In Vitro and *in Vivo* Estrogenicity of UV Screens

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Ultraviolet (UV) screens are increasingly used as a result of growing concern about UV radiation and skin cancer; they are also added to cosmetics and other products for light stability. Recent data on bioaccumulation in wildlife and humans point to a need for in-depth analyses of systemic toxicology, in particular with respect to reproduction and ontogeny. We examined six frequently used UVA and UVB screens for estrogenicity *in vitro* and *in vivo*. In MCF-7 breast cancer cells, five out of six chemicals, that is, benzophenone-3 (Bp-3), homosalate (HMS), 4-methyl-benzylidene camphor (4-MBC), octyl-methoxycinnamate (OMC), and octyl-dimethyl-PABA (OD-PABA), increased cell proliferation with median effective concentrations (EC_{50}) values between 1.56 and 3.73 µM, whereas butyl-methoxydibenzoylmethane (B-MDM) was inactive. Further evidence for estrogenic activity was the induction of pS2 protein in MCF-7 cells and the blockade of the proliferative effect of 4-MBC by the estrogen antagonist ICI 182,780. In the uterotrophic assay using immature Long-Evans rats that received the chemicals for 4 days in powdered feed, uterine weight was dose-dependently increased by 4-MBC (ED_{50} 309mg/kg/day), OMC (ED_{50} 935 mg/kg/day), and weakly by Bp-3 (active at 1,525 mg/kg/day). Three compounds were inactive by the oral route in the doses tested. Dermal application of 4-MBC to immature hairless (hr/hr) rats also increased uterine weight at concentrations of 5 and 7.5% in olive oil. Our findings indicate that UV screens should be tested for endocrine activity, in view of possible long-term effects in humans and wildlife. *Key words*: benzophenone-3, estrogenic activity, MCF-7 cell proliferation, 4-methylbenzylidene camphor, octylmethoxycinnamate, pS2 protein, rat, uterotrophic assay, UV screens. *Environ Health Perspect* 109:239–244 (2001). [Online 28 February 2001]
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Organic chemicals that absorb UVA (400–315 nm) or UVB (315–280 nm) radiation are added in concentrations up to 10% to sunscreen products for skin protection. Some of the compounds are also included in other cosmetics such as beauty creams, lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and bubble baths for product stability and durability.

Because of growing public concern about skin damage by UV light, the use of UV screens is increasing, even though the benefit with respect to prevention of melanoma remains controversial (*1,2*). Like other cosmetics such as musk fragrances (*3,4*), these chemicals are highly lipophilic and therefore can be expected to bioaccumulate in the environment. In 1991 and 1993, six different UV screens were identified in fish of the Meerfelder Maar lake (Eifel, Germany) at total concentrations of 2 mg/kg lipid in perch (summer 1991) and 0.5 mg/kg lipid in roach (1993) (*5*). Both fish species were contaminated with sunscreens, polychlorinated biphenyls and DDT at comparable levels. From these results it appeared that UV screens are relevant environmental contaminants (*5*).

Humans can be exposed to UV screens by dermal absorption (*6–9*) or through the food chain. The UV screen benzophenone-3 (Bp-3) and its metabolite 2,4-dihydroxybenzophenone have been detected in human urine from 4 hr after application of commercially available sunscreen products to the skin (*7,10*). Bp-3 has also been found to be readily absorbed from the gastrointestinal tract (*11*). Evidence for bioaccumulation in humans stems from analyses of human milk (*12*). In five out of six samples of human milk, Bp-3 and/or octyl methoxycinnamate were present in detectable amounts.

At present, the toxicologic classification of UV screens is rather heterogenous; they are classified as over-the-counter drugs in the United States, cosmetic ingredients in the European Union, and either cosmetics or quasi-drug products in Japan (*13*). Acute and subchronic systemic toxicity of these compounds is considered to be rather low (*7,14,15*), although some problems have arisen with photoallergic reactions (*16*). No values of acceptable daily intake or maximal tolerated intake of UV screens have been defined. However, the bioaccumulation potential of these lipophilic chemicals does not appear to have been considered in earlier published toxicologic long-term studies. The evidence of bioaccumulation in wildlife and humans raises the possibility of long-term exposure, including effects on reproduction and ontogeny. As a consequence, these compounds should be tested for endocrine activity.

We analyzed six frequently used UVA- or UVB-absorbing UV screens for estrogenic activity *in vitro* in MCF-7 breast cancer cells and *in vivo* in the immature rat uterotrophic assay. Estrogenic activity was demonstrated for five out of six compounds *in vitro* and for three out of six compounds *in vivo* by the oral route. The orally most active compound also increased uterine weight following dermal application.

Materials and Methods

Chemicals

The UV screens Bp-3 (2-hydroxy-4-methoxybenzophenone, oxybenzone, Eusolex 4360); butyl methoxydibenzoylmethane (B-MDM, Eusolex 9020); homosalate (HMS, 2-hydroxybenzoic acid-3,3,5-trimethylcyclohexyl ester, Eusolex HMS); 3-(4-methylbenzylidene) camphor (4-MBC, Eusolex 6300); octyldimethyl-*p*-aminobenzoic acid (OD-PABA, Eusolex 6007); and octyl-methoxycinnamate (OMC, Eusolex 2292) were purchased from Merck (Dietikon, Switzerland). 17β-Estradiol (E_2) and 17α-ethinylestradiol were obtained from Calbiochem (Lucerne, Switzerland), and ICI 182,780 (Astra-Zeneca) was purchased from ANAWA (Dübendorf, Switzerland).

