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Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells

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Abstract

In order to protect consumers from ultraviolet (UV) radiation and enhance light stability of the product, three to eight UV filters are usually added to consumer sunscreen products. High lipophilicity of the UV filters has been shown to cause bioaccumulation in fish and humans, leading to environmental levels of UV filters that are similar to those of PCBs and DDT. In this paper, estrogen-regulated pS2 gene transcription in the human mammary tumor cell line MCF-7 was used as a measure of estrogenicity of four individual UV filters. Since humans are exposed to more than one UV filter at a time, an equipotent binary mixture of 2-hydroxy-4-methoxy-benzophenone (BP-3) and its metabolite 2,4-dihydroxy benzophenone (BP-1), as well as an equipotent multi-component mixture of BP-1, BP-3, octyl methoxy cinnamate (OMC) and 3-(4-methylbenzylidene) camphor (4-MBC), were also evaluated for their ability to induce pS2 gene transcription in order to examine additivity. An estrogen receptor-mediated mechanism of action was expected for all UV filters. Therefore, our null-hypothesis was that combined estrogenic responses, measured as increased pS2 gene transcription in MCF-7 cells after exposure to mixtures of UV filters, are additive, according to a concentration-addition model.

Not all UV filters produced a full concentration–response curve within the concentration range tested (100 nM–1 μM). Therefore, instead of using EC_{50} values for comparison, the concentration at which each compound caused a 50% increase of basal pS2 gene transcription was defined as the C50 value for that compound and used to calculate relative potencies. For comparison, the EC_{50} value of a compound is the concentration at which the compound elicits an effect that is 50% of its maximal effect. Individual UV filters increased pS2 gene transcription concentration-dependently with C50 values of 0.12 μM, 0.5 μM, 1.9 μM, and 1.0 μM for BP-1, BP-3, 4-MBC and OMC, respectively. Estradiol (E2) had a C50 value of 4.8 pM. Experiments with equipotent mixtures all supported our null hypothesis that mixtures of UV filters act additively to activate the estrogen receptor (ER). In view of our results and observed plasma levels it cannot be excluded that daily exposure to sunscreen formulations may have estrogenic effects in humans.

Keywords: UV filters; Benzophenone; OMC; 4-MBC; pS2; MCF-7

Introduction

Ultraviolet (UV) filters are organic chemicals that absorb UVA (315–400 nm) or UVB (280–315 nm) radiation. These chemicals are added to consumer sunscreen products in concentrations up to 10% (Schreurs et al., 2002). They are highly lipophilic and can therefore accumulate in humans and the environment. Six different UV filters were identified in fish in the Maarfelder Lake (Eifel, Germany) at total concentrations of 2 mg/kg lipid in perch and 0.5 mg/kg lipid in roach (Nagtegaal et al., 1997). Both fish species were contaminated with UV filters, PCBs, and DDT at similar levels. Therefore, UV filters should be considered relevant environmental contaminants.

Human exposure to UV filters can occur via dermal absorption (Hagedorn-Leweke and Lippold, 1995; Hayden et al., 1997; Jiang et al., 1999) and through the food chain, for example by consumption of contaminated fish. 2-Hydroxy-4-methoxybenzophenone (BP-3) is well absorbed via the dermal and oral routes (Nakagawa and Suzuki,

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2002). Both BP-3 and its metabolite 2,4-dihydroxybenzophenone (benzophenone-1, BP-1) have been detected in human urine 4 h after application of commercial products to the skin (Felix et al., 1998; Hayden et al., 1997). Analyses of human milk indicate bioaccumulation of UV filters in humans after prolonged exposure. BP-3 and/or octyl methoxy cinnamate (OMC) were present in detectable amounts (Hany, 1995).

