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Short communication

Activation of estrogen receptor α and ERβ by 4-methylbenzylidene-camphor in human and rat cells: comparison with phyto- and xenoestrogens

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Abstract

4-Methylbenzylidene-camphor (4-MBC) is an organic sunscreen that protects against UV radiation and may therefore help in the prevention of skin cancer. Recent results on the estrogenicity of 4-MBC have raised concerns about a potential of 4-MBC to act as an endocrine disruptor. Here, we investigated the direct interaction of 4-MBC with estrogen receptor (ER) α and ERβ in a series of studies including receptor binding, ER transactivation and functional tests in human and rat cells. 4-MBC induced alkaline phosphatase activity, a surrogate marker for estrogenic activity, in human endometrial Ishikawa cells. Interestingly, 4-MBC induced weakly ERα and with a higher potency ERβ mediated transactivation in Ishikawa cells at doses more than 1 μM, but showed no distinct binding affinity to ERα or ERβ. In addition, 4-MBC was an effective antagonist for ERα and ERβ. In an attempt to put 4-MBC's estrogenic activity into perspective we compared binding affinity and potency to activate ER with phyto- and xenoestrogens. 4-MBC showed lower estrogenic potency than genistein, coumestrol, resveratrol, bisphenol A and also camphor. Analysis of a potential metabolic activation of 4-MBC that could account for 4-MBC's more distinct estrogenic effects observed in vivo revealed that no estrogenic metabolites of 4-MBC are formed in primary rat or human hepatocytes. In conclusion, we were able to show that 4-MBC is able to induce ERα and ERβ activity. However, for a hazard assessment of 4-MBC's estrogenic effects, the very high doses of 4-MBC required to elicit the reported effects, its anti-estrogenic properties as well as its low estrogenic potency compared to phytoestrogens and camphor has to be taken into account.

Keywords: ERα; ERβ; Phytoestrogens; Xenoestrogens; UV-filter; Ishikawa; Primary hepatocytes

1. Introduction

4-Methylbenzylidene-camphor (4-MBC) is an organic sunscreen that is used in several cosmetic

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products for the protection against UV radiation. During the past decades, the incidence of human UV-induced skin cancer has dramatically increased and UV filters are, therefore, widely used and accepted as efficient protection against prolonged sun exposure (Ziegler et al., 1994; Boyle et al., 1995). More recently, organic UV filters including 4-MBC have been investigated for their potential estrogenic activity (Bolt et al., 2001; Schlumpf et al., 2001; Schreurs et al., 2002; Tinwell et al., 2002) and first results on the estrogenicity of 4-MBC by Schlumpf et al. (2001) have raised concerns about the potential of 4-MBC to act as an endocrine disruptor.

Endocrine disruptors comprise a variety of structurally diverse endogenous and xenobiotic compounds that may lead to disruption of endocrine signaling and possibly to adverse health effects. Estrogenic action is mediated mainly through the estrogen receptors (ERα and ERβ), which function as ligand-inducible transcription factors for genes involved in cell growth, proliferation and differentiation (Mueller and Korach, 2001). Studies using knock-out mice for the two ER subtypes have revealed that each receptor plays a unique role in estrogen biology in a wide variety of tissues (Couse and Korach, 1999). Furthermore, in vitro studies indicated that ERα and ERβ display marked differences in binding affinity and activation by natural and synthetic ER ligands (Kuiper et al., 1997, 1998).

Recent studies on the estrogenic activity of 4-MBC in vitro and in the rat uterotrophic assay (Bolt et al., 2001; Schlumpf et al., 2001; Schreurs et al., 2002; Tinwell et al., 2002) revealed that 4-MBC has distinct activities in vivo (Schlumpf et al., 2001; Tinwell et al., 2002). In vitro, 4-MBC exerted no binding affinity for ERα and no significant activity in a yeast based ERα transactivation assay (Tinwell et al., 2002), although Schreurs et al. (2002) detected activation of ER at doses of 10 μM in transient transactivation assays in human kidney cells. In addition, 4-MBC showed a more pronounced activity to induce proliferation of human MCF7 cells when compared with its activity in other in vitro assays measuring direct interaction with ER (Schlumpf et al., 2001; Tinwell et al., 2002).

Although weak estrogenic compounds show usually greater activity in vitro than in vivo, the opposite was reported for 4-MBC. This prompted us to investigate in more detail the direct interaction of 4-MBC with ERα and ERβ in a series of tests including receptor binding, ER transactivation and functional tests in the estrogen responsive human endometrial Ishikawa cell line (Holinka et al., 1986b). In addition, we compared 4-MBC with the phytoestrogens genistein, coumestrol and resveratrol as well as the xenoestrogen bisphenol A (BPA) and camphor (Fig. 1) for its binding affinity to ERα and ERβ and its potency to activate or inhibit ERα and ERβ-mediated transcription. Assuming that the greater estrogenic effects observed in vivo may be due to a metabolite, we pursued this hypothesis by analysis of 4-MBC's induction of ERα or ERβ mediated activity in metabolically competent primary rat and human hepatocytes. Our studies comprehensively analyzed the direct interaction of 4-MBC with ERα and ERβ, a potential metabolic activation and compared its estrogenic potency with phyto- and xenoestrogens to permit a ranking of a relevant estrogenic potential of 4-MBC.

2. Materials and methods

2.1. Materials and biochemicals

Media, serum, supplements, enzymes and biochemicals were purchased from Sigma (Deisenhofen, Germany) unless otherwise stated. Ishikawa cells were obtained from ECACC (Order Number 99040201, Salisbury, UK). Steroid deprived dextran-coated charcoal stripped fetal bovine serum (DCC/FBS) was from Hyclone (Lot AKD11642A, Perbio Science, Bonn, Germany). Primary human hepatocytes were obtained from In Vitro Technologies (Order number F00995-P, Lot 130, Baltimore, MD).