In Vitro Studies on MCF-7 Cells

Cell line. MCF-7 human breast cancer cells (MCF7-Bos, originally from the Michigan Cancer Foundation, Detroit, MI, USA) were kindly provided by A. Soto (Tufts University, Boston, MA, USA). Cells were frozen every 10 passages. In the present experimental series, we used samples from frozen stock for a maximum of 6–13 passages. Mycoplasma status, which was regularly checked by the Institute of Virology of the Veterinary Faculty of the University of Zurich, was negative. Cells were cultured in Dulbecco's modified Eagle Medium (DME) supplemented with 5% fetal bovine serum (FBS; Gibco, Life Technologies, Basel, Switzerland) in 5% CO_2/95% air at 37°C under saturated

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humidity in 50 mL Falcon flasks. Sex steroids were removed from the serum by charcoal dextran treatment [steroid hormone-free FBS (CD-FBS)] (*17*).

E-SCREEN. The E-SCREEN was performed according to Soto and co-workers (*17,18*). Briefly, MCF-7 cells were trypsinized, mechanically dissociated, and plated into 24-well plates (Costar; INTEGRA Biosciences, Wallisellen, Switzerland) at an initial concentration of 40,000 cells/well. Cells were allowed to attach in the seeding medium (DME supplemented with 5% FBS) for 24 hr. The seeding medium was then aspirated and replaced by the experimental medium containing phenol red-free DME with 10% CD-FBS. Cells were incubated with test compound (final concentrations 10^{-4}–10^{-7} M), E_2 as positive control (10^{-8}–10^{-13} M), or chemical-free medium (control) (Table 1). For each concentration, 4 wells per plate were used. The number of independent *in vitro* experiments is given in Table 1 and in figures representing *in vitro* data. Each experiment was accompanied by a positive control (E_2). Concentrations of stock solutions in absolute ethanol were 2×10^{-3} M for E_2 and 10^{-2} M for UV screens; final concentrations of ethanol in culture medium were between 1.0% and 0.001% (v/v) with test compounds, and were ≤ 0.0005% (v/v) with E_2. No difference in the cell proliferation rate was observed in control experiments with chemical-free medium or medium with 1.0% ethanol. Therefore, we used chemical-free medium as a control. We tested antagonism by the pure antiestrogen ICI 182,780 (*19*) in MCF–7 cells exposed for 6 days to E_2 (10 pM) or to 4–MBC (10 µM) in the presence or absence of 1, 10, or 100 nM ICI 182,780.

Measurement of cell proliferation. Experiments were terminated after 6 days of incubation by removing the media from the wells. Cells were fixed with 10% trichloroacetic acid, washed with phosphate-buffered saline, and stained with 1% sulforhodamine blue (0.4% in 1% acetic acid, 1 mL/well) for 15 min at room temperature. Stained cells were dissolved in TRIS buffer (pH 10.6), and optical density (OD) was measured in a Anthos Labtec 2000 spectrophotometer (Anthos Labtec Instruments, Salzbury, Austria) at 492 nm. OD values were converted into cell numbers by a standard curve. Experimental readings were in the linear range of the standard curve (Figure 1).

pS2 protein assay. MCF-7 cells were incubated 72 hr with UV screens at 5, 10, and 50 µM; data are shown for 10 µM (except HMS 50 µM). E_2 (10 pM) served as a positive control. Culture media were harvested and centrifuged to avoid floating of detached cells. Samples were kept at –80°C until the radioimmunoassay for pS2 protein was performed according to the protocol of the manufacturer (ELSA - PS2; CIS Bio International, Gif-sur-Yvette, France). Media were analyzed in duplicate.

In Vivo Studies

Uterotrophic assay. Animal experiments were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Protection Ordinance under permit 190/98 of the Veterinary Office of the State of Zurich. We tested estrogenic *in vivo* activity of UV screens using the rat uterotrophic assay (*20,21*). Long-Evans rats were bred in our laboratory under controlled light and dark cycle (lights on from 0200 to 1600 hr) and temperature (22°C ± 1°C), with standard diet (chow 3430; Provimi Kliba AG, Kaiseraugst, Switzerland) and water *ad libitum*. All experiments were performed on offspring of time-pregnant rats. Receptive females were mated with a male between 1600 and 1900 hr. Sperm-positive females were housed in groups of two to three and separated 1 day before parturition.We defined the stage 24 hr after onset of the 3-hr mating period as gestational day (GD) 1 and the day of birth as postnatal day (PN) 1 (GD 23).

Peroral administration of test chemicals. From PN 16, the pups and their dam were habituated to powdered chow (chow 3430, Provimi Kliba), which continued after weaning at PN 20. Beginning on PN 21, female pups received chow 3430 containing one of several concentrations of test compound for 4 days, until 1200 hr on PN 25. For each experiment, chemicals were dissolved either in acetone or in 99% ethanol and added to powdered chow 3430. The mixture was prepared at least 48 hr before the experiment to allow for complete evaporation of the solvent. Evaporation was assisted by continuous stirring. We used ethinylestradiol (0.3–10 µg/kg) as a positive control. Vehicle controls received normal chow 3430. To limit the number of experimental animals, we adjusted the size of the various treatment groups according to statistical needs. We used the minumum group sample size of the three UV screens that we determined were inactive.

To avoid stress to the immature pups, we housed the animals in groups of four to six. We recorded body weight at the beginning and at the end of the treatment period. Animals from different litters were randomly assigned to the various treatment groups to give similar mean body weights to the various treatment groups at the onset of treatment, with no more than 15% deviation of individual animals from the mean. Mean body weights of experimental groups were in the same range as that of the controls (initial weight 38.0 ± 4.5 g, final weight 48.8 ± 3.8 g). We calculated the mean body weight of the 4-day treatment period for each animal. Food consumption of the group of four to six animals was measured for the 4-day period. The mean daily dose was calculated from the average amount of chemical consumed per animal (ingested powdered chow per animal × concentration of test compound in chow per mean body weight of a given animal). The advantage of using the average values of consumption is that the animals were not disturbed. The consistency

Table 1. Effect of UV screens and E_2 on MCF-7 cell proliferation *in vitro*.

