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Estrogenic activity and estrogen receptor β binding of the UV filter 3-benzylidene camphor Comparison with 4-methylbenzylidene camphor

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Abstract

UV filters represent new classes of estrogenic [Environ. Health Perspect. 109 (2001) 239] or antiandrogenic [Toxicol. Sci. 74 (2003) 43] chemicals. We tested 3-benzylidene camphor (3-BC), reported as estrogenic in fish [Pharmacol. Toxicol. 91 (2002) 204], and mammalian systems in comparison to 4-methylbenzylidene camphor (4-MBC), shown to be active in rats, and analyzed binding to estrogen receptor subtypes. 3-BC and 4-MBC stimulated MCF-7 cell proliferation (EC_{50}: 0.68 and 3.9 μM). The uterotrophic assay of 3-BC (oral gavage) in immature rats showed unexpected potency with ED50 45.3 mg/kg per day; lowest effective dose 2 mg/kg per day, and maximum effect with 70% of ethinylestradiol. After comparing with literature data, we found that the oral 3-BC was considerably more potent than oral bisphenol A and almost as active as subcutaneous genistein. 3-BC and 4-MBC displaced 16α^{125}I-estradiol from porcine uterine cytosolic receptors (IC_{50}: 14.5 and 112 μM), and from recombinant human estrogen receptor β (hERβ) (IC_{50}: 3-BC, 11.8 μM; 4-MBC, 35.3 μM), whereas no displacement was detected at human estrogen receptor α (hERα) up to 3 mM. This subtype selectivity makes the two camphor derivatives interesting model compounds. Their activity on immature rat uterus is not easily explained by ERβ activation. It cannot be excluded that active metabolites with possibly different receptor binding characteristics are formed in vivo.

Keywords: 3-Benzylidene camphor (3-BC); 4-Methylbenzylidene camphor (4-MBC); Estrogen receptor α; Estrogen receptor β; MCF-7 cells; Uterotrophic assay

1. Introduction

UV filters used in sun protection products and other cosmetics are of interest in human toxicology and ecotoxicology because these lipophilic high production volume chemicals are released into the environment and reach animals via the food chain and humans via a dual exposure: the food chain and the direct applications (Hany and Nagel, 1995; Nagtegaal et al., 1997). Several frequently used UV filters have been identified as estrogenic (Schlumpf et al., 2001,2003) or antiandrogenic (Ma et al., 2003). They

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represent new classes of endocrine active chemicals. 4-Methylbenzylidene camphor (4-MBC) exhibited the highest in vivo activity of the first series of UV filters studied (Schlumpf et al., 2001). Its estrogenic activity has meanwhile been confirmed in several in vitro (Schreurs et al., 2002; Mueller et al., 2003) and in vivo systems (Tinwell et al., 2002). A closely related camphor derivative, 3-benzylidene camphor (3-BC), was first reported to be estrogenic in fish (Holbech et al., 2002). We tested 3-BC on mammalian in vitro and in vivo systems for estrogenic activity in comparison with 4-MBC and steroidal estrogens. In addition, we analyzed binding of the two camphor derivatives to a cytosolic uterine estrogen receptor (ER) preparation and to recombinant human estrogen receptor α (hERα) and estrogen receptor β (hERβ) using iodinated estradiol as radioligand. Binding of 4-MBC to ER has so far not been demonstrated unequivocally and has remained a point of controversy (Tinwell et al., 2002; Mueller et al., 2003), whereas binding of 3-BC has not yet been studied.

We now report a remarkable estrogenic activity of 3-BC in an acute mammalian in vivo test system, the uterotrophic assay in immature rats, and we provide data indicating that both the camphor derivatives—3-BC and 4-MBC—are selective ERβ ligands.

2. Material and methods

2.1. Chemicals

3-Benzylidene camphor (CAS No. 15087-24-8, molecular weight 240.0, purity 100%) was purchased from Induchem AG (CH-8604 Volketswil, Switzerland), 4-methylbenzylidene camphor (3-(4-methylbenzylidene) camphor, Eusolex 6300, CAS No. 36861-47-9, molecular weight 254.37, purity 99.7–99.9%) from Merck (Darmstadt, Germany), and estradiol-17β and ethinylestradiol-17α from Calbiochem (Lucerne, Switzerland).

2.2. Estrogenic activity in vitro (E-SCREEN)

MCF-7 human breast cancer cells, kindly provided by Soto (Tufts University, Boston, USA) were frozen every 10 passages; samples from frozen stock were used for a maximum of 6–13 passages. Mycoplasma checks were regularly done (Institute of Virology, Veterinary Faculty, University of Zurich). Cells were cultured in Dulbeccos-Modified Eagles Medium (DME) with phenol red (Cat. No. 22320-022, GIBCo, Life Technologies, Basel, Switzerland) supplemented with 5% heat-inactivated (56 °C, 30 min) fetal calf serum (FCS, Cat. No. 10081-164, GIBCo), 2 nM L-glutamin (Cat. No. 25030-024, GIBCo) in 25 cm^2 canted neck tissue culture flasks (Cat. No. 2500105, Falcon, Oxnard, CA) in 5% CO_2/95% air, saturated humidity, at 37 °C.

