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Estrogenic activity of UV filter mixtures

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Abstract

UV-absorbing chemicals (UV filters) are widely used for protection against UV radiation in sunscreens and in a variety of cosmetic products and materials. Depending on the breadth and factor of UV protection, they are added as single compounds or as a combination thereof. Some UV filters have estrogenic activity, but their activity and interactions in mixtures are largely unknown. In this work, we analyzed 8 commonly used UV filters, which are pure or partial hERα agonists, for their estrogenic activity in equieffective mixtures in a recombinant yeast assay carrying the human estrogen receptor alpha (hERα). Mixtures of two, four and eight UV filters alone, or in combination with 17 β estradiol (E2), were assessed at different effect levels and no-observed-effect-concentrations (NOEC). Predictions of the joint effects of these mixtures were calculated by employing the concentration addition (CA) and independent action (IA) model. Most binary mixtures comprising of pure hERα agonists showed a synergistic activity at all mixture combinations. Only in combination with benzophenone-1, antagonistic activity was observed at some effect levels. All mixtures of four or eight, pure or pure and partial hERα agonists, alone or including E2, showed synergistic activity at concentrations giving an increase of 10% of basal activity (BC10). This occurred even at concentrations that were at the NOEC level of each single compound. Hence, there were substantial mixture effects even though each UV filter was present at its NOEC level. These results show that significant interactions occur in UV filter mixtures, which is important for the hazard and risk assessments of these personal care products.

Keywords: UV filter; Estrogenicity; Mixtures activity; Synergism; Antagonism; Concentration addition; Independent action; Recombinant hERα yeast assay

Introduction

Sunscreens and cosmetics including lipsticks, skin lotions, hair sprays, hair dyes, shampoos and numerous other products like moldings, jet ink, tires and fabrics contain increasing amounts of UV filters, which scatter and reflect UV light. Increasing numbers of materials contain UV filters in order to prevent degeneration by UV radiation. Increased sunlight protection factors are used for preventing negative effects on the human skin. Generally this requires higher percentages of UV filters in the products and a growing use of combinations of different UV filters for absorbing UVA, UVB and UVC light.

UV filters may penetrate the human skin when applied in sunscreens or cosmetics and indeed in human urine, benzophenone-3 (BP3) and its metabolite benzophenone-1 (BP1) have been detected 4 h after application of commercially available sunscreen products to the skin (Felix et al., 1998). Half-lives of UV filters in humans, mammals or fish are not known, however. Residues of BP3 and octyl methoxycinnamate (OMC) were also detected in human breast milk samples up to 445 ng/g lipid (Hany and Nagel, 1995). Commonly, combinations of several different UV filters are added to sunscreens, cosmetics and other materials, depending on the desired protection factor and range. Thus, mixtures of UV filters with distinct estrogenic activities (Schlumpf et al., 2001) may be applied on human skin and

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human exposure can occur via dermal absorption or through the food chain, namely fish consumption.

Little is known about the fate of UV filters in the environment. They may ultimately reach aquatic systems directly via recreational activities (bathing), and indirectly via wastewater. 4-Methylbenzylidene camphor (4MBC), BP3, OMC and octocrylene (OC) were detected in lakes and wastewater in Switzerland (Balmer et al., 2005; Buser et al., 2006). They occurred up to 19 μg/L (EHMC or OMC) in raw wastewater, but were considerably lower in treated wastewater, indicating both sorption into sewage sludge (Plagellat et al., 2006) and aerobic biodegradation. 4MBC was most frequently found (up to 2.7 μg/L), followed by BP3, EHMC and OC (Balmer et al., 2005). In raw wastewater, OMC and BP3 were found in the range of 0.11 to 10.4 μg/L, and in raw drinking water OMC was in the range of 0.56 to 5.61 μg/L in southern California (Loraine and Pettigrove, 2006). Residues of several UV filters have also been detected at concentrations of 21–3100 ng/g lipid in fish (Nagtegaal et al., 1997; Balmer et al., 2005; Buser et al., 2006). Recently, a bioconcentration factor of 313 has been determined for 3-benzylidene camphor (3BC) in fish (Kunz et al., 2006a). Once UV filters enter the aquatic ecosystems, they join in a mixture of other potential xenoestrogens.

In recent years, it has become evident that some UV filters are estrogenic *in vitro* as demonstrated in MCF-7 cells (Schlumpf et al., 2001), recombinant cell lines (Schreurs et al., 2002; Mueller et al., 2003) and recombinant yeast systems carrying the human estrogen receptor (h ERα) (Routledge and Sumpter, 1997; Schultz et al., 2000; Kunz and Fent, 2006). Moreover, a high proportion of commonly used UV filters has been found to exhibit multiple hormonal activities *in vitro* (Kunz and Fent, 2006). Estrogenic activity has also been observed in rats (Durrer et al., 2005; Schlumpf et al., 2001; Seidlová-Wuttke et al. 2004) and fish (Holbech et al., 2002; Inui et al., 2003; Kunz et al., 2006a, b).

As UV filters are usually applied as compound mixtures, it is important to understand their activity in mixture combinations. Moreover, this is the general situation in the environment. Currently, however, the interactions in UV filter mixtures are largely unknown. A previous study using mixtures of estrogenic compounds including 2,4-dihydroxybenzophenone combined at concentrations below the no-observed-effect-concentrations (NOEC) demonstrated significant mixture effects in the yeast hERα assay (Rajapakse et al., 2002; Silva et al., 2002). The effect of mixtures of four estrogenic UV filters on pS2-gene transcription was studied recently in MCF-7 cells. Mixtures of two (BP1, BP3) and four compounds (BP1, BP3, OMC, 4MBC) showed additive activity (Heneweer et al., 2005). Currently, it remains unclear whether and how the estrogenic and/or antiestrogenic activity of single UV filters at low concentrations contributes to mixture effects. In our study, we focus on these questions as such knowledge is needed for evaluating the potential hazards and risks of estrogenic UV filters for humans and on aquatic life.

The question of mixture activity has gained increasing attention in the last few years (Kortenkamp and Altenburger, 1998). Mixture effects of estrogenic compounds can be calculated based on the activities of individual mixture components. The joint action of weak estrogenic compounds was recently shown to be based on the concept of concentration addition (CA) *in vitro* (Payne et al., 2000; Rajapakse et al., 2002; Silva et al., 2002) and *in vivo* in fish (Brian et al., 2005). In our study, we applied two competing pharmacological concepts for the calculation of expected additive mixture effects. The concept of CA (Loewe and Muischnek, 1926) assumes that components of a mixture act in a similar way, such that one compound can be replaced by an equal fraction of an equieffective concentration of another, without weakening the overall mixture effect. Synergistic or antagonistic additivity can then be determined by applying the method of isoboles (Loewe and Muischnek, 1926; Kortenkamp and Altenburger, 1998). The concept of independent action (IA) on the other hand assumes that mixture effects are the result of combined effects of individual mixture components with different modes of action. A mixture components that are present below zero effects are not expected to contribute to the total mixture effect (Bliss, 1939).

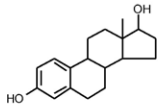
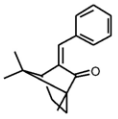
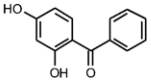
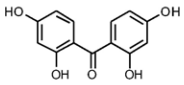
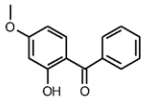
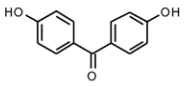
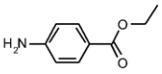
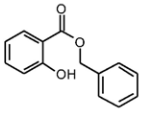
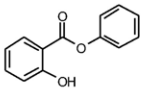
In the present work, we investigate the activities of 2, 4 and 8 UV filters alone and combined with 17β-estradiol (E2) at different effect levels and at the NOEC. We selected commonly used UV filters, which were demonstrated to be pure hERα agonists or partial hERα agonists *in vitro* (Kunz and Fent, 2006). Our UV filter selection was based more on mechanistic grounds (i.e. pure or parital hERα agonists) than on practical considerations (analyzing actually occurring mixtures in sunscreens), although some of the analyzed binary mixtures may occur in cosmetics. We addressed the influence of partial agonism for the mixture activity, as little attention is given to this property when estrogenic chemicals are assessed in mixtures. By investigating multi-component mixtures of commonly used pure and partial hERα agonistic UV filters, we test the hypothesis that mixtures comprising of pure hERα agonists follow the model of CA, leading to additive or even synergistic estrogenic activity. In addition, we hypothesize that mixtures comprising of pure and partial hERα agonists also follow the CA model, but rather lead to an antagonistic overall mixture effect, due to the antiestrogenic properties of the partial hERα agonists.

Material and methods

Chemicals. 17β-Estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). UV filters (Table 1) were obtained as follows: benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), 4,4′-dihydroxybenzophenone (DHB), benzylsalicylate (BS), phenlysalicylate (PS) and ethyl-4-aminobenzoate (Et-PABA) were obtained from Fluka AG (Buchs, Switzerland). 3-Benzylidene-camphor (3BC) was purchased from Induchem (Volketswil, Switzerland). All compounds used were >99% pure. Stock solutions were made in ethanol and stored in the dark at 4 °C. Analytical grade ethanol (free of UV filter) was purchased from T.J. Baker (Stehelin AG, Basel, Switzerland). Bidestilled water was produced using a Jencons Autostill double D-ionstill (Renggli AG, Rotkreuz, Switzerland).

