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Topic 1.12

Interactions of exogenous endocrine active substances with nuclear receptors*

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Abstract: Nuclear receptors function as ligand-regulated transcription factors and modulate the expression of sets of genes in response to varying concentrations of ligands. The ligand modulators can be endogenous metabolites that function as hormones, or they can be exogenous substances, such as pharmaceutical agents or environmental substances of natural or man-made origin, which in some cases can cause endocrine disruption. Ligands modulate nuclear receptor activity by binding to their ligand-binding domains and stabilizing conformations that lead either to transcriptional activation or repression. The ligand-binding pocket is somewhat flexible, and binding affinities can be measured over a 10-million-fold range (i.e., with equilibrium dissociation constant values ranging from ca. 0.01 nM to 100 μM). Thus, it is not surprising that by binding a large variety of structures, some nuclear receptors can appear to be promiscuous; however, when affinity is considered, the binding patterns are more restricted. The spectrum of ligands that bind to the estrogen receptor has been most thoroughly investigated. Those from natural sources include natural products in food, such as soy isoflavones and whole grain lignans, as well as microbial products and components from wood. Aside from pharmaceuticals, man-made estrogen ligands can be found in industrial products, such as alkyl phenols from nonionic detergents, bisphenols from plastics, indicator dye impurities, polymer chemicals, and chlorinated aromatics and pesticides. Exogenous ligands are also known for the androgen and progesterone receptors. While it is possible that endocrine disruption can result from exogenous chemicals acting directly as ligands for the nuclear receptors, endocrine disruption needs to be considered in the broader context; thus, compounds also need to be assessed for their effects at other levels, such as on endogenous hormone production, transport, metabolism, and clearance, and at points in signal transduction cascades that are beyond the ligand–receptor interaction.

FUNDAMENTAL ASPECTS OF NUCLEAR RECEPTOR STRUCTURE AND DYNAMICS THAT DETERMINE LIGAND-BINDING AFFINITY, POTENCY, SPECIFICITY, AND EFFICACY

Nuclear receptors, sensors of endogenous small molecule ligands

Nuclear hormone receptors form a system of ligand-modulated transcription factors that control developmental programs and regulate physiological function (see ref. [1] and other articles in this topic section). The original members of this gene superfamily, the steroid, thyroid hormone, retinoid, and vita-

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min D receptors, were identified through their high-affinity binding of known endogenous hormones. The remaining members, identified by genetic sequence similarity of cloned receptors, were first considered orphan receptors, because their function and regulating ligands were unknown. Over the past several years, however, ligand regulators for many of these orphans have been discovered and their functions have been elucidated, so that most of them have now become adopted [2–5].

Most of the human nuclear hormone receptors are regulated by small molecule ligands [6,7], although some receptors can also be activated in a ligand-independent fashion that typically involves covalent modifications (i.e., phosphorylation, acetylation, glycosylation, etc.) regulated through other signal transduction pathways (i.e., growth factor receptors; see Topic 1.8). The focus of this review, however, will be strictly on nuclear receptor interaction and activation by the small molecule ligands.

The small molecule activators of most nuclear hormone receptors are generally endogenous hormonal substances, (although some receptors, the pregnane X receptor (PXR)/steroid and xenobiotic receptor (SXR) and the constitutive androstane receptor (CAR), are thought to function as xenobiotic sensors [5]). Variations in the concentration of these substances are sensed by their cognate nuclear receptors and used to regulate developmental or functional programs, or homeostatic metabolic feedback or feedforward cycles. Some of the receptors that normally respond to endogenous ligands, however, can also respond to exogenous substances, both of natural or man-made origin. It is this exogenously induced activation or inhibition that, in some cases, might negatively affect the normal pathways of development or physiological regulation, thereby resulting in endocrine disruption.

Ligand-binding affinity and the molecular basis of hormonal specificity

Some of the nuclear hormone receptors have evolved to be very discriminating, high-affinity sensors for their cognate ligands, binding them with nanomolar or subnanomolar equilibrium dissociation constants (K_d). This is the case with the steroid, thyroid hormone, retinoid and vitamin D receptors, and discrimination between these receptors for the heterologous or cross binding their ligands are typically very high. For example, the estrogen receptor discriminates against androgens with a factor of 100 000 [8]. These discrimination factors are less pronounced among receptors that share closer evolutionary relatedness, such as the members of the glucocorticoid family, which include the progesterone, androgen, glucocorticoid, and mineralocorticoid receptors. Still, discrimination factors between members of this family are typically in the range of 100–1000. Many of the adopted orphan nuclear receptors bind their endogenous ligands with much lower affinity, having K_d values typically in the micromolar range, yet they too show high specificity for interaction with their own ligands. It is of note that some orphan receptors that normally bind their endogenous ligands with micromolar affinities can, in fact, bind certain synthetic ligands with nanomolar affinities. Although the receptors have developed this high level of discrimination, many still bind a wide variety of exogenous ligands (see Topic 1.5).

Molecular basis of ligand efficacy: How ligand structure stabilizes particular receptor conformations and how this relates to agonism vs. antagonism

The manner in which ligand binding regulates the activity of nuclear receptors is intriguing: they induce distinct conformations that then engender a certain biocharacter of the receptor response. The ligand-binding domains of hormone receptors are large, consisting of around 250 amino acids [9–14]. The upper half of these domains (as they are typically oriented in representations of crystal structures) appears to be a stable, rather rigid structure, made up of three layers of criss-crossed α-helices. By contrast, the lower half of these domains, in which the ligand is accommodated, appears to be more flexible and dynamic [12,15–17].

In the absence of ligand, the lower half of the domain is shown, in different crystal structures, either to be collapsed (as in RXRγ) [12] or expanded (as in PPARγ) [16]. In either case, it is likely that

apo ligand-binding domains have or can readily reach the character of a protein molten globule, that being a conformation that is condensed and partially folded, but much more conformationally dynamic a stable, fully folded protein [15]. Some receptors, in this apo state, have no effect on transcription, whereas others recruit corepressors proteins and actively repress transcription of target genes [18]. When ligands bind to this domain, they trigger the lower half of the ligand-binding domain to fold around them in various characteristic conformations that are most likely much more dynamically stable than are the apo-receptor forms [14].

The distinct conformation of the receptor that is induced by ligand binding reflects the size and shape of the ligand, and can—at least in large part—determine the agonist vs. antagonist nature of the response. Agonists typically stabilize a conformation in which the C-terminal helix, helix 12, folds back over the ligand-binding pocket and completes the formation of a deep but short hydrophobic coregulator groove into which coactivator proteins can bind via two alpha helical turns of short sequences called nuclear interaction domains [11,19]. Antagonists, by contrast, stabilize conformations in which helix 12 either is repositioned so as to block the coregulator binding groove or extends outward from the core of the domain, resulting in the expansion and widening of the coregulator binding groove in a manner that allows corepressors with three alpha helical turns of a more extended CoRNR box sequence to bind [20,21]. In these antagonist conformations, helix 12 may either be displaced actively, by a direct steric clash between large substituents on the ligand, or passively, by features of the ligand that indirectly interfere with a concatenation of surface residue interactions needed to hold helix 12 in the active conformation [22]. These agonist and antagonist conformations of helix 12, as viewed in various crystal structures, are most likely limiting conformations in a dynamic continuum that reflects the continuum of agonist vs. antagonist activities of various nuclear receptor ligands [22].

Modulation of ligand potency and efficacy by other cellular interactions

The binding affinity that a ligand has for the nuclear hormone receptor and the conformation that it induces in the receptor are probably the major determinants of its potency and biocharacter. Nevertheless, it is clear that other interactions can modulate both of these characteristics. For example, estradiol, which has a K_d for binding the estrogen receptor of ca. 0.2 nM, can stimulate the growth of MCF-7 cells at concentrations as low as 0.001 nM, whereas stimulation of the induction of progesterone receptor in these cells requires estradiol concentrations of 0.1–1 nM. Both responses require the binding of estradiol to the same estrogen receptor, but the potency of estradiol in effecting these responses, is very different. Thus, it is clear that the potency of an estrogen in inducing a specific biological response is not regulated simply by its binding affinity for the receptor, but can also be modulated by post-receptor interactions or other post-receptor rate-limiting events, which can collectively be considered part of the effector system for the estrogen receptor. The effector components are now appreciated to include a set of cellular coregulator proteins as well as promoter-specific factors [1,23]. Conceptually, the multicomponent nature of nuclear hormone receptors has been termed "tripartite receptor pharmacology" [23].