The E-SCREEN (Soto et al., 1995) was performed as previously described (Schlumpf et al., 2001), but 96-well plates were used instead of 24-well plates. This allowed to study a greater number of different treatments per plate. Briefly, MCF-7 cells were trypsinized, plated into 96-well plates (No. 3595, Costar, Corning, NY) at an initial density of 3000 cells per well in 100 μl experimental medium and allowed to attach at 37 °C. Experimental medium consisted of 500 ml phenol red-free DME (Cat. No. 11880-028, GIBCo), 50 ml charcoal–dextran-treated (steroid hormone free) FCS (CD-FCS), 10 ml HEPES (1 M) (Cat. No, 15630-056, GIBCo), 10 ml L-glutamin (200 mM) (Cat. No. 25030-024, GIBCo). After 24 h, another 100 μl experimental medium containing either 3-benzylidene camphor (stock solution:10^{-2} M; final concentrations 10^{-5} to 10^{-9} M; final ethanol concentrations between 0.1 and 0.00001% (v/v)), 4-methyl-benzylidene camphor (10^{-4} to 10^{-8} M, ethanol concentrations between 1.0 and 0.0001%), or estradiol-17β (positive control, final concentrations 10^{-8} to 10^{-13} M; ethanol concentrations $\leq$0.0001% (v/v)). One 96-well plate was used for nine different concentrations of one chemical (one column (six wells)/concentration); columns 1, 7, and 12 were filled with experimental medium alone. Column 7 served as negative control, the two outer columns 1 and 12 were discarded. No difference in proliferation rate was seen between control experiments with chemical free medium or with medium containing ethanol up to 1%. Five independent experiments (with two plates per experiment) were run, each simultaneously with 3-BC, 4-MBC, and estradiol-17β as positive control.

Experiments were terminated after 6 days of incubation by removing the media from the wells. Cells were fixed for 30 min with 80 μl cold 10% (w/v)

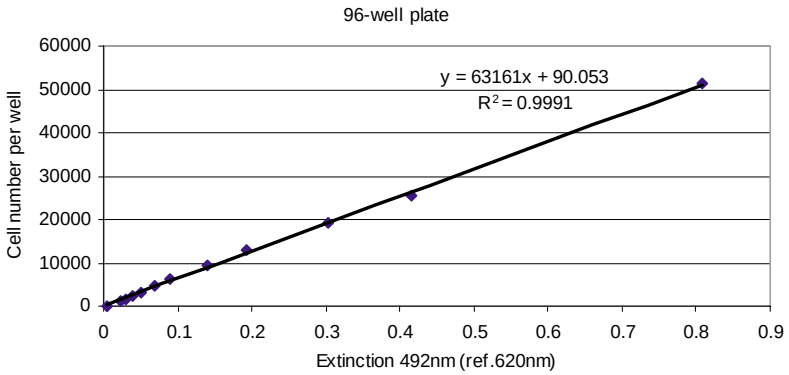

Fig. 1. Relationship between cell number and optical density (extinction at 492 nm, reference at 620 nm) in 96-well cell culture plates with the sulforhodamine B (SRB) staining method. Mean of four independent experiments.

tricholoroacetic acid (TCA, Merck) at 4 °C, washed gently three times under a slight stream of tap water and dried at 35–40 °C. Staining was performed by adding 70 μl sulforhodamine B (0.4% (w/v) SRB in 1% acetic acid/99% distilled water) (Sigma Chemical Co., St. Louis, MO, USA) for 15 min at room temperature. The supernatant was discarded and the plates were thoroughly rinsed several times with 1% acetic acid until the washing solution became colorless. After the plates were dry, the stained cells were dissolved in 100 μl Tris buffer (pH 10.6) per well.

Optical density (OD) was measured with a microplate reader (Anthos Reader 2001, Anthos Labetec instruments) at an extinction wavelength of 492 nm (reference 620 nm). A linear relationship between extinction at 492 nm and cell number was demonstrated for OD values between 0.0 and 0.8 (Fig. 1, $r = 0.9991$), with increasing numbers of cells (0–51, 200 per well, 12 different levels) seeded into 96-well plates with DMEM without phenol red, cultivated for 36 h and read according to the SRB method. For 96-well plates, the equation is cell number $= 90.053 + 63.161 \times \text{OD}$.

2.3. Estrogen receptor ligand binding assay (ER-LBA)

Subtype-specific estrogen receptor α (ERα) and estrogen receptor β (ERβ) LBAs were performed according to the recently described method of Kuiper et al. (1997), with the exception that bound and free tracer was separated by adsorption on dextran-coated charcoal (Jarry et al., 2003). Recombinant human ERα and ERβ were obtained from Panvera (Madison, USA). The radioligand 16α^{125}I-estradiol (2200 Ci/mmol) was purchased from New England Nuclear (Dreieich, Germany). Recombinant human ERα and ERβ were obtained from Panvera (Madison, USA). All other chemicals were purchased from Sigma (Deisenhofen, Germany). For the ER-LBA with uterine cytosol, porcine uteri were collected from the local slaughterhouse. With minor modifications, the method of Jarry et al. (1985) was employed to prepare a cytosolic fraction.

