



Multi-organic endocrine disrupting activity of the UV screen benzophenone 2 (BP2) in ovariectomized adult rats after 5 days treatment

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Abstract

The chemical industry has developed sun protection factor products, which contain a variety of so-called “UV screens”, among others, benzophenones (BP). Based on the structure it can be assumed, that the variant BP2 may be a potent estrogenic endocrine disrupter (ED). Only very limited data are available in the literature about such action of BP2, which focussed on the uterotrophic effect in immature rats. However, determination of ED activity in the uterus is only a restricted approach with the potential risk of missing undesirable actions. Therefore, we examined a putative multiple organ ED activity of BP2 by measuring gene expression of marker genes in the uterus, liver, vagina and pituitary after 5 days oral application in adult ovariectomized (ovx) rats. An effect on lipid metabolism was assessed by determination of cholesterol, high- and low-density lipoproteins (HDL and LDL) in the blood. As control compound, estradiol (E2) was included in the study. A dose dependent E2-agonistic activity was observed in the uterus (increased weight), vagina (increased IGF1 expression), pituitary (reduced LH synthesis), liver (increased IGF1 expression) and lipid parameters (reduction). A non-E2-like action of BP2 was observed on T4- and T3-levels, which were significantly reduced. Except for the action of BP2 on thyroid hormone levels where it may inhibit thyroid peroxidase, this UV screen exerts clear E2-agonistic actions. Application of BP2 for 5 days proved to be a sufficient treatment period to unravel a multi-organic endocrine disrupting activity of this UV screen.

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1. Introduction

Over the last decades, the effects of high-energy short wave solar radiation on human skin has received

much publicity as the major cause of accelerated skin ageing and of skin cancers. To meet public demand, the cosmetic industry has developed sun protection factor products, which contain a variety of so-called “UV screens”, among others benzophenone (BP). Besides direct protection of the skin, UV screens are also used to prevent ultraviolet light from damaging scents and colours in a variety of cosmetic products.

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A third use of UV filters is the protection of plastic products against light-induced degradation. There are many variants of BP in use. The variations change features like the solubility and the absorbance range of UV light. Frequently used variants are BP3 (2-hydroxy-4-methoxybenzophenone) as a sun protection factor in skin care products and BP2 (2,2',4,4'-tetrahydroxybenzophenone), which is used in perfumes, lipsticks and plastics used for packaging of food.

Recently, BPs have been accused to act as endocrine disrupters (ED). For example, BP3 was shown to exert an uterotrophic action in rats, to stimulate the proliferation of the breast cancer cell line MCF7 and to enhance secretion of the tumour marker pS2 (Schlumpf et al., 2001), all effects which are similar to those of estradiol (E2). Binding to and activation of estrogen receptors (ER) by BP3 was further substantiated in ER-reporter gene assays (Takatori et al., 2003).

However, *in vivo* BP3 itself may be only precursor of more potent estrogenic compounds since metabolization of BP3 with the liver extract S9 caused formation of 2,3,4-trihydroxybenzophenone (BP1), which is significantly more potent than BP3 in activation ER-driven reporter gene expression (Takatori et al., 2003). Thus, O-dealkylation and multiple hydroxylation of the BP-backbone appears to increase the estrogenic potency of these UV screens.

Among the commercially available and used UV screens of the BP-type, BP2 carries most hydroxyl groups such that it can be assumed, that this chemical may be a potent estrogenic ED. However, only very limited data are available in the literature about such an action of BP2, which focussed on the uterotrophic effect in immature rats (Yamasaki et al., 2003). Since virtually all cells of the body express one or both types of the currently known subtypes of ERs (ER α and β , Kuiper et al., 1998) and various splice variants of these receptors as well as enhancers and repressors of their transcriptional activity, determination of ED activity in the uterus only is a restricted approach with the potential risk of missing undesirable actions outside the urogenital tract. Therefore, we examined a putative multiple organ ED activity of BP2 by measuring gene expression of estrogen regulated marker genes in the uterus, liver, vagina and pituitary after 5 days oral application of BP2 in adult ovariec-

tomized (ovx) rats. An effect on the lipid metabolism was assessed by determination of cholesterol, high- and low-density lipoproteins (HDL and LDL) in the blood. As control compound, estradiol was included in the study.