Over the past few years, the list of identified endocrine disrupting environmental contaminants has grown rapidly. Many man-made chemicals are able to bind to the estrogen receptor (ER), and subsequently elicit estrogenic effects. BP-3 was shown to be slightly estrogenic in a yeast bioassay, in which yeast was transfected with estrogen receptor α (ERα), estrogen responsive elements (ERE), and a *lac*Z reporter gene (Miller et al., 2001) and Schlumpf (Schlumpf et al., 2001) observed various estrogenic effects after in vitro and in vivo exposure to several UV filters. Schreurs showed that UV filters can also bind to ER beta (Schreurs et al., 2002). Five out of six tested UV filters increased MCF-7 cell proliferation with EC_{50} values ranging from 1.56 to 3.73 μM, making them six orders of magnitude less potent than E2. For comparison, E2 had a EC_{50} value of 1.22 pM. pS2 is a secretorial protein from MCF-7 cells which is estrogen regulated (Masiakowski et al., 1982; Olsen et al., 2003) and its synthesis in and secretion from MCF-7 cells was also induced by UV filter exposure (Schlumpf et al., 2001). Additionally, BP-3 showed anti-androgenic activity in vitro (Ma et al., 2003). In vivo, female immature rats that received UV filters for 4 days in feed showed a dose-dependent increase in uterine weight in response to 3-(4-methylbenzylidene) camphor (4-MBC), OMC, and BP-3. Dermal application of 4-MBC to immature hairless rats also increased uterine weight (Schlumpf et al., 2001).

In this paper, pS2 gene expression in the human mammary tumor cell line MCF-7 was used as a measure of estrogenicity of 4 UV filters. Since humans are exposed to more than one UV filter at a time, a binary mixture (BP-1 and BP-3) and a multi-component mixture of 4 UV filters (BP-1, BP-3, 4-MBC, and OMC) were also evaluated for their ability to induce pS2-gene expression. The combination BP-1 and BP-3 was chosen because BP-3 is a UV filter that is used frequently in commercially available products. BP-1 is its main metabolite in humans. From a survey that we conducted, we concluded that BP-3, 4-MBC, and OMC were the three most frequently used UV filters in products used by humans. Therefore we also included that combination of 4-MBC, OMC, BP-3, and BP-1. To our knowledge, this is the first report on estrogenic effects of mixtures of UV filters. The estrogenic effect of a mixture can be calculated on the basis of the estrogenic effects of its individual components. Non-additive interactive effects of multiple mixture components would cause deviation from the expected effects (Thorpe et al., 2001). Two main analytical methods to predict the expected effects of a mixture, concentration addition and response addition, have been described. Concentration addition assumes similar mechanisms of action of all components in the mixture (Payne et al., 2001; Silva et al., 2002; Thorpe et al., 2001). In case of similarly acting components, there is a consensus that concentration addition is a suitable and valid concept for the prediction of mixture effects. In contrast, response addition assumes that the compounds act via independent pathways (Silva et al., 2002; Thorpe et al., 2001). This method is thought to be unsuitable for assessment of interactive effects of estrogenic compounds (Payne et al., 2001; Rajapakse et al., 2001; Thorpe et al., 2001). In case of sigmoidal concentration–response curves, it is not possible to calculate expected additive effects by arithmetic summation of individual responses (Berenbaum, 1985; Kortenkamp and Altenburger, 1998). However, an ER-mediated mechanism of action was expected for all UV filters in the mixture and therefore our null-hypothesis was that the model of concentration additivity would be a suitable model to predict the interactive effects of our multi-component mixture.

In addition, we wanted to validate pS2 gene expression as a method to assess estrogenicity, in addition to MCF-7 cell proliferation. pS2 gene expression experiments have several advantages above cell proliferation assays because they are more rapid requiring shorter incubation times, and may in specific instances avoid non-ER mediated mitogenic effects on the cells or interferences with the MTT assay.

Materials and methods

Chemicals. Estradiol (Sigma, E2758) was dissolved in 70% EtOH. UV filters were obtained from Merck (Darmstadt, Germany). 4-MBC (Eusolex 6300, CAS-No 36861-47-9), OMC (Eusolex 2292, CAS-No 5466-77-3), BP-3 (Eusolex 4360, CAS-No 131-57-7), and BP-1 (CAS-No 131-56-6) were dissolved in 70% EtOH and stored at −20 °C. Each compound was reported to be >98% pure by the manufacturer.