Compound/ concentration (M)	Cells/well
E_2	
0	44,501 ± 5,079 (13)
1×10^{-13}	61,093 ± 12,200 (4)
5×10^{-13}	61,563 ± 2,090 (6)
1×10^{-12}	333,970 ± 51,026 (11)*
1×10^{-11}	743,296 ± 88,655 (11)#
1×10^{-10}	677,115 ± 91,301 (10)#
1×10^{-9}	655,969 ± 77,928 (10)#
1×10^{-8}	623,608 ± 72,292 (11)#
4-MBC	
0	49,028 ± 15,924 (6)
1×10^{-7}	132,292 ± 33,478 (6)
1×10^{-6}	147,396 ± 46,267 (6)
5×10^{-6}	583,299 ± 51,178 (6)#
1×10^{-5}	661,597 ± 66,740 (6)#
5×10^{-5}	330,157 ± 68,896 (6)**
OMC	
0	55,504 ± 13,373 (6)
1×10^{-7}	147,033 ± 25,657 (6)
1×10^{-6}	229,688 ± 65,150 (6)
5×10^{-6}	594,809 ± 74,438 (6)#
1×10^{-5}	566,945 ± 88,253 (6)#
5×10^{-5}	215,985 ± 58,542 (6)
Bp-3	
0	40,292 ± 2,422 (5)
1×10^{-7}	99,605 ± 28,489 (5)
1×10^{-6}	71,438 ± 29,796 (5)
5×10^{-6}	448,750 ± 78,557 (5)#
1×10^{-5}	704,750 ± 53,108 (5)#
5×10^{-5}	680,354 ± 63,914 (5)#
HMS	
0	96,292 ± 15,512 (5)
1×10^{-7}	155,771 ± 20,505 (5)
1×10^{-6}	279,833 ± 22,404 (5)**
5×10^{-6}	573,042 ± 50,308 (5)#
1×10^{-5}	652,917 ± 35,943 (5)#
5×10^{-5}	586,479 ± 14,416 (5) #
OD-PABA	
0	47,726 ± 14,068 (6)
1×10^{-7}	113,229 ± 24,761 (6)
1×10^{-6}	76,719 ± 6,740 (6)
5×10^{-6}	290,181 ± 59,554 (6)**
1×10^{-5}	435,827 ± 35,692 (6)#
5×10^{-5}	326,021 ± 63,239 (6)#
B-MDM	
0	28,125 ± 5,381 (5)
1×10^{-7}	99,104 ± 35,589 (5)
1×10^{-6}	29,833 ± 6,559 (5)
5×10^{-6}	73,250 ± 46,864 (5)
1×10^{-5}	120,846 ± 79,925 (5)
5×10^{-5}	54,297 ± 19,109 (5)

Values shown are mean ± SEM (number of independent experiments).
* $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ (ANOVA plus Bonferroni pairwise comparisons) as compared to control.

of the condition is indicated by an SD/mean ratio of uterine weights of 5.6% in controls and 9.8% in treated groups.

At the end of the treatment period, pups were decapitated under light ether anesthesia.The uterus was excised, trimmed free of fat and connective tissues, and blotted with sterile gauze to remove adherent fluid.The uterus was cut just above the junction between the cervix and vagina and at the top of the uterine horns. It was then weighed (wet weight) and either frozen in liquid nitrogen or fixed in buffered 4% formaldehyde for further analysis.

Dermal application of test chemicals. We studied possible effects of dermal application of UV screens in immature females of the Rat Nu (hairless) strain (Ico: OFA hr/hr). Parent animals were obtained from IFFA CREDO (Labresle, France) and kept under the same conditions as the Long-Evans rat colony (see above). One hr+/hr+ male was caged with three adult hr+/hr+ females. Pregnant dams were identified by weight gain, and the date of parturition (PN 1) was registered. Because of difficulties of the lactating hr+/hr+ dams to produce sufficient milk, the dam was replaced on PN 2 by a lactating Long-Evans dam. Pups were weaned at PN 20.

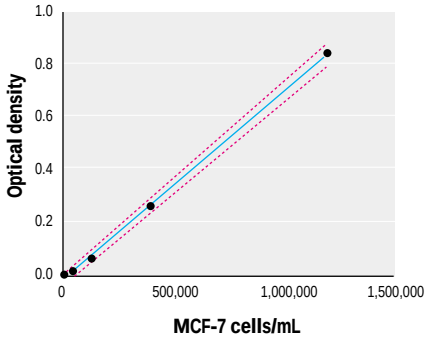

Figure 1. Representative standard curve of MCF-7 cell density (cells/mL) versus optical density (1% sulforhodamine blue staining). Linear regression with 95% confidence limits (r^2 = 0.9065). Circles represent mean values of 16 replicates.

Female rat pups were treated on 6 consecutive days, from PN 21 to PN 26. 4-MBC [2.5%, 5.0%, 7.5% (w/w) in olive oil] or olive oil (vehicle control) was applied twice daily at an interval of 3–4 hr. At 30°C ambient temperature, the animal was gently held by the neck and immersed up to its shoulders into a glass beaker containing olive oil with 4-MBC or pure olive oil for 15 sec. The pup was then transferred into a plastic box (one animal per box), where it remained on a paper towel for 30 min. During the 30-min period, an additional amount of the solution was applied twice onto the back of the animal with a soft brush. After 30 min, the skin appeared dry; the animal was transferred onto a clean paper towel to remove remaining solution and then returned to its home cage. We used separate plastic boxes for 4-MBC-treated pups and controls. The animals were continuously observed; they did not lick their skin, but remained in a quiet position. On PN 27, pups were weighed and decapitated under light ether anesthesia. The uterus was removed and weighed as described above. The treatment group was unknown to the person dissecting the uterus.