The ER binding buffer used for dilution of the receptor preparations, the uterine cytosol, and the radioligand consisted of 25 mM Tris–Cl (pH 7.4), 1.5 mM EDTA, 10% glycerol, and 1 mg/ml bovine serum albumin. The charcoal buffer was prepared with 25 mM Tris–Cl (pH 7.4), 1.5 mM EDTA, 5 mg/ml dextran T70 and 5 mg/ml charcoal. The reaction mixture contained 5 μl of test sample in 70% EtOH, 40 μl ER solution (0.5 pmol of either human recombinant ERα or ERβ), 45 μl tracer solution (adjusted to 20,000 cpm) and 350 μl of ER binding buffer. The volumes of the reagents ER-LBA with uterine cytosol were 5 μl of test sample in 70% ethanol, 100 μl tracer solution, and 150 μl cytosol. The incubations were carried out at 4 °C for 12 h, then 500 μl of cold charcoal buffer was added, and the tubes were incubated on ice for 15 min after vortexing them every 5 min. Finally, tubes were centrifuged at 2000 × *g* for 25 min at 4 °C. The supernatant was transferred in a new tube. Radioactivity was measured in a gamma counter.

2.4. Estrogenic activity in vivo (uterotrophic assay in immature rats)

Long Evans (LE) rats were purchased from Centre d'Elevage R. Janvier (F-53940 Le Genest-St. Isle, France). Litters (9–10 pups) together with their mothers were shipped to Zurich 1–2 weeks before the experiment, with date of birth indicated on the transport cages. In our animal facility, dams and their pups were kept under controlled conditions (lights on 02.00–16.00, 22 ± 1 °C) with standard diet 3430 (Provimi Kliba AG, Kauseraugst, Switzerland) and water ad libitum. Microbiological checks are performed every 3 months by the Institute of Laboratory the Animal Science of the University of Zurich which runs the animal facility. On postnatal day (PN) 20 (day of birth = PN 1), the pups were weaned. Five to six littermates, all the females and, if necessary, 1–2 males to adjust to the desired number, were transferred in their home cage to a Tecniplast-ventilated storage cabinet for rat cages (Indulab AG, CH-9473 Gams, Switzerland). This cabinet was located in the experimental room, where the animals were again kept under the same light cycle and temperature conditions.

3-Benzylidene camphor or ethinylestradiol-17α was administered on postnatal days 21, 22, and 23 (days 20, 21, and 22 of the OECD protocol). This time the window was well outside the puberty-induced rise in uterine weight, which in LE rats of our colony starts at PN 27 (Schlumpf et al., 2001). The chemicals were dissolved in olive oil at different concentrations (nine doses of 3-BC, three doses of ethinylestradiol), and given to female pups once daily by oral gavage (0.04 ml/10 g body weight), using a soft Teflon tube connected to a syringe. The dose range of ethinylestradiol was chosen from earlier data (Schlumpf et al., 2001). Pups were weighed daily, the dose was adjusted to the body weight. Vehicle controls received olive oil, and each dosage group was accompanied by vehicle controls. Twenty-four hours after the last gavage, on PN 24 (OECD day 23), the immature females were sacrificed by decapitation under ether anesthesia. The uterus was dissected with cuts between uterine cervix and vagina, and at the top of the uterine horns, trimmed free of fat and connective tissue, blotted with sterile gauze to remove the adherent fluid, and weighed (wet weight) again.

2.5. Data analysis

2.5.1. MCF-7 cells

Each experiment consisted of two plates per chemical with six wells/concentration per plate. The values of the 2 × 6 wells were averaged yielding one value per treatment (concentration of chemical or negative control) per experiment. Differences between treatment groups were analyzed by two-way ANOVA followed by Bonferroni pairwise comparisons (SYSTAT 5.01 software). For every independent experiment, non-linear regression was performed based on the two mean values/concentration from the two plates, and a median effective concentration (EC_{50}) was calculated (GraphPad Prism 3.03, GraphPad Software, Inc., San Diego, CA, USA). A mean EC_{50} was then obtained from the EC_{50}s of the five experiments. The figure shows curves averaged across data from the five experiments.

2.5.2. Estrogen receptor competition experiments

GraphPad Prism 3.03 was used for non-linear regression analysis. IC_{50} is defined as the concentration of estradiol or UV filter required to reduce the specific radioligand binding by 50%. Mean values of $IC_{50} \pm$ S.D. were calculated from individual IC_{50} values of independent experiments.

2.5.3. Uterotrophic assay

Differences between treatment groups were analyzed by two-way ANOVA followed by Bonferroni pairwise comparisons, with values of uterine weight and body weight of individual rats (SYSTAT 5.01 software). Controls from the period of June–August 2002 differed in body weight from controls of September 2002, but showed identical uterine weights. Therefore, body weights of treated groups were compared with controls of their respective experimental period. For uterine weight, the following ANOVAs were run: (1) data from all treated animals and controls of the entire experimental period (June–September 2002), (2 and 3) separate analyses of treated groups and controls from the periods June–August and September. In addition, possible correlations between uterine weight and body weight of individual rats on the day of sacrifice were studied by regression analysis (GraphPad Prism 3.03, GraphPad Software Inc., San Diego, CA, USA) within June–August and Septem-

ber controls as well as for the entire sample of control and treated animals. Non-linear regression analysis of uterine weight versus 3-BC dose, and the calculation of ED50 was based on the values of individual rats of the entire experimental period, using the same software.