2. Materials and methods

2.1. Animals and treatments

Adult 2 months old female Sprague Dawley rats were purchased from Winkelmann (Borchen, Germany). All animals were bilaterally ovariectomized and kept under standard conditions (soy-free rat diet and water *ad libitum*, illumination from 6:00 a.m. until 6:00 p.m., room temperature 23 °C, relative humidity 55%). Fourteen days after ovx, animals were treated orally via gavage once per day either with olive oil (CTL), 600 μ g/kg body weight (BW) estradiol valerate (E2) or with 250 or 1000 mg/kg BW BP2. The applied volume was 1 ml. Each group consisted of 11 rats. The treatments were conducted between 5:30 and 6:30 a.m. During the 5-day treatment, the body weight and food consumption were daily recorded. At day 5, a vaginal smear was taken and 3–4 h after the last application animals were decapitated under deep CO₂ anaesthesia and blood was collected from the trunk. From each rat, the brain, anterior pituitary and vagina were collected and immediately frozen in liquid nitrogen. In addition, the uterus, spleen, liver and one adrenal was removed and, after recording the wet weights, also snap frozen. Samples were stored at –70 °C until RNA preparation or immunological analysis.

2.2. Extraction of RNA and real time-PCR

Extraction of total RNA from the organs or microsamples of the preoptic area of the hypothalamus and the protocol of the real time-PCR are described in detail, previously (Roth et al., 2001; Seidlova-Wuttke et al., 2003). The reactions were run on an ABI Prism 7700 Sequence Detection System (TaqManTM, PE Applied Biosystems Foster City, CA, USA). The primers and probes were synthesised according to published validated systems (IGF1: Seidlova-Wuttke et al., 2003; GnRH: Roth et al., 2001). Oligonu-

Table 1

Mean daily food intake per animal during the 5-day treatment period and body weight at day 5 (means \pm S.E.M., $n = 11$, $*P < 0.05$ vs. CTL)

	CTL	E2	BP-L	BP-H
Food intake (gram/day)	16.1 \pm 1.1	8.5 \pm 1.3*	9.4 \pm 1.5*	8.1 \pm 1.5*
Body weight (gram)	299.5 \pm 3.9	269.9 \pm 2.9*	277.2 \pm 5.5*	274.8 \pm 6.2*

cleotides were purchased from Eurogentec (Seraing, Belgium).

2.3. Pituitary cell cultures

Random cycling 3 months old female Sprague Dawley rats were used as donors of pituitaries. The preparation of cell cultures has been described in detail previously (Jarry et al., 1994). The incubation procedure followed the protocol of Ortmann et al. (1999).

2.4. Serum analyses

Concentrations of TSH and LH in the serum samples and LH levels in the pituitary cell culture supernatants were determined with reagents kindly provided by the National Hormone and Pituitary Program of the NIH as described previously (Baur et al., 2000; Roth et al., 2001). The lipid parameters cholesterol, HDL and LDL were determined with commercially available kits (Roche Diagnostics, Mannheim). Serum total T4- and T3-levels were measured with kits purchased from DSL (Sinsheim). The kits were used according to the instructions of the manufacturers.

2.5. Statistical evaluation

In the present study, relative changes of mRNA levels were analysed. The mean value of the absolute data measured in the control group was set 100% and all other values determined in the respective assay were expressed in relation to this average value. Data were expressed as means \pm S.E.M. Significant differences between the control and treatment groups were analysed by one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons (PrismTM, Graph Pad, San Diego, USA). P values < 0.05 were considered significant.