2.2. Chemicals

Diethylstilbestrol (DES), 17β-estradiol (E_2), (1R)-(+)-camphor (99% purity), genistein, resveratrol, and coumestrol were from Sigma (Deisen-

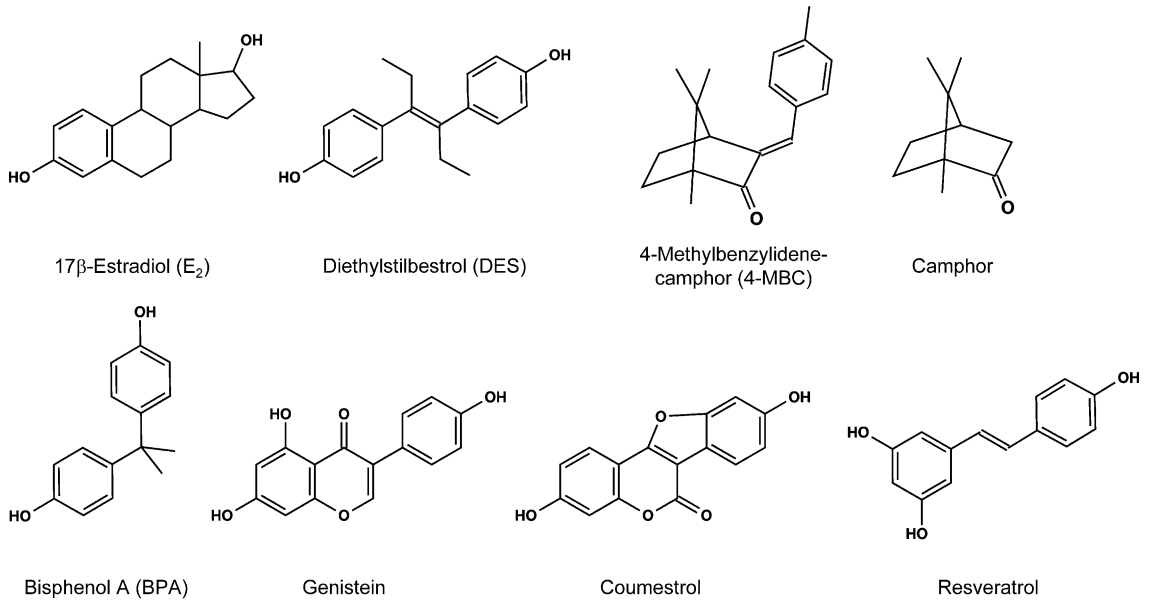


Fig. 1. Chemical structures of the investigated compounds.

hofen, Germany). BPA (99% purity) was purchased from Aldrich (Deisenhofen, Germany) and 3-(4-Methylbenzylidene) camphor (4-MBC, 99.9% purity) was from Merck KGaA (Darmstadt, Germany). ICI 182,780 (ICI) was from Tocris (Bristol, UK).

2.3. Cell culture

Ishikawa cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% FBS, 1 mM sodium pyruvate and antibiotics. Cells were cultured at 37 °C/5% CO_2 in air in a humidified atmosphere.

2.4. Isolation of primary hepatocytes from rat liver

Primary rat hepatocytes were prepared freshly from male Wistar rats (220–300 g) by the in situ perfusion procedure according to Seglen (Seglen, 1973, 1976). Livers were perfused with 0.5 mg/ml collagenase, the liver capsule removed and the released hepatocytes filtered through a 250 μm mesh followed by a second filtration step using a 100 μm mesh nitex membrane. The filtered hepatocytes were resuspended in phenol red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin. The cell number and viability of the suspension were assessed by trypan blue exclusion. Fifty microliter trypan blue was mixed with 50 μl cell suspension and the number of viable cells (cells that were not stained with trypan blue) were determined in a hemacytometer. Cell viability was always more than 80%. The cell suspensions were adjusted to 0.4×10^6 viable cells/ml using phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin. For better attachment and viability of primary hepatocytes 24-well plates used for transfection were coated with collagen I. One hundred and twenty microliter collagen I solution (100 μg/ml) was added to each well and the plates were dried under sterile conditions for 15 h. Five hundred microliter of the cell suspension was then dispensed per well in collagen-coated 24-well plates. For attachment, rat hepatocytes were cultivated at 37 °C/5% CO_2 in a humidified atmosphere for 4 h prior to transfection.

2.5. Thawing of cryopreserved human hepatocytes

Cryopreserved human hepatocytes were thawed according to the procedure provided by the manufacturer (In Vitro Technologies, Baltimore, MD). Briefly, two vials were rapidly thawed and poured into 40 ml of phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and centrifuged at $60 \times g$ for 5 min. The cell pellet was resuspended in 5 ml phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin. The cell number and viability was assessed as described for rat hepatocytes and was always more than 80%. 0.2×10^6 viable cells were dispensed per well in collagen-coated 24-well plates. For attachment, human hepatocytes were cultivated at 37 °C/5% CO_2 in a humidified atmosphere for 3 h prior to transfection.

2.6. Alkaline phosphatase assay

Forty-eight hours prior to alkaline phosphatase (AP) assay, Ishikawa cells were plated in 96-well plates in phenol-red free DMEM/F12 supplemented with 5% DCC/FBS. Forty-eight hour after plating, test compounds were added and AP activity measured after a 4-day treatment as described (Littlefield et al., 1990). Absorbance of control wells was set as 1. Increase over control was calculated and results were expressed as mean ± standard error mean from five independent experiments. The results were analyzed by the Levene's test (homogeneity of the variances) followed by Kruskall-Wallis and Wilcoxon non-parametric tests. Statistical analysis was performed using the SAS software version 6.12 (SAS, Cary, NC). Sigmoidal curve analysis of fold increase vs. log concentrations were performed using GRAPHPAD PRISM software version 3.0 (GraphPad, San Diego, CA) to determine the concentration of test compound that induced 50% of the maximal AP stimulation (EC_{50}).

2.7. Ligand-binding studies

Ligand binding was analyzed by competition of the test compound against 17β-[^{3}H]-estradiol ([^{3}H]-E_2, 71 Ci/mmol; NEN, Boston, MA). For each experiment, Ishikawa cells were rinsed twice with ice-cold phosphate-buffered saline. Then, they were scraped with a rubber cell lifter in presence of ice-cold buffer containing 10 mM Tris, 10 mM sodium molybdate, 1.5 mM ethylenediamine-tetraacetic acid, and 1 mM dithiothreitol (pH 7.4) and finally homogenized using a glass potter. The cell homogenates were centrifuged at $105\,000 \times g$ at 4 °C for 1 h. The supernatant, which represented the cytosolic fraction, was used for incubation with test compounds. Cytosolic fractions were incubated for 4.5 h at 25 °C with 2.5 nM [^{3}H]-E_2 and increasing concentrations of E_2 or 4-MBC. Unbound [^{3}H]-E_2 was removed by adding DCC (0.1% dextran, 1% charcoal). Samples were spun at $3000 \times g$ and the remaining radioactivity in the supernatant was measured in a scintillation counter (total binding, TB). Non-specific binding (NSB) was measured in the presence of 1000-fold excess of DES. Specific binding was calculated (TB-NSB) and set to 100%. Then, the percentage of specific binding for each tested compound concentration was calculated. Non-linear best fits between these percentages and the logarithm of concentrations were performed using GraphPad Prism version 3.0 (GraphPad, San Diego, CA, USA) to determine the E_2 concentration giving 50% of the specific binding (IC_{50}). The IC_{50} values were determined from five independent experiments and are given as mean ± standard error mean.