In the absence of toxicokinetic *in vivo* data, it is not possible to exactly determine the dose of 4-MBC taken up by dermal application. However, we determined the amount of olive oil applied during one treatment by weighing the animal before and after each manipulation. The average amount of oil retained after 15 sec immersion was 1.35 ± 0.13 g, and the additional amount applied by the brush was 1.4 ± 0.08 g. Thus, the total amount of oil was 2.75 g/treatment or 5.5 g/day, yielding total amounts of 4-MBC applied to the skin per day of 137.5, 275, and 412.5 mg for 2.5, 5.0, and 7.5% 4-MBC concentrations, respectively. According to *in vitro* data (*8*), the penetration of 4-MBC through hairless rat skin is 0.6% from oily gels or 0.4% from a water in oil (W/O) emulsion. Assuming 0.6% penetration, the dose absorbed from a 5% 4-MBC solution in oil can be tentatively calculated as 37 mg/kg/day based on mean body weight of the 5% group.

Data Analysis

In vitro studies. We calculated cell counts per well from optical density as described above. In every experiment, we analyzed each concentration in quadruplicate. From these values, we calculated the mean cell count of a given concentration of chemical or of chemical-free medium for each experiment. Cell counts from different independent experiments were compared using analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons (SYSTAT software; SYSTAT Intelligent Software, Evanston, IL, USA). The proliferative effect (PE) of a compound was defined as (maximal mean cell count obtained with the test chemical)/(mean cell count in the chemical-free medium), the relative proliferative effect (RPE) in relation to that of E_2 as (PE of test compound –1)/(PE of E_2 – 1) × 100 (*17*). For comparison with uterotrophic data, we expressed the increase in cell number as percentage of E_2 [(cell number with test compound – control)/(cell number with E_2 – control) × 100]. We calculated the median effective concentration (EC_{50}) values from the ascending part of the concentration–response curve using Graph Pad Prism2 software (Graph Pad Software, Inc., San Diego, CA, USA). For pS2 protein, treated groups and the chemical-free medium group were compared by ANOVA followed by Bonferroni pairwise comparisons (SYSTAT).

In Vivo Studies

Uterine weights of individual animals from different dose groups and vehicle controls were compared by ANOVA followed by Bonferroni pairwise comparisons. We calculated ED_{50} values using Prism2.

Table 2. Comparison of *in vitro* and *in vivo* activity of UV screens and steroidal estrogens.

	MCF-7 cell proliferation				Uterotrophic effect in immature rats		
Compound	PE[a]	RPE[b]	Maximal cell count increase (% of E_2)[c]	EC_{50}	Increase of uterine weight over control[d]	Maximal weight increase (% of EE_2)[e]	ED_{50}
E_2	16.70	100	100	1.22 pM			
EE_2					4.08	100	0.818 µg/kg/day
Bp-3	17.49	105.0	95.09	3.73 µM	1.23	7.60	1,000–1,500 mg/kg/day
4-MBC	13.49	79.54	87.66	3.02 µM	2.09	35.51	309 mg/kg/day
OMC	10.72	61.90	77.18	2.37 µM	1.68	22.21	934 mg/kg/day
OD-PABA	9.13	51.77	55.54	2.63 µM	1.04	1.15	Inactive
HMS	6.78	36.81	79.65	1.56 µM	1.12	3.79	Inactive
B-MDM	4.30	21.01	13.27	Inactive	1.06	2.01	Inactive

EE_2, ethinylestradiol.

[a]Proliferative effect over control; PE = (maximal cell count of experimental group)/(cell count of control). [b]Maximal proliferative effect (% of E_2); RPE = (PE of experimental group – 1)/(PE of estradiol – 1) × 100. [c](Cell count of experimental group – cell count of control)/(cell count of estradiol – cell count of control) × 100. [d](Uterine weight of experimental group)/(uterine weight of control). [e]Maximal weight increase (% of ethinylestradiol) = (uterine weight of experimental group – uterine weight of control)/(uterine weight of ethinylestradiol – uterine weight of control) × 100.

Results

Effect of UV Screens on MCF-7 Cells *in Vitro*

MCF-7 cell proliferation. Cell proliferation was dose-dependently increased by all UV screens tested except for B-MDM, with a bell-shaped dose–response curve (Tables 1 and 2, Figure 2). The effective concentration range (1–50 µM) and the maximum effect concentration (at around 10 µM) was similar for the various compounds. *In vitro* EC_{50} values of UV screens range between 1.56 µM (HMS) and 3.73 µM (Bp-3) (Table 2). According to their maximum effects on cell proliferation in relation to E_2, 4-MBC, OMC, OD-PABA, and HMS acted as partial agonists, whereas the maximum activity of Bp-3 reached the level of E_2. The proliferative effects of 4-MBC and the positive control E_2 were completely blocked by the pure estrogen receptor antagonist ICI 182,780 (Figure 3).

pS2 protein. Secretion of the estrogen-regulated protein pS2 into the culture medium was significantly increased by 4-MBC, HMS, and Bp-3 (Figure 4). Levels were also above control after incubation with OMC and OD-PABA, but the difference was not significant; B-MDM was clearly negative. At the concentration tested, 4-MBC induced the greatest response (43.9% of E_2). The correlation between the increase in proliferation and in pS2 secretion at the concentration used for pS2 protein induction was low ($r^2 = 0.6046$, not significant).

Effect of UV Screens on the Immature Rat Uterus *in Vivo*

Peroral administration. After administration in powdered feed for 4 days, three of the five chemicals active *in vitro* and the positive control ethinylestradiol elicited dose-dependent increases in uterine weight of immature Long-Evans rats (Table 3, Figure 2). The rank order of potency, 4-MBC > OMC > Bp-3, differed from the one observed *in vitro*; 4-MBC exhibited a significant increase in uterine weight at a dose of 119 mg/kg/day and an ED_{50} of 309 mg/kg/day (Tables 2,3). Two of the compounds with *in vitro* activity, HMS and OD-PABA, as well as B-MDM, were inactive *in vivo* at the doses tested. Mean body weights of the various treatment groups were similar (data not shown) and in the range of the vehicle control group (mean ± SD of 38.0 ± 4.5 g at PN 21 and 48.8 ± 3.8 g at PN25).