Yeast estrogen assay expressing human estrogen receptor alpha (hERα assay). The estrogen-inducible expression system used was kindly gifted by J. Sumpter (Brunel University, U.K.) and is described in detail by Routledge

Table 1
Chemical structures, molecular weights and CAS numbers of compounds analyzed

Compound	Chemical structure	Molecular weight (g/L)	CAS
17β-Estradiol (E2)		272.39	50-28-2
3-Benzylidene camphor (3BC)		240.34	15087-24-8
Benzophenone-1 (BP1)		214.22	131-56-6
Benzophenone-2 (BP2)		246.22	131-55-5
Benzophenone-3 (BP3)		228.25	131-57-7
4,4′-Dihydroxybenzophenone (4DHB)		214.22	611-99-4
Ethyl-4-aminobenzoate (Et-PABA)		165.19	94-09-7
Benzyl salicylate (BS)		228.25	118-58-1
Phenyl salicylate (PS)		214.22	118-55-8

Abbreviations: CAS, Chemical Abstracts Service.

and Sumpter (1996). In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα). Yeast cells also contain expression plasmids carrying estrogen responsive elements (ERE), regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand (i.e. 17β-estradiol or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesized and secreted into the medium, leading to a color change of chromogenic substrate chlorophenol red β-D-galactopyranoside (CPRG) from yellow to red.

Preparation of assay media. Preparation of medium compounds was done as previously described by (Routledge and Sumpter, 1996). All components except $Fe_2(SO_4)_3$, inositol, copper (II) sulfate (Fluka, Buchs, Switzerland) and CPRG (Roche, Basel, Switzerland), were purchased from Sigma (Glattbrugg, Switzerland). A ten-times concentrated stock culture of the yeast strain was stored at −20 °C in 0.5 mL aliquots. Every 4 months (shelf life), the yeast strain was replaced with new −20 °C stock culture. Prior to the experiments the growth medium was inoculated with 125 μL 10-times concentrated yeast stock, and incubated at 28 °C for 24 h on an orbital shaker. The final assay medium was prepared by seeding 50 mL fresh growth medium with 4×10^7 yeast cells. Then 0.5 mL CPRG was added.

hERα assay procedure. The yeast assay was carried out within a type II laminar flow. Stock solutions of standards and chemical mixtures were serially diluted in ethanol. Aliquots of 10 μL were then transferred to 96-well optically flat-bottomed microtiter plates (Greiner Bio-One, Huber AG, Basel, Switzerland) and ethanol was allowed to evaporate to dryness. All experiments were repeated at least twice and each plate contained a positive control with E2 in triplicates. The tested mixtures were analyzed in quadruplicates. A blank row with ethanol was added in order to control for a possible CPRG conversion due to the medium components or materials alone. After adding aliquots of 200 μL of the final assay medium to the plates, they were sealed with plate sealers (Micronic, Vitaris AG, Baar, Switzerland) and shaken vigorously for 2 min on a titer plate shaker before incubation at 32 °C. After the 72 h of incubation, plates were shaken vigorously for 2 min and left for 1 h to allow yeasts to settle. The plates were than read at absorbances of 540 nm and 620 nm, using a Tecan GENios plate reader (Tecan AG, Männedorf, Switzerland).

Calculations. The absorbance-measurement at 540 nm (CPRG) and 620 nm (turbidity) allowed for subsequent correction for turbidity (yeast growth), as follows:

$$\begin{aligned}\text{Corrected absorbance} = {} & \text{chemical absorbance}_{540\ \text{nm}} \\ & - [\ \text{chemical absorbance}_{620\ \text{nm}} \\ & - \ \text{blank absorbance}_{620\ \text{nm}}] \\ & - \ \text{blank absorbance}_{540\ \text{nm}}\end{aligned}$$

Statistical analysis. For curve fitting and EC50 calculations, the corrected absorbance values versus the logarithm of concentration were plotted, whereby the best fit from a number of non-linear regression models was selected for final data analysis. In this study, we used the four-parameter logistic equation (Hill equation) to fit full dose–response curves according to

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC50} - X)^{*}\text{HillSlope}}},$$

where X is the logarithm of concentration and Y is the response showing a sigmoid shape. LogEC50 is the concentration of the mixture yielding half maximal effects and HillSlope is the slope parameter. Coefficient of determination (R^2), residuals and 95% confidence intervals were calculated so as to verify that the fitted curve represents the data correctly. The runs test was carried out in order to ensure that the model chosen to fit the curve does not significantly deviate from the data. Submaximal dose–response curves were fitted using the best fit from a number of non-linear regression models. Curve fitting was carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, USA).

Efficacies, i.e. curve heights of full and submaximal dose–response curves were calculated as follows for single compounds and mixtures:

$$\text{Efficacy}_{\text{UV filter}} = \left(\frac{1}{\text{Top}_{\text{Standard}} - \text{Bottom}_{\text{Standard}}}\right) * \text{Top}_{\text{UV filter}} - \text{Bottom}_{\text{UV filter}}$$

where the curve height ($\text{Top}_{\text{UV filter}} - \text{Bottom}_{\text{UV filter}}$) of the compound was compared with the curve height of the corresponding standard ($\text{Top}_{\text{Standard}} - \text{Bottom}_{\text{Standard}}$, efficacy set at 100%). Estrogenic activities were calculated for dose–response curves of single compounds and mixtures by normalizing the data (blank or basal absorbance = 1) and using non-linear regression analysis to determine the concentration at which each compound elicits 50% of its maximal activity (EC50). Subsequently relative potencies (RP), i.e. estrogenic activities were derived for each compound and mixture by dividing the EC50 for E2 by the EC50 values of each single compound or mixture (Table 2).

Mixture design and testing. The compounds used for the mixture experiments were previously found to be either pure or partial hERα agonists in the hERα assay (Kunz et al., 2006b; Kunz and Fent, 2006). BP1, BP2, DHB and Et-PABA are full hERα agonists exhibiting full dose–response curves (Fig. 1A) with no antiestrogenic activity (Fig. 1B). BP3, BS, PS and 3BC are partial hERα agonists with submaximal dose–response curves (Fig. 1C). They show full antiestrogenic activity when co-exposed with E2 (Fig. 1D) (Kunz and Fent, 2006). Table 2 gives data about their dose–response curves and relative potencies (RP), which served as a basis for our mixture experiments.

In our experiments, the compounds were mixed at equieffective concentrations; this means that the concentrations used for the single compounds would lead all the same effect when tested alone. Mixture experiments with combinations of two and four compounds with or without E2 were conducted with pure hERα agonistic UV filters only (Figs. 1A, B). In order to investigate mixture activities of these UV filters, binary mixture experiments were conducted first by mixing effect concentrations of the single UV filters for the effect levels of EC25, EC50 and EC75, respectively. Experiments with mixtures of four pure hERα agonists and mixtures of 8 UV filters (±E2), comprising of pure and partial hERα agonists (Figs. 1A, C), were then conducted at lower mixture levels in order to investigate activities at low concentrations (10% effect level, BC10) and at the NOEC (NOEC).

In order to get equipotent multi-component mixtures of 4 or 8 UV filters, with and without E2, we used non-linear regression analysis for the normalized dose–response curves of each mixture component to determine the concentration at which each compound caused a 10% (BC10) increase of basal hERα activity. The NOEC was defined as an increase of 0.3% of basal hERα activity. We used equieffective concentrations based on absorbance and baseline instead of EC-mixture levels for the multi-compound mixtures, because thereby we bypassed differences in curve height and slope. We used this approach to calculate NOECs, because they generally denote the highest concentration at and below which the response of exposed organisms does not depart significantly from untreated controls. They are calculated by the Dunnett's

Table 2
Estrogenic effects of 8 UV filters and E2 in the yeast hERα assay and their concentrations in equieffective mixtures of two, four and eight compounds

	Efficacy [a]	RP [b]	Asymmetric hill function parameters			Binary mixture ratios (M)			Multiple mixture ratios (M)	
		1/...	Hill slope	Max [c]	EC50 (M)	EC25	EC50	EC75	BC10	NOEC
E2	100%	1	1.63±0.38	1.53±0.16	2.59±1.19E−10	1.08E−10	2.11E−10	4.14E−10	3.91E−11	6.32E−12
BP1	96%	5'000	1.300	1.604	1.15E−06	4.96E−07	1.15E−06	2.69E−06	1.93E−07	1.68E−08
BP2	91%	21'000	2.107	1.336	1.09E−05	6.47E−06	1.09E−05	1.85E−05	3.55E−06	6.16E−07
DHB	91%	170'000	1.902	1.256	7.34E−05	4.12E−05	7.34E−05	1.29E−04	2.39E−05	3.28E−06
Et-PABA	87%	3'500'000	2.395	1.490	8.96E−04	5.49E−04	8.69E−04	1.37E−03	2.82E−04	6.39E−05
3BC	21%	1'300'000	1.000	0.326	3.10E−04	–	–		1.75E−03	1.56E−06
BP3	18%	45'000	2.322	0.324	1.86E−05	–	–		2.49E−05	1.28E−06
BS	12%	860'000	1.000	0.157	1.66E−04	–	–		5.05E−03	8.38E−07
PS	32%	480'000	1.699	0.529	1.10E−04	–	–		6.89E−05	3.69E−06

Abbreviations: For abbreviations of listed compounds see Table 1; EC75, EC50 and EC25, concentration of the compound exhibiting 75%, 50% and 25%, respectively, of its total effect; BC10, concentration of the compound exhibiting a 10% increase of the baseline absorbance, used instead of EC10 because of too large differences in curve steepness and height; NOEC, calculated as a 0.3% increase of the baseline absorbance. EC, BC and NOEC values of compounds derived from three experiments with four replicates each. –, not calculated. Values for E2-standard are given in mean±SEM (n=9). Value of compounds from three experiments with four replicates each.

