TOXICOLOGICAL SCIENCES **83**, 264–272 (2005)
doi:10.1093/toxsci/kfi035
Advance Access publication November 10, 2004

Interaction of Polycyclic Musks and UV Filters with the Estrogen Receptor (ER), Androgen Receptor (AR), and Progesterone Receptor (PR) in Reporter Gene Bioassays

Richard H. M. M. Schreurs,* Edwin Sonneveld,† Jenny H. J. Jansen,† Willem Seinen,*,[1] and Bart van der Burg†

*Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands, and †BioDetection Systems B.V., Badhuisweg 3, 1031 CM, Amsterdam, The Netherlands

Received August 20, 2004; accepted November 8, 2004

Two important ingredients of personal care products, namely polycyclic musk fragrances and UV filters, can be found in the environment and in humans. In previous studies, several compounds of both classes have been tested for their interaction with the estrogen receptor. Two polycyclic musk fragrances, namely AHTN and HHCB, turned out to be anti-estrogenic both *in vitro* and *in vivo* in a transgenic zebrafish assay. Several UV filters have been shown to exert estrogenic effects *in vitro* and in some *in vivo* studies. Here, we assessed the interaction of five polycyclic musk compounds and seven UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone (PR) receptor, using sensitive and specific reporter gene cell lines. Four polycyclic musks (AHTN, HHCB, AETT, and AHMI) were found to be antagonists toward the ERβ, AR and PR. The UV filters that showed estrogenic effects (benzophenone-3, Bp-3; 3-benzylidene camphor, 3-BC; homosalate, HMS; and 4-methylbenzylidene camphor, 4-MBC) were found to be antagonists toward the AR and PR. The ERα agonistic UV filter octyl-dimethyl-*p*-aminobenzoic acid (OD-PABA) did not show activity toward the AR and PR. Octyl methoxy cinnamate (OMC) showed weak ERα agonism, but potent PR antagonism. Butyl methoxydibenzoylmethane (B-MDM) only showed weak ERα agonism and weak AR antagonism. Most effects were observed at relatively high concentrations (above 1 μM); however, the anti-progestagenic effects of the polycyclic musks AHMI and AHTN were detected at concentrations as low as 0.01 μM. The activity of anti-progestagenic xenobiotics at low concentrations indicates the need to undertake more research to find out about the potential endocrine disrupting effects of these compounds *in vivo*.

Key Words: **polycyclic musks; UV filters; estrogen receptor; androgen receptor; progesterone receptor.**

Personal care products, such as fragrances, cosmetics, or sunscreen agents, are used in significant quantities all over the world. Two classes of ingredients that are used in personal care products, namely ultraviolet (UV) filters and polycyclic musks, have been found in the aquatic environment and in humans. UV filters are organic compounds that can absorb UV-A and/or UV-B radiation. They are used in sunscreens in order to block the negative effects of sunlight on the skin, but they are also used in other cosmetics for product stability. UV filters have been found in surface waters and fish (Nagtegaal *et al*., 1997), and also in human urine and milk (Felix *et al*., 1998; Hany and Nagel, 1995; Hayden *et al*., 1997). Polycyclic musks are essential fragrance ingredients that are used in household cleaning products, perfumes, cosmetics, and laundry detergents. These chemicals have been detected in marine and estuarine waters, surface waters, sediment, fish, and mussels (Bester *et al*., 1998; Draisci *et al*., 1998; Fromme *et al*., 1999, 2001; Gatermann *et al*., 1999; Heberer *et al*., 1999; Rimkus *et al*., 1999) and also in human milk, adipose tissue, and blood (Bauer and Frössl, 1999; Muller *et al*., 1996; Rimkus and Wolf, 1996).

Recently, parabens, which are used as preservatives in cosmetics, have been found in human breast tumors. It has been suggested that these chemicals might contribute to the rising incidence of breast cancer (Darbre, 2003). In several laboratory experiments, parabens have shown hormone-disrupting properties, including weak estrogenic activity *in vitro* and estrogenic activity *in vivo* (Byford *et al*., 2002; Darbre *et al*., 2002, 2003; Lemini *et al*., 1997; Routledge *et al*., 1998). In recent years, UV filters and polycyclic musks have been analyzed for possible hormone-related activity. Several UV filters have been found to be estrogenic in *in vitro* systems (Miller *et al*., 2001; Mueller *et al*., 2003; Nakagawa and Suzuki, 2002; Schlumpf *et al*., 2001; Schreurs *et al*., 2002a) and also *in vivo* (Ashby *et al*., 2001; Holbech *et al*., 2002; Schlumpf *et al*., 2001; Tinwell *et al*., 2002). The polycyclic musks AHTN and HHCB were found to be only weakly estrogenic, but more potent anti-estrogens in an *in vitro* reporter gene assay (Schreurs *et al*., 2002b; Seinen *et al*., 1999). In an *in vivo* transgenic zebrafish assay, anti-estrogenic effects were observed for both compounds (Schreurs *et al*., 2004). Interaction with androgen receptors has been investigated for UV filters (Ashby *et al*., 2001, Ma *et al*., 2003), some of which

[1] To whom correspondence should be addressed at Institute for Risk Assessment Sciences, Utrecht University, PO Box 80176, 3508 TD, Utrecht, The Netherlands. Fax. +31 30 2535077. E-mail: w.seinen@iras.uu.nl

some exerted anti-androgenic effects (Ma *et al*., 2003). As far as we know, interaction of polycyclic musks and UV filters with the progesterone receptor has never been reported.

In this paper we assess seven UV filters: benzophenone-3 (Bp-3), octyl-methoxycinnamate, 4-methylbenzylidene camphor (4-MBC), butyl-methoxydibenzoylmethane (B-MDM), homosalate (HMS), octyl-dimethyl-*p*-aminobenzoic acid (OD-PABA), 3-benzylidene camphor (3-BC), and five polycyclic musk fragrances (Tonalide®, Galaxolide®, Celestolide®, Versalide®, Phantolide®) for their (anti)androgenic and (anti)-progestagenic activity in newly developed and sensitive *in vitro* reporter gene assays.