Dermal application of 4-MBC. Following dermal application of 4-MBC in olive oil twice daily for 6 days, immature rats of the hr/hr (hairless) strain exhibited a dose-dependent increase in uterine weight, with a significant increase above control induced by 5% and 7.5% 4-MBC (Figure 5). The mean uterine weight of the 5% 4-MBC group was also significantly higher than that of the 2.5 or 7.5% groups, yielding a bell-shaped dose–response curve. The control uterine weight of this strain appeared to be slightly lower than that of Long-Evans rats, even though the animals were 2 days older. Mean body weights (± SD) of the various groups were similar at the beginning (control, 34.78 ± 3.15; 2.5% 4-MBC, 32.54 ± 1.40; 5% 4-MBC, 34.44 ± 4.10; 7.5% 4-MBC, 34.51 ± 1.92) and at the end of the treatment period (control, 52.52 ± 7.41; 2.5% 4-MBC, 43.63 ± 0.81; 5% 4-MBC, 55.99 ± 5.38; 7.5% 4-MBC, 51.11 ± 5.07).

Discussion

The present study demonstrates *in vitro* and *in vivo* estrogenic activity for a number of UV screens with different chemical structures. The compounds tested are frequently used in sunscreens and cosmetics and have the potential for bioaccumulation.

In the *in vitro* system, five out of the six UV screens tested displayed significant dose-dependent estrogenic activity on MCF-7 cells. Bp-3 was most active on cell proliferation, followed by 4-MBC, HMS, OMC, and OD-PABA, whereas B-MDM was inactive. With maximum effects on MCF-7 cell proliferation at 5–10 µM and EC_{50} values between 1.5 and 3.7 µM (Table 2), the estrogenic activity of the five UV screens is in the range of other industrial chemicals identified as environmental estrogens (*17*,*22*). The strain of MCF-7 cells used displayed good sensitivity to E_2, with a maximum proliferation rate at 10 pM, comparable to previously published data (*17*). This enabled good discrimination between test chemicals. It cannot be determined whether the effects of the UV screens were caused by the parent compounds and/or by possible metabolites because MCF-7 cells express constitutive and inducible cytochrome P450 enzymes (*23*).

Complete blockage of the proliferative effect of 4-MBC by the pure estrogen receptor antagonist ICI 182,780 (*19*) indicates an estrogen receptor-mediated effect. The estrogenic activity of these chemicals is further demonstrated by the induction of the estrogen-regulated pS2 protein (*24*). The correlation between the effects of the six chemicals on cell proliferation and pS2 protein (expressed as percentage of E_2) was low ($r^2 = 0.6046$, not significant); however, the same compounds (4-MBC, Bp-3, and HMS) were most active on proliferation as well as pS2 protein induction, and B-MDM was clearly inactive on both parameters. It should be noted that the effect on pS2 protein was only analyzed for one concentration of test chemicals in the range of the maximum proliferative effect. The dose–response relationship and, hence, the maximum effective dose may be different for the two parameters.

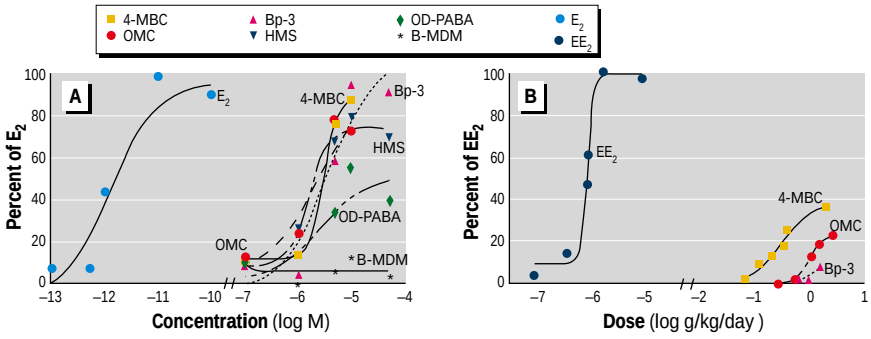

Figure 2. Dose–response relationship of estrogenic activity of different UV screens. (*A*) *In vitro* effect of UV screens on MCF-7 cell proliferation (cell number/well) as a percentage of the maximum effect of E_2. (*B*) Effect of oral UV screens on immature rat uterine weight as a percentage of the maximum effect of ethinylestradiol (EE_2).

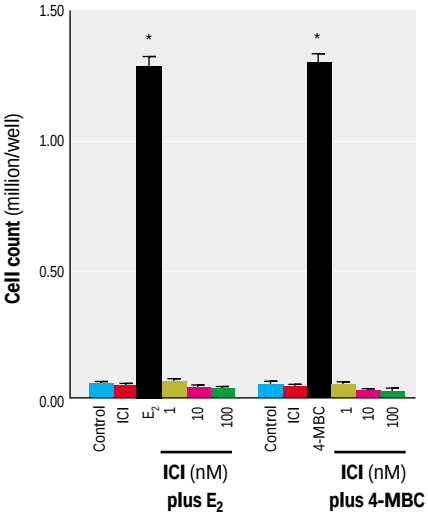

Figure 3. Antagonism of the proliferative effect of 4-MBC (10 µM) or E_2 (10 pM) on MCF-7 cells by increasing concentrations of the pure estrogen receptor antagonist ICI 182,780. Controls received chemical-free medium. ICI 182,780 alone was 100 nM. Values shown are mean ± SEM of four independent experiments.