Tripartite receptor pharmacology is a complex issue, but one tenet is that the potency of a hormonal ligand is determined not only by its affinity for the receptor, but also by the manner in which the ligand-receptor complex is coupled with the effector system required to produce a given response. Coregulator binding has, in fact, been shown to modulate the kinetic stability of ligand-receptor complexes [24] and of receptor dimers (Tamarzi, et al., in press). Promoter context has an influence on receptor conformation [25] and coregulator selection by ligand-receptor complexes [26].

A second tenet of tripartite receptor pharmacology is that the biocharacter of a ligand, that is its activity as an agonist vs. antagonist, is likewise not simply a direct consequence of the nature of its interaction with its receptor, but can also be modulated by the cellular context of coregulator proteins and by the nature of the promoter system through which the response is mediated [23]. Again, this can be illustrated with ligands for the estrogen receptor, where the biocharacter of a compound such as ta-

moxifen varies from that of a nearly pure antagonist in the breast, to a partial agonist in the uterus, to a nearly pure agonist in liver, bone and the cardiovascular system [27]. Thus, the action of tamoxifen in these tissues is mediated through the estrogen receptor, yet the responses, in terms of agonism vs. antagonism, differ in a tissue/response-specific manner [26,28].

Thus, according to tripartite receptor pharmacology, both the potency and the biocharacter of a ligand are not fully determined just by the way in which the ligand interacts with the receptor, but they are also modulated by the manner in which the whole receptor-effector-response unit is set up to interpret the ligand.

Nature of the ligand-binding pocket and the molecular basis for hormone disruption

Whereas the effect that the ligand induces on the *exterior* topology of the ligand-binding domain is thought to be the principal determinant of ligand efficacy and its interaction with other cellular constituents, it is the details of ligand interaction with the *interior* of the ligand-binding pocket that underlies, fundamentally, ligand-binding affinity and hormonal potency. As was mentioned above, the ligand-binding pocket is ill formed in the absence of ligand [12,15,16], and the ligand itself forms a core unit—typically hydrophobic—around which the lower zone of the ligand-binding domain folds as the ligand is bound [14]. Ligand binding is obviously a complex and dynamic process that involves a lot of protein movement; this is reflected in the slow rates of ligand association with receptors. On the scale of protein–small molecule association rates, which are typically diffusion limited (k_{assoc} ca. 10^8 $M^{-1}s^{-1}$), the rate at which small molecule ligands associate with nuclear receptors is very slow (k_{assoc} ca. 10^4 $M^{-1}s^{-1}$) [29].

To some degree, the size and shape of the pocket that forms around the ligand is determined by the size and shape of the ligand. The interior of the ligand-binding pocket exhibits a considerable degree of plasticity, and reshapes itself around the contours of the ligand. The packing between protein and ligand, however, is not uniformly tight, and considerable voids can exist [9]. The sum of the volumes of these preformed or residual pockets can, in some cases, be comparable to that of the ligand. For example, with the estrogen receptor-α, X-ray structures show empty pockets above the 11β and below the 7α sites of the endogenous ligand estradiol that have a total volume of 200 $Å^3$, whereas the ligand volume is not much larger (250 $Å^3$) [9]. The existence of these preformed pockets around the ligand had been predicted by a global analysis of the binding affinity of substituted steroidal estrogens [30]. The ligand-binding pockets of nuclear hormone receptors are also typically very hydrophobic, with the few internal polar residues being positioned to interact with the few polar functions on the hormones.

It is not surprising, then, that the nuclear hormone receptors, with their large, flexible, and hydrophobic ligand-binding pockets, are able to interact with ligands that span a wide range of sizes and structures. It is this "specificity tolerance", which has sometimes been called "promiscuity" [31], that underlies the sensitivity of this receptor system to disruption by exogenous ligands [32]. The affinity with which exogenous ligands bind to these receptors depends, of course, on their structure and their functional nature, and the degree to which they deviate in size, shape, functional nature, and hydrophobilicity from the natural high-affinity ligands. The relationship between ligand structure and binding affinity in nuclear hormone receptors, however, is not always a smooth one, where predictions can be made with confidence. In some cases, small changes in structure and stereochemistry can have large effects on ligand binding, yet molecules of different size and shape can have comparable affinities [30,33–36]. Thus, the structure-binding affinity relationships for these receptors are perhaps best termed "eclectic", rather than promiscuous.

IMPLICATIONS OF THE 10-MILLION-FOLD RANGE OF ENDOCRINE BINDING AND ACTIVITY ASSAYS

In considering the potential health effects of endocrine-disrupting substances on humans and wildlife, it is important to keep a focus on the issue of effective dose, which is, in essence, the product of potency and exposure. The exposure factor (see other contributions to this volume) will not be considered in this review, but one needs to appreciate that long and/or high exposure to a low-affinity ligand might be as effective as short or low exposure to a high-affinity ligand. Potency (as well as its biochemical cousin, affinity) is a critical factor to understand; it can be estimated by binding and bioactivity assays. These assays are not difficult to devise (for those receptors that bind ligands with nanomolar affinity). It is important to note that such assays are typically capable of detecting compounds that differ from one another in affinity and potency by a *factor of up to 10 000 000* (10^7) (Fig. 1).

For example, a simple competitive binding assay with the estrogen receptor might use [^{3}H]estradiol as tracer, a natural ligand that has a K_d of 0.2 nM or a relative affinity of 100 %. Such an assay can detect very high affinity estrogens, such as 11β-chloromethylestradiol, having a K_d of 6.7 pM and a relative affinity of 3000 %, or very low affinity estrogens, such as nonylphenol, with a K_d of 70 μM and a relative affinity of 0.0003 %. Similarly, a cell-based assay for estrogens based on the proliferation rate of breast cancer (MCF-7) cells shows a response to a potent compound such as estradiol at 1 pM, whereas the response to weak estrogens can often be measured by raising concentrations to as high as 10 μM without encountering nonspecific toxic effects. Certain physiological responses to estrogens that form the basis of whole animal assays, such as the uterine weight gain assay in immature female rats, are sensitive to potent estrogens such as 17α-ethinyl estradiol at doses as low as 0.01 μg, whereas doses as high as 10 mg of many compounds can be safely administered animals.

The point to reemphasize here is the following: The size of the universe of compounds that might be considered active "endocrine disruptors" or "exogenous endocrine substances" depends on the potency or affinity range that is considered. For example, the number of compounds and the range and diversity of structures of the compounds that are capable of activating the estrogen receptor are rather small if one considers only those that have a potency within a factor of 10 that of estradiol, whereas they are enormous if one considers all compounds whose potency is within a factor of 10^5 or 10^6 that of estradiol. Thus, comments that a receptor such as the estrogen receptor is "promiscuous" because it responds to such a wide range of compounds has little meaning if these statements are made without considering potency.

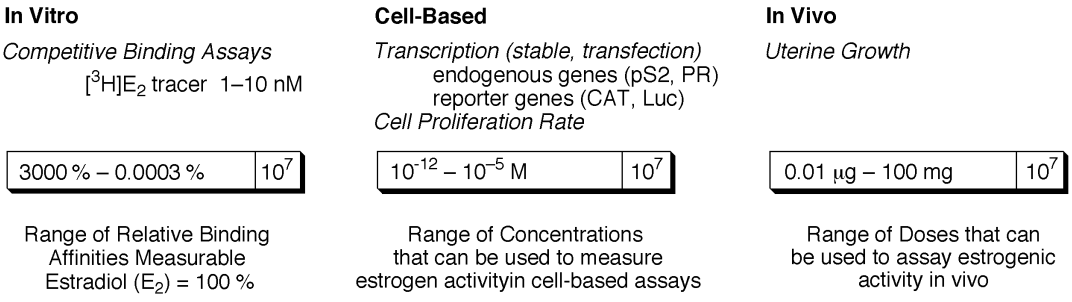

Fig. 1 Assays for estrogenic activity have high sensitivity and a wide working range.