MATERIALS AND METHODS

Chemicals and reagents. 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), flutamide, and mifepristone (RU486) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Vinclozolin was purchased from Riedel-de Haën (Seelze, Germany). ICI 182,780 was purchased from Tocris (Bristol, UK). Org 2058 was supplied by the Department of Medicinal Chemistry of N.V. Organon (Oss, The Netherlands). The UV filters benzophenone-3 (Bp-3, Eusolex 4360), butyl-methoxydibenzoylmethane (B-MDM, Eusolex 9020), homosalate (HMS, Eusolex HMS), 4-methylbenzylidene camphor (4-MBC, Eusolex 6300), octyl-dimethyl-*p*-aminobenzoic acid (OD-PABA, Eusolex 6007), and octyl-methoxycinnamate (OMC, Eusolex 2292) were obtained from Merck (Darmstadt, Germany), and 3-benzylidene camphor (3-BC, Unisol S-22) was obtained from Permcos GmbH (Arisdorf, Switzerland). The polycyclic musks Tonalide, Versalide and Phantolide were kind gifts from PFW-Aroma Chemicals (Barneveld, The Netherlands). Galaxolide and Celestolide were kind gifts from International Flavours and Fragrances (Hilversum, The Netherlands). 4-Hydroxytamoxifen was a kind gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Table 1 shows the chemical structures of the polycyclic musks and the UV filters.

Cell culture. HEK293 and U2-OS cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) (Integro, Linz, Austria). The cell lines were cultured at 37°C and 7.5% CO_2.

AR and PR CALUX® bioassays. The generation of stable hAR and hPR transfectants of U2-OS cells is described elsewhere (Sonneveld *et al*., in press; Sonneveld *et al*., in press). In short, these cells contain a pSG5-neo-hAR or pSG5-neo-hPR expression vector in combination with a 3×ARE-TATA-Luc-reporter construct, respectively. AR CALUX® cells were trypsinized and suspended in phenol-red-free DF medium, supplemented with 5% dextran charcoal stripped FCS. Cells were plated in 96-well tissue culture plates (Nunc, Roskilde, Denmark) (6000 cells/well) at a volume of 200 µl per well. After 48 h the medium was changed, and the compounds to be tested (dissolved in ethanol) were added directly to the medium in a 1:1000 dilution. After 24 h, the cells were lysed in 30 µl Triton-lysis buffer (1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO4, 4 mM EGTA and 1 mM DTT). A 25-µl portion of cell lysate was transferred to a black 96-well plate to which 25 µl luciferine substrate (LucLite reporter gene assay kit, Packard Instruments, Meriden, CT) was added. Luciferase activity was measured in a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min per well. The PR CALUX® bioassay is performed in exactly the same way as the AR CALUX® bioassay.

Gene expression assay in stable ERα and ERβ reporter cell lines. The generation of stable hERα and hERβ transfectants of HEK293 cells is described previously (Lemmen *et al*., 2002). The performance of the assay is similar to the AR and PR CALUX® assays.

Data analysis. Luciferase activity per well was measured as light units. In every experiment, each concentration was analyzed in triplicate. From these values, fold induction was calculated by dividing the mean value of light units in exposed and nonexposed wells. ARE-luc activity as a percentage of maximal dihydrotestosterone induction was calculated by setting the highest fold induction of dihydrotestosterone at 100%. The same was done for the ER- and PR-reporter cell lines, using estradiol and ORG2058 as reference compounds, respectively.

Dose-response curves were fitted using the sigmoidal function: $y = y0 + a/[1 = \exp(-(x - x0)/b)]$ in SigmaPlot 2002 for Windows version 8.02 (SPSS Inc., Chicago, IL, USA). Using this fit, the values of EC_{50} and IC_{50} were calculated. The figures were drawn using GraphPad Prism 4.00 (GraphPad Software, Inc.).

RESULTS

ER Transactivation by UV Filters and Suppression of E2-Induced Transactivation by Polycyclic Musks in Stably Transfected 293HEK Reporter Cell Lines

The test compounds were analyzed for their (anti)estrogenic activity using 293HEK cells, stably transfected with either hERα or hERβ, and a 3×ERE-tata-Luc-reporter gene construct, To measure anti-estrogenicity, cells were incubated with both the chemical to be tested and an E2 concentration of 3 and 100 pM for hERα and hERβ, respectively. This E2 concentration was the approximate EC_{50}, taken from the dose-response curves (Figs. 1A and 1C). As positive controls for ER antagonism, we used 4-hydroxytamoxifen (OHT) and ICI 182,780. Both compounds could completely abolish E2-induced transactivation at both receptor subtypes (Figs. 1B and 1D).

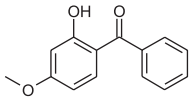
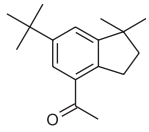
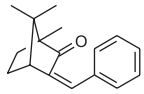
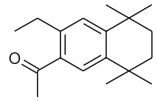
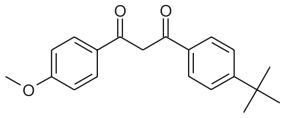
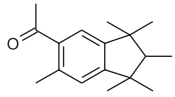
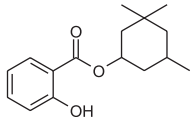
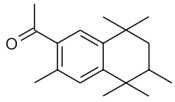
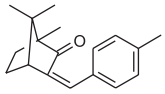
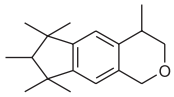
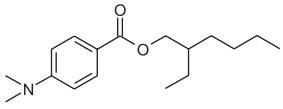
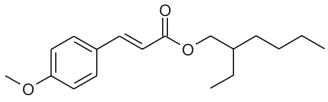
Data on the UV filters (except for 3-benzylidene camphor, 3BC) have been published previously (Schreurs *et al*., 2002a). In short, all UV filters showed agonism toward hERα, and additionally, a number were found to show agonism toward hERβ as well. None of the UV filters showed anti-estrogenic effects. Figures 1A and 1C show the agonistic dose-response curve of 3BC toward hERα and hERβ. Table 2 shows the EC_{50} values on both estrogen receptors.

Consistent with previous data in 293HEK cells (Schreurs *et al*., 2002b), none of the five tested musks showed clear ER agonism (above 10% relative to estradiol) on both ERα and ERβ receptors in this cell line (data not shown). Anti-estrogenic effects were found only on hERβ (Fig. 2). AHTN, HHCB, AHMI, and AETT show rather weak antagonism at concentrations above 1 µM. IC_{50} values can be found in Table 3.