3. Results

3.1. MCF-7 cell proliferation in vitro

Optical density as an index of cell number was dose dependently increased by 3-benzylidene camphor and by 4-methylbenzylidene camphor, which was re-investigated for comparison (Schlumpf et al., 2001) (Fig. 2). 3-BC was 5.8 times more potent than 4-MBC as indicated by EC_{50} values of 0.68 μM for 3-BC and 3.99 μM for 4-MBC (Table 1). The EC_{50} values of 4-MBC and of the positive control, estradiol, were almost identical to those observed in our previous study (Schlumpf et al., 2001) conducted with 24-well plates (4-MBC 3.02 μM, estradiol 1.22 pM). 3-BC concentrations between 600 nM and 10 μM yielded a significant increase in optical density. The maximum effect of 3-BC, at 6–10 μM, was similar to that of estradiol, while 4-MBC did not reach this level.

3.2. Binding to uterine cytosolic estrogen receptors and estrogen receptors α and β

3-BC and 4-MBC were tested in competition experiments with 16α^{125}I-estradiol and estradiol as positive control. IC_{50} values of estradiol were in the low nanomolar range as previously observed with the same ERα and ERβ preparations (Jarry et al., 2003). In a cytosolic ER preparation obtained from porcine uterus, 3-BC and 4-MBC displaced the radioligand in a concentration-dependent manner (Fig. 3a, Table 1). A marked difference was observed in binding experiments on human recombinant ERα and ERβ. Neither 3-BC nor 4-MBC were able to displace the radioligand from ERα at concentrations up to 1 mM (Fig. 3b), but both UV filters bound to ERβ (Fig. 3c, Table 1). 3-BC was more potent than 4-MBC, as indicated by IC_{50} values of 14.5 μM versus 112 μM for the cytosolic receptor preparation and of 11.8 μM versus 35.3 μM for ERβ (Table 1). At ERβ, the relative binding affinity (RBA) of 3-BC was in the range reported for bisphenol A and alkylphenols (Kuiper et al., 1997), but these chemicals also bind to ERα.

3.3. Uterotrophic assay in immature rats

When immature rats were given three daily oral applications of 3-benzylidene camphor by gavage

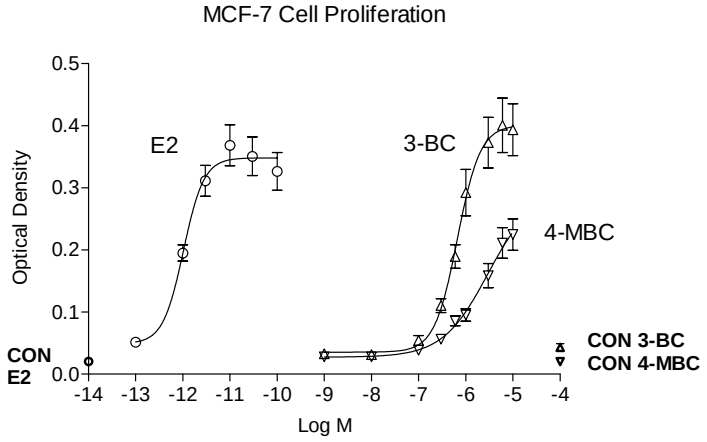

Fig. 2. Effect of estradiol-17β (E2), 3-benzylidene camphor (3-BC) and 4-methylbenzylidene camphor (4-MBC) on MCF-7 cell proliferation in vitro. Mean ± S.E.M. (optical density) of five independent experiments with E2, 3-BC and 4-MBC run in parallel. Significant difference from negative control: 3-BC 0.6 μM, $P = 0.028$ and 1–10 μM, $P < 0.001$; 4-MBC 1 μM, $P = 0.024$ and 3–10 μM, $P < 0.001$. CON E2, CON 3-BC, CON 4-MBC: negative controls for E2, 3-BC and 4-MBC.