Cell culture and experimental design. The MCF-7 malignant human mammary epithelial cancer cell line was obtained from the American Type Culture Collection (ATCC No. HTB-22) and cultured in phenol red-free RPMI 1640 medium containing glutamine (GibcoBRL 11835-030), 10% heat inactivated FCS (GibcoBRL 10099-141), 1% penicillin/streptomycin (GibcoBRL 15140-114), and 1 mg/ml insulin (Sigma, St. Louis, MO, USA) in a 5% CO_2 atmosphere at 37 °C. MCF-7 cells were placed on steroid-free medium (containing dextran-coated charcoal-treated FCS) 72 h prior to the start of the estrogenicity assays.

Then, for MCF-7 cell proliferation experiments, cells were plated in 24-well plates containing 1×10^5 cells per well. The following day, after cells had attached to the bottom of the wells, medium was refreshed and 1 μl of

compound was added to the cells for an incubation period of 6 days. Final solvent concentration was 0.1%. After 3 days, medium and compounds were refreshed. After 6 days, an MTT-test was performed as an indicator for cell numbers, as described previously (Heneweer et al., 2004).

For pS2 gene transcription experiments, cells were transferred to 12-well plates containing 2 ml steroid-free medium. The following day, after cells had attached to the bottom of the wells, medium was refreshed and 2 μl of test compounds were added to the cells for an incubation period of 24 h. Final solvent concentration was 0.1%. Concentration–response curves for the induction of the pS2 gene transcription by E2 were determined at different exposure times, indicating that an exposure of 24 h was sufficient for accurate measurements (data not shown). A concentration–response curve for E2 was included in each experiment as internal control.

RNA isolation and PCR conditions. After 24 h, RNA was isolated from MCF-7 cells using the RNA Instapure System (Eurogentec, Liège, Belgium) according to enclosed instructions. Purity of RNA was assessed by measuring 260/280 nm absorbance ratio. Absorbance at 260 nm was used to calculate the concentration of RNA. RNA was stored at −70 °C at a concentration of 10 ng/μl. For pS2 transcripts, primers designed by Lee (Lee et al., 2003) were used. Primer sequences were 5′-GCGAAGCTTGGCCAC-CATGGAGAACAAGG-3′ and 5′-GCGGATCCACGAAC-GGTGTCGTCGAA-3′. The PCR-product for pS2 was 189 bp. Reverse transcriptase polymerase chain reactions (RT-PCR) were performed using the Access RT-PCR System (Promega, Madison, WI, USA) according to the supplied protocol. In order to optimize the protocol and obtain the appropriate number of cycles, we repeated the RT-PCR a number of times with increasing number of cycles. We decided to use the number of cycles at the linear part of the curve, which was the case at 25 cycles. A 20-ng amount of RNA was added to the RT-PCR reaction. Annealing temperature was 55 °C, extension lasted 45 s at 72 °C, and 25 cycles were performed. Mg^{2+} concentration was 1 mM. The expected amplification product of 189 bp was detected using 2% agarose gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Molecular Dynamics, USA) and ImageQuant software (Amersham Biosciences, USA). Because β-actin mRNA expression was also stimulated by estrogens, we chose 36B4 as internal control.

Effects of individual UV filters and mixtures on pS2 gene transcription and calculation of relative potencies. First, concentration–response curves for individual compounds were established. In order to get an equipotent multi-component mixture of the 4 UV filters, we used non-linear regression analysis to determine the concentration at which each compound caused a 50% increase of basal pS2 gene transcription and defined this concentration as C50. Subsequently, relative potencies (RPs) were derived for each compound by dividing the C50 value for E2 by the C50 values of each single compound. These RPs were used to calculate respective fractions of the compounds in the equipotent mixtures.