AR Transrepression by UV Filters and Polycyclic Musks in the AR CALUX® Bioassay

The newly developed AR CALUX® bioassay was used to measure the agonistic and antagonistic effects of UV filters and polycyclic musks at the androgen receptor. In this assay, U2-OS cells stably contain a 3×ARE-TATA-Luc-reporter construct in

TABLE 1
Chemical names and structures of the tested UV filters and polycyclic musks

UV-filters		Polycyclic musks	
Benzophenone-3 (Bp-3)		4-acetyl-1,1-dimethyl-6-*tert*-butylindane (ADBI) (Celestolide®)	
3-Benzylidene camphor (3-BC)		7-acetyl-6-ethyl-1,1,4,4-tetramethyltetralin (AETT) (Versalide®)	
Butyl methoxydibenzoylmethane (B-MDM)		6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI) (Phantolide®)	
Homosalate (HMS)		7-acetyl-1,1,3,4,4,6-hexamethyl-tetralin (AHTN) (Tonalide®)	
4-Methylbenzylidene camphor (4-MBC)		1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-γ-2-benzopyrane (HHCB) (Galaxolide®)	
Octyl dimethyl-*p*-aminobenzoic acid (OD-PABA)			
Octyl-methoxycinnamate (OMC)			

combination with a hAR expression plasmid (Sonneveld *et al*., in press). The natural AR ligand dihydrotestosterone (DHT) was used as a positive control for agonism, while flutamide and vinclozolin were used as controls for AR antagonism. A DHT concentration of 0.1 nM (EC_{50}) was used for the measurement of anti-androgenicity. Figure 3 shows the agonistic dose-response curve of DHT and the antagonistic effect curves of flutamide and vinclozolin.

None of the test compounds showed AR transactivation (data not shown), however, nearly all test compounds were found to be AR antagonists (Fig. 4). The UV filters OD-PABA and OMC did not repress DHT-induced transactivational activity, while the polycyclic musk ADBI was only a very weak antagonist. The IC_{50} values are depicted in Table 4. The antagonistic effects of all of the compounds tested were reversed by coincubation with excess DHT (100 times the EC_{50} value), showing the specificity of the response (data not shown). In contrast, we have found previously, using the same assay, that the inhibitory effects of high levels of individual brominated flame retardant congeners could not be reversed by excess DHT (Hamers *et al*., manuscript in press). This coincided with cytotoxicity of these ligands, as assessed through inhibition of expression of a constitutively expressed reporter gene and a positive response in the MTT assay (Hamers *et al*., manuscript in press).

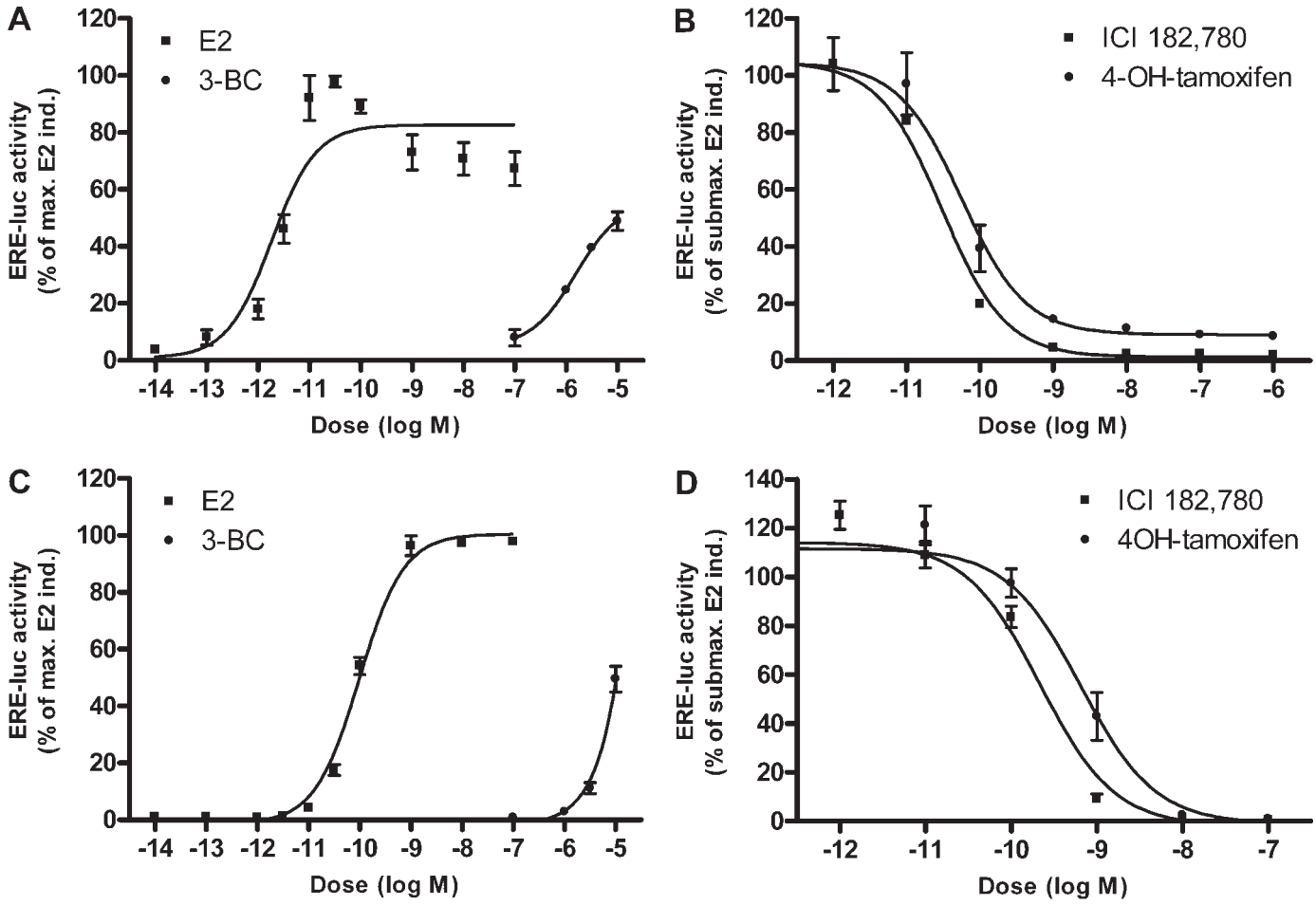


FIG. 1. Activation of transcription of hERα (A) or hERβ (C) by E2 and 3-BC in stably transfected HEK293 cells. Results are expressed as a percentage of maximal E2 induction. Repression of transcription of hERα (B) or hERβ (D) by ICI 182,780 and 4-OH-tamoxifen in stably transfected HEK293 cells. Results are expressed as a percentage of submaximal E2 induction (hERα, 0.003 nM; hERβ, 0.1 nM). Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

TABLE 2
Activation of Transcription for hERβ and hERβ in Stably Transfected HEK293 Cells by UV Filters and 17β-Estradiol

Compound	EC_{50} hERα	EC_{50} hERβ
17β-Estradiol	2.1 pM	83 pM
Bp-3	2.9 μM	25 μM
3-BC	13 μM	10 μM
B-MDM	[a]	—
HMS	1.6 μM	[c]
4-MBC	6.2 μM	14 μM
OD-PABA	11 μM	—
OMC	[b]	—

[a]Dose-response curve of B-MDM on hERα reached its plateau level at 37%.
[b]Dose-response curve of OMC on hERα reached its plateau level at 42%.
[c]Dose-response curve of HMS on hERβ reached its plateau level at 32%.