[a] Efficacy, effect (curve height) of a compound given as percentage of the effect of E2.

[b] RP, relative potency gives ratio of the EC50 of a compound divided by the EC50 of E2.

[c] Max, curve-height = top–bottom.

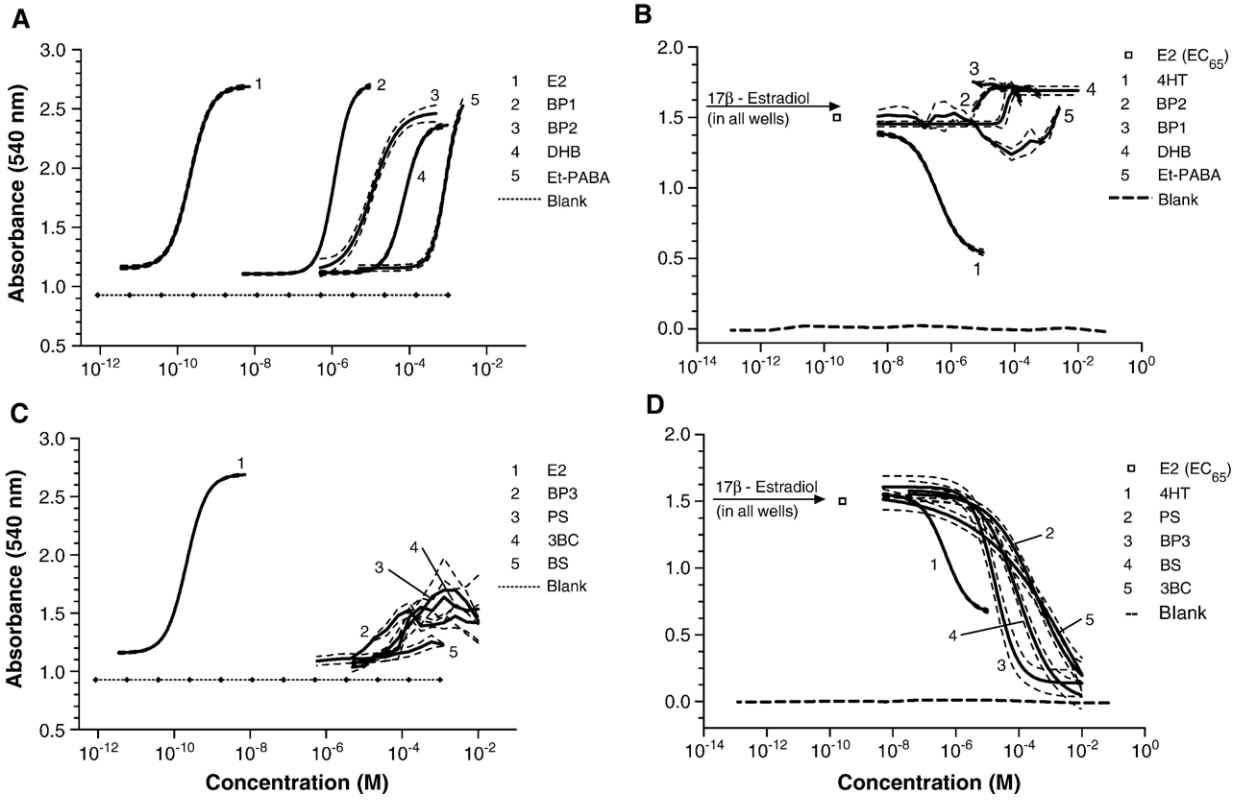


Fig. 1. Estrogenic and antiestrogenic activities of single compounds in the hERα assay. (A) Estrogenic activity of pure hERα agonists. (B) Antiestrogenic activity of pure hERα agonists measured by decrease of E2-induced (65% of maximal E2 activity) absorbance. 4-hydroxytamoxifen (4HT) serves as positive control. (C) Estrogenic activity of partial hERα agonist. (D) Antiestrogenic activity of partial hERα agonists. Data shown are means±95% CI band (three experiments with four replicates each). Data from Kunz and Fent, 2006.

statistical test. Hence, the NOEC may not represent a true "zero-effect", but the effect may not be detected in the assay, because it is too weak. In order to prevent testing weak undetectable effects instead of NOEC, we used the EC*x* point estimates, which are discussed to replace NOEC in risk assessment (Van der Hoeven, 1997).

Calculations of predicted mixture effects. The joint effects of a mixture with known composition were calculated on the basis of the Hill regression models for each UV filter in the mixture by using the concepts of concentration addition (CA) and independent action (IA).

In the concentration addition (CA) model (Loewe and Muischnek, 1926), compounds act similarly, on the same receptor site and can replace each other. Each compound in the mixture contributes individually to the mixture-effect in proportion to its concentration. Therefore the mixture ratio (the relative abundance of the compound in the mixture) has to be known and dose–response data for each individual compound have to be available. Thus assuming that the combined effect of the mixture with n components is concentration additive, the following equation will hold for any effect level:

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_i} = 1, \tag{1}$$

where c_i denotes the concentration of the compound i in a mixture yielding to an effect E and EC_i the concentration of i needed to produce the same effect on its own.

The concentration c_i of the compound i in the mixture is related to the total mixture concentration by:

$$c_i = p_i \times \mathrm{EC}_{\mathrm{mix}} \tag{2}$$

where the ratio p_i is the concentration of i relative to the total mixture concentration $\mathrm{EC}_{\mathrm{mix}}$ producing the total mixture effect E. Substitution of c_i in Eq. (1) gives:

$$\sum_{i=1}^{n} \frac{(p_i \times \mathrm{EC}_{\mathrm{mix}})}{\mathrm{EC}_i} = 1 \tag{3}$$

and rearranging yields

$$\mathrm{EC}_{\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{EC}_i} \right)^{-1} \tag{4}$$

The concentrations EC_i where calculated from the dose–response curves of the single compounds by using the inverse expression of the asymmetric Hill function.

Mixtures that were found to follow the concept of CA were further investigated for additivity, synergism or antagonism by using the method of isoboles (Loewe and Muischnek, 1926) and the toxic unit approach (Berenbaum, 1985; Kortenkamp and Altenburger, 1998). For binary mixtures, the isobole method was carried out by constructing graphs that show curves describing combinations of two compounds A and B, which produce the same specific effect (isobole). The axes of the graph are the doses of the two compounds on a linear scale. A line is drawn between the isoeffective doses A and B, representing additivity of the mixture. In addition, the toxic units were calculated for all binary mixtures.

For multi-compound mixtures of four components and more, the isobole method is no more applicable, because of the problem of graphical visualization

of multi-dimensional isobolograms. Thus instead of the isobole method, we assessed combinations of four or more components by the toxic unit approach (Berenbaum, 1985; Kortenkamp and Altenburger, 1998) using Eq. (5).

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_i} = 1 \tag{5}$$

Thereby additivity (zero interaction) of a mixture is expressed if Eq. (5) equals 1. Each fraction (c_i/EC_i) of Eq. (5) 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).

If a given mixture deviates from 1, a synergistic mixture effect is indicated by values <1, whereas an antagonistic mixture effect results in values >1. This method is suitable to analyze combinations of compounds, irrespective of the shape of their individual dose–response curves and can also be used in the case of partial agonism (Kortenkamp and Altenburger, 1998).

In the concept of independent action (IA, Bliss, 1939), it is assumed that compounds act on different subsystems and show different modes of action. This leads to effects in mixtures, which are based on individual compound-effects that are greater than zero. This model allows predicted effects of mixtures of known composition to be calculated using the expression:

$$1 - \prod_{i=1}^{n} [1 - E(c_i)], \tag{6}$$

where $E(c_i)$ is the effect E produced by the compound i at concentration c. Inherent in this expression is the fact that $E(c_i)$ cannot exceed 1, i.e. $E(c_i)$ is a fraction of a maximal possible effect, making IA a probabilistic model. Therefore when applying this model to our assay effects $\mathrm{AE}(c_i)$, a maximal effect $E_{\max}$ has to be defined. We used in our assays E2, which gave the highest absorbance value as a reference. The effect of the mixture is expressed relative to the maximal effect of E2:

$$E(c_i) = \frac{\mathrm{AE}(c_i)}{E_{\max}} \tag{7}$$

If the concentration–response relationships of all mixture compounds i are described by an appropriate regression model F_i (asymmetric Hill function), the assay effect $\mathrm{AE}(c_i)$ can be estimated from the mean effect $F_i(c_i)$, predicted by the regression model. Thus,

$$\mathrm{AE}(c_i) = F_i(c_i), \text{ and } E(c_i) = \frac{F_i(c_i)}{E_{\max}} \tag{8}$$

substitution of $E(c_i)$ in this equations yields

$$e_{\mathrm{mix}} = 1 - \prod_{i=1}^{n} \left[1 - \frac{F_i(c_i)}{E_{\max}}\right]. \tag{9}$$

In order to assure comparability of the IA predictions with those of CA, the fractional effect in the above equation was rescaled by multiplication with $E_{\max}$. Thus

$$E_{\mathrm{mix}} = E_{\max} \times e_{\mathrm{mix}}, \text{ and } E_{\mathrm{mix}} = E_{\max}\left[1 - \prod_{i=1}^{n} \left[1 - \frac{F_i(c_i)}{E_{\max}}\right]\right]. \tag{10}$$

Results

Binary mixtures of pure hERα agonists

In all mixture experiments, UV filters were added at non-cytotoxic concentrations only. The hERα agonistic UV filters (BP1, BP2, DHB and Et-PABA) were first assessed in binary mixtures at three different effect levels each (EC25, EC50 and EC75). The dose–response curves are shown in Fig. 2. The estimates calculated for all binary mixtures produced almost identical curves for the concept of CA and IA (not shown in Fig. 2 for clarity reasons). Two examples of estimated CA and IA curves in binary mixtures is shown in Fig. 3. When the mixture was strongly synergistic, the observed curve was shifted considerably to lower concentrations (Fig. 3A), whereas the dose–response curves for the observed and expected mixture effects agreed well, when the mixture was close to additivity and displayed weak antagonism (Fig. 3B).