SEX STEROID RECEPTOR INTERACTIONS WITH EXOGENOUS ENDOCRINE SUBSTANCES

In the sections that follow, we present a brief summary of compounds, from a variety of sources, which have been reported to be exogenous endocrine active substances through interaction with various nu-

clear hormone receptors. An attempt has been made to illustrate the range of structures of such substances and the diverse sources from which they arise. In this short review, however, it has not been possible to critically evaluate the validity of all of the potencies and each of the activities that are reported. Where information on potency or affinity is readily available (and appears to be reliable), some values are noted in the figures. For ease of comparison, binding affinity values have been converted to a relative binding affinity (RBA) scale, where the affinity of a compound is given as a percent of a reference ligand, which is considered to have a relative affinity of 100.

Estrogen receptor as a target for endocrine disruption

By far, the most intensively studied target for interaction with exogenous ligands and endocrine disruption is the estrogen receptor [32]. This receptor has a general tolerance for binding appropriately substituted phenols and other classes of lipophilic compounds [30,33–38]. There are two subtypes of the estrogen receptor (ERα and ERβ), but most studies on estrogens were done before the second subtype (ERβ) was known [39]. Binding affinity values of compound for ER are given on an RBA scale, and unless they are associated specifically with ERα or ERβ (i.e., numbers denoted with an α or β), they refer to studies done on extracts from estrogen target tissues (typically uterus), which is contains predominantly ERα.

Mammalian estrogens and estrogen pharmaceuticals

Estradiol, estrone, and estriol are the principal endogenous mammalian estrogens, though some more highly hydroxylated estrogens (estetrol, produced by the fetus) and some B-ring unsaturated estrogens (equine estrogens) are known. Many synthetic estrogens, both steroidal and nonsteroidal, have been prepared in the search for estrogen pharmaceuticals, and a variety of structural alterations have been made. The ER tolerates certain substitutions on the steroidal estrogen skeleton very well; in fact, some substitutions increase estrogenic potency by enhancing binding to the estrogen receptor (e.g., 11β-ethyl or 11β-chloromethyl), or by retarding metabolism (e.g., 17α-ethynyl or 11β-methoxy). The estrogen receptor will also tolerate very large substitutents at the 7α position, as is manifest in certain antiestrogens. Curiously, a phenolic ring is not an absolute requirement for an estrogen receptor ligand; certain androgen derivatives have significant binding affinity [40–42]. Examples of natural estrogens and estrogen pharmaceuticals are given in Fig. 2.

There are many classes of synthetic, nonsteroidal estrogens. From this variety of ligand structures, it may appear that the estrogen receptor system is unique among the steroid hormone receptors in its tolerance for nonsteroidal ligands, but this is more likely a quirk of history: It was shown as early as 1936 that the simple bisphenol diethystilbestrol was a potent estrogen [43], and this finding—nearly 60 years ago—prompted an active search for other nonsteroidal estrogens that has spanned many decades and produced many active agents, some estrogens and some antiestrogens [33–36]. In contast, the investigation of nonsteroidal ligands for other steroid hormone receptors (see below) has been a more recent endeavor, although it is clear that many potent nonsteroidal ligands exist for these other system, as well [44].

A number of nonsteroidal estrogens have been developed as tissue-selective estrogens, now better known as selective estrogen receptor modulators or SERMs [27]. These agents are used for breast cancer prevention and treatment [45], and they hold promise for menopausal hormone replacement with a better benefit/risk balance [27].

Because endogenous estrogens are hormones produced naturally and are essential for development, health, and reproduction, and because estrogen pharmaceuticals are administered for their hormonal effect in regulating fertility and for menopausal hormone replacement, these compounds are not typically thought of as potential endocrine disruptors in humans. Nevertheless, there is evidence that some of these compounds can find their way into rivers and streams and can affect aquatic wildlife [46].

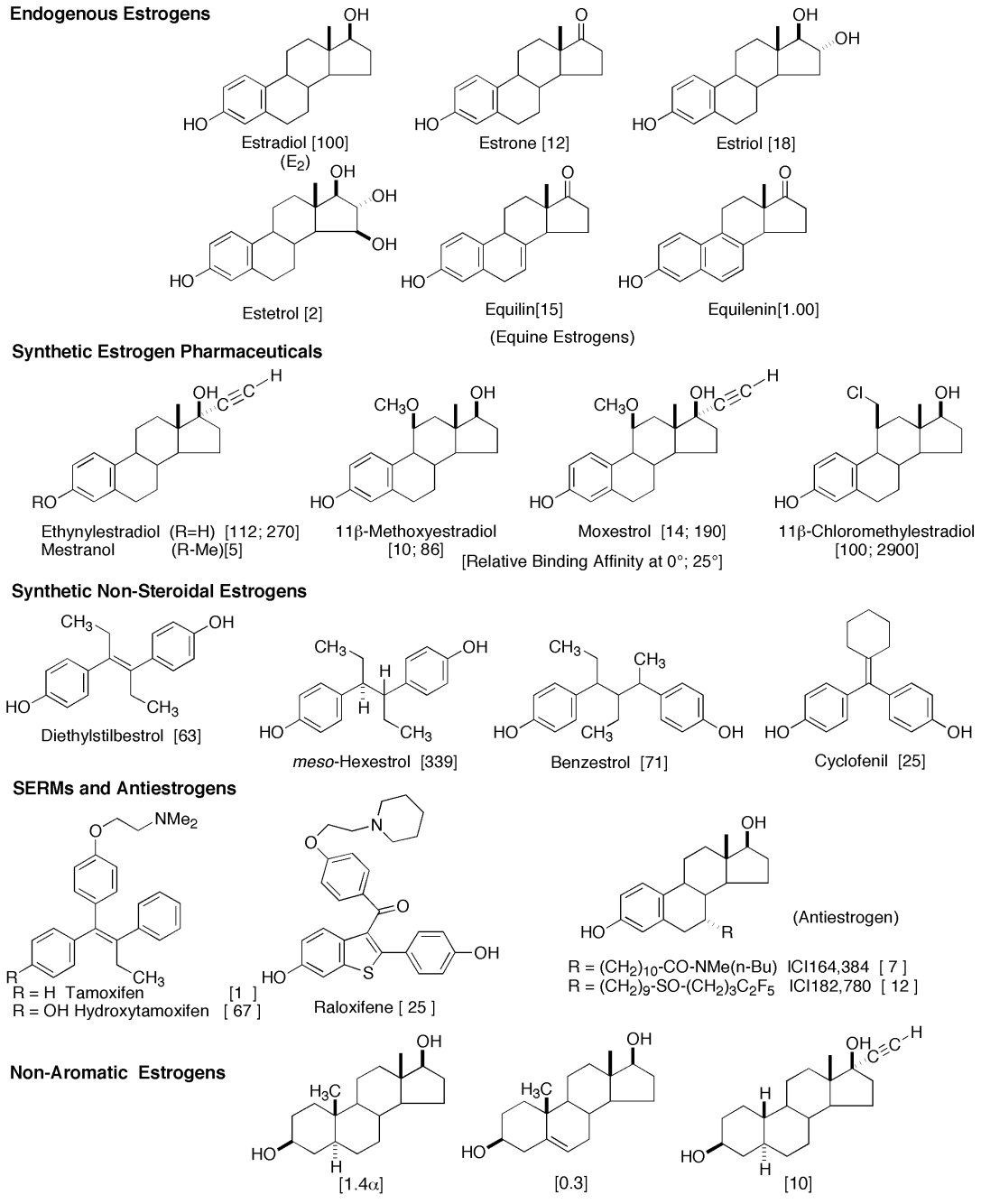

Fig. 2 Endogenous estrogens and estrogen pharmaceuticals.

Typical sewage treatment processes do not consume all estrogen pharmaceuticals (such as mestranol) and their metabolites that are present in human excrement; even endogenous human estrogens (such as estradiol and estriol) do not seem to be fully degraded by the standard methods of sewage treatment [46,47]. The use of certain other estrogen pharmaceuticals such as diethylstilbestrol and hexestrol and mycoestrogens (such as zeranol, see below) as anabolic agents in livestock [48,49] or as chemical cas-

tration agents in fowl can result in residual levels of hormone in meat or animal byproducts through which other animals and humans can be exposed [50,51].