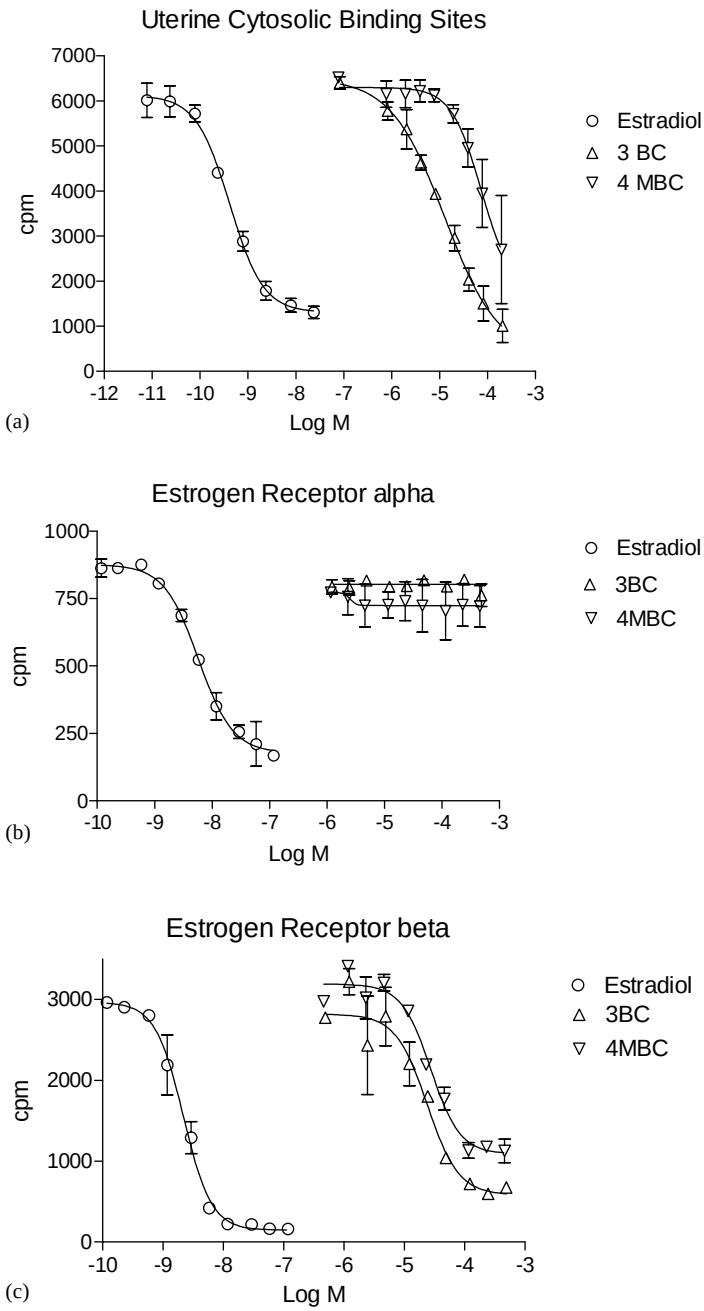

Fig. 3. Displacement of 16α[125]I-estradiol by estradiol-17β (E2), 3-benzylidene camphor (3-BC), and 4-methylbenzylidene camphor (4-MBC) in ligand binding assays (LBA). (a, top) Porcine uterine cytosolic binding sites. (b, center) Human recombinant estrogen receptor α (ERα). (c, bottom) Human recombinant estrogen receptor β (ERβ). Each value represents the mean ± S.E.M. of three independent experiments.

Table 1
Estrogenic activity and estrogen receptor α and β affinity of 3-benzylidene camphor (3-BC) and 4-methylbenzylidene camphor (4-MBC)[a]

	3-BC	4-MBC	Estradiol-17β
In vitro activity			
MCF-7 cell proliferation EC_{50} (M)	$6.84 \times 10^{-7} \pm 0.89 \times 10^{-7}$ (5)	$3.99 \times 10^{-6} \pm 2.95 \times 10^{-6}$ (5)[b]	$1.03 \times 10^{-12} \pm 0.058 \times 10^{-12}$ (5)
Maximum increase as percent of estradiol[c] (%)	102.8	58.7	100
In vitro competition with $16\alpha^{125}$I-estradiol			
Cytosolic binding sites from porcine uterus IC_{50} (M)	$1.45 \times 10^{-5} \pm 0.70 \times 10^{-5}$ (3)	$1.12 \times 10^{-4} \pm 0.74 \times 10^{-4}$ (3)	$4.27 \times 10^{-10} \pm 0.80 \times 10^{-10}$ (3)
Human estrogen receptor α IC_{50} (M)	No displacement at 10^{-3} (3)	No displacement at 10^{-3} (3)	$7.13 \times 10^{-9} \pm 2.31 \times 10^{-9}$ (3)
RBA[d]	$\ll$0.0007	$\ll$0.0007	100
Human estrogen receptor β IC_{50} (M)	$1.18 \times 10^{-5} \pm 0.97 \times 10^{-5}$ (3)	$3.53 \times 10^{-5} \pm 2.04 \times 10^{-5}$ (3)	$2.13 \times 10^{-9} \pm 0.72 \times 10^{-9}$ (3)
RBA	0.02	0.006	100
	3-BC	4-MBC[e]	Ethinylestradiol[e]
In vivo activity			
Uterotrophic activity ED50 (mg/kg per day)	45.3	309	0.000818
Maximum increase as percent of ethinylestradiol[c] (%)	70.0	35.5	100

[a] Mean EC_{50}, and IC_{50} values ± S.D. and number of independent experiments.
[b] Cf. Schlumpf et al. (2001): 3.02×10^{-6} M.
[c] (Maximum level of experiment group − control level of experiment group)/[(maximum level of estradiol/ethinylestradiol) − (control level of estradiol/ethyinylestradiol)] × 100.
[d] Relative binding affinity (RBA) was calculated as the ratio of IC_{50} of estradiol and IC_{50} of the competitor, with RBA of estradiol set at 100.
[e] Uterotrophic data for 4-MBC and ethinylestradiol from Schlumpf et al. (2001).

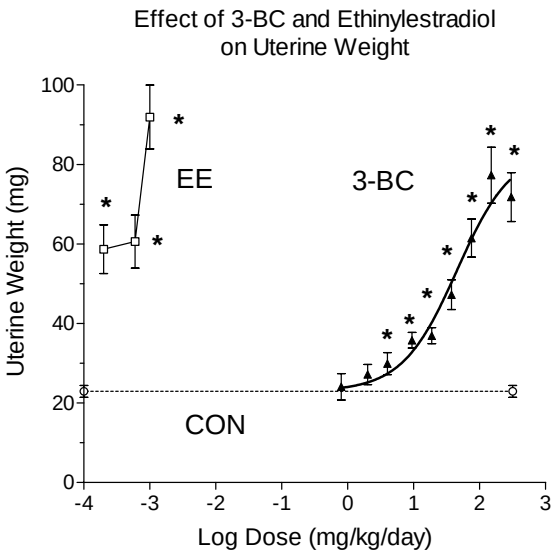

Fig. 4. Uterine weight of immature Long Evans rats on postnatal day (PN) 24, following three applications of 3-benzylidene camphor (3-BC) or ethinylestradiol (EE) by oral gavage on PN 21, 22 and 23 (day of birth = PN 1). CON: vehicle control (olive oil). Mean ± S.D., $n = 5$–9 animals per dose. Asterisk (∗) means different from vehicle control $P < 0.001$ (see Table 2 for details).