In order to evaluate binary mixtures for additivity, synergism or antagonism, we have plotted the results using the isobole method and the results are shown in Fig. 4. Moreover, toxic units were calculated for all mixtures and effect levels (Fig. 5). Binary mixtures with BP1 exhibited antagonism at EC25 (mixture with DHB, Figs. 2D, 4D, 5) or at EC75 (mixture with BP2, Figs. 2E, 4E, 5), or at both mixture levels (mixture with Et-PABA, Figs. 2C, 4C, 5). However, these were exceptions. For all other binary mixtures and effect levels, we observed synergistic interactions (Figs. 4D–F, 5). Mixtures at EC25 of BP2 with DHB and BP1, and DHB with Et-PABA (Figs. 2A, E, F and 4A, E, F), indicate that lower mixture levels elicited stronger synergism.

Mixtures of four UV filters and E2

All mixtures of 4 pure hERα agonists (BP1, BP2, DHB and Et-PABA) followed the CA model better than the IA model (Fig. 6). The deviations between the observed and expected curves are due to synergistic activities (Figs. 6, 7). Comparison of their relative potencies with those of single UV filters revealed that mixtures of pure hERα agonists at BC10 and NOEC level led to enhanced relative potencies. Mixture activities reached values similar to the EC50 value of BP1 (1.15×10^{-6} M, Table 2), the most estrogenic UV filter (Fig. 8B).

To our surprise the mixture of four pure hERα agonists (BP1, BP2, DHB and Et-PABA) that were added at concentrations at their NOEC showed a full dose–response curve (Fig. 6C). It exhibited a stronger relative potency (Fig. 8) than the mixture of these 4 UV filters at the effect level of BC10. This mixture produced a dose–response curve of lesser height (Fig. 6A).

Mixtures of eight UV filters and E2

The combination of eight UV filters (BP1, BP2, DHB, Et-PABA, BP3, BS, PS and 3BC) mixed at their NOEC displayed submaximal dose–response curves that were higher than those of the same compounds mixed at their BC10 (Fig. 8A). The activities of mixtures consisting of 4 pure (BP1, BP2, DHB and Et-PABA) and 4 partial (BP3, BS, PS and 3BC) hERα agonists, alone or in combination with E2, differed from the CA and IA model because of their considerable synergistic activity (Fig. 9). This was the case for both the mixtures at the BC10 and NOEC level. As with binary mixtures, the calculated estimates of the mixtures of 8 UV filters produced very similar curves for the CA and IA model. Surprisingly, the combined activity of these eight UV filters was synergistic (Figs. 7, 9). When they were mixed with E2 at their NOEC, they showed relative potencies that were only 8'373-times weaker than E2, compared to the same mixture at the effect level BC10, which was 244'241-times weaker than E2 (Table 3,

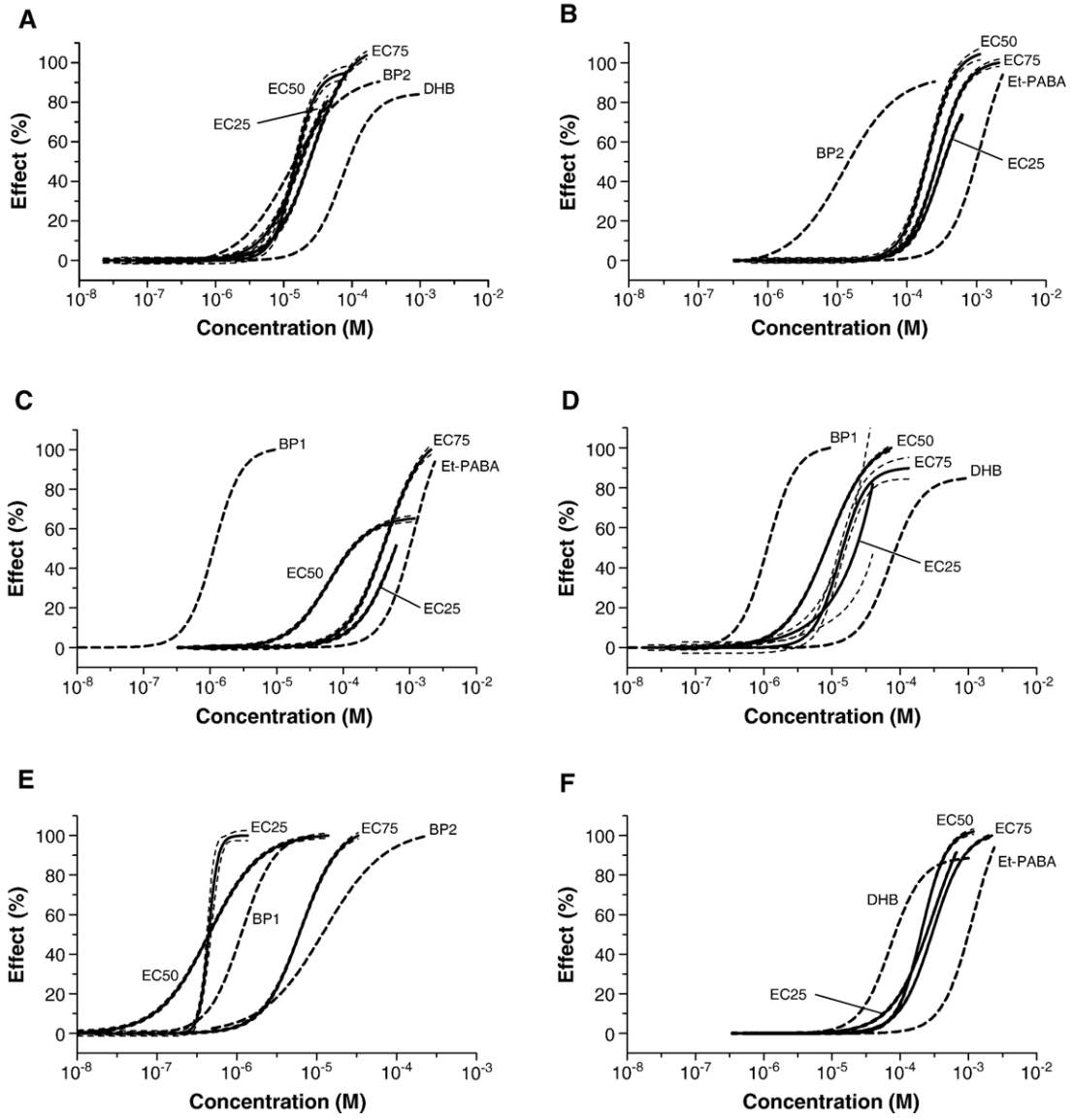


Fig. 2. Binary mixtures of pure hERα agonistic UV filters, showing dose–response curves for single compounds (dotted lines) and for mixtures at effect levels EC25, EC50 and EC75 (solid lines). Dose response curve of single mixture compounds are data from Kunz and Fent (2006). Results are presented in panels A–F for clarity reasons. Equieffective mixtures of BP2 and DHB (A), BP2 and Et-PABA (B), BP1 and Et-PABA (C), BP1 and DHB (D), BP1 and BP2 (E) and DHB and Et-PABA (F). Data shown are means ±95% CI band (three experiments with four replicates each). For clarity reasons, estimated CA and IA curves are not shown.

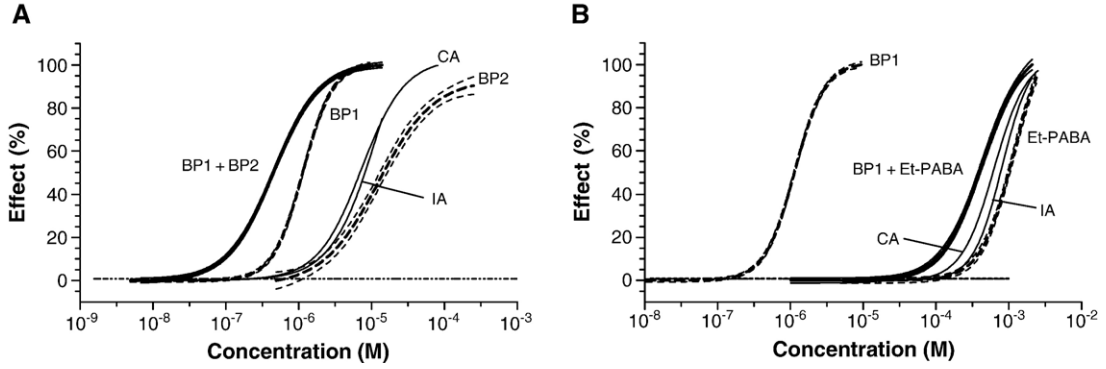


Fig. 3. Two examples for estimated CA and IA curves for binary mixtures. (A) Binary mixture of BP1 and BP2 at EC50 with synergistic mixture activity. (B) Binary mixture of BP1 and Et-PABA at EC75 with weakly antagonistic mixture activity. Data shown are means ±95% CI band (three experiments with four replicates each). Dotted lines represent dose–response curve of single mixture compounds (data from Kunz and Fent, 2006).