*$p < 0.001$ (ANOVA followed by Bonferroni pairwise comparisons) as compared to all other experimental groups (control, ICI 182,780 alone, and E_2 or 4-MBC plus ICI 182,780 at all concentrations).

Three UV screens, 4-MBC, OMC, and Bp-3, were active by the oral route in an acute mammalian *in vivo* model for estrogenicity (*21*), eliciting dose-dependent increases of uterine weight in immature rats. Differences between individual UV screens were more pronounced than *in vitro*. 4-MBC was most active, with a significant increase in uterine weight at 119 mg/kg/day and an ED_{50} of 309 mg/kg/day; it also had the greatest maximal effect (Table 2). In contrast, Bp-3 displayed only weak activity *in vivo*. This compound has recently been reported to be inactive in the uterotrophic assay (*25*). The dose level used was also ineffective in our investigation; thus, the two data sets are in agreement. The *in vitro* (proliferation) and *in vivo* dose–response curves of 4-MBC and OMC suggest that they are partial agonists. Bp-3 is difficult to judge because it reached the level of the full agonist, E_2, *in vitro*, whereas the *in vivo* dose–response curve is incomplete.

Weak binding to estrogen receptors has been reported for unsubstituted benzophenone (*26*). One of the main metabolites of Bp-3, 2,4-dihydroxybenzophenone, binds to estrogen receptors with micromolar affinity, in contrast to its parent compound (*27*). This metabolite was detected in human urine after dermal application of a commercial sunscreen product (*10*). *O*-dealkylation also appears to be the major metabolic pathway of Bp-3 in rats (*28*). However, the relative roles of parent compounds and metabolites for *in vivo* estrogenic activities of the various UV screens remain to be clarified.

A comparison of *in vitro* and *in vivo* data indicates that the *in vitro* assay was useful for identifying estrogenic activity, but was of limited predictive value for the mammalian *in vivo* situation. Although all three chemicals that exhibited *in vivo* activity were strongly active *in vitro*, two compounds with high or moderate *in vitro* activity, HMS and OD-PABA, were completely inactive in the uterotrophic assay at the doses tested. The rank orders of activity also differed between *in vitro* and *in vivo* experiments. This may have resulted from pharmacodynamic and/or pharmacokinetic differences. A precise quantitative comparison of *in vitro* and *in vivo* effects is not possible because different tissues served as end points and different estrogens were used as positive controls. The differences between *in vitro* and *in vivo* data support the need for *in vivo* testing of chemicals after identification of endocrine activity *in vitro*.

The investigated chemicals are diverse in structure, but they share a common use as UVA or UVB screens and they have potential for bioaccumulation. Four of the UV screens with estrogenic activity, 4-MBC, Bp-3, HMS, and OMC, have been detected in fish (*5*), and so far two compounds, Bp-3 and OMC, have been detected in human milk (*12*). The total concentration of estrogenic UV screens in fish, where a larger data set is available, ranged between 1.6 µM and 7.8 µM in fat, or between 0.02 and 0.2 µM in whole fish (roach and perch, respectively). UV screens thus may contribute to the total body burden of endocrine active compounds in wildlife and humans.

The effective dose range of oral 4-MBC, OMC, and Bp-3 in the rat uterotrophic assay (119 mg/kg/day for 4-MBC to 1,500 mg/kg/day for Bp-3) compares with daily oral doses of bisphenol A (400 mg/kg) (*29*), methoxychlor (100–500 mg/kg), nonylphenol (190–1,000 mg/kg), and *o,p´*-DDT (1,000 mg/kg) (*21,30*) that increase rodent uterine weight. Uterine epidermal growth factor receptor was induced by 500 mg/kg/day methoxychlor (*31*). In one study on bisphenol A, Gould et al. (*32*) were unable to detect changes in uterine weight, but they reported an increase in peroxidase activity at 100 mg/kg/day and increased progesterone receptor levels at lower doses. These doses cannot be compared with actual exposure levels, as it is generally agreed that the acute high-dose rodent model cannot serve as a basis for risk assessment, but rather for identification of *in vivo* activity. Thus, bisphenol A has been found to disturb developmental processes at doses that are several orders of magnitude lower (2–50 µg/kg) (*33,34*).

As UV screens, the chemicals tested in this study present two different toxicologic aspects: On one hand, they may play an ecotoxicologic role in wildlife and humans, probably resulting mainly from intake via the food chain. On the other hand, they may also be transdermally active in humans when they are used as sunscreens. We observed an increase in uterine weight after dermal application of

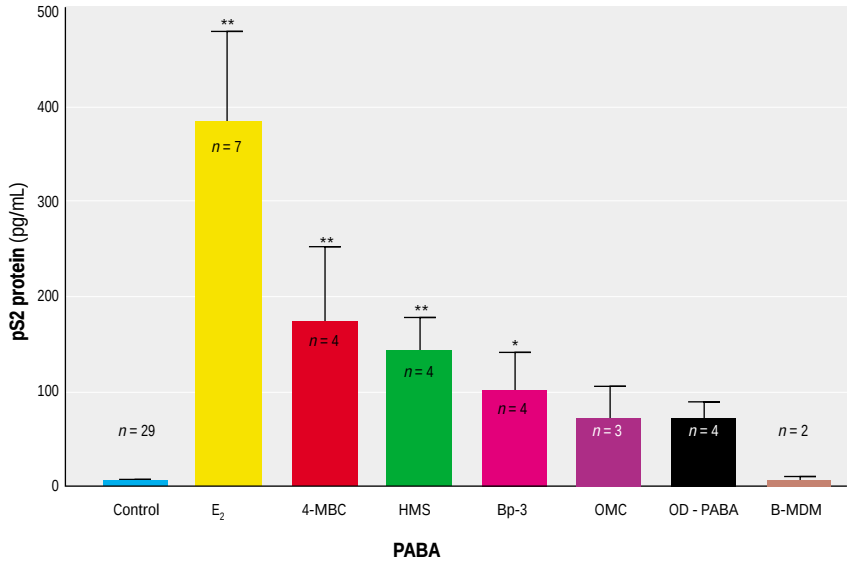

Figure 4. Effect of UV screens 4-MBC (10 µM), HMS (50 µM), Bp3 (10 µM), OMC (10 µM), OD-PABA (10 µM), B-MDM (10 µM), and E_2 (10 pM) on pS2 protein secretion by MCF-7 cells. Bars indicate mean pS2 protein concentration in culture medium (pg/ml) ± SEM; numbers inside the bars indicate the number of independent experiments).