MCF-7 cells were exposed to 7 dilutions of an equipotent mixture consisting of 4 UV filters (solvent control, 0.01×, 0.03×, 0.1×, 0.3×, 3×, and 10×) to obtain a concentration–response curve for pS2 induction. For the each dilution of the mixture, estrogen equivalents (EEQ, RP of individual compound × concentration in mix) was calculated. The effect on pS2 transcription was plotted against the sum of the individual EEQ values for the mixture and compared with the E2 curve. Overlapping curves suggest an additive mechanism of estrogenicity. A statistically significant shift of the EC_{50} values obtained from the mixture curve to the left or right indicates an synergistic or antagonistic effect, respectively.

Additionally, we used the toxic unit approach to confirm results obtained by the EEQ approach. Additivity can be expressed algebraically by the following equation (Berenbaum, 1985):

$$\sum_{i=1}^{n} \frac{ci}{\mathrm{EC}xi} = 1,$$

where n is the number of mixture components, $\mathrm{EC}x_i$ is the concentration of the i^{th} mixture component that elicits x% effect when applied individually. ci is the concentration of the respective component in the mixture that elicits x% effect. Each fraction ($c_i/\mathrm{EC}x_i$) represents the concentration of a mixture component scaled for its relative toxicity and is generally referred to as the toxic unit of that component (Altenburger et al., 2000). When this equation results in values <1, synergism is suggested. When values >1 are observed, the components can be considered antagonists. This method can be used to analyze combinations of compounds, irrespective of the shape of their individual concentration–response curves. Also in the case of partial agonism, this method can be used (Kortenkamp and Altenburger, 1998).

Equipotent binary mixtures of BP-1 and BP-3, based on RPs for single compounds, were added to the cells (final solvent concentration was 0.1%). The concentration of each compound when tested alone (0:100 or 100:0) was approximately equal to the C50 value. The concentration ratio of each binary mixture was varied, so that each mixture would produce the same effect as the C50 response of each individual component, assuming additivity. Ratios of observed and expected responses were calculated and deviations from 1 indicate synergism (ratio > 1) or antagonism (ratio < 1).

Data analysis and statistics. In each experiment, each concentration was tested in triplo. Each independent experiment was repeated 2–4 times and found to be reproducible. Fig. 2 illustrates the mean of all experiments. Maximum

response was defined as 100%. Concentration–response curves were plotted using Prism 3.0 (GraphPad Software Inc. San Diego, CA, USA). All error bars represent standard error of the mean (SEM). Statistically significant differences among means were identified using a one-way ANOVA followed by Tukey's posteriori test.

Results

pS2 gene transcription and MCF-7 cell proliferation after exposure to E2

After a 6-day E2 exposure, a maximum increase in cell proliferation of 3-fold above basal levels was observed, which was similar to the maximum increase of 3- to 4-fold in pS2 gene transcription experiments where exposures were for 24 h (Fig. 1). Average EC_{50} values for E2 were 14 and 12 pM in cell proliferation and in pS2 gene transcription experiments, respectively. The observed difference was not statistically significant. Results with UV filters did not indicate differences in sensitivity between cell proliferation experiments and pS2 gene transcription (data not shown).

Effects of individual UV filters on E2-mediated pS2 gene transcription

Concentration–response curves for the UV filters were obtained after a 24-h exposure of MCF-7 cells to each compound, individually.

Exposure of MCF-7 cells to BP-1, BP-3, 4-MBC, or OMC each resulted in concentration-dependent increases of pS2-gene transcription (Fig. 2). Maximum increase of pS2 gene transcription was approximately 3- to 4-fold above control levels, which was similar to that of E2. For 4-MBC, a full concentration–response curve was not obtained.