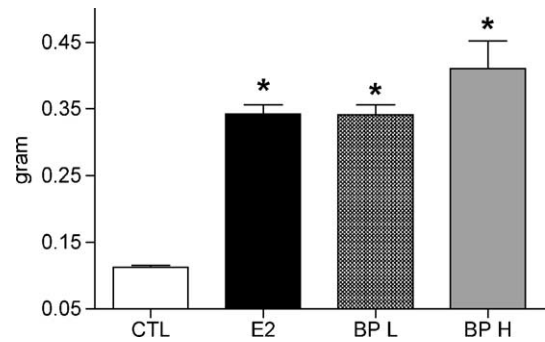


Fig. 1. Mean uterus weights in the treatment groups. Note the strong increase in E2 and BP2 treated animals ($*P < 0.05$ vs. CTL).

3. Results

The average food intake during the 5-day treatment period is significantly reduced in rats treated with either E2 or BP2, which is accompanied with a reduced body-weight at the end of the experiment (Table 1). However, neither the weight of livers, adrenals or spleens differ among the experimental groups (data not shown). In contrast, E2 application increased uterine weight significantly, an effect which was also achieved with both doses of BP2 (Fig. 1).

The E2-like activity of BP2 was also observed in vaginal cytology (Fig. 2), i.e. it clearly induced cornification of epithelial cells. Because of the missing variability of data in the control and E2-group (all animals showed score 1 or 3, respectively), a statistical analysis is not applicable.

LH levels in the blood were significantly reduced by E2 and the high dose of BP2 (Fig. 3). This reduction of LH levels may be due to direct actions of these compounds on gonadotrope cells in the pituitary or may be caused by an inhibition of the secretion of the hypothalamic releasing factor GnRH. Since GnRH secretion cannot be measured directly, GnRH mRNA levels were determined as an indirect measure of secretion of this releasing factor. No changes in neither experimental group were detected with regard to the mRNA levels

of GnRH in the preoptic area of the hypothalamus (data not shown), i.e. reduced LHB biosynthesis is not the consequence of reduced GnRH release from the hypothalamus. The direct action of E2 and BP2 at the level of the pituitary is demonstrated by the stimulatory action of both compounds on *in vitro* LH release from rat pituitary cells (Fig. 4). Under cell culture conditions, a direct estrogenic action is reflected in the amplification of GnRH stimulated LH release (Ortmann et al., 1999).

Though TSH levels in the serum were identical in all groups (Fig. 5), T4 concentrations, however, were dose dependently suppressed by both doses of BP2. Similarly, T3-levels were reduced by BP2, an effect, which reached significance in the BP-H group. This effect on thyroid hormone levels was not observed for E2.

The parameters of lipid metabolism cholesterol, HDL and LDL were altered by E2 and BP2 in an iden-

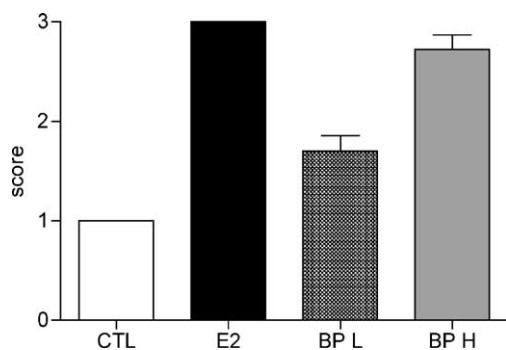


Fig. 2. Evaluation of the vaginal cytology. Smears were staged according to: 1: "diestrus-like", 2: "proestrus-like", 3: "estrus-like". The missing error bars in groups CTL and E2 are due to the fact that all animals in the respective group were scored identical.

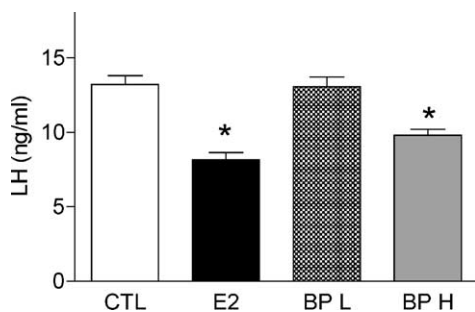


Fig. 3. Mean LH levels in the blood were significantly reduced by E2 and the high dose of BP2 ($P < 0.05$ vs. CTL).