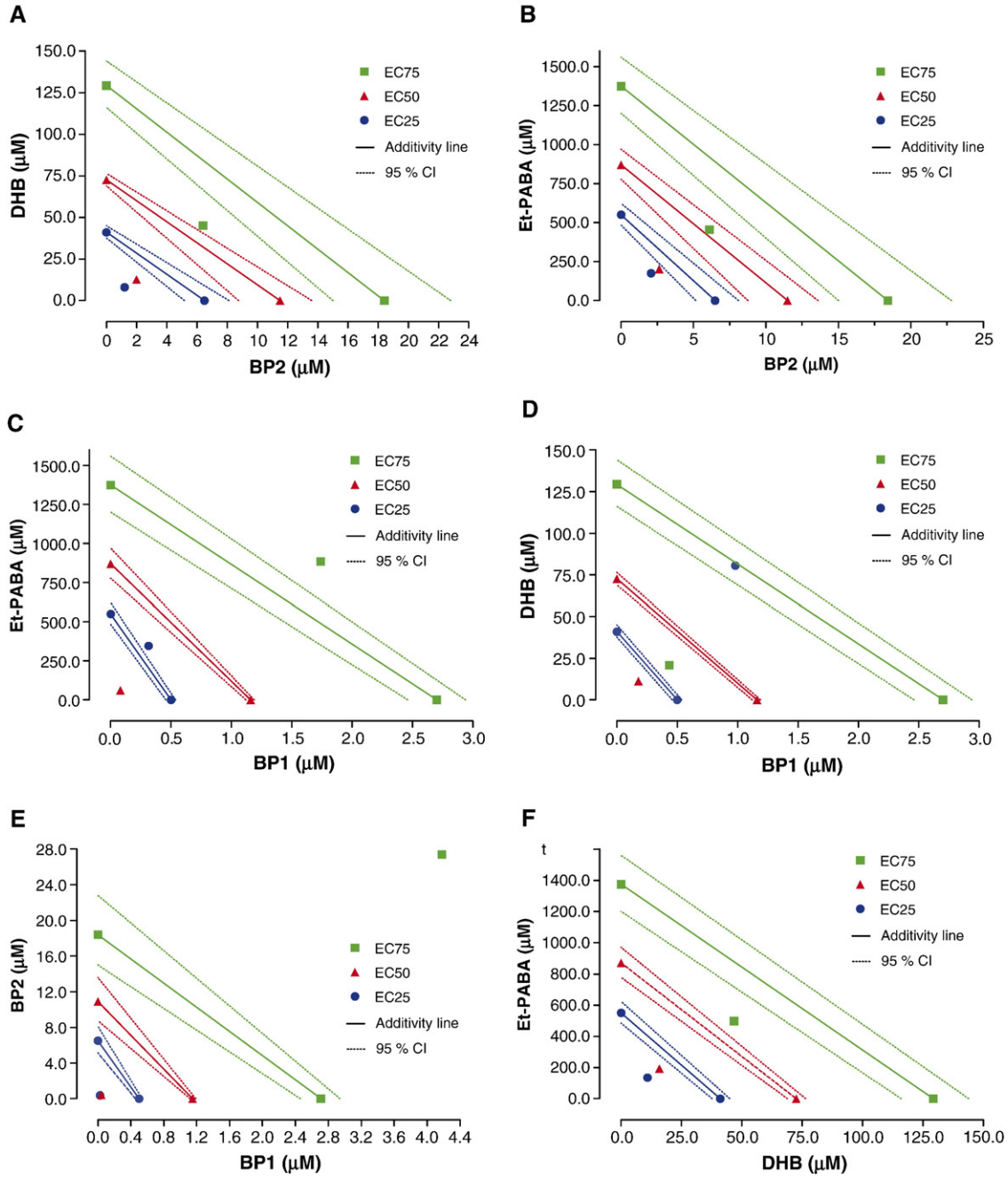


Fig. 4. Equieffective isobole graphs for binary mixtures of pure hERα agonistic UV filters at effect levels EC25, EC50 and EC75. Solid lines represent the additivity line with the 95% confidence intervals (CI) band for the single compounds. Results are presented in panels A–F for clarity reasons. Equieffective mixtures of BP2 and DHB (A), BP2 and Et-PABA (B), BP1 and Et-PABA (C), BP1 and DHB (D), BP1 and BP2 (E) and DHB and Et-PABA (F).

Fig. 8B). Estimated relative potencies are summarized in Fig. 8B. It is important to note that the relative potencies of the NOEC mixtures were again in the same range as the most estrogenic UV filter (BP1) found in our study (Fig. 8B).

Discussion

UV filters are commonly applied as mixtures in cosmetics and consequently, humans and aquatic organisms are exposed to mixtures of UV filters. In order to better understand the combined mixture activity, we analyzed 8 commonly used UV filters with either pure or partial hERα agonistic activity for their estrogenicity. To our surprise, the multi-component mixture experiments revealed pronounced synergistic activities of these UV filters when mixed at low effect concentrations. This marked effect was observed for mixtures with pure and partial hERα agonists, even when the individual UV filter concentrations alone were at their NOEC.

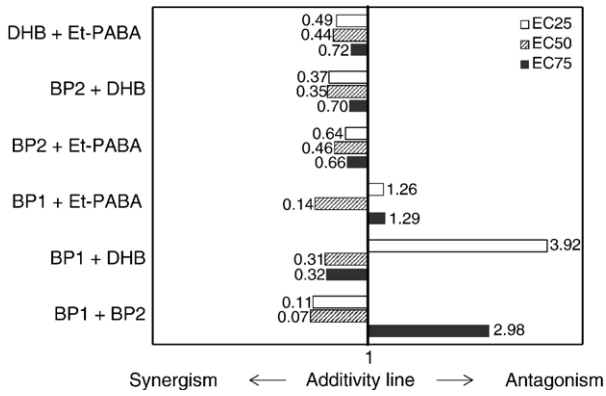


Fig. 5. Toxic unit calculations for binary mixtures of pure hERα agonistic UV filters, at effect levels EC25, EC50 and EC75. If the calculated toxic unit equals 1, additivity is assumed; if it is >1, antagonism, and if it is <1, synergism is assumed. Data shown are means±SEM (three experiments with four replicates each).

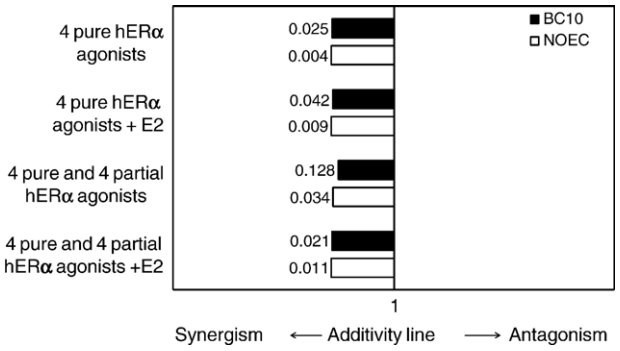


Fig. 7. Toxic unit calculations for mixtures of 4 agonistic (BP1, BP2, DHB, Et-PABA), or 4 agonistic and 4 antagonistic (BP3, 3BC, BS, PS) UV filters, with/without E2, at effect levels BC10 and NOEC. If the calculated toxic unit equals 1, additivity is assumed; if it is >1, antagonism, and if it is <1, synergism is assumed.

Concentration addition concept

The hypothesis that binary and multi-component mixtures of commonly used UV filters would follow the model of CA was rather supported by our data, although a distinction between the CA and IA model was difficult or even impossible. The estimates for all binary and multi-component mixtures calculated on the assumption of additive interaction produced almost identical curves for CA and IA. This was already observed for mixtures with the same mode of action (hERα agonists) using the same assay (Payne et al., 2000) and for mixtures of compounds with different modes of action in the E-screen (Payne et al., 2001). As the hERα assay is based on the compound's interactions with hERα and does not recognize any other effects, the CA model should produce valid calculations of additive effects. Indeed mixtures of 4 and 8 environmental chemicals in the hERα assay corresponded well with the predictions of CA (Payne et al., 2000;