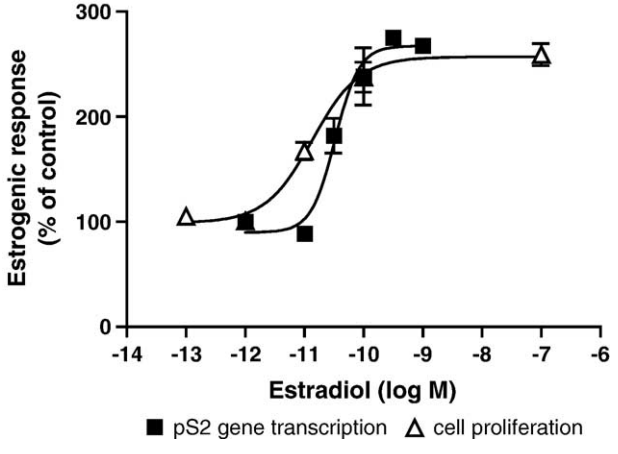

Fig. 1. Estrogenic response (% of control) in MCF-7 cells after exposure to estradiol. △: Data of cell proliferation experiments. ■: Data of pS2 gene transcription experiments. Data points are averages of three measurements and error bars represent SEM.

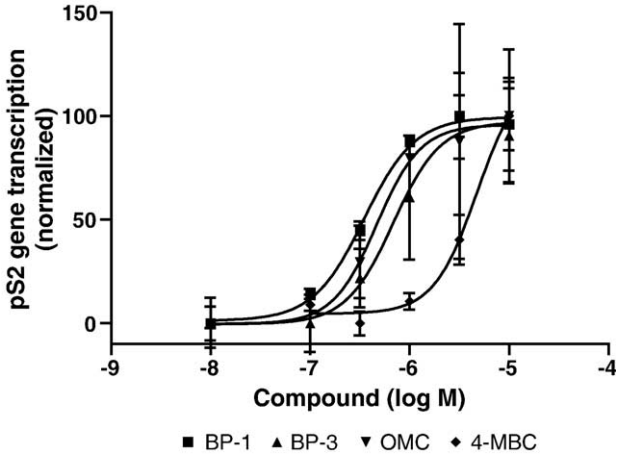

Fig. 2. Normalized concentration–response curves for induction of pS2 gene transcription in MCF-7 cells after a 24-h exposure to individual UV filters. Data points represent averages of all measurements. Error bars are SEM.

Therefore, to compare potencies, we determined concentrations at which each compound caused a 50% increase of basal pS2 gene transcription (C50 value). Similar approaches have been used by Soto (Soto et al., 1997) and Birkhøj (Birkhoj et al., 2004) who also used an arbitrary effect level for comparison of potencies. For E2, BP-1, BP-3, 4-MBC, and OMC, these C50 values were 4.8 pM, 0.12 μM, 0.5 μM, 1.9 μM, and 1.0 μM, respectively. From these values, relative potencies (RPs) for individual UV filters were calculated (Table 1).

Effects of equipotent mixtures of UV filters on E2-mediated pS2 gene transcription

When cells were dosed to dilutions of the mixture containing equipotent concentrations of BP-1, BP-3, OMC, and 4-MBC, a concentration-dependent response was obtained. The maximum increase in pS2 gene transcription was about 3-fold and similar to the maximum increase obtained by E2-exposure in this experiment (Fig. 3). Comparison of the EC_{50} values from both curves showed that these were not statistical significantly different from each other ($P = 0.1592$). The sum of the toxic units of the various mixture components was calculated to be 1.35 at the C50 effect level.

Table 1
Concentrations (C50) of the compounds causing a 50% increase of basal pS2 transcription levels, their potencies (RPs) relative to estradiol and concentrations of the compounds in the 1× mix

	C50 values (M)	RP	Concentration in 1× mix (M)
E2	4.80E−12	1	
BP-3	5.00E−07	9.60E−06	1.4E−06
BP-1	1.22E−07	3.93E−05	3.5E−07
4-MBC	1.85E−06	2.59E−06	5.3E−06
OMC	1.04E−06	4.62E−06	3.0E−06