on PN 21, 22, and 23, uterine weight (wet weight) was dose dependently increased 24 h after the last dose (Fig. 4). 3-BC had an ED50 of 45.3 mg/kg per day (Table 1), showed significant effects at doses of 2 mg/kg per day and above (Table 2), and reached 70% of the maximal increase in uterine weight produced by ethinylestradiol (Table 1). The uterine weight changes observed after the positive control ethinylestradiol were in the range of a previous study (Schlumpf et al., 2001). 4-Methylbenzylidene camphor (4-MBC) was also given by gavage at a dose of 300 mg/kg per day (close to ED50, Schlumpf et al., 2001) in an additional group. Uterine weight was increased to 44.64 ± 6.45 mg (body weight at PN 24 45.93 ± 7.15 g, $n = 8$), similar to the weight level previously observed after administration of the substance in the chow. The vehicle control level of this additional treatment series was 24.13 ± 1.23 mg ($n = 6$).

3-BC-treated animals did not show signs of general toxicity. Body weight increased at a similar rate as in controls, except for an initial transient reduction at the three highest doses (Fig. 5). However, also with these doses, final body weights were at the level of control weights. Controls and treated animals of an experiment run in September 2002 had somewhat lower body weights than those of the major part of the study performed between June and August 2002 (Fig. 5, Table 2). Therefore, the two time periods (June–August and September) were also analyzed separately. Body weights of 3-BC-treated animals did not differ significantly, at PN 21 and 24, from body weights of controls of the respective period. Mean uterine weights of controls of the two time periods were identical in spite of different mean body weights (Table 2). Also, linear regression analysis, performed separately for data of the time periods June–August and September, did not show any correlation between uterine weight and body weight (at PN 24), neither within the control groups nor for the entire set of treated and control animals of the two experimental periods (Fig. 5). ANOVA analyses of uterine weight differences done separately for the periods of June–August and September, and for the entire period June–September, yielded the same results (Table 2).

4. Discussion

The present data demonstrate a significant estrogenic activity of the UV filter 3-benzylidene camphor in mammalian systems, in vitro in a human breast cancer cell line and in vivo in the uterotrophic assay in immature rats. 3-BC has been reported to exhibit estrogenic activity in fish (Holbech et al., 2002). Both, 3-BC and 4-methylbenzylidene camphor (4-MBC), previously identified as UV filter with estrogenic activity (Schlumpf et al., 2001), appear to bind preferentially to ERβ.

3-BC is 5.8 times more potent than 4-MBC in vitro on MCF-7 cells, and 6.8 times more potent in vivo; the ED50 of 45.3 mg/kg per day in the uterotrophic assay is close to the value reported for vitellogenin induction by 3-BC in rainbow trout (37.5 mg/kg) after a comparable treatment period (3 days, Holbech et al., 2002). In the mammalian in vivo model, 3-BC turned out to possess remarkable estrogenic activity as compared to other industrial chemicals known as xenoestrogens. Its minimum effective dose in the uterotrophic assay is 2 mg/kg with three daily applications. When the doses of bisphenol A (BPA) and 3-BC required to produce a given increase in uterine weight are compared,

Table 2
Uterine weight and body weight of immature rats following three daily oral doses of 3-benzylidene camphor (3-BC), 4-methylbenzylidene camphor (4-MBC), or ethinylestradiol

	Body weight[a] (g) PN 21[b]	Body weight[a] (g) PN 24	Uterine weight[a] (wet weight, mg) PN 24
Vehicle controls			
Controls June–August	35.41 ± 1.80 (17)	43.64 ± 2.62 (17)	23.05 ± 1.50 (17)
Controls September	31.9 ± 2.69 (7)	37.46 ± 2.61 (7)	22.66 ± 1.52 (7)
Controls combined	34.39 ± 2.61 (24)	41.84 ± 3.85 (24)	22.94 ± 1.48 (24)
3-BC (mg/kg per day p.o.)			
300	42.43 ± 2.16 (6)	46.63 ± 4.07 (6)	71.8 ± 6.15 (6)**
150	39.38 ± 1.54 (6)	45.23 ± 1.54 (6)	77.3 ± 7.025 (6)**
75	39.83 ± 1.40 (4)	48.23 ± 0.88 (4)	61.48 ± 4.80 (4)**
37.5	36.58 ± 5.14 (8)	41.73 ± 6.05 (8)	47.25 ± 3.73 (8)**
18.75	34.88 ± 1.51 (5)	43.36 ± 2.05 (5)	36.94 ± 2.02 (5)**
9.4	35.62 ± 1.88 (5)	42.96 ± 2.07 (5)	35.78 ± 1.97 (5)**
4.0	35.75 ± 2.87 (8)	42.58 ± 3.34 (8)	30.20 ± 0.77 (8)**
2.0	30.62 ± 1.61 (6)	36.70 ± 2.81 (6)	27.17 ± 2.53 (6)+
0.8	34.27 ± 3.86 (9)	41.73 ± 5.45 (9)	24.09 ± 3.32 (9)
Ethinylestradiol (mg/kg per day p.o.)			
0.001	31.24 ± 1.35 (5)	38.02 ± 2.54 (5)	91.86 ± 8.03 (5)**
0.0006	41.75 ± 1.74 (6)	48.25 ± 2.61 (6)	60.60 ± 6.67 (6)**
0.0002	28.82 ± 2.91 (5)	36.58 ± 4.99 (5)	58.70 ± 6.12 (5)**