PR Transrepression by UV Filters and Polycyclic Musks in the PR CALUX® Bioassay

To investigate the agonistic and antagonistic effects of UV filters and polycyclic musks at the human progesterone receptor, the newly developed PR CALUX® bioassay was used (Sonneveld *et al.*, manuscript in press). This assay consists of U2-OS cells containing a 3×PRE-TATA-Luc-reporter construct in combination with a hPR expression plasmid. ORG2058 was used as a stable PR agonist, while RU486 was used as a control for PR-antagonism. An ORG2058 concentration of 30 pM (EC_{50}) was used for the measurement of antiprogestagenic activity. Figure 5 shows the agonistic dose-response curve of ORG2058 and the antagonistic effect curve of RU486.

None of the test compounds showed PR transactivation (data not shown); however, several test compounds were found to be PR antagonists (Fig. 6). The IC_{50} values are given in Table 5. The

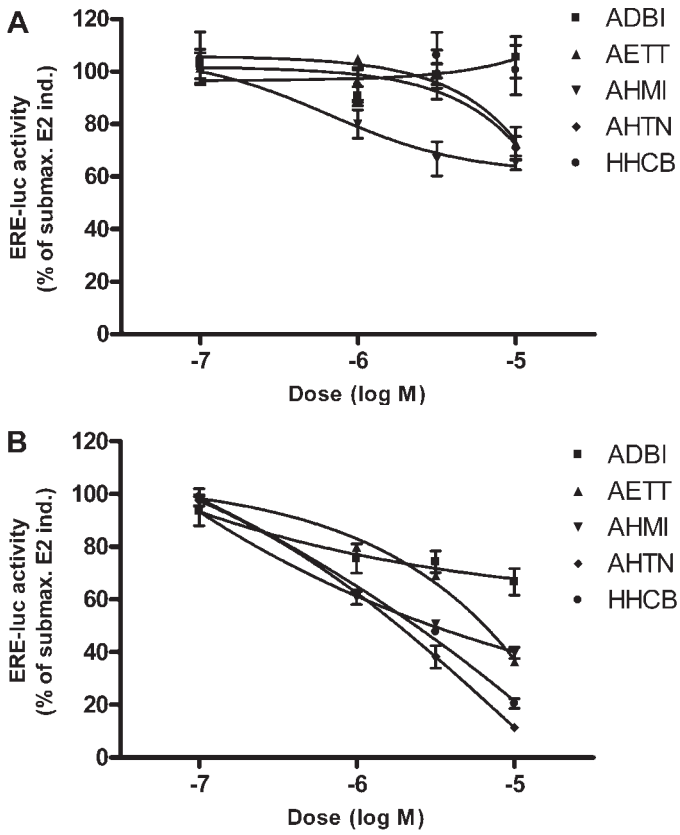


FIG. 2. Repression of transcription of hERα (A) or hERβ (B) by polycyclic musks in stably transfected HEK293 cells. Results are expressed as a percentage of submaximal E2 induction (hERα, 0.003 nM; hERβ, 0.1 nM). Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

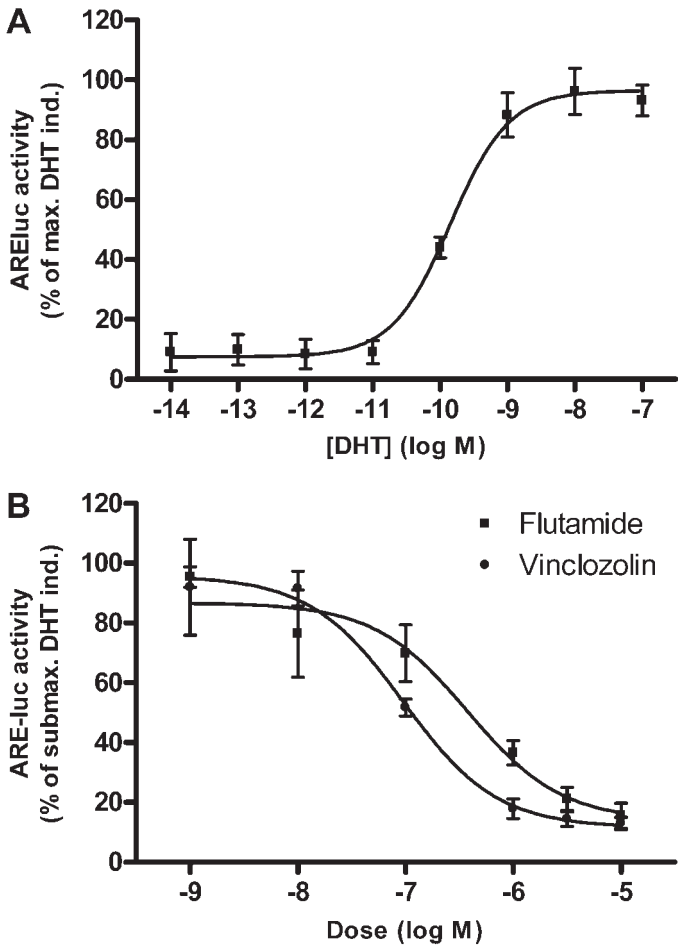


FIG. 3. Activation of transcription of hAR (A) by DHT in AR CALUX® cells. Results are expressed as a percentage of maximal DHT induction. Repression of transcription of hAR (B) by flutamide and vinclozolin in AR CALUX® cells. Results are expressed as a percentage of 0.1 nM DHT induction. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

TABLE 3
Repression of Transcription or hERα and hERβ in Stably Transfected HEK293 Cells by Polycyclic Musks, 4-OH-Tamoxifen, and ICI 182,780

Compound	IC_{50} hERα	IC_{50} hERβ
4-OH-tamoxifen	74 pM	803 pM
ICI 182,780	32 pM	239 pM
ADBI	—	—
AETT	—	6.5 μM
AHMI	—	3.0 μM
AHTN	—	1.9 μM
HHCB	—	2.4 μM

five tested polycyclic musks showed antagonism, but with different activities. AHTN and AHMI were the strongest antagonists (IC_{50} values of 24 and 28 nM, respectively), while ADBI was the weakest antagonist (IC_{50} of 6.4 μM). UV filters OD-PABA and B-MDM did not show PR antagonism. The other five UV filters showed antagonism in the micromolar range. The antagonistic effects of all of the compounds tested were reversed by coincubation with excess ORG2058 (100 times the EC_{50} value), showing the specificity of the response (data not shown).