2.8. Transient transfection and transactivation assay

Ishikawa cells were seeded on 24-well 15 h prior to transfection in phenol-red free DMEM/F12 supplemented with 10% DCC/FBS. Primary hepatocytes were plated on collagen-coated 24-well plates in phenol-red free DMEM/F12 supplemented with 10% DCC/FBS and 5 μg/ml insulin as described. The plasmids were transfected in phenol-red free DMEM/F12 supplemented with 5% DCC/FBS using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's protocol. Each well received 0.5 μg of reporter plasmid and 0.01 μg pRL-CMV (*Renilla* luciferase for normalization; Promega, Madison, WI). A firefly

luciferase reporter driven by three copies of the vitellogenin estrogen response element (3xERE-Luc; kindly provided by D. McDonnell, Duke University, Durham, NC) was used to measure ER transcriptional activity (Norris et al., 1997). For cotransfection of ERα or ERβ with the luciferase reporter vector, 0.09 μg of mammalian expression plasmids for human ERα (hERα) or hERβ (pcDNA3-hERα or pcDNA3-hERβ, kindly provided by K.S. Korach, NIEHS, RTP, NC, USA), 0.4 μg 3xERE-Luc and 0.01 μg pRL-CMV normalization vector were used per well. After transfection, cells were incubated in medium supplemented with 10% DCC/FBS as described above with test compounds (final concentration of vehicle ethanol 1% v/v) for 20 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocols (Promega, Madison, WI). Each value was normalized to the luciferase normalization control and each data point obtained represents the average of duplicate determinations. All experiments were repeated at least three times. EC_{50} values (ligand concentration yielding half-maximal activation) were derived by non-linear curve-fitting using ORIGIN Software (Microcal Software, Northampton, MA) from transactivation curves and are given as mean ± standard deviation of at least three independent experiments.

2.9. Ligand binding studies by fluorescence polarization

Binding affinities to purified human ERα and hERβ were determined by competition binding of the test compounds against fluorescein-labeled estradiol (ES2) using an ER screening kit according to the manufacturer's protocol (Panvera, Göttingen, Germany). Ten nanomolar of recombinant human ERα or ERβ were incubated with the test compounds for 2 h in the presence of 0.5 nM ES2. Fluorescence polarization was measured using a Polarion multiwell plate reader (Tecan, Crailsheim, Germany). Ligand concentration yielding 50% inhibition of binding of fluorescently labeled E_2 to ER (IC_{50}) was derived by logarithmic curve-fitting using ORIGIN Software (Microcal Software, Northampton, MA) from competition binding curves. The IC_{50} were determined from at least three independent experiments and are given as mean ± standard deviation.

3. Results

3.1. 4-MBC shows weak estrogenic activities in human endometrial Ishikawa cells

We first examined the ability of 17β-estradiol (E_2) and 4-MBC to activate AP in human endometrial Ishikawa cells that express endogenous ER (Holinka et al., 1986b,a; Hata et al., 1992). Activation of AP in Ishikawa cells is a well-characterized surrogate marker for estrogenic activity (Littlefield et al., 1990). E_2 caused a distinct induction of AP activity at doses of 0.1 nM, whereas 4-MBC lacked any activity at doses up to 1 μM (Fig. 2A). At the highest dose of 10 μM, 4-MBC resulted in a significant induction of AP activity (2.8 ± 0.5-fold over solvent control, see Fig. 2A). Addition of the antiestrogen ICI 182,780 (ICI) (Wakeling and Bowler, 1992) completely inhibited AP activity induced by E_2 or 4-MBC confirming that the observed induction of AP is mediated by ER (Fig. 2B).

We then analyzed the binding affinity of 4-MBC to endogenous ER in Ishikawa cells. We determined the ligand concentration yielding 50% inhibition of specific binding of labeled E_2 to ER (IC_{50}) by non-linear curve-fitting from competition binding curves (Fig. 3). These experiments revealed that 4-MBC did not exhibit any significant binding affinity to ER up to the highest dose tested ($IC_{50} > 8.2$ μM) whereas the IC_{50} value of the positive control E_2 was 2.0 ± 0.2 nM (Fig. 3).

3.2. 4-MBC is a weak agonist but effective antagonist for ERα and ERβ

Since binding to the ER does not necessarily result in activation of the ER and AP activity measures an unspecific response, we analyzed the ability of 4-MBC and the potent estrogen DES (Fig. 1) to activate ERα or ERβ in Ishikawa cells. Transactivation studies were performed using mammalian expression plasmids for human ERα

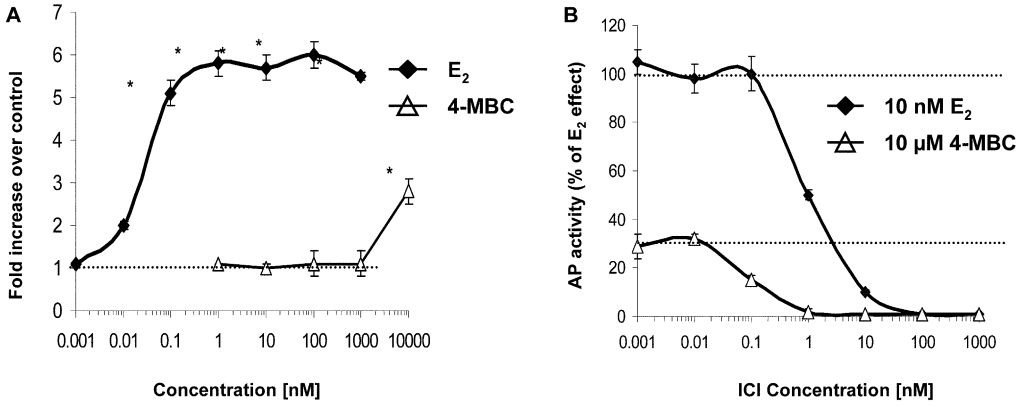


Fig. 2. Induction of AP activity in Ishikawa cells by 4-MBC and E_2. Ishikawa cells were treated for 4 days with E_2 (solid diamonds) or 4-MBC (white triangles) as indicated and the activity of AP was measured. (A) Ishikawa cells were treated with each compound at the indicated concentrations for 4 days. The dotted line indicates the value for the solvent control. (B) Ishikawa cells were treated with 10 nM E_2 or 10 μM 4-MBC in the presence of the indicated concentration of ICI for 4 days. The dotted line indicates the value for 10 nM E_2 or 10 μM 4-MBC, respectively. The induction by 10 nM E_2 was set as 100. Each value represents the mean ± standard error mean of five independent experiments. Asterisks indicate statistical significance ($P \leqslant 0.05$).