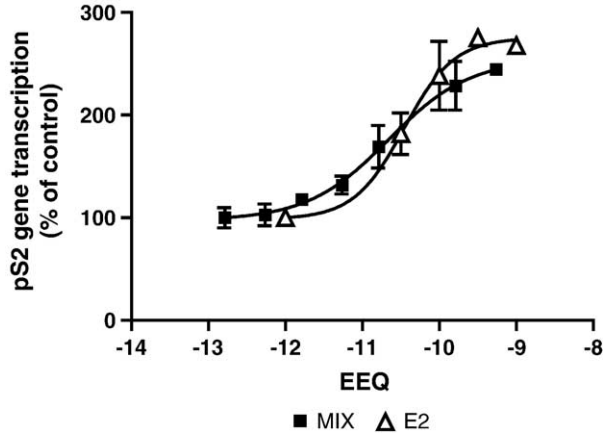

Fig. 3. Concentration-dependent curves for induction of pS2 gene transcription after a 24-h exposure of MCF-7 cells to estradiol (E2) or different dilutions of an equipotent mixture of UV filters. Dilutions of the mixture were expressed in EEQs for comparison with E2. Data points represent averages of three measurements. Error bars are SEM.

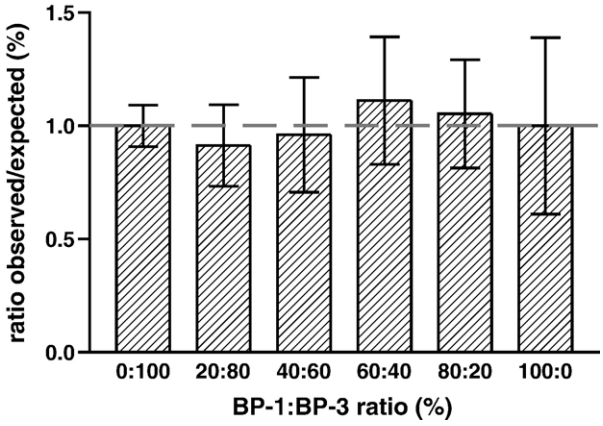

Fig. 4. Ratio between observed and expected effects on pS2 transcription after exposure of MCF-7 cells to equipotent mixtures of BP-1 and BP-3 in various ratios. The concentration of each component alone was equal to the concentration (C50) that produces a 50% increase of pS2 transcription above control levels. Bars represent averages of three measurements. Error bars are SEM.

An equipotent binary mixture of BP-1 and BP-3, based on their respective RP, was tested in the MCF-7 cell line for effects on induction of pS2-gene expression (Table 2).

The concentration ratio was varied and the concentration of each compound alone (0:100 or 100:0) was approximately equal to the C50 value. No statistically significant differences between observed and expected responses were observed (Fig. 4).

Discussion

Comparison of pS2 gene transcription and MCF-7 cell proliferation as endpoint for estrogenicity

EC_{50} values for E2 were 14 pM in an in vitro MCF-7 cell proliferation experiment. The average EC_{50} value, obtained from several pS2 gene transcription experiments, was 12 pM. Both endpoints had similar maximum estrogenic responses after E2-exposure, since a 3-fold increase above basal levels in cell number in the cell proliferation experiment and in pS2 gene transcription was observed. Therefore, we conclude that, also in our hands, pS2 gene transcription is a suitable alternative to cell proliferation for assessing estrogenicity in MCF-7 cells.

Table 2
Concentrations of BP-1 and BP-3 in equipotent binary mixtures with varying concentration ratio

(BP-1:BP-3)	BP-1 (M)	BP-3 (M)
0:100	0	5.0E−07
20:80	2.4E−08	4.0E−07
40:60	4.9E−08	3.0E−07
60:40	7.3E−08	2.0E−07
80:20	9.8E−08	1.0E−07
100:0	1.2E−07	0

The concentration of each component alone was equal to the concentration (C50) that produces a 50% increase of pS2 transcription above control levels.