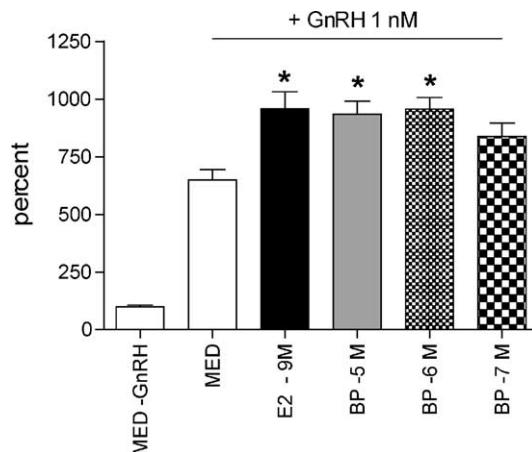


Fig. 4. Mean LH levels in rats pituitary cell cultures after 48h incubation. Note the stimulatory action of the brief GnRH exposure on LH release, which is significantly enhanced by E2 and BP2 ($*P < 0.05$ vs. MED).

tical manner (Fig. 6). E2 and BP2 (in a dose dependent manner) significantly reduced serum levels of these parameters.

Expression of IGF1 in the vagina is profoundly stimulated by both, E2 and BP2 (Fig. 7). A reduction of IGF1 mRNA occurred in the liver of E2 treated rats, and in a dose dependent fashion, also in BP2 exposed animals.

4. Discussion

The present data describe for the first time a multi-organic endocrine activity of BP2 in the adult ovx rat upon a 5 days acute treatment. Except for the action of BP2 on thyroid hormone levels in the blood, this UV screen exerts clear E2-like actions on body weight, food intake, uterine weight, pituitary LH synthesis without changing hypothalamic GnRH mRNA levels, IGF1 expression in liver and vagina and on lipid parameters. The acute application of BP2 for 5 days proved to be a sufficient treatment period to unravel a multi-organic endocrine disrupting activity of this UV screen.

In an accompanying study (Seidlova-Wuttke and Wuttke, 2004), BP2 was applied via the food for 3 months to assess long-term effects. Also, this approach revealed an estrogenic action in the classical target organs, uterus and vagina. These corresponding data obtained with two different treatment regimens allows

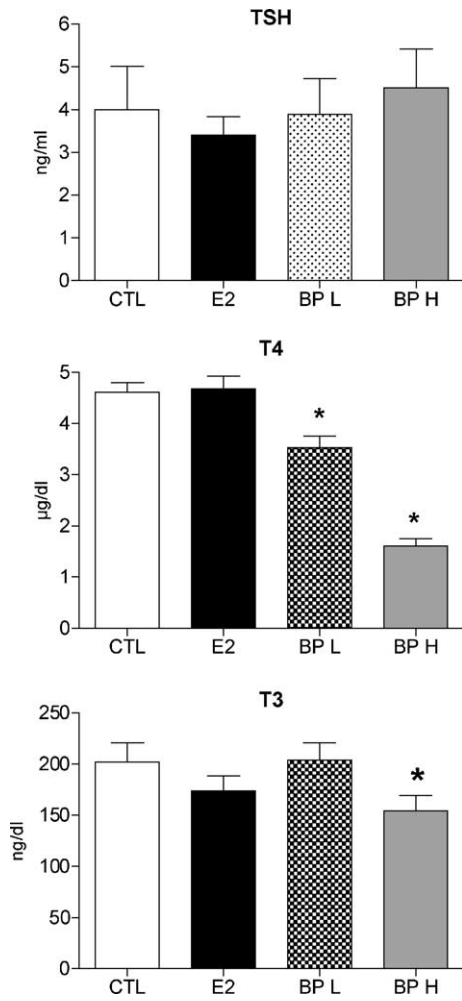


Fig. 5. Effects of E2 and BP2 on serum TSH, total T4- and T3-levels (* $P < 0.05$ vs. CTL).

the conclusion, that for evaluation of multi-organic ED actions of a test compound, the time- and cost-efficient application of 5 days is a reliable tool to study effects in many but not in all organs. If for example, effects on bones are expected, longer treatment intervals are necessary. Whether oral application versus subcutaneous injections should be preferred may be a matter of debate, however, treatment per gavage ensures reproducible dosage and includes all possible metabolic pathways of conversion of the parent compound to more active compounds as reported for BP3 which can be metabolized to the more active BP1 (Takatori et al., 2003).