DISCUSSION

In the past decade, much and increasing research on endocrine disruption has been undertaken and reported in the scientific literature. The interaction of compounds interacting with nuclear hormone receptors is widely believed to be a key factor in inducing endocrine disruption. In many laboratories all over the world, many compounds have been tested for their interaction and activity toward estrogen receptors, both *in vitro* and *in vivo*. However, in addition to estrogen receptors, the nuclear steroid hormone receptor family also consists of the androgen receptor

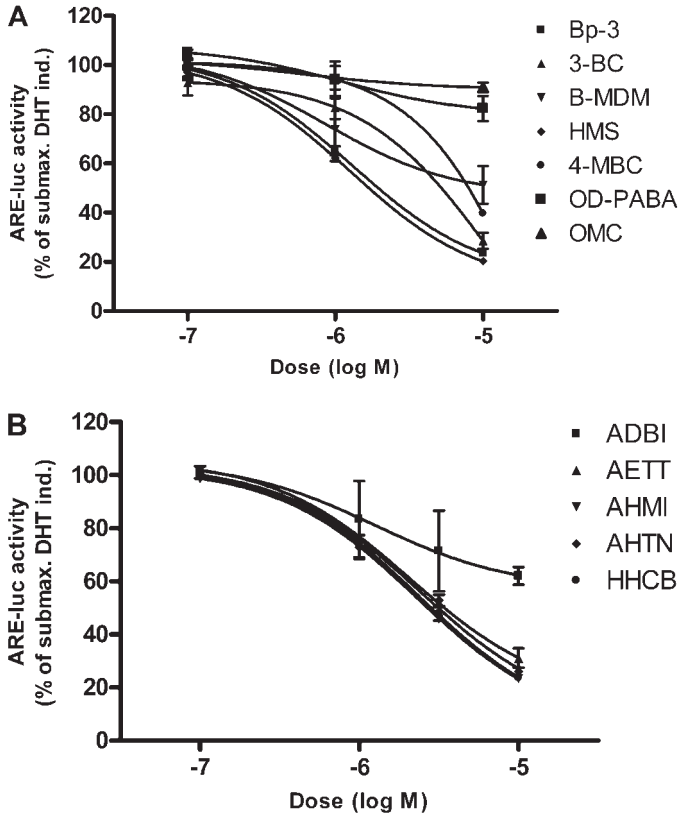


FIG. 4. Repression of transcription of hAR by UV-filters (A) and polycyclic musks (B) in AR CALUX® cells. Results are expressed as a percentage of 0.1 nM DHT induction. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

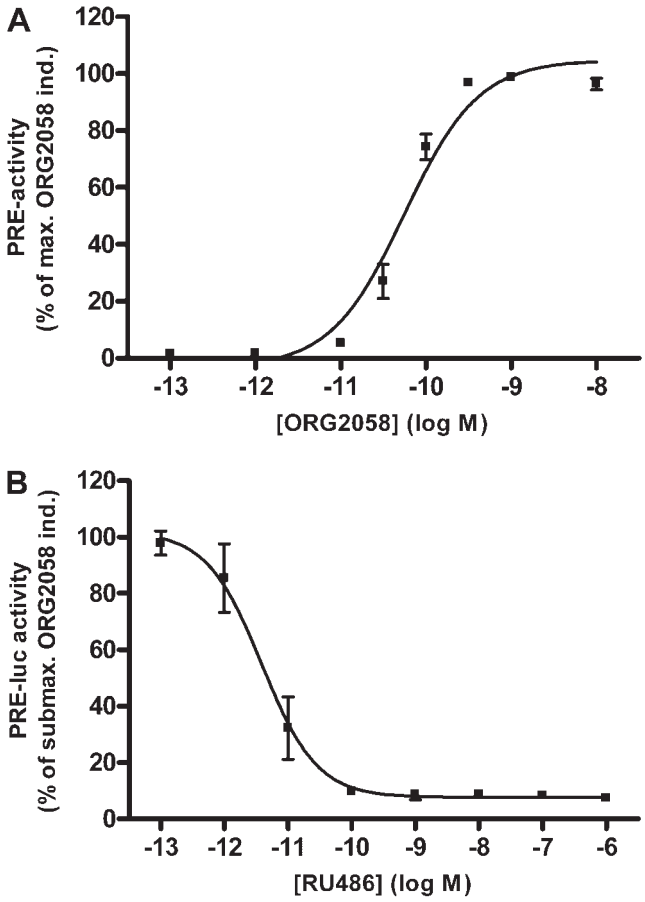


FIG. 5. Activation of transcription of hPR (A) by ORG2058 in PR CALUX® cells. Results are expressed as a percentage of maximal ORG2058 induction. Repression of transcription of hPR (B) by RU486 in PR CALUX® cells. Results are expressed as a percentage of 30 pM ORG2058 induction. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

TABLE 4
Repression of Transcription of hAR in AR CALUX® Cells by Polycyclic Musks, UV-Filters, Flutamide, and Vinclozolin

Compound	IC_{50} AR	Compound	IC_{50} AR
Flutamide	0.5 μM	Bp-3	2.0 μM
Vinclozolin	0.1 μM	3-BC	4.6 μM
ADBI	—	B-MDM	[a]
AETT	3.5 μM	HMS	1.7 μM
AHMI	2.7 μM	4-MBC	7.1 μM
AHTN	3.6 μM	OD-PABA	—
HHCB	2.9 μM	OMC	—

[a]Extrapolation outside the tested dose range resulted in an IC_{50} value of 11 μM.

(AR), progesterone receptor (PR), glucocorticoid receptor (GR), retinoic acid receptor (RAR), thyroid receptor (TR), mineralocorticoid receptor (MR), and vitamin D receptor (VDR). These other receptors have been scarcely studied in researching suspected endocrine-disrupting chemicals. In this research, two classes of cosmetic ingredients, namely polycyclic musks and UV filters, have been tested for their interaction with the ER, AR, and PR. Several compounds from both classes have been tested previously for (anti)estrogenic activity. UV filters have been assessed both *in vitro* and *in vivo* by, amongst others, Holbech *et al*. (2002), Mueller *et al*. (2003), Schlumpf *et al*. (2001), Schreurs *et al*. (2002a), and Tinwell *et al*. (2002). Polycyclic musks, predominantly AHTN and HHCB, have been researched by Seinen *et al*. (1999), Schreurs *et al*. (2002b, 2004), and Bitsch *et al*. (2002). Interaction of UV filters with the AR has only been found by Ma *et al*. (2003).