*$p < 0.05$, **$p < 0.001$ (ANOVA followed by Bonferroni pairwise comparisons) as compared to controls that received chemical-free medium.

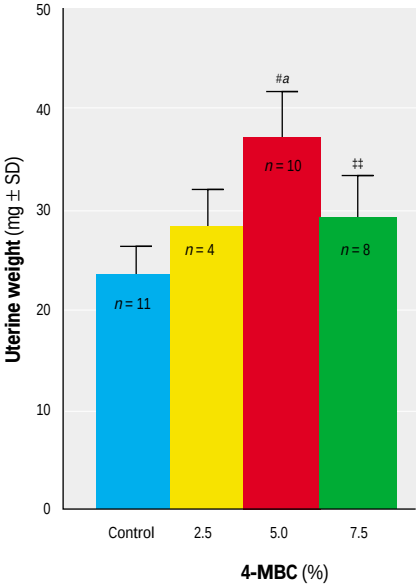

Figure 5. Effect of dermal application of 4-MBC (2.5, 5.0, or 7.5 % in olive oil, twice daily for 6 days) on uterine weight of immature hairless (hr+/hr+) rats. Controls received vehicle (olive oil). Bars indicate mean ± SD; numbers inside the bars indicate the number of animals.

[a]5% 4-MBC different from 2.5%, $p < 0.005$; different from 7.5%, $p < 0.001$ [#]$p < 0.001$, [‡‡]$p < 0.025$ as compared to controls (ANOVA followed by Bonferroni pairwise comparisons).

4-MBC in olive oil to immature hairless rats at concentrations allowed in sunscreen products. The dose–response curve of uterine weight was bell-shaped, suggesting more complex interactions. With 5% 4-MBC, the increase (159% of control) corresponded to the increase (154% of control) produced by an oral dose of 337 mg/kg/day, which is close to the oral ED_{50}. 4-MBC exhibits significant penetration through skin of hairless rats from a 6% solution in either a W/O emulsion or oily gels (*8*). BP-3 is also dermally absorbed in rats (*35*). Evidence for absorption by human skin has been presented for 4-MBC, Bp-3, and OD-PABA (*6,7,9,10*); 4-MBC also penetrates a folioxane membrane, a model for human skin (*8*). Skin penetration may vary between compounds, as indicated by lower penetration of OMC as compared to Bp-3 (*9,36*), and appears to be influenced by the formulation (*8,36*). Such kinetic differences may be of importance from a toxicologic point of view, but present knowledge is too incomplete to provide a picture of the general human exposure to UV screens.

Table 3. Effect of oral UV screens and ethinylestradiol on uterine weight of immature rats.

Dose	Uterine weight (mg)
Ethinylestradiol (µg/kg/day)	
0.085	29.15 ± 3.59 (6)
0.342	37.02 ± 1.05 (6)##
0.780	61.82 ± 5.00 (5)##
0.856	72.80 ± 5.77 (5)##
1.648	102.86 ± 13.09 (5)##
8.631	100.95 ± 3.28 (6)##
4-MBC (mg/kg/day)	
66	27.25 ± 1.72 (10)
119	32.43 ± 3.61 (13)‡
211	35.24 ± 5.84 (19)##
337	38.78 ± 6.36 (18)##
402	45.22 ± 8.23 (9)##
1,980	52.80 ± 11.8 (4)##
OMC (mg/kg/day)	
268	24.95 ± 2.50 (10)
522	26.81 ± 1.64 (13)
1,035	35.46 ± 8.74 (10)##
1,518	39.59 ± 7.58 (7)##
2,667	42.48 ± 1.25 (5)##
Bp-3 (mg/kg/day)	
611	26.84 ± 1.87 (5)
937	26.94 ± 2.26 (9)
1,525	31.14 ± 3.13 (5)##
HMS (mg/kg/day)	
491	28.18 ± 1.64 (6)
892	23.36 ± 0.96 (5)
OD-PABA (mg/kg/day)	
596	26.05 ± 0.95 (4)
761	24.75 ± 1.29 (6)
1,419	26.13 ± 3.10 (6)
B-MDM (mg/kg/day)	
421	26.80 ± 1.08 (6)
636	26.05 ± 0.97 (6)
Vehicle control	
0	25.24 ± 1.41 (28)

Values shown are mean ± SD (number of rats).
##$p < 0.0001$, ‡$p < 0.002$ (ANOVA plus Bonferroni pairwise comparisons) as compared to controls.

Conclusions

Our investigation revealed that several frequently used UV screens possess estrogenic activity *in vitro* and *in vivo*, in the range of other known xenoestrogens. With the exception of some benzophenones, these chemicals do not appear to have been considered as potential environmental endocrine disruptors (*37*). Considering the widespread use of UV screens, we suggest that toxicokinetics, in particular skin penetration, and systemic toxicology of these chemicals should be investigated more extensively. In view of possible long-term effects, screening for endocrine activity seems important. From our data and from observations in other fields (see above), it appears that there is a need to reconsider the potential benefits of extensive UV screen use both from a medical and an ecologic perspective.

