive result in either of these *in vivo* assays is therefore not necessarily any more significant than a demonstration of receptor binding *in vitro*, coupled with evidence of associated transcrip-

tional activation, especially if these latter endpoints were applied in systems incorporating both phase I and phase II metabolism, and were to be interpreted in conjunction with biokinetic and ADME (absorption, distribution, metabolism and excretion) information. It is therefore urgent that such *in vitro* assays are validated for use as the first part of a sequential testing strategy, i.e. to indicate potential for ED activity.

### Endocrine Disruptors: Summary, Conclusions and Recommendations

The mechanisms whereby EDs may act are complex and diverse, and this has hindered efforts to develop reliable and relevant animal and non-animal tests for their detection. The emergence of relevant and reliable animal tests has also been hindered by the fact that few, if any, of the available animal-test methods have been designed with the specific purpose of detecting EDs. They are, in fact, modifications of existing animal tests.

The use of animal tests for EDs poses several important welfare problems, and there are complications arising from the possible existence of a low-dose effect.

The validation of available non-animal approaches should be made a priority, so that they can be used for compound prioritisation, and incorporated into tiered testing strategies in such a way that they reduce or replace further testing in animals.

In addition, there is much research that ought to be undertaken to assess the need for non-animal assays to cover the full spectrum of possible modes of action of EDs, and to provide definitive evidence of whether a substance does or does not interact with the hormonal systems of humans and animals.

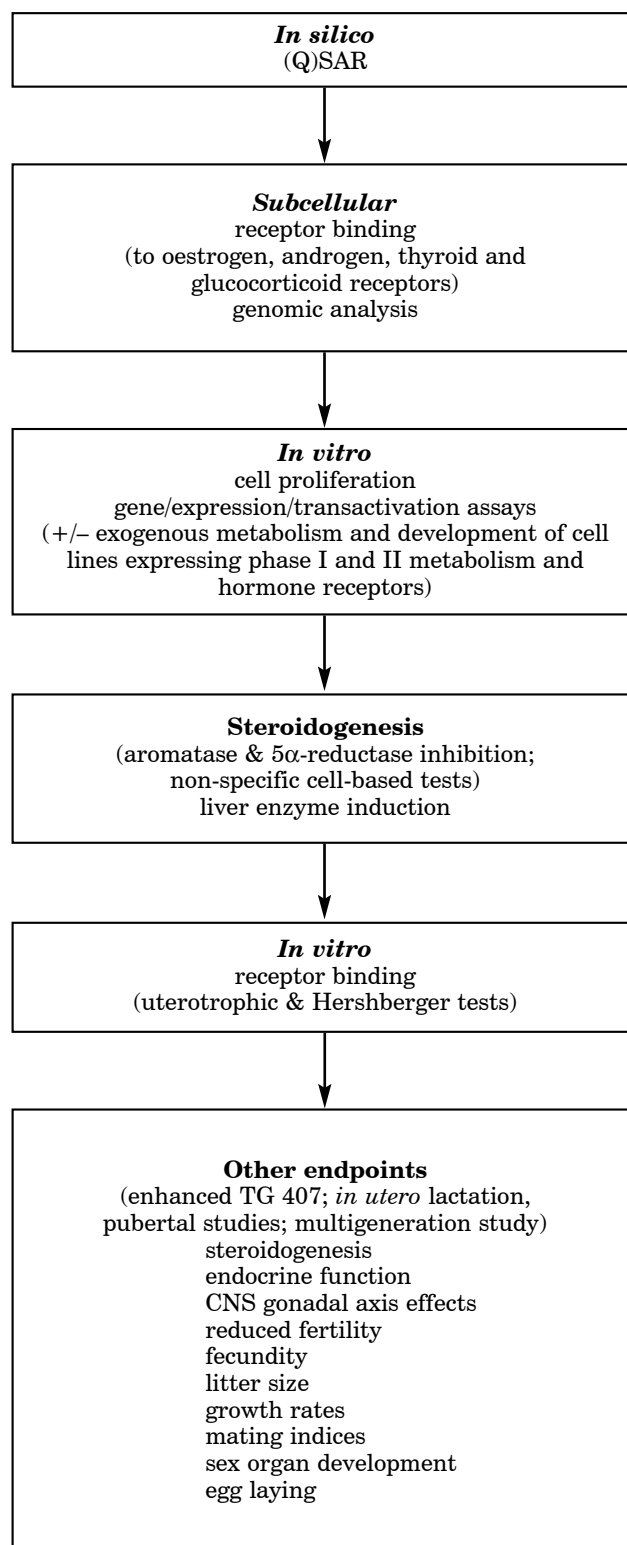
#### Short-term prospects

The validation of (Q)SAR models and cell culture systems for predicting receptor binding.

#### Recommendations for research and development

1. Investigations on the use of microarrays for screening for receptor binding and gene induction, coupled with the search for further relevant receptors.
2. Research into the use of *in vitro* receptor-binding assays in conjunction with biokinetic modelling.
3. An assessment of the potential use of biomarkers of exposure and effect for EDs.

**Figure 11.2: Elements of an integrated testing scheme for endocrine disruptors**



4. Investigations on the suitability of *in vitro* systems for yielding consistent data regarding the potential interactive effects of chemical mixtures of EDs.
5. Further research on the basic mechanisms of ED action, especially by way of non-receptor pathways, and the development of *in vitro* models for such processes.
6. The development of appropriate *in vitro* methods for measuring steroidogenesis in males and females, and for investigating endocrine function.
7. The development of approaches for detecting ED activity by using exogenous metabolising systems and cell culture systems, comprising transgenic cell lines with relevant hormone-receptor response elements, reporter gene sequences, and genes for phase I and phase II metabolism.
8. The development and application of immortalised fetal and pre-pubertal cell lines.
9. The development of an integrated testing strategy for EDs, based on the maximum use of non-animal approaches (Figure 11.2).

### Recommendations to specific organisations

ECVAM should organise a workshop, to discuss the possibilities of using *in vitro* systems for screening for EDs, and to define the roles of such tests for this purpose. The workshop should include experts on endocrine disruption, *in vitro* toxicologists, clinical endocrinologists, ecologists, chemists, experts in (Q)SARs, and regulators.

The appropriate regulatory authorities should consider the scientific merits of a test battery for EDs, either to supplement the existing reproductive toxicity test package, or to be applied as a separate set of tests specifically for ED activity.

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