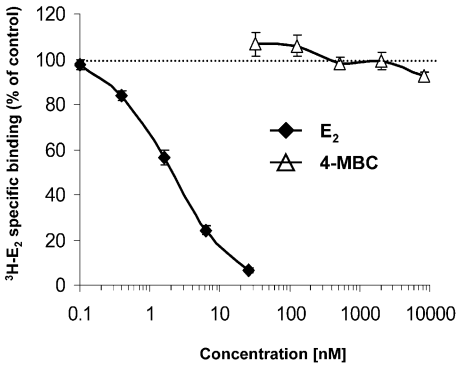


Fig. 3. ER-competition binding assay of 4-MBC and E_2 with cytosolic extracts of Ishikawa cells. Cytosolic extracts of Ishikawa cells were incubated with [^{3}H]-labeled E_2 and increasing amounts of unlabeled E_2 (solid diamonds) or 4-MBC (white triangles) as indicated. The dotted line indicates the value of the solvent control. Each value represents the mean ± standard error mean of five independent experiments.

or human ERβ and the 3xERE-Luc (containing 3 copies of the vitellogenin A2 ERE) reporter construct (Norris et al., 1997). Similar to the results obtained in the AP activity assay and receptor binding, no activity was observed up to a dose of 1 μM 4-MBC. However, in the dose range of 10 μM up to 150 μM (highest dose was limited due to cytotoxicity) a concentration-dependent increase in transactivation in parental Ishikawa cells (Fig. 4A) as well as in Ishikawa cells with additional transfected ERα (Fig. 4B) or ERβ (Fig. 4C) was observed. The parental Ishikawa cells express endogenous ER (Hata et al., 1992) and showed weak ligand-inducible transactivation of the 3xERE reporter by 4-MBC compared to DES (EC_{50} of DES 0.07 ± 0.02 nM vs. EC_{50} of 4-MBC > 150 μM; Fig. 4A). Very similar results were obtained when additional ERα was transiently transfected into Ishikawa cells (Fig. 4B, Table 1). Interestingly, 4-MBC showed a higher potency to activate ERβ compared to ERα on the 3xERE reporter in Ishikawa cells (EC_{50} > 150 μM for ERα vs. 57 ± 9 μM for ERβ; see Fig. 4C, Table 1). Overall, these results confirmed that 4-MBC has a weak potency to activate ERα and to a higher extent ERβ in Ishikawa cells.

To confirm that the measured activation of DES and 4-MBC is mediated by ERα or ERβ, we performed transactivation assays in the presence of the anti-estrogen ICI. Co-treatment of Ishikawa cells with ICI and 4-MBC or DES resulted in an abrogation of activity, thereby proving that the measured transactivation is mediated by ERα or ERβ, respectively (Fig. 5). In contrast to its weak agonistic activity on ERα and ERβ, 4-MBC effectively inhibited the activity of DES to induce ERα or ERβ in a dose-dependent fashion (Fig. 5).

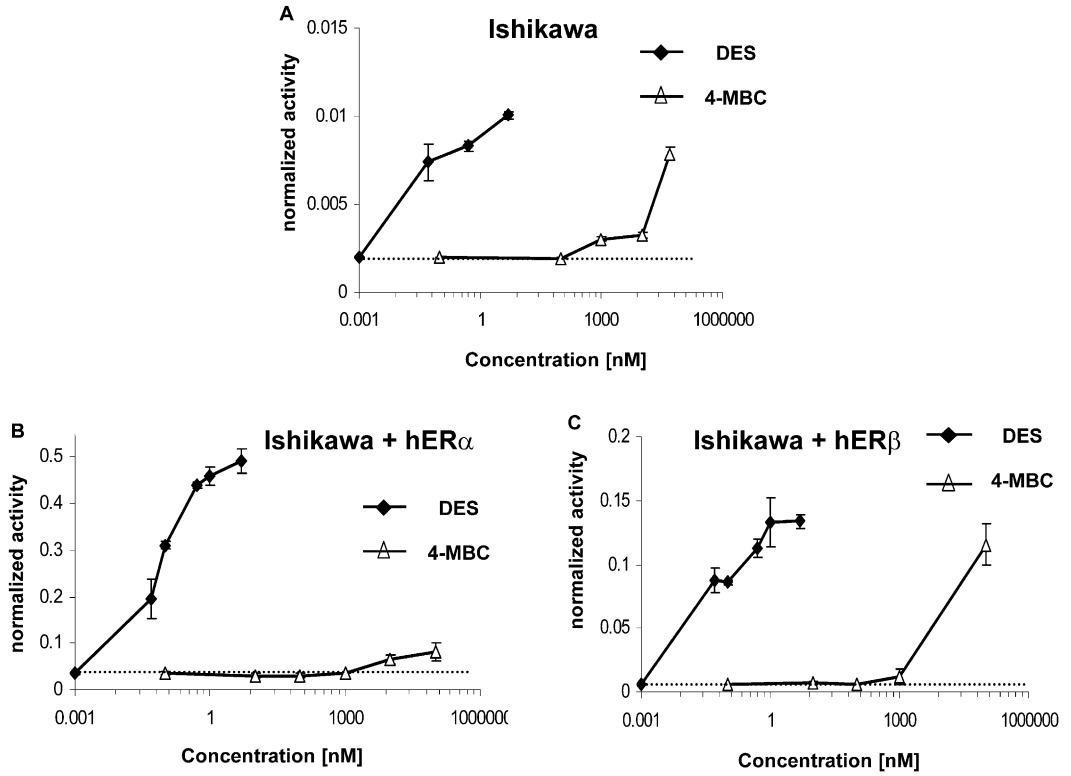


Fig. 4. Transactivation in Ishikawa cells. Cells were transiently transfected (A) with the 3xERE-Luc reporter and pRL-CMV normalization vector, (B) with mammalian expression vector for ERα (pcDNA3-hERα) along with 3xERE-Luc and pRL-CMV, or (C) with mammalian expression vector for ERβ (pcDNA3-hERβ) along with 3xERE-Luc and pRL-CMV. Following transfection, cells were treated with DES (solid diamonds) or 4-MBC (white triangles) at the indicated concentrations for 22 h. Each value was normalized to the internal luciferase control. The dotted line indicates the value of the solvent control. Each data point represents the average ± range of duplicate determinations. Experiments were repeated at least three times with reproducible results.

These results indicate that 4-MBC possesses also antagonistic properties with regard to ERα and ERβ.