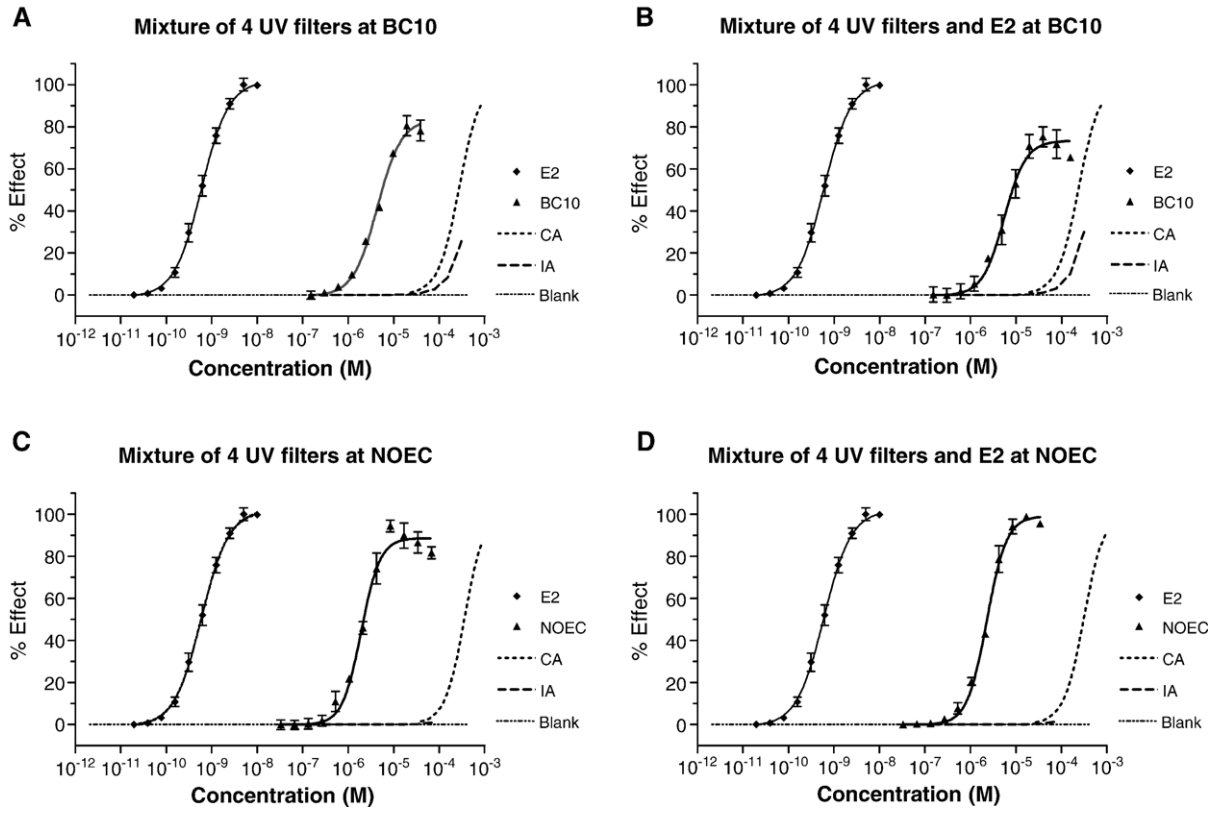


Fig. 6. Multi-component mixtures of 4 pure hERα agonistic UV filters (BP1, BP2, DHB, Et-PABA) without (A and C) and with E2 (B and panel D), at effect levels BC10 (A and B) and NOEC (C and D). Dotted lines represent estimates for CA and IA curves, respectively. Data shown are means ± SEM (three experiments with four replicates each).

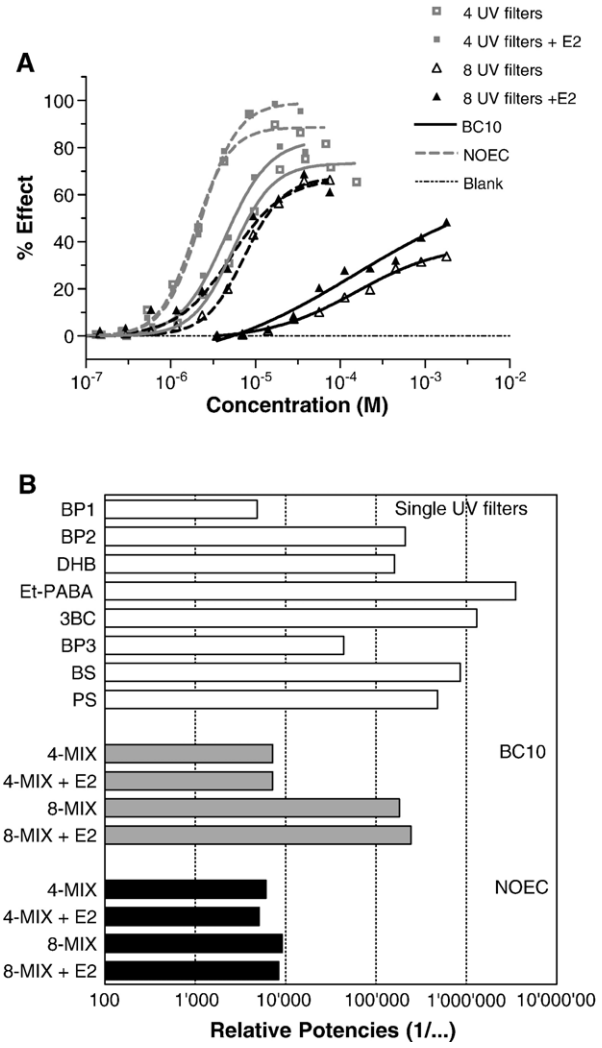


Fig. 8. Mixtures of 4 pure agonistic (BP1, BP2, DHB, Et-PABA), or 4 pure agonistic and 4 partial agonistic (BP3, BS, PS, 3BC) UV filters, with/without E2, at effect levels BC10 and NOEC. (A) Dose–response curves of multi-component mixtures at BC10 (solid lines) and NOEC (dotted lines). (B) Estimated relative potencies (RP) compared to E2 for all single compounds, binary mixtures and multi-component mixtures. Data derived from three experiments with four replicates each.

Silva et al., 2002). For our findings, we assumed CA because of the reasons mentioned above, and used the isobole method and the toxic unit approach in order to analyze synergistic or antagonistic interactions.

Binary mixtures with pure hERα agonists

In a first series of experiments, we mixed different combinations of two UV filters that are pure hERα agonists at three different effect levels. All these binary combinations displayed synergistic interactions. The only exceptions were combinations of UV filters with BP1 at some effect levels. A possible reason for this difference may be related to the large differences in relative potencies of the UV filters; BP1 possesses the highest relative potency of all investigated UV filters. Its relative potency is also considerably higher than that of the other three pure agonists. Another reason is the deviation from the equieffectivity of the mixture ratios, because the curve slopes at low and high effect levels differ.

When BP1 was combined with BP2 that shows a 4-times lower potency, antagonism was found at the effect level EC75, possibly due to system saturation. This may occur because of the high mixture effect level and/or because of differing slopes. Antagonism also occurred when BP1 was mixed with 4DHB (34-times lower potency) at EC25, or Et-PABA (700-times lower potency) at EC25 and EC75. As a consequence, we calculated the equieffective concentrations of single UV filters for mixtures of 4 and 8 compounds on the basis of equieffective absorbance values, instead of EC values. Thereby, difficulties arising from differences in curve slope and height were excluded.

Quaternary mixtures with pure hERα agonistic UV filters

Combinations of four compounds mixed at their BC10 and NOEC showed higher activities than predicted by the CA model. The toxic unit approach (Kortenkamp and Altenburger, 1998) demonstrates synergistic interactions for all mixtures at all effect concentrations. The mixture of BP1, BP2, 4DHB and Et-PABA at the NOEC level led to a full dose–response curve and to higher relative potency, as when mixed at BC10 level. This suggests that synergism increases with decreasing mixture levels. Similar to our findings, Silva et al. (2002) found substantial mixture effects and high efficacies when they mixed 8 xenoestrogens at low-dose levels.

The dose–response curves of BC10 mixtures displayed a lower efficacy of 72 to 83%, which seems to be caused by cytotoxicity to the yeasts. Toxicity occurred in the BC10 mixture at concentrations greater than 2×10^{-3} M, whereas the concentrations at which the individual compounds were added to the mixture were far from being toxic on their own. The cytotoxic concentrations of UV filters BP-2 ($>2.5\times10^{-3}$ M) and Et-PABA ($>2.5\times10^{-3}$ M) were well above those having maximal estrogenicity. This indicates that the cytotoxicity of some UV filters might also be additive or even synergistic in multi-component mixtures, whereas in binary mixtures the interaction of these compounds apparently does not lead to toxicity. The addition of E2 enhanced the efficacy of the mixture, but a stronger relative potency was only observed at the NOEC level. Similarly, a mixture of 11 xenoestrogens combined at their EC01 levels (zero effect level) with E2 led to a shift of the resulting dose–response curve to lower concentrations (Rajapakse et al., 2002).

Multi-component mixtures of pure and partial hERα agonistic UV filters

The strong synergism in mixtures incorporating pure and partial hERα agonistic UV filters was surprising. Synergistic activity was observed, when 4 pure hERα agonists were co-exposed with 4 partial hERα agonists. Due to the antiestrogenic activity of partial hERα agonists, an overall antagonistic mixture activity was expected, based on the fact that partial agonists elicit only submaximal dose–response curves on their