Comparison of estrogenic potencies of UV filters

The pS2 gene was originally identified as an estrogen-inducible transcript in MCF-7 cells (Masiakowski et al., 1982) and pS2-gene expression is a suitable marker for assessing the estrogenicity of various compounds (Soto et al., 1997). To study the estrogenicity of mixtures of UV filters, pS2 gene transcription inducing potencies of single compounds and mixtures were determined from concentration–response experiments. They were compared to the potency of estradiol (E2) in order to express the biological potency of the UV filters in E2-equivalents under these specific conditions. For one of the compounds (4-MBC), concentration–response curves could not be determined completely. Consequently, EC_{50} values could not be used as a good measure of relative potency. Therefore, in order to perform experiments with equipotent mixtures we had to use other than the EC_{50} value, for which we chose the C50 value (50% induction of pS2 gene transcription above background levels). We believe that higher concentrations of 4-MBC could elicit an even greater estrogenic effect. The concentration–response curve was not finished yet at the highest tested concentration (10 μM). We do not believe that this inability suggests that pS2 is not a good indicator of estrogenic chemicals, since it was shown to be a good indicator when other well known estrogenic compounds were used.

Based on RPs obtained from our study, the order of estrogenic potency was E2 >> BP-1 > BP-3 > OMC > 4-MBC, with E2 being approximately 5 orders of magnitude more potent than the UV filters. Jorgensen and coworkers have also described effects on pS2 gene transcription

(Jorgensen et al., 2000). They showed that bisphenol A, methoxychlor, endosulfan, and dibutylphthalate, which are widely recognized as environmental estrogens, are also 5 to 6 orders of magnitude less estrogenic than E2. Therefore, the UV filters that were tested in this study fall within the same range of estrogenic compounds and could be considered as environmental estrogens as well.

Both BP-3 and 4-MBC were shown to increase pS2 secretion in MCF-7 cells (Schlumpf et al., 2001). Our results showing increased pS2 gene transcription confirm this observation. However, OMC did not increase pS2 secretion in MCF-7 cells at a concentration of 10 μM (Schlumpf et al., 2001) in contrast to our observed 3- to 4-fold increase in pS2 gene transcription at the same concentration. Possibly, estrogenic properties of OMC are strong enough to elicit pS2 gene transcription after 24 h but too weak to stimulate pS2 protein synthesis and secretion, even after an incubation time of 72 h.

EC_{50} values obtained from a cell proliferation experiment identified OMC to be slightly more potent than 4-MBC (Schlumpf et al., 2001). In the same study, BP-3 was the only UV filter to elicit a maximal response that was similar to that of E2. Estrogenicity of UV filters has not only been found in in vitro studies, but also in in vivo experiments. A rat uterine growth experiment after oral UV filter administration showed 4-MBC to be more potent than OMC, which was in turn more potent than BP-3 (Schlumpf et al., 2001).

Equipotent binary mixture of BP-1 and BP-3

Assuming additivity, all different concentration ratios in our in vitro study with an equipotent binary mixture of BP-3 and BP-1 would produce the same effect. As no statistically significant differences between observed and expected responses were observed, we suggest that both compounds act additively to stimulate estrogenicity, confirming our null-hypothesis.

In vitro, BP-3 acted as a full ER agonist, being 5 orders of magnitude less potent than E2. However, in vivo, maximum BP-3-mediated increase of uterine weight in immature rats was not as large as after exposure to ethinyl estradiol (Schlumpf et al., 2001). This observation suggests metabolic deactivation of BP-3 within the animal. BP-3 passes through the skin in significant amounts varying from 10% (Jiang et al., 1999) to 35% (Fernandez et al., 2002) of applied dose. However, in contrast to BP-3 itself, one of its main metabolites, BP-1, binds to the ER in the micromolar range (Blair et al., 2000). BP-1 and another benzophenone metabolite, 4-hydroxybenzophenone, both elicited estrogenic effects in a yeast two-hybrid assay in which yeast cells were transfected with two expression plasmids containing an estrogen receptor ligand binding domain and a β-galactosidase reporter gene, with a potency 100-fold greater than that of BP-3 (Kawamura et al., 2003). Results from our study also indicate that BP-1 (C50 = 0.1 μM) is more estrogenic than BP-3 (C50 = 0.5 μM). These results clearly indicate that metabolism of BP-3 can lead to significant bioactivation besides deactivation to possible less estrogenic compounds, which has been confirmed for rats and humans (Mueller et al., 2003).