3.3. 4-MBC has a low potency to activate ERα and ERβ compared to several phyto- and xenoestrogens

We showed with receptor binding studies, functional assays and ER transactivation assays that 4-MBC has a weak estrogenic potency. In an attempt to put a potential hazard of 4-MBC into perspective, we compared its activity with the phytoestrogens genistein, coumestrol and resveratrol (Mueller, 2002) as well as with the well characterized xenoestrogen BPA (BenJonathan and Steinmetz, 1998) and camphor, the latter sharing the basic structural unit with 4-MBC (Fig. 1). For a comparison of the potencies of these compounds to induce ER activity, we performed receptor binding studies using purified hERα and hERβ based on fluorescence polarization as well as transactivation assays in Ishikawa cells. Relative binding affinities (RBAs) and relative transactivational potencies for ERα and ERβ were determined and compared to the potent estrogen DES (Figs. 6 and 7, Table 1). As highlighted by Fig. 6, 4-MBC and camphor had no significant binding affinity to ERα or ERβ up to the highest dose tested ($IC_{50} > 3$ mM and 10 mM, respectively), in contrast to the phytoestrogens and BPA (Fig. 6 and Table 1). Likewise, activation of ERα and ERβ in Ishikawa cells occurred only at high concentrations of 4-MBC. The potency of 4-MBC to induce ERα or ERβ was more than 1000

Table 1
Comparison of RBAs in vitro and relative transactivational potencies of phyto-/xenoestrogens and 4-MBC to DES in Ishikawa cells

	ERα				ERβ			
	Binding		Transcriptional activation		Binding		Transcriptional activation	
	IC_{50}[a]	RBA[b]	EC_{50}[c]	Rel. potency[d]	IC_{50}[a]	RBA[b]	EC_{50}[c]	Rel. Potency[d]
DES	7.4±2.8 nM	100	0.08±0.02 nM	100	14±3.5 nM	100	0.2±0.2 nM	100
Coumestrol	21±9.6 nM	35	16±4 nM	0.5	9.1±2.0 nM	151	6.6±2.4 nM	3.0
Genistein	519±318 nM	1.4	25±10 nM	0.3	16±1.5 nM	88	6.3±2.4 nM	3.2
Resveratrol	0.9±0.8 μM	0.9	1.4±0.4 μM	0.006	0.7±0.3 μM	2.1	1.6±0.2 μM	0.01
BPA	4.9±2.3 μM	0.2	1.0±0.6 μM	0.008	1.2±0.3 μM	1.2	3.3±3.3 μM	0.007
Camphor	>10 mM	<0.0001	26±7.5 μM	0.0003	>10 mM	<0.0001	22±6.9 μM	0.0009
4-MBC	>3 mM	<0.0002	>150 μM	<0.00005	>3 mM	<0.0005	57±9 μM	0.0004

[a] IC_{50} values (ligand concentration yielding 50% inhibition of binding of fluorescein labeled E_2 to ER) were derived by non-linear curve-fitting from competition binding curves (Fig. 6) and are given as mean±standard deviation of at least three independent experiments.
[b] RBAs were calculated by 100 × IC_{50} (DES)/IC_{50} (test compound). DES was set to 100.
[c] EC_{50} values (ligand concentration yielding half-maximal activation) were derived by non-linear curve-fitting from transactivation curves obtained in Ishikawa cells (Figs. 4 and 7) and are given as mean±standard deviation of at least three independent experiments.
[d] Relative potency (Rel. potency) was calculated by 100 × EC_{50} (DES)/EC_{50} (test compound). DES was set to 100.

times lower than that of genistein or coumestrol and even lower than that of camphor (Table 1 and Fig. 7).

3.4. 4-MBC shows no increased activity to induce ERα or ERβ in metabolically competent primary rat and human hepatocytes

Although we and others have provided ample evidence that 4-MBC, although acting through ERα or ERβ, has low estrogenic activity in vitro, several groups have shown distinct estrogenic activity in the uterotrophic assay in immature rats (Schlumpf et al., 2001; Tinwell et al., 2002). One major drawback of in vitro studies is the lack of metabolic activation. In our studies we used Ishikawa cells that do not have any capacity to metabolize xenobiotics. One possible explanation for the apparent higher activity of 4-MBC in the uterotrophic assay compared to the in vitro results could be due to metabolites of 4-MBC formed in rats that have a higher estrogenic potency. We therefore performed transactivation assays in metabolically competent rat and human primary hepatocytes. Cryopreserved human hepatocytes were obtained commercially, which are certified for metabolic competence. Since cryopreserved human hepatocytes maintain their metabolic capacity to a large extent at least for 24 h after thawing, we performed transactivation assays within this time period. Primary rat hepatocytes were prepared freshly according to standard procedures (Seglen, 1973, 1976). Activity of various cytochrome P450 enzymes of freshly prepared primary hepatocytes was checked by measuring methoxy-(MROD), ethoxy-(EROD), benzyloxy-(BROD) and pentoxy-(PROD) resorufin activity (Burke and Mayer, 1974; Burke et al., 1985; Lubinski et al., 1994) and testosterone metabolism according to standard procedures (Sonderfan et al., 1987). Only hepatocytes that displayed metabolic activities were used for the described studies (mean activities±standard deviation of three preparations of rat heptocytes 48 h in culture in nmol per min per mg protein: MROD 2.0±0.4; EROD 7.0±1.5; BROD 1.0±0.3; PROD 1.8±0.4; 6β and 16β-testosterone hydroxylation: 39±4 and 2.7±1.3, respectively).

4-MBC and DES were analyzed for their potency to induce ERα or ERβ activity in primary hepatocytes by transient transfection of these cells with mammalian expression plasmids for human ERα or ERβ along with the 3xERE reporter (Fig. 8). DES displayed a high potency to induce ERα

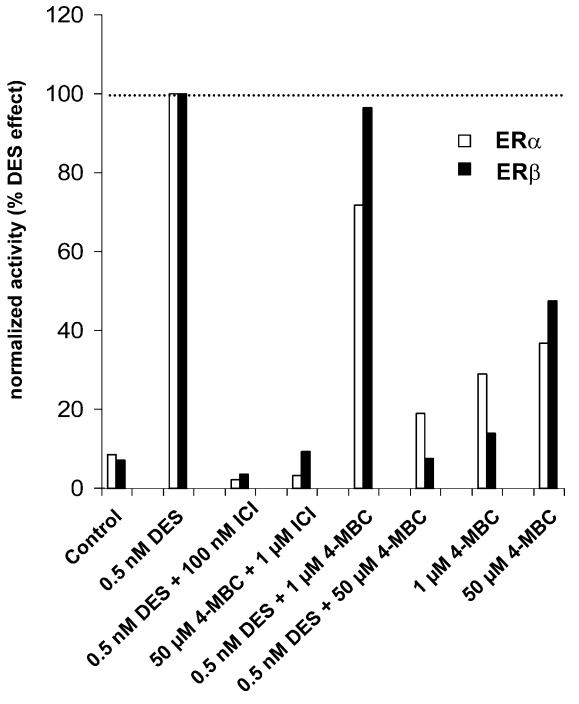


Fig. 5. Transactivation in Ishikawa cells. Antagonistic activity of 4-MBC. Cells were transiently transfected with mammalian expression vector for ERα (white columns) or ERβ (black columns) along with 3xERE-Luc and pRL-CMV. Following transfection, cells were treated with test compounds as indicated for 22 h. Each value was normalized to the internal luciferase control. The dotted line indicates the relative value of the positive control DES (set to 100%). Each data point represents the average of duplicate determinations. Experiments were repeated at least three times with reproducible results.