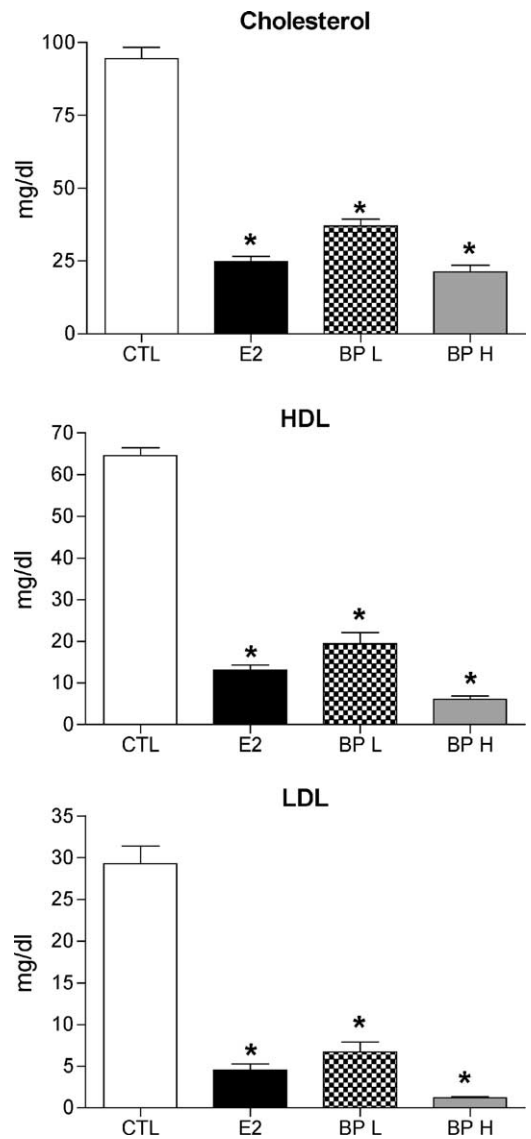


Fig. 6. Effects of E2 and BP2 on serum cholesterol, HDL- and LDL-levels (* $P < 0.05$ vs. CTL).

Only one study on an estrogenic action of BP2 in vivo was available from the literature (Yamasaki et al., 2003). Using doses up to 800 mg BP2/kg BW, an uterotrophic action of BP2 was reported in immature rats. Based on this information and the doses used by Schlumpf et al. (2001) for BP3 in immature rats, we decided to use 1000 mg/kg BP2 as maximal dose for treatment of adult ovx rats. The lower dose of 250 mg/kg proved to be already maximal in the uterus,

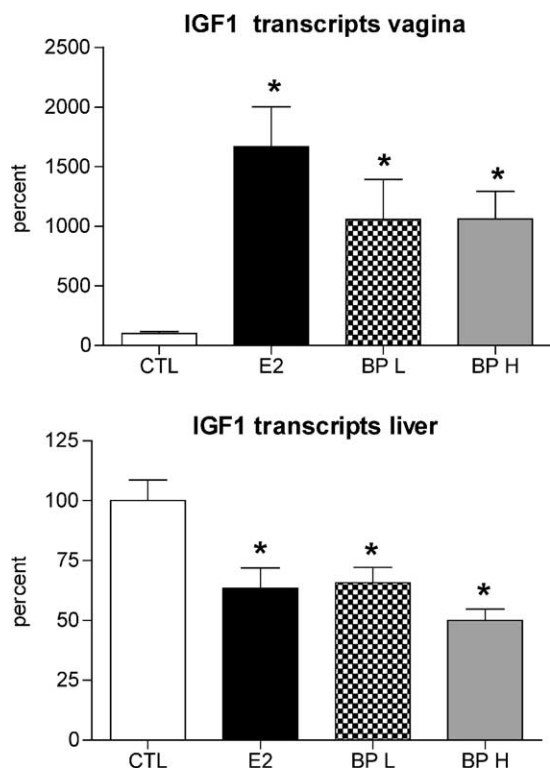


Fig. 7. Effects of E2 and BP2 on IGF 1 mRNA levels in vagina and liver (* $P < 0.05$ vs. CTL).