Estrogen pharmacophore

A general estrogen pharmacophore might be considered to be a phenol substituted in the 3 and/or 4 positions with a variety of cyclic and acyclic carbon substituents and oxygen functionalities having a total carbon count in the range of 15–20 and generally lacking in highly polar or charged groups [30,33–38]. Within this paradigm, there are nearly endless varieties of polycyclic, acyclic, macrocyclic, and heterocyclic examples [33,52]. There are also estrogens that deviate from this general pharmacophore by lacking a phenol, but these typically have a phenol surrogate, such as an alcohol, hydrated carbonyl function, or sulfoxide group, and they are also usually very hydrophobic, often the result of multiple halogen substitution (see below).

Nonphenolic estrogens are typically of low potency, though some, especially the polychlorinated compounds, might be slow to be metabolized and cleared, and thus subject to bioconcentration, thereby amplifying the potential exposure of these agents to those at the end of the food chain [53]. In addition, because of their lipophilicity, these compounds can accumulate to high levels in lipids and membranes from which they can be released slowly, so as to provide a low, persistent level of compound in blood. Such continuous dosage might be particularly effective in stimulating certain estrogenic responses [54,55].

In the figures that follow are examples of compounds, reported to be estrogens, that come from a variety of natural and man-made sources and that are potential endocrine disruptors.

Exogenous estrogens from natural sources

Phytoestrogens, naturally occurring compounds in plants that mimic steroidal estrogens [56], are comprised of several classes, isoflavones, flavones, lignans, coumestans, and stilbenes (Fig. 3). Isoflavones are moderately potent estrogens found in legumes, the best known being genistein and daidzein from soy [57,58]. Genistein is one of the few ER ligands that shows rather selective binding for ERβ over ERα [59]. Considerable quantities of these agents are ingested by humans on "Asian diets", and they are believed to be primarily responsible for the beneficial effects of soy [60]. In fact, extracts that are very rich in soy isoflavones (e.g., NovaSoy®) are being promoted as nutritive supplements. Certain reduced isoflavones, such as equol, are estrogenic substances produced endogenously from isoflavones by the action of gut microflora [57].

Lignans are compounds that contain a 2,3-dibenzylbutyrolactone or butandiol structure and exist in minor quantities in plants that use them as building blocks for preparation of lignin, the major constituent in a plant cell wall [61]. The most abundant lignans, secoisolariciresinol and matairesinol, are found as glycosides in certain oilseeds, whole grains, berries, and some vegetables. In the colon, they are deglycosylated and further converted, by the action of intestinal microflora, to the weakly estrogenic enterodiol and enterolactone, which are reported to be very weak estrogens (no affinity values are available) [61]. Resveratrol, a stilbene triol found in the skin of grapes and in red wine, is a low potency estrogen [62]. The coumestans, coumestrol and mirestrol, found in certain clover, were first recognized as potent phytoestrogens as a result of endocrine disruption on sheep [63].

Human exposure to estrogens such as phytoestrogens that are natural components in healthy food is essentially unavoidable, and compounds such as genistein are generally considered to be a health benefit [60]. These natural estrogens are low-affinity, low-potency ligands for the estrogen receptor, and it is known that certain weak, short-acting estrogens, such as estriol, as well as other "impeded" estrogens, can reduce the effect of potent estrogens such as estradiol when they are given in an appropriate dose regimen. The net effect of such treatment can be the antagonism of an estrogen-responsive system that could be protective of health [54,55,64]. Nevertheless, the health consequences of exposure to

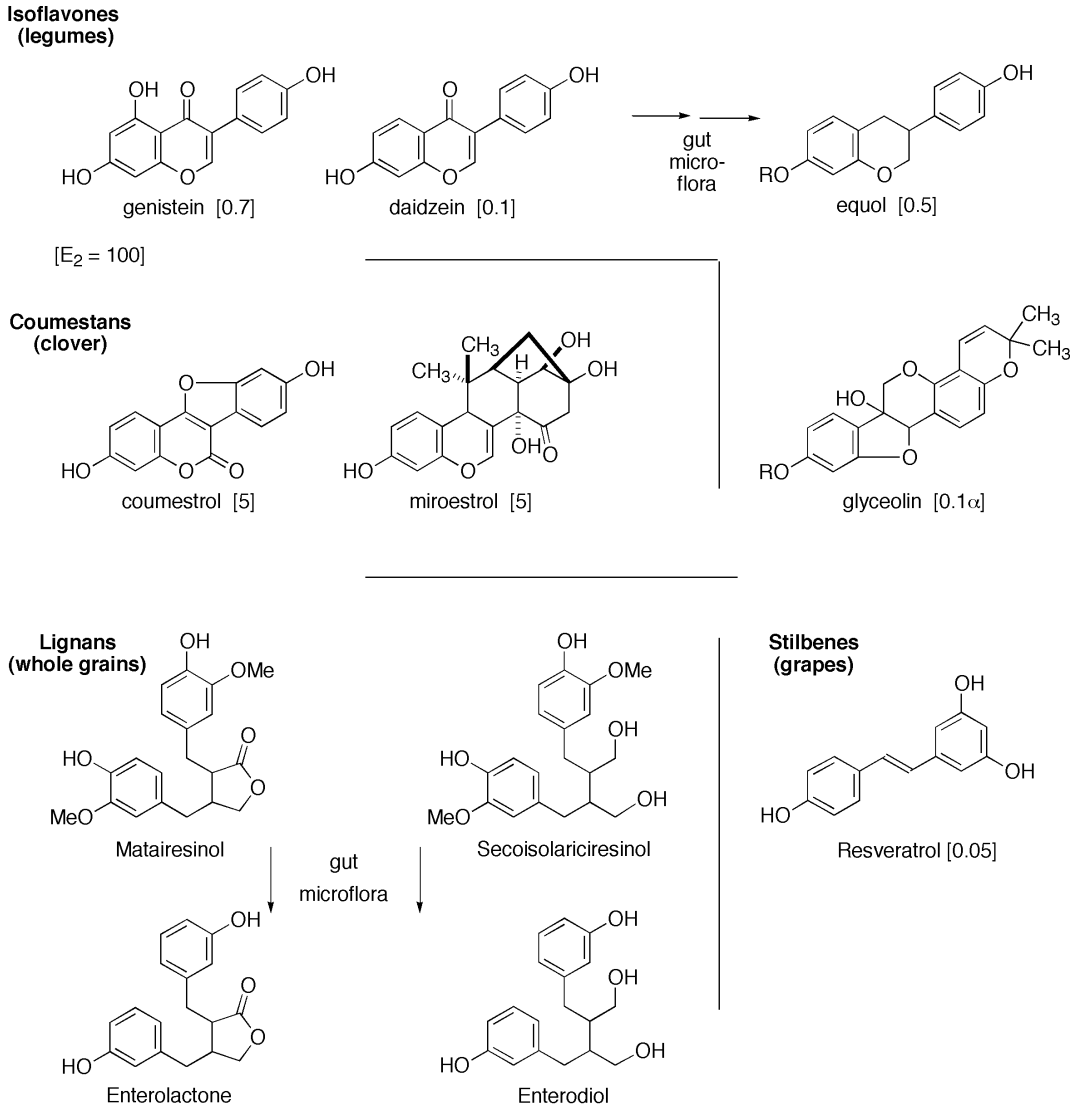

Fig. 3 Exogenous estrogens from natural foodstuff sources.

phytoestrogens is not likely to be a simple matter, and in certain situations, such as for women who have or are at high risk for breast cancer, phytoestrogen exposure could pose a risk, particularly if exposure is amplified by ingestion of large amounts of food extracts in which these substances are highly concentrated [65].

Some of these compounds also have other biological activities that might have health consequences. For example, genistein is a tyrosine kinase and topoisomoerase inhibitor and has some antiangiogenic activity so it that might be considered to have anticancer activity [60]. It should be noted, however, that the potency of genistein as an antiangiogenic agent is much less than its potency as an estrogen [66].