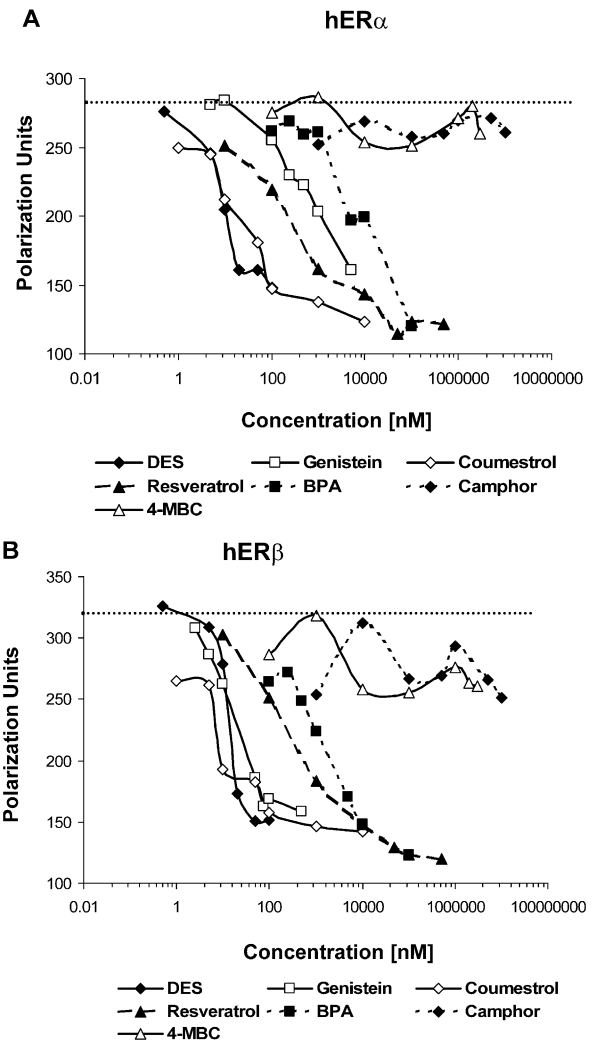


Fig. 6. ER-competition binding assay of 4-MBC, DES and phyto-/xenoestrogens using fluorescence polarization. Ten nanomolar purified human ERα (A) or human ERβ (B) were incubated with 0.5 nM fluorescein labeled E_2 (ES2) and increasing amounts of unlabeled test compound as indicated for 2 h and then fluorescence polarization was measured. High polarization values indicate binding of fluorescein labeled E_2 to the ER, low polarization values indicate free, unbound fluorescein labeled E_2. The dotted line indicates the value of the solvent control. Each data point represents the average of duplicate determinations. Experiments were repeated at least three times with reproducible results.

and ERβ activity in primary hepatocytes proving that metabolically competent primary hepatocytes are a suitable model system to test compounds for their estrogenicity. 4-MBC showed no increased estrogenic potency in rat or human hepatocytes compared to Ishikawa cells ($EC_{50} > 200$ µM for ERα and 73 ± 22 µM for ERβ in primary hepatocytes; compare Fig. 8 with Fig. 4), indicating that neither rat nor human hepatocytes were able to transform 4-MBC into an estrogenic metabolite.

4. Discussion

In recent publications, several UV filters including 4-MBC were investigated for their potential estrogenic activity in rat uterotrophic assays and also in vitro (Schlumpf et al., 2001; Schreurs et al., 2002; Tinwell et al., 2002). In most studies on the

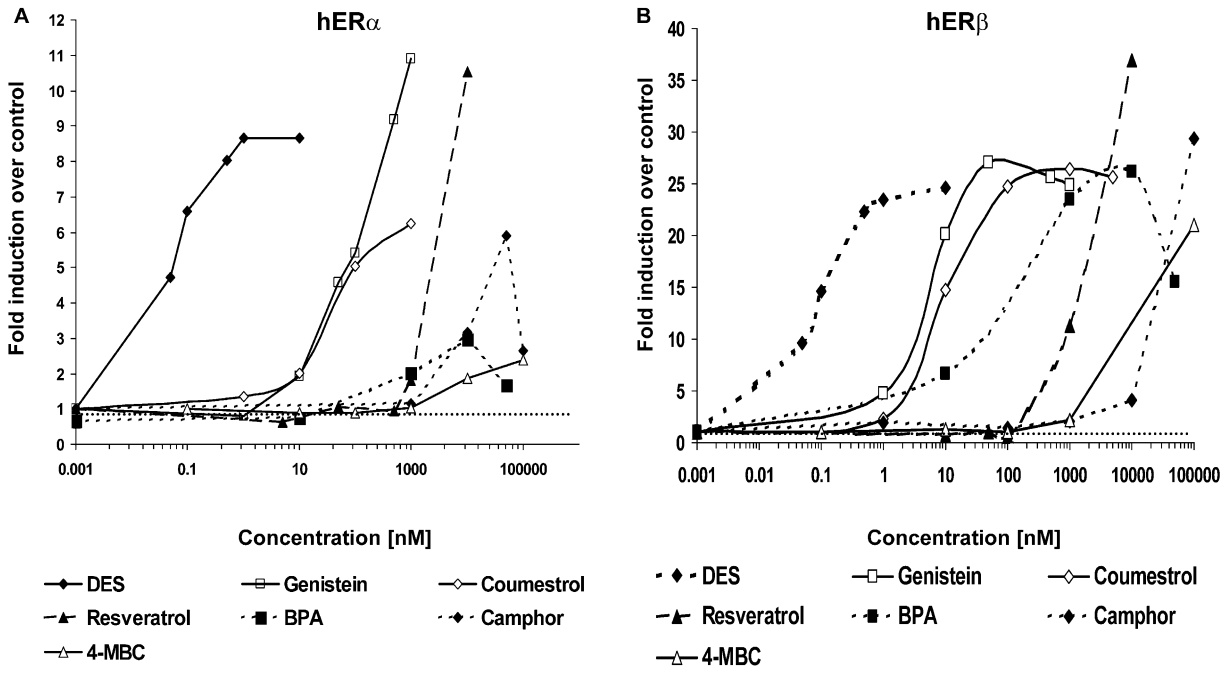
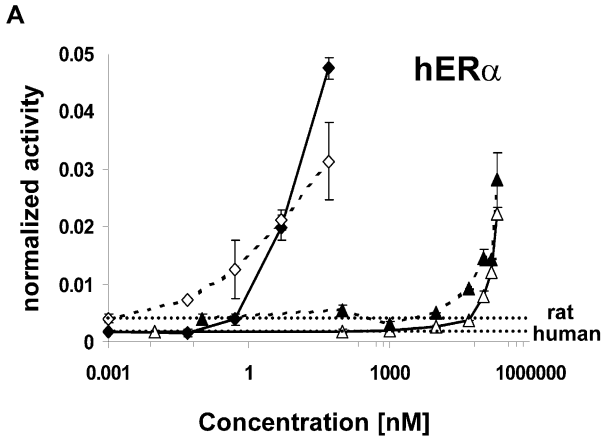