pituitary and vagina, while serum lipid parameters were even further reduced by the high dose of BP2. No signs of intoxication were observed during the 5 days treatment with BP2. The observed reduced bodyweight and food consumption in BP2-treated rats is characteristic for ovx animals exposed to an E2 agonist. Thus, the doses chosen in the present study are clearly sufficient to induce a specific ED activity of BP2. A dose response study with BP2 in the described animal model to define the no effect level is currently in progress.

The OECD validation studies demonstrate that the rodent uterotrophic bioassay protocols are robust and reliable for identifying estrogen agonists and antagonists and are transferable across laboratories (Owens et al., 2003; Kanno et al., 2003). Despite the virtually ubiquitous tissue distribution of ER in the body, the concept of multi-organic risk assessment has not yet been considered adequately by the OECD. Using the standard protocol, undoubtedly BP2 would have been identified as an estrogenic ED agent with all possible

hazardous actions in the human. However, the strong anti-thyroid action of BP2 would have been missed if the test protocol just focuses on uterine parameters. Though an estrogenic action of BP2 is undoubtedly undesirable, but the profound interference with thyroid hormone synthesis is a point of even more severe concern.

Though both types of ER are expressed in the thyroid (Egawa et al., 2001), a suppressive action of E2 on T4-levels is not known and was also not found in the present study. Thus, the suppressive action of the “estrogenic” compound BP2 must involve non-ER-mediated mechanisms. One candidate of BP2 action is thyroid peroxidase (TPO), one key enzyme of thyroid hormone biosynthesis. In vitro suicide inactivation of rat TPO has been reported for endocrine disrupting agents like genistein (Divi et al., 1997; Chang and Doerge, 2000). Other well-known examples of non-ER-mediated ED actions are the inhibition of tyrosine kinase and deiodinase activity by flavonoids (Akiyama et al., 1987; Köhrle et al., 1988). The inhibition of TPO activity by genistein at high-micromolar concentrations was recently confirmed by Schmutzler et al. (2004). In this study, BP2 was also reported as a very potent inhibitor of TPO activity with an IC_{50} of about 300 nM. Another enzyme affected by BP2 is aldose reductase (Ono and Hayano, 1982), which indicates that a broad spectrum of enzymes may be target of the ED activity of BP2. Therefore, despite EDs may act primarily via nuclear receptors, other mechanisms like interference with enzyme activity may be a common feature.

Humans can be exposed to UV screens by dermal absorption or through the food chain. BP3 and its metabolite BP1 have been detected in human urine from 4 h after application of commercially available sunscreen products to the skin (Felix et al., 1998). BP3 has also been found to be readily absorbed from the gastrointestinal tract (Kadry et al., 1995). However, no information is available about the exposure level of humans to BP2. In view of the present multi-organic ED activity of BP2 and in particular its anti-thyroid activity, studies on the bioavailability are required.

In conclusion, this study reports an E2-like activity of BP2 in the uterus, vagina, pituitary and liver, which has to be evaluated as a multi-organic ED activity. The anti-thyroid effect of BP2 demonstrates that this multi-organic action profile includes non-ER-mediated mechanisms.