In the literature, several chemical compounds have been assessed for their interaction with the androgen receptor. Only a few environmental xeno-androgens have been found, namely triphenyltin (Schulte-Oehlmann *et al*., 2000), and unidentified compounds in pulp-mill effluent (Parks *et al*., 2001). Many more environmental anti-androgens have been found, for example the pesticides vinclozolin, p,p′-DDE, DDT, procymidone, and linuron (Gray *et al*., 1999, 2001; Kelce *et al*., 1997). The phthalates diethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DBP) have been found to be nonclassical

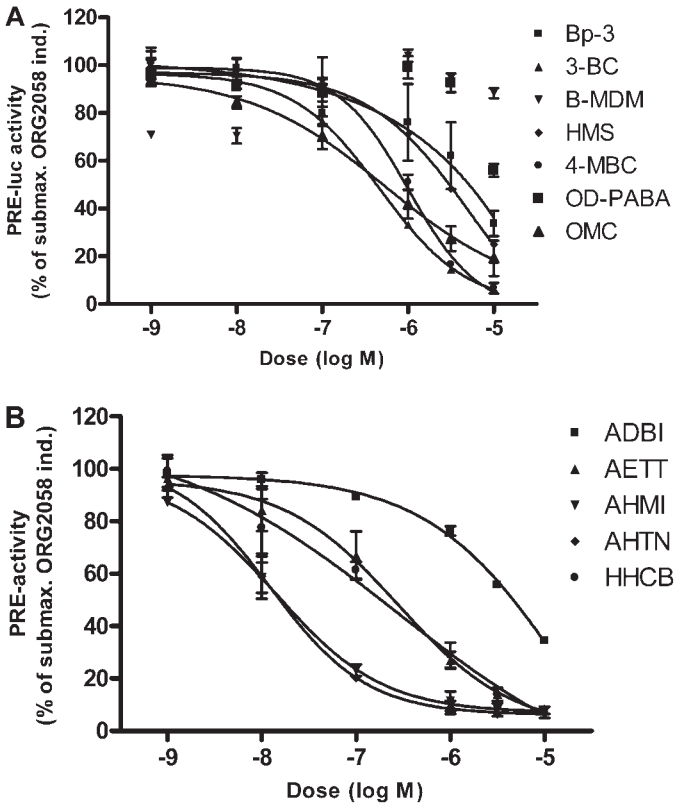


FIG. 6. Repression of transcription of hPR by UV-filters (A) and polycyclic musks (B) in PR CALUX® cells. Results are expressed as a percentage of 30 pM ORG2058 induction. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

TABLE 5
Repression of Transcription of hPR in PR CALUX® Cells by Polycyclic Musks, UV-Filters, and RU486

Compound	IC_{50} PR	Compound	IC_{50} PR
RU486	4.9 pM	Bp-3	5.2 μM
		3-BC	0.4 μM
ADBI	4.7 μM	B-MDM	—
AETT	0.3 μM	HMS	3.0 μM
AHMI	0.02 μM	4-MBC	0.9 μM
AHTN	0.02 μM	OD-PABA	—
HHCB	0.2 μM	OMC	0.5 μM

anti-androgens, in that they inhibit enzymes concerned with hormone metabolism, rather than competing with endogenous androgens for receptor binding (Parks *et al*., 2000). The same phenomenon can be observed for the progesterone receptor. As far as we know, the flavonoid apigenin is the only environmental PR agonist found (Willemsen *et al*., 2004). Several compounds have been reported to act like PR antagonists, for example DDT and its metabolites, 4-tert-octylphenol, 4-nonylphenol, lindane, and endosulfan (Jin *et al*., 1997; Klotz *et al*., 1997; Tran *et al*., 1996). These studies were undertaken using a yeast strain hPR-PRE with β-galactosidase as the read-out signal. The most potent compounds were 4-nonylphenol (IC_{50} 0.50 μM) and the DDT-metabolite DDOH (IC_{50} 0.35 μM). In this research, we also find only antagonists for the AR and PR, rather than agonists.

It is striking that such high concentrations of the clinically used anti-androgen flutamide are needed to exert an effect. It may be that flutamide is not metabolized into the more potent anti-androgen hydroxyflutamide, as occurs *in vivo*. The UV filter HMS is only a factor of two less potent than flutamide, and the polycyclic musk HHCB is roughly a factor of 5 less potent. Although some of the test compounds were found to be nearly as potent anti-androgens as flutamide, the effect concentrations are quite high and have not been found in the environment.

The most variable range of IC_{50} values were found in the PR CALUX bioassay. Apart from ADBI, the polycyclic musks were found to be the most potent PR antagonists. The IC_{50} values of AHTN and AHMI are even lower than those found for 4-nonylphenol and the DDT-metabolite DDOH, while HHCB and AETT have similar IC_{50} values. When comparing the IC_{50} values of these four polycyclic musks on the different receptors, those on the PR are roughly a factor of 10–100 lower. This means that the tested polycyclic musks are more potent antagonists on the PR than on the ER or AR, when effect concentrations are taken into account.

Some discrepancies with respect to the UV filters 3-benzylidene camphor and 4-methyl benzylidene camphor were found when comparing our results with other studies. The UV filter 3-BC has recently been investigated and compared with the UV filter 4-MBC by Schlumpf *et al*. (2004). In an E-SCREEN assay, both compounds induced MCF-7 cell proliferation, with EC_{50} values of 0.69 μM 3-BC and 3.9 μM 4-MBC. In an *in vivo* uterotrophic assay, 3-BC was found to be more potent than 4-MBC. In our study, such difference between 3-BC and 4-MBC was not observed. In an ER-binding assay, Schlumpf *et al*. showed that both compounds displaced 16α-125I-estradiol from human ERβ, but not from human ERα. This would seem to indicate that 3-BC and 4-MBC are unable to directly bind ERα, and that their agonistic effects in this study could be caused by metabolites. The fact that we do not find different potencies between 3-BC and 4-MBC may be due to differences in metabolism between experimental systems.

Another discrepancy concerns observed anti-androgenic effects. Ma *et al*. (2003) only found anti-androgenic effects for the UV filters Bp-3 and HMS, while we also found anti-androgenic effects with 3-BC and 4-MBC. These differences may be explained by the fact that Ma *et al*. used an MDA-kb2 cell line containing low endogenous AR and GR levels. In this study we used a U2-OS cell line overexpressing AR which is probably more selective and sensitive for measuring AR interaction.