Certain microbial metabolites, termed "mycoestrogens", have estrogenic activity (Fig. 4) [67]. Most notable are resorcylic acid lactones produced by *Fusarium* [68], a mold that can infect corn and other forage crops, and has caused endocrine disruption in pigs. Zearalenone, the parent compound, is

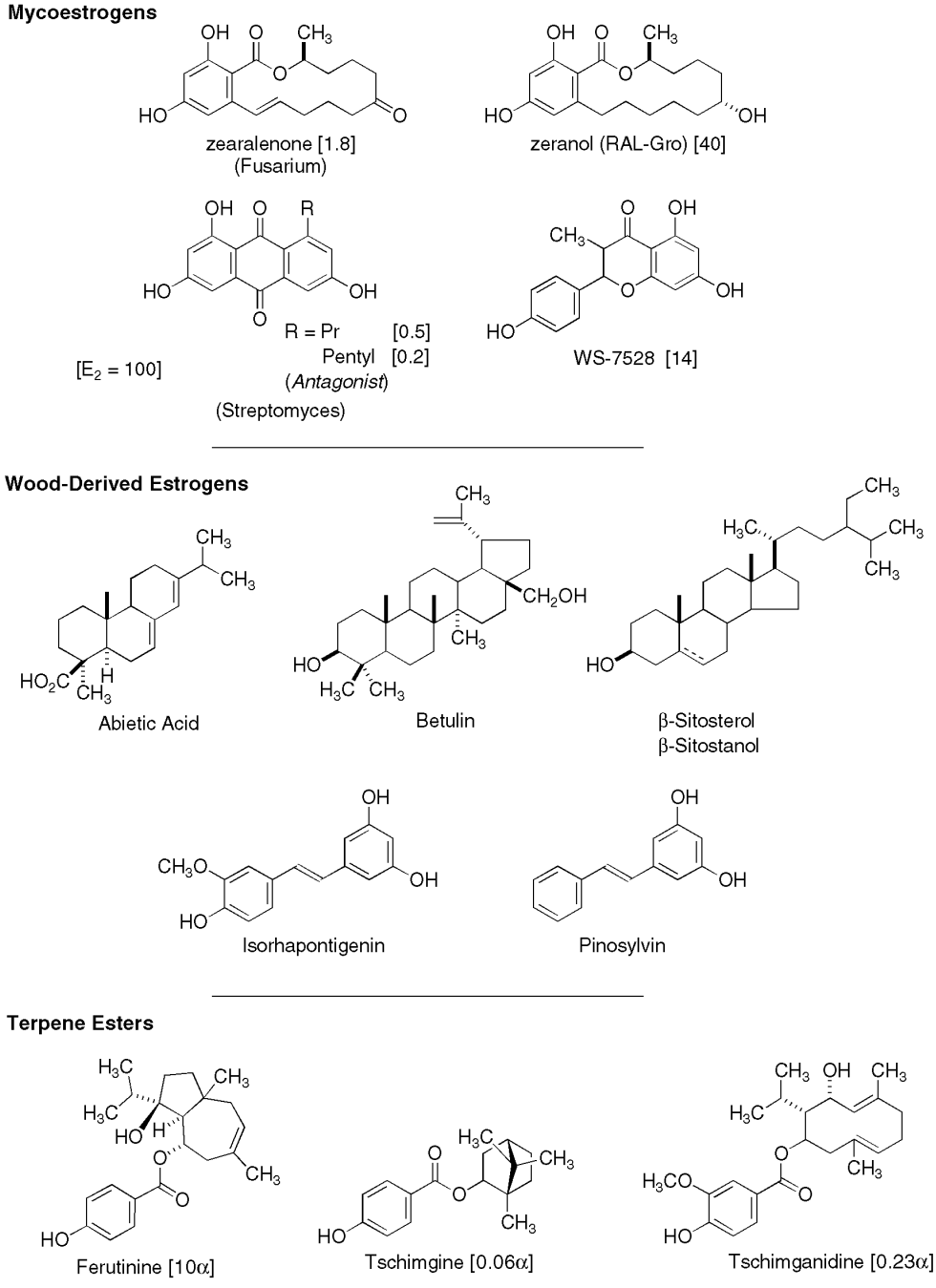

Fig. 4 Exogenous estrogens from other natural sources.

quite a potent estrogen [69], and some of its reduced analogs (Zeranol®, Ralgro®) have been used as anabolic agents in cattle [49]. Several anthraquinone alcohols from *Streptomyces* were found to bind to ER and to be antagonists (less potent than tamoxifen) [70].

Some phenolic and nonphenolic compounds derived from wood have been reported to be estrogenic in cell proliferation assays. Abietic acid and β-sitosterol are interesting nonphenolic examples,

and the stilbenes isorhapontigenin and pinosylvin are close structural analogs of resveratrol (see above) [71]. The potency reported for some of these compounds as estrogens seems higher than expected, based on their structures (affinities are not reported) [71].

Terpenoids are less well known as estrogen receptor ligands, but recently, sesquiterpenoids derived from the umbelliferae family have been added to the growing list of phytoestrogens [72]. The terpene benzoate ester ferutinine had good affinity for ERα and ERβ, but others, tschimgine and tschmiganidine, have lower affinity. Interestingly, in transcription assays, ferutinine was a full agonist for ERα and an agonist/antagonist for ERβ, whereas tschmiganidine was an agonist only on ERα and an antagonist on ERβ.

Exogenous estrogens of man-made origin

An intriguing variety of man-made compounds, or their metabolites, have been found to have estrogenic activity. Though their potency is rarely high, some are very lipophilic and potentially environmentally persistent compounds; others come from surprising sources.

Estrogenic activity among certain commercial chemicals (Fig. 5) has been found, such as *p-tert*-alkyl phenols, most notably octylphenol (a single isomer) and nonylphenol (an isomer mixture). Such phenols form the hydrophobic core of the polyethoxylate nonionic detergents. Because the free phenols are released by the action of microbes during sewage treatment, they can be present into waste streams where they can be exposed to aquatic wildlife [73]. Estrogenicity is greatest when the substituent is branched at the benzylic position, para to the phenol, and of a certain size [73].

Certain components of plastics, dialkyl phthalates used as plasticizers and bisphenol A, a component of thermostable polycarbonate polymers, have been reported to be weak estrogens [74,75]. Bisphenol A was first prepared as an estrogen [43] and only later was used in the production of plastics. It is released into the contents of polycarbonate bottles when they are heated in an autoclave sterilizer, and at the concentration thus released, it can stimulate estrogen-responsive cells in culture [76]; its presence in polycarbonate baby bottles has raised concern [77]. Curiously, bisphenol A is found in recycled paper, but not in virgin paper [78]. Similarly, a bisphenolic impurity present at very low levels in commercial grade samples of phenol red, a widely used component of cell culture media, is a high-affinity estrogen and was found to be the cause of inadvertent stimulation of estrogen-responsive cells [79–81]. Hydroxylated azobenzenes bear structural resemblance to stilbene estrogens and have significant affinity for the estrogen receptor [82].

More curious are examples of estrogenic substances found in cosmetics. In vivo estrogenic activity was reported for a cyclic tetrasiloxane, which was a minor constituent of a polymer mixture prepared as a moisturizing cream [83]. A related linear siloxane, also containing a 1,3-diphenyl moiety, has been reported to be weakly estrogenic [84]. Certain ingredients in commercially available sunscreens, notably 4-methylbenzylidene camphor and octyl 4-methoxycinnmate, have been implicated as estrogens [85–88].

Quite a number of highly chlorinated aromatic substances, insecticides and industrial fluids, have been found to have estrogenic activity, and some have been implicated as endocrine disruptors (Fig. 6). *o*,*p*′-DDT, a minor isomer in commercial DDT, its metabolite *o*,*p*′-DDE, and the nonbioaccumulating analog methoxychlor and its phenolic metabolites are all weak estrogens [89,90]. The net estrogenic activity of *o*,*p*′-DDT and DDE and their endocrine disruptive effects (as well as that of the related *p*,*p*′ isomers) may, however, be the result of their antiandrogenic, rather than their estrogenic activity [91,92]. PCBs, which are typically mixtures of many isomers with varying degrees of chlorine substitution, are reported to be estrogenic [93–95]. The highest potency is found with PCB metabolites that have a *para*-hydroxy group [93,96,97], but RBA values are still quite low. Several hydroxylated PCBs, particularly those with chlorine substitutents at positions 2′, 4′ and 6′ on the nonphenolic ring, exhibit antiestrogenic effects in MCF-7 cells [98].