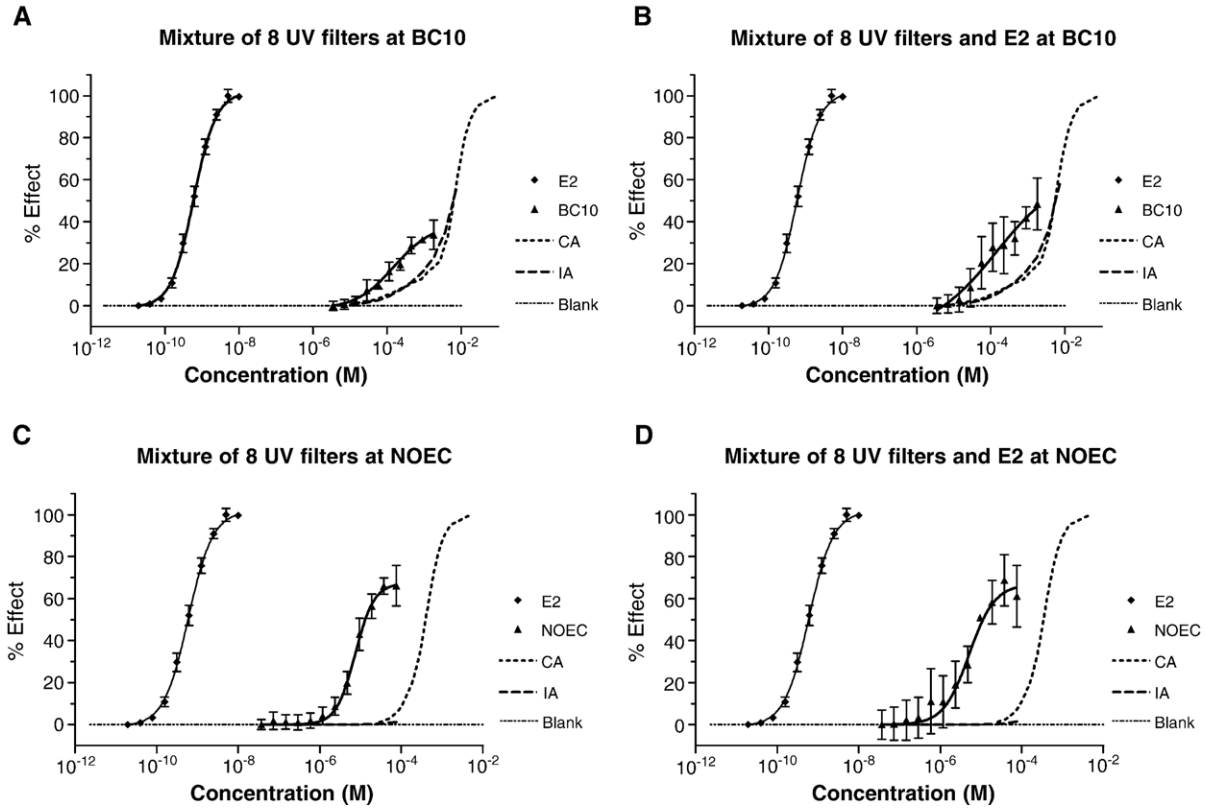


Fig. 9. Multi-component mixtures of 8 UV filters (BP1, BP2, DHB, Et-PABA, BP3, 3BC, BS PS) without (panel A and C) and with E2 (panel B and D), at effect levels BC10 (panel A and B) and NOEC (panel C and D). Dotted lines represent estimates for CA and IA curves. Data shown are means±SEM (three experiments with four replicates each).

own and block the effect of full agonist, when co-exposed with these compounds (Stephenson, 1956). Partial agonists may only induce a suboptimal conformational change of the hERα (Pike et al., 1999; Bowers et al., 2000; Gangloff et al., 2001), or bind to a secondary binding site (Dudley et al., 2000; Jensen and Khan, 2004), both of which can lead to steric or ionic interferences resulting in reduced efficacy. The only indication of a weak inhibitory action mediated by the partial agonists was found when 8 UV filters were mixed at their BC10 level. In this case, a considerably lower relative potency was observed compared to a mixture of 4 UV filters (181'421- versus 7'161-times weaker than E2).

Surprisingly, the activity of mixtures of 8 UV filters (with or without E2) combined at their NOEC possessed a stronger estrogenic potency than its counterpart at the BC10 level (8'373- versus 244'241-times weaker than E2). It showed an enhanced activity, which is highly synergistic and only slightly less potent than the NOEC mixtures with 4 pure hERα agonistic UV filters. Nevertheless, the efficacies were only submaximal, with a slightly decreasing effect at the highest concentration, indicating slight toxicity even at NOEC mixture level. The high estrogenicity and efficacy of the NOEC mixture of 8 UV filters may be explained as follows. At very low doses, components with higher affinity to the hERα may bind first compared to components having lower affinity, while these may rather act as co-activators. Thereby, partial agonism of a compound seems to have only a minor effect at NOEC mixtures. It was shown that a partial agonist exposed alone or in the presence of low concentrations of a full agonist, the resulting effect is submaximal agonistic or additive, respectively (Stephenson, 1956; Kenakin, 2004).

Possible explanations for the high incidence of synergism found in the yeast hERα assay

Why do low-dose mixtures or mixtures at NOEC result in such marked effects? Low-dose effects of endocrine disrupting chemicals are mediated by endocrine signaling pathways that evolved to act as powerful amplifiers, with the result that large changes in cell function can occur in response to extremely low concentrations (Welshons et al., 2003). Thus, in a mixture, some compounds may directly mimic E2, whereas others interfere with the production, metabolism or transport of E2 (Weltje et al., 2005), or with regard to our study, they may interfere with β-galactosidase production in the yeast hERα assay. In multi-compound mixtures at very low doses, mixture components with higher affinity to the ER may first bind to it, while other compounds rather act as co-activators. Thereby the tiniest amount of a chemical added could have an effect, because its activity adds to a chemical, which is already there, resulting in no threshold effects (Sheehan et al., 1999). Similar to our findings with UV filters, xenoestrogens at levels below individual non-significant concentrations may indeed enhance estrogenic effects in yeasts and MCF-7 cells (Payne et al., 2000; Rajapakse et al., 2002; Silva et al., 2002), but also in transfection systems

Table 3
Parameters of UV filter mixtures in the yeast hERα assay

	Effect level	Efficacy	RP	Hill function parameters			Highest mixture concentration (M)
			1/...	Hill	Max	EC50 (M)	
BP1+BP2	EC25	99%	4'389	7.448	1.624	4.456E−07	6.999E.06
	EC50	88%	1'629	1.369	1.044	4.552E−07	1.266E.05
	EC75	100%	2'256	1.000	1.862	1.046E−05	2.110E−05
BP1+4DHB	EC25	86%	2'588'699	1.000	1.481	2.445E−04	4.154E−05
	EC50	120%	18'840	1.000	1.478	2.372E−05	1.137E−05
	EC75	90%	68'690	2.302	1.636	1.321E−05	1.320E−04
BP1+Et-PABA	EC25	52%	4'094'450	1.392	0.904	7.621E−04	5.505E−04
	EC50	66%	132'538	1.441	0.983	6.102E−05	8.713E−04
	EC75	104%	2'548'848	1.552	1.853	4.370E−04	1.378E−03
BP2+Et-PABA	EC25	76%	1'420'000	2.082	1.349	3.017E−04	5.565E−04
	EC50	115%	1'062'711	2.305	1.912	2.042E−04	8.816E−04
	EC75	107%	1'462'580	2.159	1.852	2.777E−04	1.394E−03
BP2+DHB	EC25	100%	93'595	1.585	1.800	1.758E−05	4.753E−05
	EC50	96%	97'862	2.727	1.667	1.456E−05	8.406E−05
	EC75	110%	128'339	1.627	1.937	2.624E−05	1.477E−04
DHB+Et-PABA	EC25	94%	2'110'570	1.434	1.617	3.119E−04	5.910E−04
	EC50	96%	1'003'004	2.439	1.901	2.070E−04	9.427E−05
	EC75	103%	2'035'410	1.830	1.961	2.990E−04	1.505E−03
4-Mixture	BC10	72%	7'172	1.64	1.360	4.361E−06	3.093E−04
	NOEC	88%	6'083	1.53	1.360	3.060E−06	6.769E−05
4-Mixture+E2	BC10	83%	7'161	1.82	1.217	5.438E−06	3.093E−04
	NOEC	100%	5'106	2.01	1.828	2.287E−06	6.769E−05
8-Mixture	BC10	41%	181'421	0.83	0.629	1.544E−04	7.199E−03
	NOEC	69%	9'163	1.87	1.092	7.399E−06	7.507E−05
8-Mixture+E2	BC10	78%	244'241	0.48	1.343	1.459E−04	7.199E−03
	NOEC	67%	8'373	1.38	1.178	5.086E−06	7.507E−05

For abbreviations, see Tables 1 and 2; value of compounds from three experiments with four replicates each.

(Le Page et al., 2006). Also a binary mixture of E2 and ethinylestradiol was more potent than either of the individual compounds *in vivo* in fish (Thorpe et al., 2003).

However, synergistic interactions of xenoestrogen mixtures have caused particular concern, originating in a study that reported strong synergism of a mixture of weak xenoestrogens (Arnold et al., 1996), which could not be reproduced by other laboratories (Ashby et al., 1997; Gaido et al., 1997; Ramamoorthy et al., 1997). This led to the withdrawal of the original paper (McLachlan, 1997). Recently, Kortenkamp and Altenburger (1998) reinvestigated mixture studies with the isobole method and found that at some effect levels (Gaido et al., 1997; Shekhar et al., 1997) and mixture ratios (Ashby et al., 1997) synergism was overlooked. We used the method of isoboles and the toxic unit calculations in order to evaluate mixtures for additivity, synergism or antagonism. The additional calculations of CA and IA, as well as of relative mixture potencies further support our findings with the isobole method for synergistic interactions.

Thus we hypothesize that the observed synergism in most of the mixtures with UV filters – even when combined at individual NOEC's, and even when partial agonists were added – is unlikely due to the characteristics of the yeast transactivation assay, but rather caused by properties of the UV filters, which may activate and co-activate the hERα and other co-activators and factors via diverse actions. In addition, other signal transduction pathways responsible for estrogen-related effects may also play a role. Hormone receptor agonists are not only determined by ligand–receptor binding, but they may also interact with other factors such as co-regulators that are able to modulate the transcriptional activity of steroid hormone receptors (Katzenellenbogen et al., 1996). In contrast to our observations with UV filters, additive interactions were observed in the same hERα assay for mixtures consisting of other xenoestrogens at their NOEC (Rajapakse et al., 2002; Silva et al., 2002). Significant effects in multi-component mixtures were also found with estrogenic pharmaceuticals (Fent et al., 2006), and the interaction was synergistic in some cases. UV filters actually seem to have synergistic activities in mixtures, at least in the hERα assay. However, synergism may also be related to and caused by interactions with co-factors in the yeast system. Thus individual compounds in a mixture may have different activities that may influence transactivation, binding, co-activation and expression of β-galactosidase. In order to prove the synergistic interactions of UV filters in mixtures as observed in our study, further analysis in other systems such as cell-lines is necessary.