In this study we found interaction of cosmetic ingredients with estrogen, androgen, and progesterone receptors. Four polycyclic musks were found to be antagonists toward ERβ, AR, and PR. The UV filters were found to be mainly ERα-agonists, and

antagonists toward AR and PR. For a detailed risk assessment of cosmetic ingredients concerning endocrine disruption in humans, more data is needed, especially *in vivo* data. Recently, parabens, which are used in deodorants, were found to be present in human breast tumors (Darbre *et al*., 2004). Several *in vitro* studies have shown that parabens are estrogenic (Byford *et al*., 2002; Routledge *et al*., 1998). The same effect has also been shown in *in vivo* studies (Darbre *et al*., 2003; Routledge *et al*., 1998). Due to the apparent estrogenic activity of parabens and their presence in breast tumors, it has been speculated that parabens might contribute to the rising incidence of breast cancer (Darbre *et al*., 2004). However, parabens are not the only xenoestrogens that can be found in breast tissue. Polychlorinated biphenyls (PCBs), DDT, DDE, and dieldrin have been found in breast tissue too. Further, it cannot be ruled out that the tested estrogenic UV filters, which have been found in mother's milk, can also be found in breast tissue or breast tumors. The polycyclic musks AHTN and HHCB have also been found in human breast milk as well, but these compounds act as ER antagonists. The consequences of exposure to an anti-estrogenic effect induced by xenobiotics for human health so far remain unclear. In literature, anti-estrogenic effects in wildlife have been rarely found. Several estrogenic chemicals have been found to also be anti-androgenic (Sohoni and Sumpter, 1998). In this paper some UV filters were also found to have these properties. A small number of chemical-compound-induced anti-androgenic effects in animals have been reported, for example by vinclozolin and DDE (reviewed in Kelce and Wilson, 1997). As far as we know, disruption of the PR-mediated pathway has not been investigated. As these latter effects in our research were found to be relatively strong, future studies should focus on *in vivo* endocrine disruptive effects produced by anti-progestins.

This research shows that a single compound can exert different effects on several hormone receptors. However, it is not possible to predict the overall endocrine disruptive effect from *in vitro* studies. Therefore, *in vivo* studies are needed to find out whether a compound can exert endocrine-related adverse health effects. Screening chemicals in suitable *in vitro* assays and finding out whether a compound is an agonist and/or an antagonist toward a given hormone receptor is a useful and potentially important starting point in the search for environmental endocrine disruptive chemicals. Using *in vitro* assays can provide a mechanistically based, early focus on compounds of interest and a potentially significantly reduction in the number of *in vivo* studies with the associated ethical issues and costs involved. The bioassays used in this paper are sensitive, straightforward and fast to use, which makes them a suitable tool for the screening of suspected endocrine active compounds.

ACKNOWLEDGMENT

We would like to thank Justin Mason for reading the manuscript and for linguistic corrections.

REFERENCES

Ashby, J., Tinwell, H., Plautz, J., Twomey, K., and Lefevre, P. A. (2001). Lack of binding to isolated estrogen or androgen receptors, and inactivity in the immature rat uterotrophic assay, of the ultraviolet sunscreen filters Tinosorb M-active and Tinosorb S. *Regul. Toxicol. Pharmacol.* **34,** 287–291.

Bauer, K., and Frössl, C. (1999). Blutkonzentrationen von polycyclischen und nitromoschusverbindungen bei deutschen probanden. *Umweltmedizinische Praxis* **12,** 235–237.

Bester, K., Hühnerfuss, H., Lange, W., Rimkus, G. G., and Theobald, N. (1998). Results of non target screening of lipophilic organic pollutants in the German Bight II: Polycyclic musk fragrances. *Water Res.* **32,** 1857–1863.

Bitsch, N., Dudas, C., Körner, W., Failing, K., Biselli, S., Rimkus, G., and Brunn, H. (2002). Estrogenic activity of musk fragrances detected by the E-screen assay using human MCF-7 cells. *Arch. Environ. Contam. Toxicol.* **43,** 257–264.

Byford, J. R., Shaw, L. E., Drew, M. G. B., Pope, G. S., Sauer, M. J., and Darbre, P. D. (2002). Oestrogenic activity of parabens in MCF7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **80,** 49–60.

Darbre, P. D. (2003). Underarm cosmetics and breast cancer. *J. Appl. Toxicol.* **23,** 89–95.

Darbre, P. D., Aljarrah, A., Miller, W. R., Coldham, N. G, Sauer, M. J., and Pope, G. S. (2004). Concentrations of parabens in human breast tumours. *J. Appl. Toxicol.* **24,** 5–13.

Darbre, P. D., Byford, J. R., Shaw, L. E., Hall, S., Coldham, N. G., Pope, G. S., and Sauer, M. J. (2003). Oestrogenic activity of benzylparaben. *J. Appl. Toxicol.* **23,** 43–51.

Darbre, P. D., Byford, J. R., Shaw, L. E., Horton, R. A., Pope, G. S., and Sauer, M. J. (2002). Oestrogenic activity of isobutylparaben *in vitro* and *in vivo*. *J. Appl. Toxicol.* **22,** 219–226.

Draisci, R., Marchiafava, C., Ferretti, E., Palleschi, L., Catellani, G., and Anastasio, A. (1998). Evaluation of musk contamination of freshwater fish in Italy by accelerated solvent extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A.* **814,** 187–197.

Felix, T., Hall, B. J., and Brodbelt, J. S. (1998). Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. *Anal. Chim. Acta* **371,** 195–203.

Fromme, H., Otto, T., and Pilz, K. (2001). Polycyclic musk fragrances in different environmental compartments in Berlin (Germany). *Water Res.* **35,** 121–128.

Fromme, H., Otto, T., Pilz, K., and Neugebauer, F. (1999). Levels of synthetic musks; bromocyclene and PCBs in eel (*Anguilla anguilla*) and PCBs in sediment samples from some waters of Berlin/Germany. *Chemosphere* **39,** 1723–1735.

Gatermann, R., Hellou, J., Hühnerfuss, H., Rimkus, G., and Zitko, V. (1999). Polycyclic and nitro musks in the environment: A comparison between Canadian and European aquatic biota. *Chemosphere* **38,** 3431–3441.

Gray, L. E., Jr., Ostby, J., Monosson, E., and Kelce, W. R. (1999). Environmental antiandrogens: Low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. Ind. Health* **15,** 48–64.

Gray, L. E., Ostby, J., Furr, J., Wolf, C. J., Lambright, C., Parks, L., Veeramachaneni, D. N., Wilson, V., Price, M., Hotchkiss, A., *et al*. (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum. Reprod. Update* **7,** 248–264.

Hany, J., and Nagel, R. (1995). Nachweis von UV-Filtersubstanzen in Muttermilch. *Deutsche Lebensmittel-Rundschau* **91,** 341–345.

Hayden, C. G. J., Roberts, M. S., and Benson, H. A. E. (1997). Systemic absorption of sunscreen after topical application. *Lancet* **350,** 863–864.

Heberer, Th., Gramer, S., and Stan, H.-J. (1999). Occurrence and distribution of organic contaminants in the aquatic system in Berlin. Part III: Determination of synthetic musks in Berlin surface water applying solid-phase

microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). *Acta Hydrochim. Hydrobiol.* **27,** 150–156.

Holbech, H., Norum, U., Korsgaard, B., and Poul, B. (2002). The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. *Pharmacol. Toxicol.* **91,** 204–208.