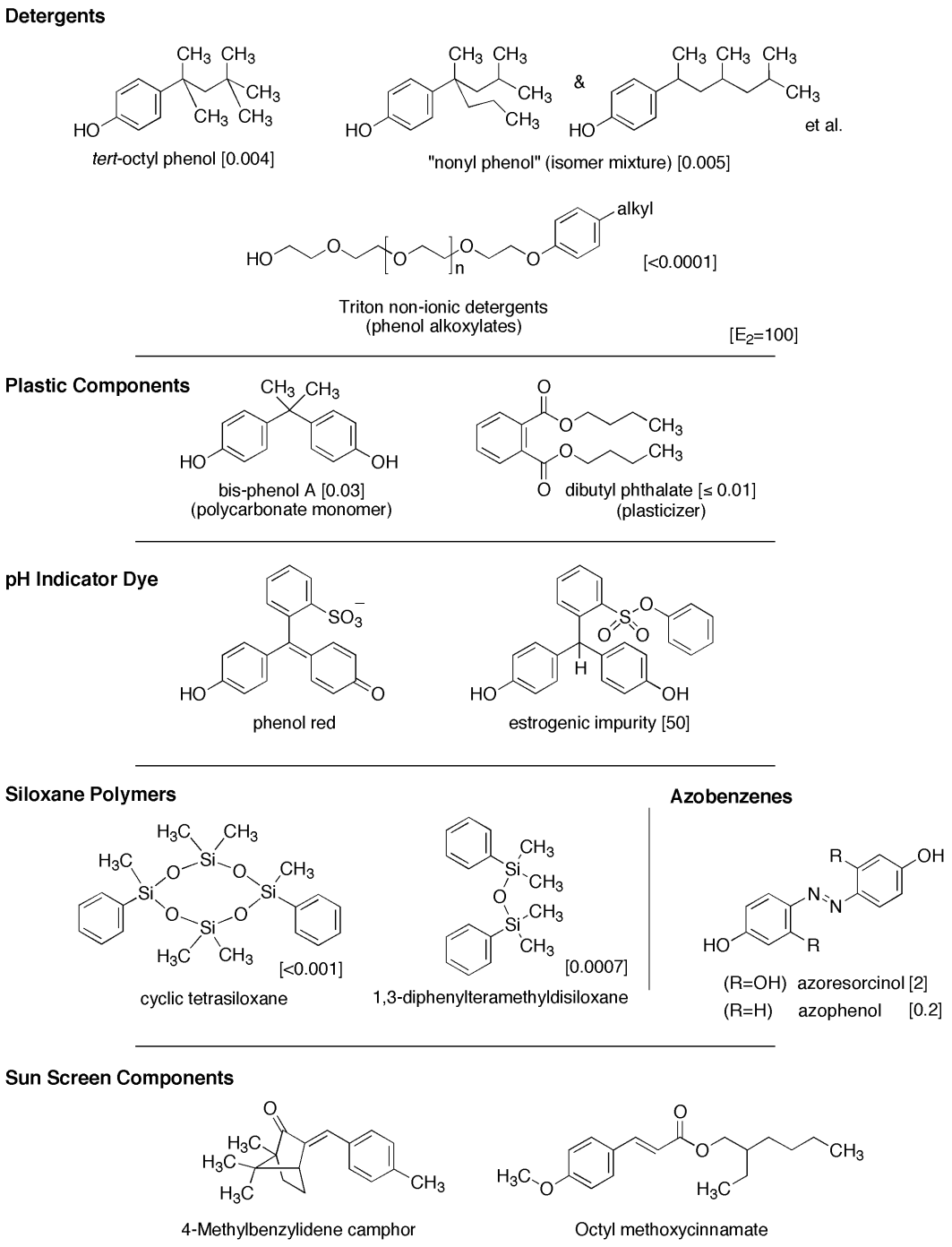

Fig. 5 Exogenous estrogens: Commercial chemicals and impurities.

A variety of nonaromatic chlorinated pesticides are reported to have estrogenic activity (Fig. 6). Most remarkable is that all of these lack not only the signature phenol of most estrogens, they are not even aromatic. Endosulfan, dieldrin, and kepone/chlordecane (a metabolite of the persistent fire ant insecticide mirex) are all polycyclic and heavily chlorinated [99,100], and though they are not phenolic,

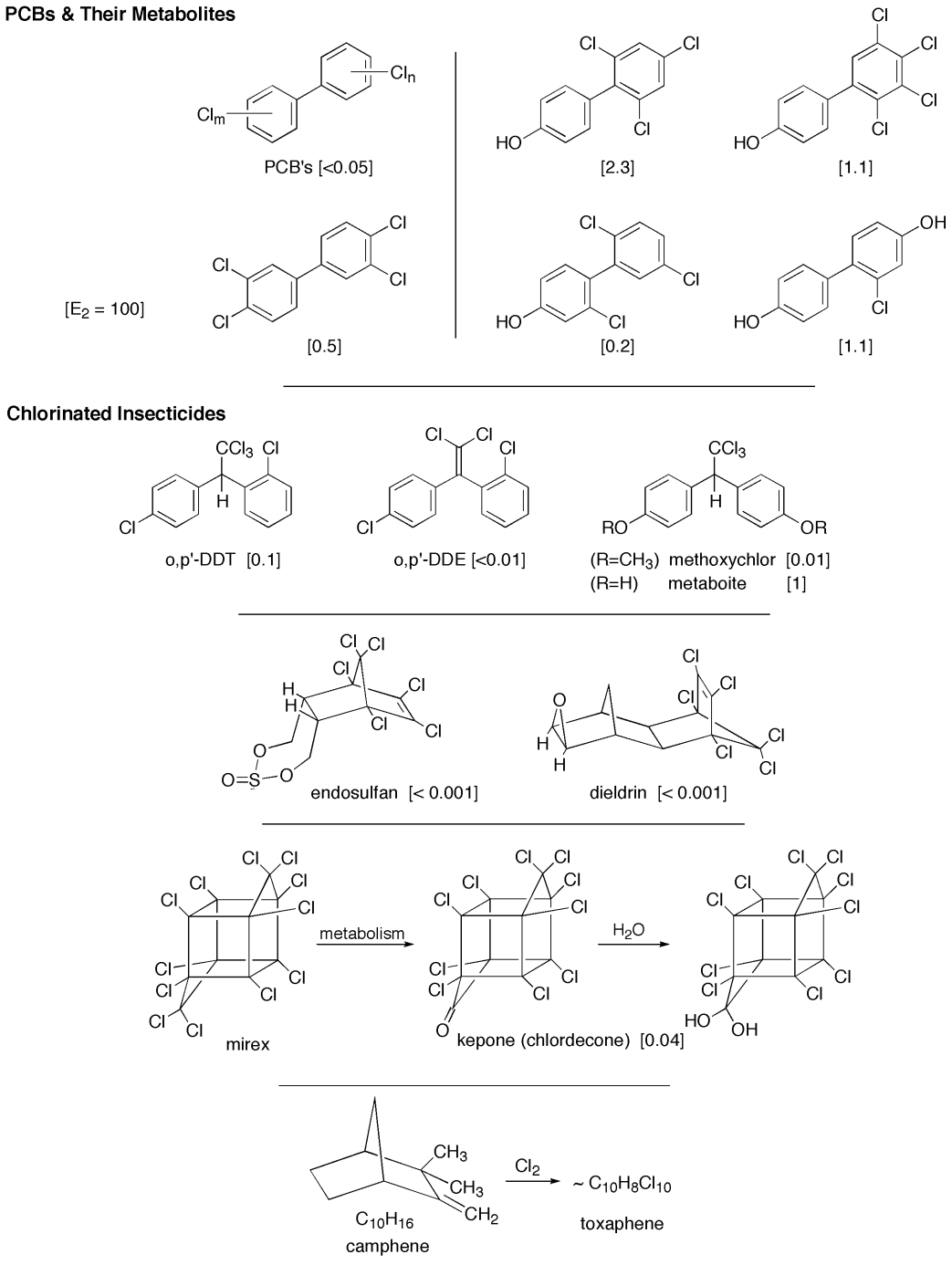

Fig. 6 Exogenous estrogens: Highly chlorinated.

they each have a polar function that might fulfill this function, e.g., a sulfite in endosulfan and an epoxide in dieldrin. The ketone function in kepone is most likely hydrated as a gem-diol, and this may be the functional equivalent of the phenol, although the overall structure of kepone certainly deviates markedly from that of typically estrogens. Lindane, a mixture of hexachlorocyclohexanes, and

toxaphene, a complex mixture of chlorinated terpenes derived from camphene, have been reported to have estrogenic and antiestrogenic activity [101–103]; however, these materials bear no obvious structural or functional relationship to other estrogens, save that they are polychlorinated like some other compounds that are weak estrogens.