[a] Mean ± S.D., number of animals.
[b] Day of birth = PN 1 (OECD protocol = PN 0).
** Uterine weight different from control, in analysis of whole experimental period June–September and in two separate analyses of experimental periods June–August and September, $P < 0.001$ (except 4 mg/kg June–August, $P = 0.011$).
+ Different from control in separate analysis of September experimental period, $P < 0.05$.

3-BC appears to be over 10 times more potent than BPA by the oral route (Table 3) (Ashby and Tinwell, 1998; Yamasaki et al., 2000), and even more potent than BPA given subcutaneously (Ashby and Tinwell, 1998; Yamasaki et al., 2000, 2002). Orally administered 4-MBC (Schlumpf et al., 2001), is slightly more potent than oral BPA (Table 3), but less active than subcutaneous BPA. In contrast to their activity on estrogen receptors, 3-BC and 4-MBC are devoid of androgenic or antiandrogenic activity (Ma et al., 2003).

The effects of 3-BC on uterine weight were independent of changes in body weight. The daily rate

Table 3
Comparison of uterotrophic activities of bisphenol A (BPA), 3-BC and 4-MBC (three applications) in immature rats

Effect of BPA		Equipotent dose of UV filters	
BPA[a] mg/kg per day p.o.	Uterine weight increase (fold control)	3-BC mg/kg per day p.o.	4-MBC mg/kg per day p.o.
160 (2)	1.13	2.5	78.5
400 (1)	1.31	6.4	156.7
600 (1)	1.38	8.0	189.2
800 (2)	1.46	10.0	230.7
800 (1)	2.18	36.8	1980

In the absence of detailed dose–response curves for BPA, the comparison could not be based on ED50 values. Instead, the doses of 3-BC and 4-MBC required to produce the increase in uterine weight reported for a given dose of BPA, were calculated from the dose–response curves of the UV filters obtained in this study and by Schlumpf et al. (2001).
[a] *Source of data*: (1) Ashby and Tinwell (1998) and (2) Yamasaki et al. (2000).

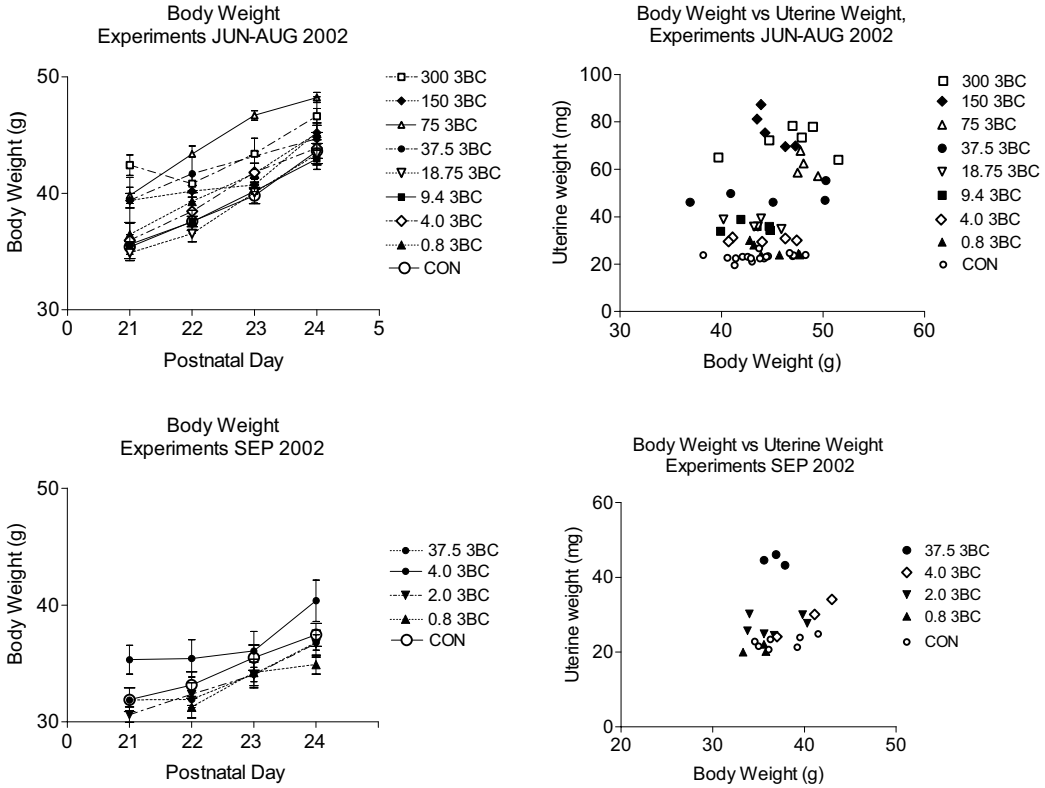

Fig. 5. Body weight and uterine weight of immature Long Evans rats. Top: Period June–August 2002, bottom: period September 2002. Left panel: Development of mean body weight (±S.E.M.) on postnatal days (PN) 21, 22, 23, and 24 (day of birth = PN 1). Right panel: Uterine weight vs. body weight of individual animals on PN 24.