Jin, L., Tran, D. Q., Ide, C. F., McLachlan, J. A., and Arnold, S. F. (1997). Several synthetic chemicals inhibit progesterone receptor-mediated transactivation in yeast. *Biochem. Biophys. Res. Commun.* **233,** 139–146.

Kelce, W. R., Lambright, C. R., Gray, L. E., Jr., and Roberts, K. P. (1997). Vinclozolin and p,p′-DDE alter androgen-dependent gene expression: *In vivo* confirmation of an androgen receptor-mediated mechanism. *Toxicol. Appl. Pharmacol.* **142,** 192–200.

Kelce, W. R., and Wilson, E. M. (1997). Environmental antiandrogens: Developmental effects, molecular mechanisms, and clinical implications. *J. Mol. Med.* **75,** 198–207.

Klotz, D. M., Ladlie, B. L., Vonier, P. M., McLachlan, J. A., and Arnold, S. F. (1997). O,p′-DDT and its metabolites inhibit progesterone-dependent responses in yeast and human cells. *Mol. Cell. Endocrinol.* **129,** 63–71.

Lemini, C., Silva, G., Timossi, C., Luque, D., Valverde, A., González-Martínez, M., Hernández, A., Rubio-Póo, C., Chávez Lara, B., and Valenzuela, F. (1997). Estrogenic effects of *p*-hydroxybenzoic acid in CD1 mice. *Environ. Res.* **75,** 130–134.

Lemmen, J. G., Van den Brink, C. E., Legler, J., Van der Saag, P. T., and Van der Burg, B. (2002). Detection of oestrogenic activity of steroids present during mammalian gestation using oestrogen receptor α- and oestrogen receptor β-specific *in vitro* assays. *J. Endocrinol.* **174,** 435–446.

Ma, R., Cotton, B., Lichtensteiger, W., and Schlumpf, M. (2003). UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. *Toxicol. Sci.* **74,** 43–50.

Miller, D., Wheals, B. B., Beresford, N., and Sumpter, J. P. (2001). Estrogenic activity of phenolic additives determined by an *in vitro* yeast bioassay. *Env. Health Persp.* **109,** 133–138.

Muller, S., Schmid, P., and Schlatter, C. (1996). Occurrence of nitro and non-nitro benzenoid musk compounds in human adipose tissue. *Chemosphere* **33,** 17–28.

Mueller, S. O., Kling, M., Arifin Firzani, P., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Broschard, T., and Kramer, P. J. (2003). Activation of estrogen receptor alpha and ERbeta by 4-methylbenzylidene-camphor in human and rat cells: Comparison with phyto- and xenoestrogens. *Toxicol. Lett.* **142,** 89–101.

Nagtegaal, M., Ternes, T. A., Baumann, W., and Nagel, R. (1997). UV-Filtersubstanzen in Wasser und Fischen. *UWSF-Z Umweltchem. Ökotox.* **9,** 79–86.

Nakagawa, Y., and Suzuki, T. (2002). Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells. *Chem. Biol. Interact.* **139,** 115–128.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D, Klinefelter, G. R., Barlow, N. J., and Gray, L. E., Jr. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.* **58,** 339–349.

Parks, L. G., Lambright, C. S., Orlando, E. F., Guillette, L. J., Jr., Ankley, G. T., and Gray, L. E., Jr. (2001). Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol. Sci.* **62,** 257–267.

Rimkus, G. G. (1999). Polycyclic musk fragrances in the aquatic environment. *Toxicol. Lett.* **111,** 37–56.

Rimkus, G. G., and Wolf, M. (1996). Polycyclic musk fragrances in human adipose tissue and human milk. *Chemosphere* **33,** 2033–2043.

Routledge, E. J., Parker, J., Odum, J., Ashby, J., and Sumpter, J. P. (1998). Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **153,** 12–19.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., and Lichtensteiger, W. (2001). *In vitro* and *in vivo* estrogenicity of UV screens. *Env. Health Perspect.* **109,** 239–244.

Schlumpf, M., Jarry, H., Wuttke, W., Ma, R., and Lichtensteiger, W. (2004). Estrogenic activity and estrogen receptor beta binding of the UV filter 3-benzylidene camphor; Comparison with 4-methylbenzylidene camphor. *Toxicology* **199,** 109–120.

Schreurs, R., Lanser, P., Seinen, W., and Van der Burg, B. (2002a). Estrogenic activity of UV filters determined by an *in vitro* reporter gene assay and an *in vivo* transgenic zebrafish assay. *Arch. Toxicol.* **76,** 257–261.

Schreurs, R. H. M. M., Legler, J., Artola-Garicano, E., Sinnige, T. L., Lanser, P. H., Seinen, W., and Van der Burg, B. (2004). *In vitro* and *in vivo* antiestrogenic effects of polycyclic musks in zebrafish. *Environ. Sci. Technol.* **38,** 997–1002.

Schreurs, R. H. M. M., Quaedackers, M. E., Seinen, W., and Van der Burg, B. (2002b). Transcriptional activation of estrogen receptor ERα and ERβ by polycyclic musks is cell type dependent. *Toxicol. Appl. Pharmacol.* **183,** 1–19.

Schulte-Oehlmann, U., Tillmann, M., Markert, B., Oehlmann, J., Watermann, B., and Scherf, S. (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyltin as a xeno-androgen. *Ecotoxicology* **9,** 399–412.

Seinen, W., Lemmen, J. G., Pieters, R. H., Verbruggen, E. M., and Van der Burg, B. (1999). AHTN and HHCB show weak estrogenic – but no uterotrophic activity. *Toxicol. Lett.* **111,** 161–168.

Sohoni, P., and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158,** 327–339.

Sonneveld, E., Jansen, H. J., Riteco, J. A., Brouwer, A., and Van Der Burg, B. (2004). Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. *Toxicol. Sci.* (in press).

Tinwell, H., Lefevre, P. A., Moffat, G. J., Burns, A., Odum, J., Spurway, T. D., Orphanides, G., and Ashby, J. (2002). Confirmation of uterotrophic activity of 3-(4-methylbenzylidene)camphor in the immature rat. *Environ. Health Perspect.* **110,** 533–536.

Tran, D. Q., Klotz, D. M., Ladlie, B. L., Ide, C. F., McLachlan, J. A., and Arnold, S. F. (1996). Inhibition of progesterone receptor activity in yeast by synthetic chemicals. *Biochem. Biophys. Res. Comm.* **229,** 518–523.

Willemsen, P., Scippo, M.-L., Kausel, G., Figueroa, J., Maghuin-Rogister, G., Martial, J. A., and Muller, M. (2004). Use of reporter cell lines for detection of endocrine-disruptor activity. *Anal. Bioanal. Chem.* **378,** 655–663.