References and Notes

1. Autier P, Doré J-F, Cattaruzza MS, Renard F, Luther H, Gentiloni-Silverj F, Zantedeschi E, Mezzetti M, Monjaud I, Andry M, et al. Sunscreen use, wearing clothes, and number of nevi in 6- to 7-year-old European children. J Natl Cancer Inst 90:1873–1880 (1998).
2. Bigby M. The sunscreen and melanoma controversy. Arch Dermatol 135:1526–1527 (1999).
3. Suter-Eichenberger R, Altorfer H, Lichtensteiger W, Schlumpf M. Bioaccumulation of musk xylene (MX) in developing and adult rats of both sexes. Chemosphere 36:2747–2762 (1998).
4. Suter-Eichenberger R, Boelsterli UA, Conscience-Egli, M, Lichtensteiger W, Schlumpf M. CYP450 enzyme induction by chronic oral musk xylene in adult and developing rats. Toxicol Lett 115:73–87 (2000).
5. Nagtegaal M, Ternes TA, Baumann W, Nagel R. UV-Filtersubstanzen in Wasser und Fischen. UWSF-Z Umweltchem Ökotox 9:79–86 (1997).
6. Hagedorn-Leweke U, Lippold BC. Absorption of sunscreens and other compounds through human skin *in vivo*: derivation of a method to predict maximum fluxes. Pharm Res 12:1354–1360 (1995).
7. Hayden CGJ, Roberts MS, Benson HAE. Systemic absorption of sunscreen after topical application. Lancet 350:863–864 (1997).
8. Aghazarian V, Tchiakpe L, Reynier JP, Gayte-Sorbier A. Release of benzimidazole and benzylidene camphor from topical sunscreen formulations. Drug Dev Ind Pharm 25:1277–1282 (1999).
9. Jiang R, Roberts MS, Collins DM, Benson HAE. Absorption of sunscreens across human skin: an evaluation of commercial products for children and adults. Br J Clin Pharmacol 48:635–637 (1999).
10. Felix T, Hall BJ, Brodbelt JS. 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 (1998).
11. Kadry AM, Okereke CS, Abdel-Rahman MS, Friedman MA, Davis RA. Pharmacokinetics of benzophenone-3 after oral exposure in male rats. J Appl Toxicol 15:97–102 (1995).
12. Hany J, Nagel R. Nachweis von UV-Filtersubstanzen in Muttermilch. Deutsche Lebensmittel-Rundschau 91:341–345 (1995).
13. Pittet G, Ferrer F. UV filters regulatory status in the European Union, Japan and the USA – 1996. Seifen Öle Fette Wachse-J 123:510–519 (1997).
14. Final report on the safety assessment of benzophenones-1, -3, -4, -5, -9, and -11. J Am Coll Toxicol 2:35–77 (1983).
15. Okereke CS, Barat SA, Abdel-Rahman MS. Safety evaluation of benzophenone-3 after dermal administration in rats. Toxicol Lett 80:61–67 (1995).
16. Schauder S, Ippen H. Contact and photocontact sensitivity to sunscreens. Review of a 15 year experience and of the literature. Contact Dermatitis 37:221–132 (1997).
17. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(suppl 7):113–122 (1995).
18. Soto AM. Personal communication.
19. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867–3873 (1991).
20. Reel JR, Lamb JC, Neal BH. Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol 34:288–305 (1996).
21. Odum J, Lefevre PA, Tittensor S, Paton D, Routledge EJ, Beresford NA, Sumpter JP, Ashby J. The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:176–188 (1997).
22. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263 (1998).
23. Christou M, Savas U, Spink DC, Gierthy JF, Jefcoate CR. Co-expression of human CYP1A1 and a human analog of cytochrome P450-EF in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the human mammary carcinoma-derived MCF-7 cells. Carcinogenesis 15:725–732 (1994).
24. Nunez AM, Jakowlev S, Briand JP, Gaire M, Krust A, Rio MC, Chambon P. Characterization of the estrogen-induced pS2 protein secreted by the human breast cancer cell line MCF-7. Endocrinology 121:1759–1765 (1987).
25. Baker VA, Jones PA, Lea LJ. Assessment of the oestrogenic activity of benzophenone sunscreen agents [Abstract]. Toxicologist 54:262 (2000).
26. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persisting chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect 103:582–587 (1995).
27. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54:138–153 (2000).
28. Okereke CS, Kadry AM, Abdel-Rahman MS, Davis RA, Friedman MA. Metabolism of benzophenone-3 in rats. Drug Metab Dispos 21:788–790 (1993).
29. Ashby J, Tinwell H. Uterotrophic activity of bisphenol A in the immature rat. Environ Health Perspect 106:719–720 (1998).
30. Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* assays. Environ Health Perspect 104:1296–1300 (1996).
31. Metcalf JL, Laws SC, Cummings AM. Methoxychlor mimics the action of 17β-estradiol on induction of uterine epidermal growth factor receptors in immature female rats. Reprod Toxicol 10:393–399 (1996).
32. Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Safe S, McDonnell DP, Gaido KW. Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Mol Cell Endocrinol 142:203–214 (1998).
33. Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 105:70–76 (1997).
34. Gupta C. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc Soc Exp Biol Med 224:61–68 (2000).
35. Okereke CS, Abdel-Rahman MS, Friedman MA. Disposition of benzophenone-3 after dermal administration in male rats. Toxicol Lett 73:113–122 (1994).
36. Gupta VK, Zatz JL, Rerek M. Percutaneous absorption of sunscreens through micro-Yucatan pig skin *in vitro*. Pharm Res 16:1602–1607 (1999).
37. European Commission.Towards the Establishment of a Priority List of Substances for Further Evaluation of Their Role in Endocrine Disruption. Final Report. DG ENV M0355008/1786Q. Delft, The Netherlands:BKH Consulting Engineers (in association with TNO Nutrition and Food Research, Zeist, The Netherlands), 2000.