of increase in body weight of 3-BC-treated animals was similar to that of controls in the lower part of the dose-range studied. At the three highest doses, 3-BC caused an initial retardation of body weight increase, but final values were at control level. Also, there was no correlation between uterine weight and body weight at the end of the treatment period. As described above, the analysis was done separately for two experimental subsets that had been performed with rats of slightly different initial body weights. Neither a comparison of the control groups of the two subsets, nor a combined analysis of treated and control rats yielded indications for a relationship between the two parameters. These observations support the idea that the influence of body weight on uterine weight is a minor factor during the defined prepubertal treatment window (Kanno et al., 2001).

While estrogenic activity of both camphor derivatives and transactivational effects of 4-MBC at estrogen receptors (see below) have been documented, unequivocal evidence for binding of either 4-MBC or 3-BC to estrogen receptors has so far been lacking. In a rat uterine receptor preparation, very weak displacement of ^{3}H-estradiol was observed at and above 50 μM 4-MBC (Tinwell et al., 2002), whereas no binding of this compound to cytosolic extracts of human Ishikawa cells was detected at concentrations up to 10 μM (Mueller et al., 2003). Using an iodinated radioligand and a receptor preparation from porcine uterus, we now could demonstrate concentration-dependent displacement of the radioligand by both, 3-BC and 4-MBC. More interestingly, both the camphor derivatives effectively displaced the radioligand from a recombinant hERβ preparation, but did not exhibit any binding to hERα at concentrations up to 1 mM. The IC_{50} values for the porcine uterine cytosolic preparation and for hERβ are very similar for 3BC (14.5 and 11.8 μM, respectively),

while the IC_{50} of 4-MBC for hERβ (35.4 μM) is somewhat lower than that for the cytosolic preparation (112 μM). Binding of 4-MBC to hERα and hERβ has been tested by Mueller et al. (2003), but these authors failed to detect significant displacement using fluorescein-labeled estradiol.

Our data provide evidence for a high degree of binding selectivity of 3-BC and 4-MBC for ERβ. This observation is in line with data from transactivation experiments in Ishikawa cells, where 4-MBC exhibited a lower EC_{50} at hERβ (57 μM) than at hERα (>150 μM) (Mueller et al., 2003). 4-MBC also was found to be ineffective in the yeast ER transactivation assay which again may be viewed as an argument against a significant activity at ERα (Tinwell et al., 2002). However, in another transactivation assay using HEK 293 cells, no clear-cut difference in the effect of 4-MBC on ERα and ERβ was evident (Schreurs et al., 2002). 3-BC which is far more active and exhibits a higher binding affinity for ERβ than 4-MBC, has not yet been studied in such systems. So far, relatively few ligands with pronounced ERβ selectivity have been described. The two camphor derivatives are structurally quite different from that of ERβ ligands such as genistein or diarylpropionitrile (DPN) (Meyers et al., 2001).

The role of ERβ in uterus is still little understood. In adult uterus, its expression level is lower than that of ERα (Kuiper et al., 1997; Wang et al., 1999; Matsuzaki et al., 1999), but a more balanced expression of the two subtypes has been reported for prepubertal mouse uterus (Weihua et al., 2000), suggesting an increased responsiveness to ERβ-mediated effects. In rat uterus, however, we so far have no evidence for significant differences in ERα/ERβ mRNA ratios between adult and prepubertal animals (Durrer and Gaille, unpublished observations). Indirect evidence from immature ERβ knockout (BERKO) mice suggests that ERβ-mediated activity would rather have antiproliferative effects (Weihua et al., 2000). In spite of that, genistein, a preferential ERβ ligand (Kuiper et al., 1997), is uterotrophic in immature rats (Yamasaki et al., 2002). Whether this effect is due to ERβ-mediated activity cannot be decided because genistein also displayed significant transactivational activity at ERα (Kuiper et al., 1997). Orally administered 3-BC is almost as active as subcutaneous genistein in the lower dose range, with 1.15- and 2.45-fold increase in uterine weight produced by 2 and 20 mg/kg genistein s.c. (Yamasaki et al., 2002) and 2.9 and 54.7 mg/kg 3-BC p.o. This in vivo potency would not be suspected from binding data, since the relative binding affinity of 3-BC is several orders of magnitude lower than that of genistein also at ERβ. One possible explanation for the discrepancy may be the formation of an active metabolite of 3-BC which possibly might be less ERβ-specific than the parent compound, but given the complexity of ER signaling, the effectiveness of 3-BC in vivo might also result from different mechanisms.

In conclusion, the camphor derivative 3-BC exhibits considerable estrogenic potency in a mammalian test system, the uterotrophic assay in immature rats, as compared to other nonsteroidal xenoestrogens. Both, 3-BC and the related camphor derivative 4-MBC are characterized by preferential binding to ERβ. How this feature relates to the uterotrophic activity, remains to be elucidated.

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