Fig. 7. Transactivation of ERα and ERβ by phyto-/xenoestrogens in Ishikawa cells. Cells were transiently transfected with 3xERE-Luc and pRL-CMV along with mammalian expression vector for (A) ERα or (B) ERβ. Following transfection, cells were treated with each compound at the indicated concentrations for 22 h. Each value was normalized to the internal luciferase control and results are expressed as fold induction compared to the solvent control. The dotted line indicates the value of the solvent control (set to 1). Each data point represents the average of duplicate determinations. Experiments were repeated at least three times with reproducible results.

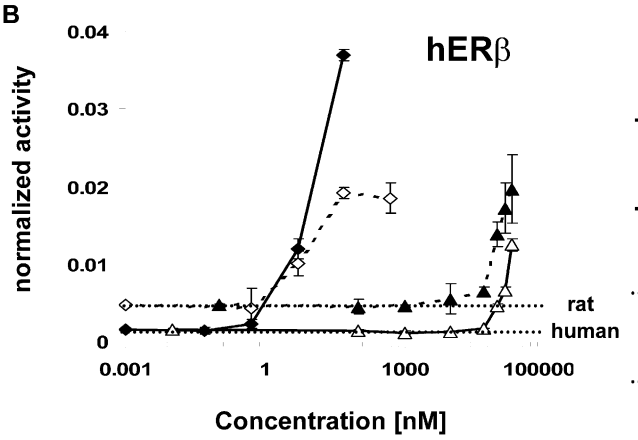
direct interaction with ER (ER binding, receptor activation in yeast or zebrafish (Schreurs et al., 2002; Tinwell et al., 2002)) the activity of 4-MBC was equivocal or very weak. In cell proliferation studies in MCF7 cells as marker for estrogenicity, 4-MBC showed a distinct effect (Schlumpf et al., 2001; Tinwell et al., 2002). Thus, 4-MBC showed apparent differences to elicit rather unspecific estrogenic effects as observed in the proliferation assay compared to its direct interaction with ER as determined in ER binding studies. We aimed, therefore, to investigate the extent of a direct interaction of 4-MBC with ERα and also with ERβ in a series of test systems covering receptor binding, transactivation of ERα and ERβ and functional assays (Mueller, 2002).

4-MBC showed weak but distinct activation of AP in Ishikawa cells at doses of 10 μM. This result confirmed the positive effects of 4-MBC on proliferation in MCF-7 cells, although in the latter test system 4-MBC showed significant activity already at doses of 0.1 μM (Schlumpf et al., 2001; Tinwell et al., 2002). Since these effects correlate with estrogenicity but do not necessarily require ER action, we performed the AP assay in the presence of the antiestrogen ICI (Wakeling and Bowler, 1992). ICI dose-dependently inhibited AP activity induced by 4-MBC proving that the measured effects are at least in part mediated by the ER. However, receptor binding studies did not show any significant binding affinity of 4-MBC to either ERα or ERβ.

Although our results support a weak interaction of 4-MBC with the ER, no definite activation of 4-MBC of either ERα or ERβ has been shown. Schreurs et al. (2002) were first to show that 4-MBC induces ERα and ERβ activity in kidney cells at doses of 10 μM and higher. Here, we also showed that 4-MBC is able to induce ERα and to a higher extent ERβ in endometrial Ishikawa cells. In the published study no potency or efficacy was reported (Schreurs et al., 2002). In the study



	EC_{50}	Rel. Potency
DES human hep.	9.5 ± 0.1 nM	100
4-MBC human hep.	> 200 μM	< 0.005
DES rat hep.	8.7 ± 0.9 nM	100
4-MBC rat hep.	> 200 μM	< 0.004



	EC_{50}	Rel. Potency
DES human hep.	6.9 ± 4.7 nM	100
4-MBC human hep.	> 200 μM	< 0.004
DES rat hep.	11 ± 5.9 nM	100
4-MBC rat hep.	73 ± 22 μM	0.015

Fig. 8. Transactivation in primary human and rat hepatocytes. Cells were transiently transfected with 3xERE-Luc and pRL-CMV along with mammalian expression vector for (A) ERα or (B) ERβ. Following transfection, human (solid lines) or rat (dotted lines) hepatocytes (hep.) were treated with DES (diamonds) or 4-MBC (triangles) at the indicated concentrations for 22 h. Each value was normalized to the internal luciferase control. The dotted line indicates the value of the solvent control. Each data point represents the average ± range of duplicate determinations. Experiments were performed twice with reproducible results. EC_{50} values (ligand concentration yielding half-maximal activation) were derived by non-linear curve-fitting from transactivation curves as shown and are given as average ± range of two independent experiments on the right. Relative (Rel.) potency was calculated by $100 \times EC_{50}$ (DES)/EC_{50} (test compounds). DES was set to 100.

presented here, we determined the potency of 4-MBC to transactivate ERα and ERβ and showed that 4-MBC exhibits a more than 2 000 000-fold and 250 000-fold lower potency than DES to induce ERα and ERβ activity, respectively. Interestingly, 4-MBC is a more potent agonist for ERβ compared to ERα and, moreover, 4-MBC showed also distinct antagonistic properties on ERα and ERβ. These mixed agonist/antagonist properties have been reported also for other weakly estrogenic compounds like resveratrol (Bowers et al., 2000).