All of these compounds are very low potency estrogens, but they are exceedingly lipophilic agents (based on water–octanol partition coefficient estimates), and they are very persistent in the environment. It was proposed that these low-potency estrogens might engage in a potency-intensifying synergy [104]; however, this contention was not widely supported, and it has been withdrawn [105]

Androgen receptor as a target for endocrine disruption

The androgen receptor is also recognized as a target for endocrine disruption [91,92,106]. As with the estrogens, steroidal ligands for this receptor with improved potency and in vivo stability have been developed as pharmaceuticals agents, and a number of nonsteroidal ligands are known to be androgen antagonists, some of which are used clinically for the treatment of prostate cancer (Fig. 7) [44]. Certain phytochemicals have been shown to have weak androgenic and antiandrogenic activity [107]. Curiously, the fungicide vinclozolin is an androgen antagonist, and one of its metabolites whose struc-

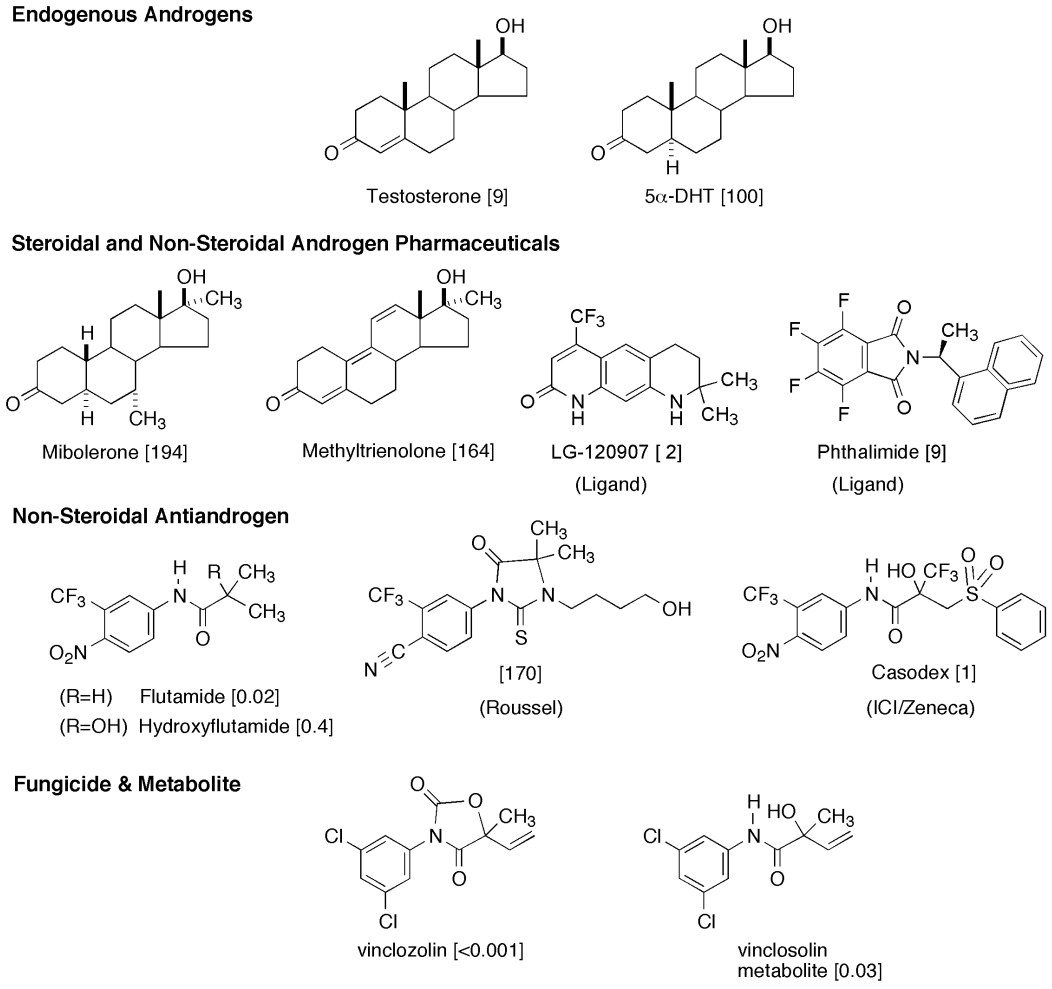

Fig. 7 Androgen receptor ligands.

ture resembles that of some nonsteroidal antiandrogens has significant affinity for the androgen receptor [108]. Also, as was mentioned above, the endocrine disruptive activity of the DDT and DDE isomers is thought to be the result of their antiandrogenic activity [92,106,108]. The waste water from wood pulping has been associated with androgenization of aquatic species [109], and the causative agents appears to be steroidal androgens produced by the action of bacteria on sterols from wood [110,111]. For illustrative purposes, various ligands for the progesterone receptor, both natural, synthetic, and nonsteroidal, are shown to illustrate the diversity of chemical structures that bind to this receptor with high affinity (Fig. 8). As far as we are aware, there are no examples of exogenous progestins causing endocrine disruption.