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References

- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y., 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262, 5592–5595.
- Baur, A., Bauer, K., Jarry, H., Köhrle, J., 2000. Effects of proinflammatory cytokines on anterior pituitary 5'-deiodinase type I and type II. *J. Endocrinol.* 167, 505–515.
- Chang, H.C., Doerge, D.R., 2000. Dietary genistein inactivates rat thyroid peroxidase in vivo without an apparent hypothyroid effect. *Toxicol. Appl. Pharmacol.* 168, 244–252.
- Divi, R.L., Chang, H.C., Doerge, D.R., 1997. Anti-thyroid isoflavones from soybean: isolation, characterization, and mechanisms of action. *Biochem. Pharmacol.* 54, 1087–1096.
- Egawa, C., Miyoshi, Y., Iwao, K., Shiba, E., Noguchi, S., 2001. Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in normal and malignant thyroid tissues by real-time polymerase chain reaction. *Oncology* 61, 293–298.
- 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 371, 195–203.
- Jarry, H., Leonhardt, S., Gorkow, C., Wuttke, W., 1994. In vitro prolactin but not LH and FSH release is inhibited by compounds in extracts of *Agnus castus*: direct evidence for a dopaminergic principle by the dopamine receptor assay. *Exp. Clin. Endocrinol.* 102, 448–454.
- Kadry, A.M., Okereke, C.S., Abdel-Rahman, M.S., Friedman, M.A., Davis, R.A., 1995. Pharmacokinetics of benzophenone-3 after oral exposure in male rats. *J. Appl. Toxicol.* 15, 97–102.
- Kanno, J., Onyon, L., Peddada, S., Ashby, J., Jacob, E., Owens, W., 2003. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dose-response studies. *Environ. Health Perspect.* 111, 1530–1549.
- Köhrle, J., Spanka, M., Irmscher, K., Hesch, R.D., 1988. Flavonoid effects on transport, metabolism and action of thyroid hormones. *Prog. Clin. Biol. Res.* 280, 323–340.
- Kuiper, G.G., Shughrue, P.J., Merchenthaler, I., Gustafsson, J.A., 1998. The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. *Front. Neuroendocrinol.* 19, 253–286.
- Ono, H., Hayano, S., 1982. [2,2', 4,4']-Tetrahydroxybenzophenone as a new aldose reductase inhibitor. *Nippon Ganka Gakkai Zasshi.* 86, 353–357.
- Ortmann, O., Asmus, W., Diedrich, K., Schulz, K.D., Emons, G., 1999. Interactions of ovarian steroids with pituitary adenylate cyclase-activating polypeptide and GnRH in anterior pituitary cells. *Eur. J. Endocrinol.* 140, 207–214.
- Owens, W., Ashby, J., Odum, J., Onyon, L., 2003. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dietary phytoestrogen analyses. *Environ. Health Perspect.* 111, 1559–1567.
- Roth, C., Schrickler, M., Lakomek, M., Strege, A., Heiden, I., Luft, H., Munzel, U., Wuttke, W., Jarry, H., 2001. Autoregulation of the gonadotropin-releasing hormone (GnRH) system during puberty: effects of antagonistic versus agonistic GnRH analogs in a female rat model. *J. Endocrinol.* 169, 361–371.
- 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.
- Schmutzler, C., Ambrugger, P., Huhne, K., Grüters, A., Köhrle, J., 2004. Endocrine Disrupters inhibit human thyroid peroxidase activity. *Exp. Clin. Endocrinol. Diab.* 112 (Suppl. 1), 54.
- Seidlova-Wuttke, D., Hesse, O., Jarry, H., Christoffel, V., Spengler, B., Becker, T., Wuttke, W., 2003. Evidence for selective estrogen receptor modulator activity in a black cohosh (*Cimicifuga racemosa*) extract: comparison with estradiol-17beta. *Eur. J. Endocrinol.* 149, 351–362.
- Seidlova-Wuttke, D., 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*, in press.
- Takatori, S., Kitagawa, Y., Oda, H., Miwa, G., Nishikawa, J., Nishihara, T., Nakazawa, H., Hori, S., 2003. Estrogenicity of metabolites of benzophenone derivatives examined by a yeast two-hybrid assay. *J. Health Sci.* 49, 91–98.
- Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Takatsuki, M., 2003. Comparison of the reporter gene assay for ER-alpha antagonists with the immature rat uterotrophic assay of 10 chemicals. *Toxicol. Lett.* 142, 119–131.