Conclusions and possible environmental consequences

Recent studies demonstrate that UV filters possess endocrine disrupting properties at rather high concentrations (Schlumpf et al., 2001; Holbech et al., 2002; Inui et al., 2003; Kunz et al., 2006b). Our findings on the pronounced synergistic effects of multi-component mixtures of UV filters combined at their

individual NOEC indicate that low UV filter concentrations present on the human skin and in the environment may produce relevant estrogenic activity on their own, or lead to enhanced estrogenic activities of other xenoestrogens or E2, depending on the mixture components.

Indeed, concentrations of single UV filters in the NOEC mixtures were mostly in the μg/L range, when eliciting highest estrogenic activities. These effect concentrations are close to residual concentrations of UV filters found in the environment. In drinking water in southern California, OMC and BP3 were found in the range of 0.45 to 5.61 μg/L (Loraine and Pettigrove, 2006). Residues of BP3 and OMC were found in human breast milk samples up to 445 ng/g lipid (Hany and Nagel, 1995) and in fish 4 to 6 different UV filters were identified in the low mg/kg range. Our findings suggest that partial agonistic UV filters do not reduce the overall mixture activity due to antagonism. This seems also to be the case at very low concentrations, were these compounds also seem to add to the overall mixture effect.

Our study reveals a novel and more detailed picture of the hormonal activity of UV filter mixtures *in vitro* and discloses unexpected synergistic properties of these compounds. In order to assess, whether our *in vitro* findings will translate into other *in vitro* systems and to *in vivo* activity, further studies are needed. The consequences of mixture activities of UV filters found in this study are of significant scientific and practical interest. For an adequate risk assessment, it seems unavoidable to consider compound mixtures, when investigating endocrine disrupting properties of UV filters towards humans and aquatic organisms. Ongoing *in vivo* studies in our laboratory will show whether *in vitro* mixture activities of UV filters will translate into the activity in fish at environmentally relevant concentrations.

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References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures in *Vibrio fischeri* mixtures composed of similar acting chemicals. Environ. Toxicol. Chem. 19, 2341–2347.

Arnold, S.F., Robinson, M.K., Notides, A.C., Guillette, L.J., McLachlan, J.A., 1996. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environ. Health Perspect. 104, 544–548.

Ashby, J., Lefevre, P.A., Odum, J., Harris, C.A., Routledge, E.J., Sumpter, J.P., 1997. Synergy between synthetic oestrogens? Nature 385, 494.

Balmer, M., Buser, H.R., Müller, M.D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953–962.

Berenbaum, M.C., 1985. What is synergism? Pharmacol. Rev. 41, 93–141.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585–615.

Bowers, J.L., Tyulmenkov, V.V., S.C.J., Klinge, C.M., 2000. Reservatrol acts as a mixed agonist/antagonist for estrogen receptors α and β. Endocrinology 141, 3657–3667.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A., Sumpter, J. P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721–728.

Buser, H.R., Balmer, M.E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427–1431.

Dudley, M.W., Sheeler, C.Q., Wang, H., Khan, S., 2000. Activation of the human estrogen receptor by the antiestrogens ICI 182,780 and tamoxifen in yeast genetic systems: implications for their mechanism of action. Proc. Nat. Acad. Sci. U.S.A. 97, 3696–3701.

Durrer, S., Maerkel, K., Schlumpf, M., Lichtensteiger, W., 2005. Estrogen target gene regulation and coactivator expression in the rat uterus after developmental exposure to the ultraviolet filter 4-methylbenzylidene camphor. Endocrinology 146, 2130–2139.

Felix, T., Hall, B.J., 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.

Fent, K., Escher, C., Caminada, D., 2006. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reprod. Toxicol. 22, 175–185.

Gaido, K.W., Leonard, L.S., Lovell, S., Gould, J.C., Babaï, D., Portier, C.J., McDonnel, D.P., 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol. Appl. Pharm. 143, 205–212.

Gangloff, M., Ruff, M., Eiler, S., Duclaud, S., Wurtz, J.M., Moras, D., 2001. Crystal structure of a mutant hERα ligand-binding domain reveals key structural features for the mechanism of partial agonism. J. Biol. Chem. 276, 15059–15065.

Hany, J., Nagel, R., 1995. Nachweis von UV-Filtersubstanzen in Muttermilch. Deut. Lebensm.-Rundsch. 91, 341–345.

Heneweer, M., Musse, M., Van den Berg, J., Sanderson, T., 2005. Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. Toxicol. Appl. Pharmacol. 208, 170–177.

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

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43–50.

Jensen, E.V., Khan, S.A., 2004. A two-site model for antiestrogen action. Mech. Ageing Dev. 125, 679–682.

Katzenellenbogen, J.A., O'Mallay, B.W., Katzenellenbogen, B.S., 1996. Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. Mol. Endocrin. 10, 119–131.

Kenakin, T., 2004. Principles: receptor theory in pharmacology. Trends Pharmacol. Sci. 186–192.

Kortenkamp, A., Altenburger, R., 1998. Synergisms with mixtures of xenoestrogens: a reevaluation using the method of isoboles. Sci. Total Environ. 221, 59–73.

Kunz, P.Y., Fent, K., 2006. Multiple hormonal activities of UV filters and comparison on in vitro and in vivo activity of ethyl 4-aminobenzoate. Aquat. Toxicol. 79, 305–324.

Kunz, P.Y., Gries, T., Fent, K., 2006a. The ultraviolet filter 3-benzylidene camphor adversely affects reproduction in fathead minnow *(Pimephales promelas)*. Toxicol. Sci. 93, 311–321.

Kunz, P.Y., Galicia, H.F., Fent, K., 2006b. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349–361.

Le Page, Y., Scholze, M., Kah, O., Pakdel, F., 2006. Assessment of xenoestrogens using three distinct estrogen receptors and the zebrafish

brain aromatase gene in a highly responsive glial cell system. Environ. Health Perspect. 114, 752–758.
Loewe, S., Muischnek, H., 1926. Über Kombinationswirkungen. Arch. Exp. Pathol. Phramakol. 114, 313–326.
Loraine, G.A., Pettigrove, M.E., 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. Environ. Sci. Technol. 40, 687–695.
McLachlan, J.A., 1997. Synergistic effect of environmental estrogens: report withdrawn. Science 277, 462–463.
Mueller, S.O., Kling, M., Firzani, P.A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., Kramer, P.J., 2003. Activation of estrogen receptor α and ERβ 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., Nagel, R., 1997. UV-Filtersubstanzen in Wasser und Fischen. UWSF-Z Umweltchem. Ökotoxikol. 9, 79–86.
Payne, J., Rajapakse, N., Wilkins, M., Kortenkamp, A., 2000. Prediction and assessment of effects of mixtures of four xenoestrogens. Environ. Health Perspect. 108, 983–987.
Payne, J., Scholze, M., Kortenkamp, A., 2001. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environ. Health Perspect. 109, 391–397.
Pike, A.C.W., Brzozowski, A.M., Hubbart, R.E., Bonn, T., Thorsell, A.G., Engström, O., Ljunggren, J., Gustafsson, J.A., Carquist, M., 1999. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full agonist. EMBO J. 18, 4608–4618.
Plagellat, C., Kupper, T., Furrer, R., de Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland. Chemospere 62, 915–925.
Rajapakse, N., Silva, E., Kortenkamp, A., 2002. Combining xenoestrogens at levels below individual no-observed effect concentrations dramatically enhances steroid hormone action. Environ. Health Perspect. 110, 917–921.
Ramamoorthy, K., Vyhlidal, C., Wang, F., Chen, I.-C., Safe, S., McDonnell, D.P., Leonard, L.S., Gaido, K.W., 1997. Additive estrogenic activities of a binary mixture of 2′,4′,6′-trichloro- and 2′,3′,4′,5′-tetrachloro-4-biphenylol. Toxicol. Appl. Pharmacol. 147, 93–100.
Routledge, E.J., Sumpter, J.P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15, 241–248.
Routledge, E.J., Sumpter, J.P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J. Biol. Chem. 272, 3280–3288.
Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. *In vitro* and *in vivo* estrogenicity of UV screens. Environ. Health Perspect. 109, 239–244.
Schreurs, R.H., Lanser, P., Seinen, W., Van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257–261.
Schultz, T.W., Seward, J.R., Sinks, G.D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301–304.
Seidlová-Wuttke, D., Jarry, H., Wuttke, W., 2004. Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. Toxicology 205, 103–112.
Sheehan, D.M., Willingham, E., Gaylor, D., Bergeron, J.M., Crews, D., 1999. No threshold dose for estradiol-induced sex reversal of turtle embryos: How little is too much? Environ. Health Persp. 107, 155–159.
Shekhar, P.V.M., Werdell, J., Basrur, V.S., 1997. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J. Natl. Cancer Inst. 89, 1774–1782.
Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing"-eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36, 1751–1756.
Stephenson, R.P., 1956. Modification of receptor theory. Brit. J. Pharmacol. Chemoth. 11, 379–393.
Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 15, 1142–1149.
Van der Hoeven, N., 1997. How to measure no effect: Part III. Statistical aspects of NOEC and ECx estimates. Environmetrics 8, 241–248.
Welshons, W.V., Thayer, K.A., Judy, B.M., Taylor, J.A., Curran, E.M., Vom Saal, F.S., 2003. Large effects from small exposures: I. Mechanisms for endocrine disrupting chemicals with estrogenic activity. Environ. Health Perspect. 111, 994–1006.
Weltje, L., Vom Saal, F.S., Oehlmann, J., 2005. Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. Hum. Exp. Toxicol. 24, 431–437.