Equipotent multi-component mixture of BP-1, BP-3, OMC, and 4-MBC

Exposure of MCF-7 cells to different dilutions of an equipotent mixture of 4 UV filters resulted in a concentration-dependent increase in pS2 gene transcription with a maximal increase similar to that elicited by E2. After mathematical transformation of the dilutions of the mixture to estrogen equivalents (EEQs), both curves overlapped with similar maximum effects and EC_{50} values that did not differ statistically significantly, not rejecting our null hypothesis that mixtures of UV filters act additively on the ER. The C50 value of the multi-component mix corresponded with a 0.12 dilution of the multi-component mix.

In order to predict responses to a multi-component mixture of 4 UV filters using the model of concentration addition, it is assumed that all chemicals have similar mechanism of action, resulting in parallel concentration–response curves with similar shape. Our experimental results showed differences in slope and efficacy for some compounds. However, this phenomenon is not unique and observed both for compounds that are easily biotransformed such as PAHs and biopersistent compounds like dioxins and PCBs. As MCF-7 cells contain significant CYP1A1 and 1B1 activity, it is rather likely that this enzyme activity is also involved in hydroxylation of UV filters and metabolism can have a significant influence on the final ER-mediated response.

These effects clearly hamper an ideal approach to determine concentration additivity of mixtures that are composed of different compounds. However, based on the assumption that UV filters act through a similar mode of action (Schreurs et al., 2002), concentration additivity was expected, in spite of the experimental limitations described above. Therefore, deviation from the concentration additivity situation for this mixture was assessed. The multi-component mixture (based on relative potency derived from individual C50 values) does not provide any evidence to justify the conclusion that deviations from the additivity concept have taken place. Furthermore, the toxic unit approach suggested that our mixture components interacted in a close to additive manner. Also, in the binary mixture, no statistical and visible deviation from the additivity concept was observed as has been shown in Fig. 4.

The overall conclusion is that our experiments indicate that the tested UV filters, under these experimental conditions, do follow the concentration additivity concept for compounds with a similar mechanism of action.

Biological relevance

The C50 value of the multi-component mixture equals concentrations of 0.21, 0.05, 0.78, and 0.44 μM for BP-3, BP-1, 4-MBC, and OMC, respectively. The total concentration of estrogenic UV filters in fish ranged from 1.6 to 7.8 μM in fat, or from 0.02 to 0.2 μM in whole fish (Schlumpf et al., 2001). The sum of the 4 concentrations in our undiluted mixture falls well within the range of concentrations presently found in fish. Furthermore, BP-3, 4-MBC, and OMC can be found in a high number of commercial UV-screens in Europe.

75% or less of the skin surface is, often daily, treated with sunscreens during a period of 3 to 4 weeks in summer (Nohynek and Schaefer, 2001). For a number of these UV filters this application is sufficient to cause bioaccumulation in the human body. Maximum plasma levels of BP-3, 4-MBC, and OMC in postmenopausal women were 200 ng/ml (0.9 μM), 20 ng/ml (0.1 μM), and 10 ng/ml (0.03 μM), respectively, 3–4 h after application of a formulation containing 10% (wt/wt) of each compound (Janjua et al., 2004). The C50 values of 0.5, 1.9, and 1 μM for BP-3, 4-MBC, and OMC, respectively, fall within the range of the reported plasma levels after daily exposure to sunscreens. In view of our results and observed plasma levels it cannot be excluded that daily exposure to sunscreen formulations may have estrogenic effects in humans. To our knowledge there are no epidemiologic studies that studied the association between sunscreen use and any estrogenic or adverse responses in humans. However, several mechanistic studies, both in vitro and in vivo, have been published that show that several UV filters are able to elicit estrogenic effects. Therefore, further (epidemiological) studies have to be performed in order to elucidate whether there is indeed a risk of using these UV filters for humans. The observed estrogenic effects of these commercially available cosmetic compounds in human breast carcinoma cells requires further investigations, particularly in relation to the development of estrogen-responsive breast tumors in high risk women.

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