The ranking of 4-MBC to bind to and to induce ERα or ERβ activity (Table 1) clearly demonstrated that the potential estrogenic hazard of 4-MBC is markedly less than that of the phytoestrogens coumestrol and genistein and also lower than that of the xenoestrogen BPA. Similar to the phytoestrogens genistein and coumestrol, 4-MBC preferentially activated ERβ (Kuiper et al., 1998).

Interestingly, 4-MBC had also lower or at most equal binding affinity and transactivational potency for ERα and ERβ compared to camphor, which is the basic structural unit of 4-MBC and is considered to lack any relevant endocrine properties. Tinwell et al. (2002) also investigated the activity of camphor in a yeast ERα transactivation assays and reported that camphor lacked any activity, whereas 4-MBC showed some, equivocal activity. Taken together, the assays analyzing direct interaction of 4-MBC with ERα and ERβ showed extremely weak to equivocal activity compared to phytoestrogens and camphor.

The fact that 4-MBC was unequivocally active in vivo as shown in rat uterotrophic assays (Schlumpf et al., 2001; Tinwell et al., 2002) in contrast to its equivocal or weak direct interaction with the ER (data reported here and Schreurs et al., 2002; Tinwell et al., 2002) intrigued us, since weakly estrogenic compounds in vivo exert usually more pronounced effects in vitro. One possible reason for this discrepancy could be that 4-MBC is metabolized in vivo to more estrogenic intermediates that elicit the effects observed. However, our results obtained in rat and human hepatocytes did not indicate any metabolic activation of 4-MBC to more estrogenic intermediates that could explain the more pronounced effects observed in vivo.

Since we found no evidence for a possible metabolic activation of 4-MBC to estrogenic intermediates, non-estrogenic effects may be responsible for the more pronounced effects seen in uterotrophic assays. Since thyroid hormones are known to enhance uterotrophic effects by endogenous hormones and to mimic estrogens in vitro (Gardner et al., 1978; Nogueira and Brentani, 1996) and a thyroid hormone imbalance has been reported for 4-MBC (SCCNFP, 1998), such a non-ER pathway may explain the estrogenic activity of 4-MBC observed in vivo. Therefore, for a better understanding of 4-MBC's endocrine activity non-ER mediated mechanisms should also be investigated.

In conclusion, the results presented here agree with previously published studies (Schlumpf et al., 2001; Schreurs et al., 2002; Tinwell et al., 2002) and established that 4-MBC is able to interact directly with ERα, to a higher extent with ERβ and to elicit weak estrogenic effects in vitro. Given the very high doses of 4-MBC required to induce estrogenic effects, its anti-estrogenic properties as well as its low potency compared to phytoestrogens, 4-MBC is unlikely to induce adverse estrogenic effects in humans or wildlife.

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References

BenJonathan, N., Steinmetz, R., 1998. Xenoestrogens: the emerging story of bisphenol A. Trends Endocrinol. Metab. 9, 124–128.

Bolt, H.M., Guhe, C., Degen, G.H., 2001. Comments on "In vitro and in vivo estrogenicity of UV screens". Environ. Health Perspect. 109, A358–A361.

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

Boyle, P., Maisonneuve, P., Dore, J.F., 1995. Epidemiology of malignant melanoma. Br. Med. Bull. 51, 523–547.

Burke, M.D., Mayer, R.T., 1974. Etoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2, 583–588.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer, R.T., 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologoues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem. Pharmacol. 34, 3337–3345.

Couse, J.F., Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine. Rev. 20, 358–417.

Gardner, R.M., Kirkland, J.L., Ireland, J.S., Stancel, G.M., 1978. Regulation of the uterine response to estrogen by thyroid hormone. Endocrinology 103, 1164–1172.

Hata, H., Kuramoto, H., Holinka, C.F., Pahuja, S.L., Hochberg, R.B., Gurpide, E., Gravanis, A., 1992. Immunocytochemical determination of estrogen and progesterone receptors in human endometrial adenocarcinoma cells (Ishikawa cells). J. Steroid Biochem. Mol. Biol. 42, 201–210.

Holinka, C.F., Hata, H., Kuramoto, H., Gurpide, E., 1986a. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). Cancer. Res. 46, 2771–2774.

Holinka, C.F., Hata, H., Kuramoto, H., Gurpide, E., 1986b. Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa line). J. Steroid. Biochem. 25, 85–89.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–4263.

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J., 1997. Comparison of the ligand binding specifity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.

Littlefield, B.A., Gurpide, E., Markiewicz, L., Mac Kinley, B., Hochberg, B., 1990. A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of D5 adrenal steroids. Endocrinology 127, 2757–2762.

Lubinski, J., Flint, O.P., Durham, S.K., 1994. In vivo and in vitro studies of rat liver cytochrome P450 induction: 2. In vitro induction by phenobarbital and 3-methylcholanthrene measured in an automated 24-well plate assay for cytochrome P450-dependent activity (PROD and EROD). In Vitro Toxicol. 7, 13–23.

Mueller, S.O., 2002. Overview of in vitro tools to assess the estrogenic and antiestrogenic activity of phytoestrogens. J. Chromatogr. B 777, 155–165.

Mueller, S.O., Korach, K.S., 2001. Mechanisms of estrogen receptor-mediated agonistic and antagonistic effects. In: Metzler, M. (Ed.), Endocrine Disruptors. Springer, Berlin, pp. 1–25.

Nogueira, C.R., Brentani, M.M., 1996. Triiodothyronine mimics the effects of estrogen in breast cancer cell lines. J. Steroid Biochem. Mol. Biol. 59, 271–279.

Norris, J.D., Fan, D., Kerner, S.A., McDonnell, D.P., 1997. Identification of a third autonomous activation domain within the human estrogen receptor. Mol. Endocrinol. 11, 747–754.

SCCNFP, 1998. Opinion of the scientific committee on cosmetic products and non-food products intended for consumers concerning 3-(4′-methylbenzylidene)-D,L-camphor adopted by the plenary session of the SCCNFP of January 21, 1998.

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

Seglen, P.O., 1973. Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. Exp. Cell. Res. 82, 391–398.

Seglen, P.O., 1976. Preparation of isolated rat liver cells. Methods Cell Biol. 13, 29–83.

Sonderfan, A.J., Arlotto, M.P., Dutton, D.R., McMillen, S.K., Parkinson, A., 1987. Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. Arch. Biochem. Biophys. 255, 27–41.

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

Wakeling, A.E., Bowler, J., 1992. ICI 182,780, a new antioestrogen with clinical potential. J. Steroid Biochem. Mol. Biol. 43, 173–177.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T., Brash, D.E., 1994. Sunburn and p53 in the onset of skin cancer. Nature 372, 773–776.