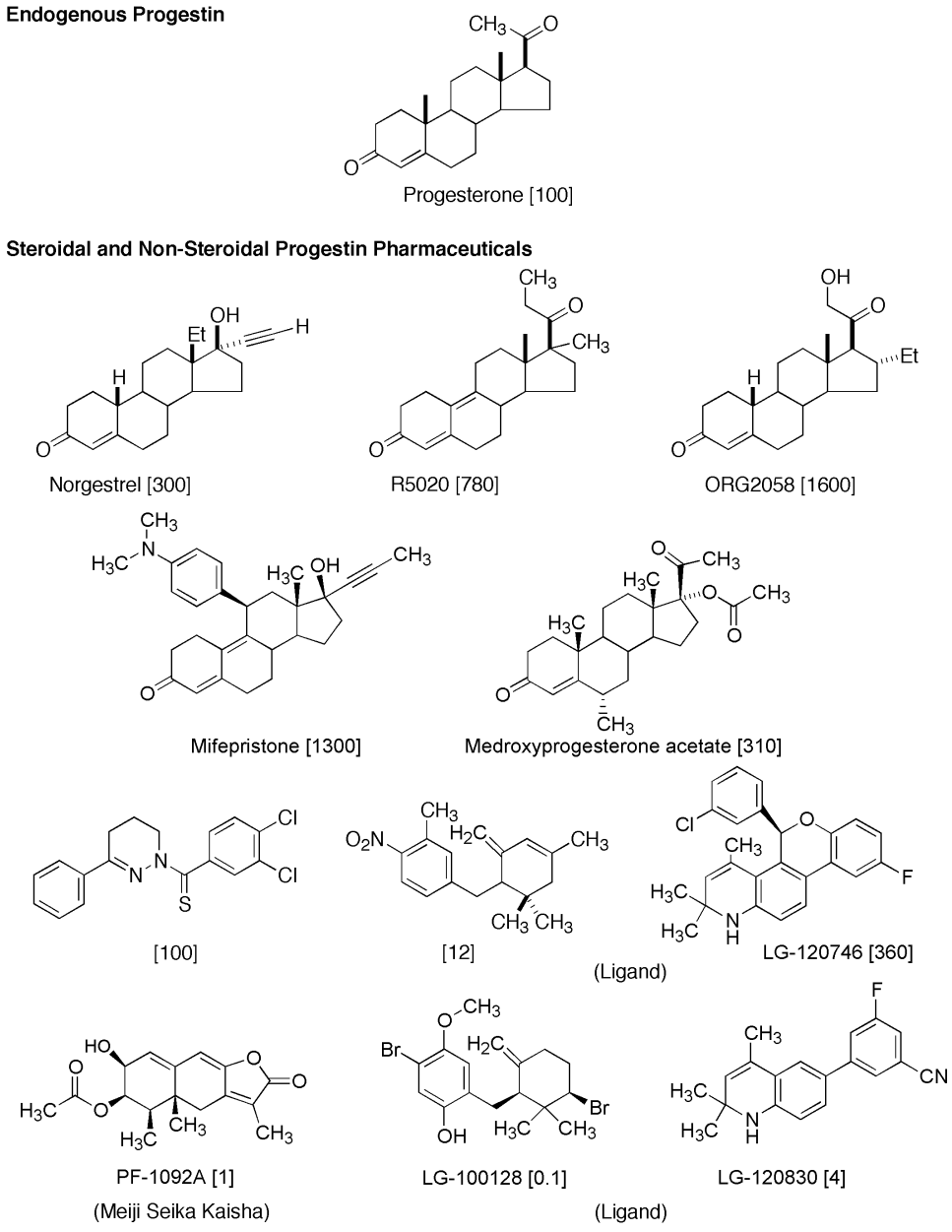

Fig. 8 Progesterone receptor ligands.

Other nuclear receptors as targets for endocrine disruption

There is much less in the literature on endocrine disruption through other nuclear hormone receptors. PCBs and other halogenated aromatics affect the thyroid hormone system, but this appears to result from the displacement of endogenous thyroid hormones from plasma carrier proteins and activation of liver enzymes, both of which enhance the rate of hormone metabolism and clearance, rather than from direct binding of these agents to the thyroid hormone receptor [97,112–114]. Certain phytochemicals are reported to affect the progesterone receptor [107,115]. It has been proposed that developmental abnormalities in frogs arise from endocrine disruption acting through the retinoid receptors. Paper mill effluent is reported to inhibit retinoid signaling, but the causative agents have not been identified [116,117].

The Ah receptor is also a transcription regulator whose activity is regulated by small molecule ligands, though it is from a completely different structural class than the nuclear hormone receptors. It also appears to be a target for endocrine disruption, and there is evidence for negative cross talk between the Ah receptor and other nuclear hormone receptors, such as the estrogen receptor. (See Topic 1.13.)

NUCLEAR RECEPTORS IN CONTEXT: THE MULTIPLE LEVELS AT WHICH EXOGENOUS SUBSTANCES CAN EXERT AN EFFECT BY PERTURBING AN ENDOGENOUS ENDOCRINE SYSTEM

The preceding review displaying the wide variety of compounds—from different chemical classes and widely divergent sources—that have been reported to have activity on one or another nuclear hormone receptor should not cause one to become myopic and focus on these proteins as the only target for endocrine disruption. Hormone regulatory systems are complex, and though the receptors are at the center, the overall function of these interwoven webs also involves the processes of hormone production, transport in the blood, metabolism, and then clearance, as well as the cross talk that occurs between different cellular signal transduction pathways (Fig. 9).

In principle, hormone disruption could arise from the interaction of small molecules at any of these levels: by inhibiting the enzymes involved in hormone biosynthesis, displacing hormones from transport proteins, and activating or inhibiting the enzymes involved in hormone metabolism and clear-

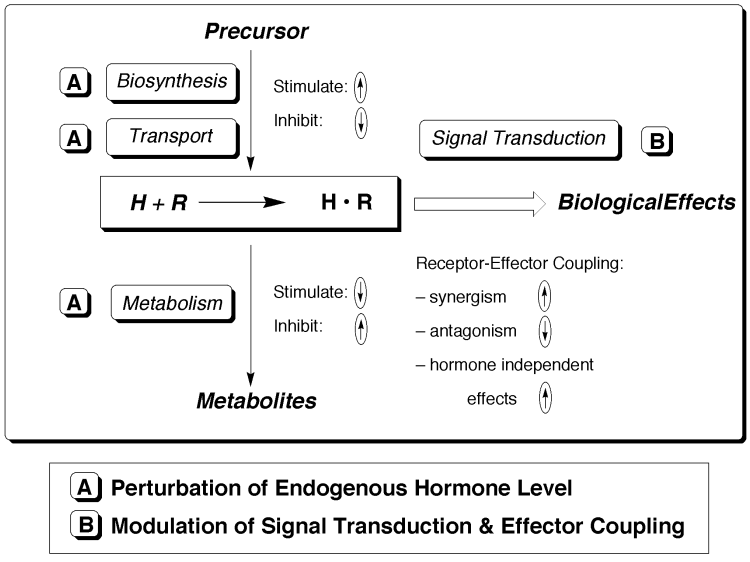

Fig. 9 Modulation of endocrine activity not involving ligand binding to receptors.

ance. Certain pharmaceuticals target hormone production: aromatase inhibitors block the conversion of androgens to estrogens [118], 5α-reductase inhibitors prevent the bioactivation of testosterone to 5α-dihydrotestosterone [119], and aminoglutethamide blocks the early stage of the conversion of cholesterol to corticosteroids [120]. Other drugs, antibiotics, and natural products (such as hyperforin found in the widely consumed herb St. John's Wort) interact with the PXR/SXR system [121], activating a series of drug-metabolizing enzymes that can cause "drug interactions" [122]. In fact, the use of antibiotics in the treatment of human infections, which cause profound alterations in gut microflora, can affect the potency of endogenous and exogenous hormones, and this has been cited as a cause of the failure of estrogen oral contraceptives [123].

No doubt, there are other examples of endocrine disruption that occurs at "extra-receptor sites", such as was noted for the effect of halogenated aromatics on the thyroid system via disruption of hormone transport and acceleration of clearance [112–114] and as has been reported with some halogenated pesticides on estrogen biosynthesis [99]. The hormonal ("estrogenic") effects of certain hepatoxins, such as carbon tetrachloride, ethylene glycol, or dimethylformamide [124,125], are also most likely the indirect result of disrupted hormone clearance and elevated blood levels of endogenous hormones.

One must, therefore, keep a broad perspective in seeking the target or processes through which an endocrine disruptive event is mediated. The nuclear hormone receptors are just one of many possible targets. The regulation of homeostasis, development, and reproduction involves complex and autoregulated endocrine cycles whose sensitivity to exogenous hormonal substances may vary widely, depending on the phase of the cycle. Thus, one also needs to be most alert to assessing the effects of endocrine disruption at the most sensitive phases of these cycles [126].

Experiments can be carefully designed to evaluate whether a binding or biological effect of an endocrine disruptor is the result of interaction with a specific receptor: Specific receptor binding should be competitive with the binding of specific tracers, and agonism or antagonism through a specific receptor should be reversed by receptor-specific antagonist or agonists, respectively. Endocrine disruption that occurs indirectly, by alterations in hormone production, transport, or clearance, may be evident through altered levels of endogenous hormones, which can also be assayed by standard immunoassay and spectroscopic methods. Experiments in animals in which the organs of endogenous hormone production are removed can also be revealing of indirect endocrine disruption.

These varied considerations notwithstanding, one should keep in sight a number of simple facts about human exposure to hormone disruptors, and of estrogens in particular: Estrogens may be as good as they are bad, but human exposure to estrogenic substances (even if one considers only natural sources) is, by any reasonable standard, inescapable; this is true not only for adults but during embryonic development as well. Most human diets contain large amounts of weakly estrogenic substances (or their precursors) as natural components of food. Estrogens are produced endogenously by both males and females, and, aside from unusual cases, the major lifetime exposure that humans have to potent estrogens is to those of their own production.

The bottom line is simply this: On the one hand, one should remain aware of the widespread potential for compounds having varying structures, but sharing some common structural motifs and physical properties, as outlined above, to have nuclear hormone receptor activity. On the other hand, one must try—on a case-by-case basis—to come to a better understanding of whether the activity of these compounds and our exposure to them is a detriment or a benefit to animal and human health, or is, perhaps, inconsequential.

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