Estrogenic and Anti-Androgenic Activities of Benzophenones in Human Estrogen and Androgen Receptor Mediated Mammalian Reporter Gene Assays

Yoko Kawamura,*,[a] Motoh Mutsuga,[a] Teruhisa Kato,[b] Mitsuru Iida,[b] and Kenichi Tanamoto[a]

[a]National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan and [b]Otsuka Life Science Initiative, Otsuka Pharmaceutical Co., Ltd., 224–18 Ebisuno, Kawauchi-cho, Tokushima 771–0195, Japan

(Received September 8, 2004; Accepted November 26, 2004)

The estrogenic and anti-androgenic activities of benzophenone and 19 hydroxylated derivatives were measured in reporter gene assays using transfected human estrogen and androgen receptors in Chinese hamster ovary cells. Eighteen benzophenones had estrogenic activity and seventeen also had anti-androgenic activity. In both assays, 2,4,4′-trihydroxybenzophenone and 2,2′,4,4′-tetrahydroxybenzophenone showed the strongest activity which were comparable to bisphenol A or 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE). The structure-activity relationships of the estrogenic activity in this mammalian reporter gene assay were mostly similar to the yeast two-hybrid assay as previously reported. Benzophenones hydroxylated at the 3 or 4-position showed the estrogenic activity, while the others showed negative or weakly positive activities. Moreover, a hydroxyl group added at the 2-position of the 4-hydroxylated benzophenone enhanced activity, but reduced activity at the 3-position. In contrast, different results were obtained when a hydroxyl group was added to another benzene ring. The added hydroxyl group enhanced the activity in this reporter gene assay, but reduced it in the yeast two-hybrid assay. Results from the reporter gene assay corresponded with the *in vivo* uterotrophic assay. On the other hand, a hydroxyl group at the 2-position generally enhanced the anti-androgenic activity, though the effect of other hydroxyl groups was less clear. Meanwhile, these benzophenones had no or very weak androgen agonistic activities.

Key words ——— hydroxylated benzophenone, estrogenic activity, anti-androgenic activity, mammalian reporter gene assay, 2,4,4′-trihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone

INTRODUCTION

Benzophenone is used in medicines, UV stabilizers, aroma retention reagents and others, and also found in waste water. It is listed among "the chemicals suspected of having endocrine disrupting effects,"[1] however, it has been reported to have no estrogenic activity.[2–6] In contrast, several hydroxylated benzophenones had a higher estrogenic activity in a variety of assays. These assays included the MCF-7 cell assay,[2,3] the competitive binding assay,[4,5] the reporter gene assay[6–8] and the uterotrophic assay.[5,8,9] One derivative, 2-hydroxy-4-methoxybenzophenone was also reported to be an androgenic antagonist.[10] Hydroxylated benzophenones are used as UV-stabilizers in a variety of commercial products, such as sunscreens, cosmetics, and plastics. Some are also produced in the body as the metabolites of benzophenone and its derivatives.[2,3,11,12]

In the previous paper,[13] we tested the estrogenic activities of benzophenone and 19 hydroxylated benzophenones using the yeast two-hybrid assay. Among them, fifteen expressed estrogenic activity, some showed a higher activity than bisphenol A, and the results provided an indication of the structure-activity relationships. While the results raised questions concerning the ability of the yeast two-hybrid assay to predict the results in living animals.

A number of *in vitro* mammalian cell-based assays have been developed to evaluate endocrine disruption, and recently the genetically engineered mammalian stable cell lines, the so-called ER-

*To whom correspondence should be addressed: National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan. Tel.: +81-3-3700-9413; Fax: +81-3-3700-9403; E-mail: kawamura@nihs.go.jp

Table 1. Estrogenic Activities of Positive Chemicals and Benzophenones

Abbr.	Chemical name	Estrogenic activity		
		Mammalian reporter gene assay		Two-hybrid assay[13]
		EC_{50} (M)	PC_{50} (M)	REC_{10} (M)
E_2	17β-Estradiol	1.5×10^{-11}	1.5×10^{-11}	3.4×10^{-10}
BPA	Bisphenol A	2.0×10^{-7}	1.4×10^{-7}	1.1×10^{-5}
NP	4-Nonylphenol	2.9×10^{-7}	3.2×10^{-7}	4.6×10^{-7}
BP-1	Benzophenone	3.0×10^{-5}	6.5×10^{-5}	$> 1 \times 10^{-3}$
2	2-Hydroxybenzophenone	9.9×10^{-6}	7.7×10^{-6}	6.2×10^{-4}
3	3-Hydroxybenzophenone	1.7×10^{-6}	9.3×10^{-7}	1.0×10^{-5}
4	4-Hydroxybenzophenone	2.7×10^{-6}	1.2×10^{-6}	4.5×10^{-6}
5	2,2′-Dihydroxybenzophenone	3.4×10^{-5}	9.6×10^{-5}	$> 1 \times 10^{-3}$
6	2,4-Dihydroxybenzophenone	1.5×10^{-6}	7.7×10^{-7}	1.8×10^{-6}
7	4,4′-Dihydroxybenzophenone	4.8×10^{-6}	4.6×10^{-7}	3.8×10^{-5}
8	2,3,4-Trihydroxybenzophenone	1.8×10^{-5}	3.0×10^{-5}	9.0×10^{-6}
9	2,4,4′-Trihydroxybenzophenone	9.2×10^{-7}	1.6×10^{-7}	1.8×10^{-5}
10	2,2′,4,4′-Tetrahydroxybenzophenone	5.5×10^{-6}	1.2×10^{-7}	1.4×10^{-5}
11	2,3,4,4′-Tetrahydroxybenzophenone	2.8×10^{-6}	2.6×10^{-6}	3.6×10^{-5}
12	2,3′,4,4′-Tetrahydroxybenzophenone	2.0×10^{-6}	1.4×10^{-6}	2.4×10^{-4}
13	2-Hydroxy-4-methoxybenzophenone	2.6×10^{-5}	1.5×10^{-5}	6.6×10^{-4}
14	2,2′-Dihydroxy-4-methoxybenzophenone	2.8×10^{-5}	9.3×10^{-5}	1.0×10^{-3}
15	2,2′-Dihydroxy-4,4′-dimethoxybenzophenone	2.7×10^{-5}	1.9×10^{-5}	$> 2 \times 10^{-3}$
16	4-Hydroxy-2′,4′-methoxybenzophenone	2.7×10^{-6}	1.8×10^{-6}	4.0×10^{-5}
17	2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$	$> 1 \times 10^{-3}$
18	2-Hydroxy-4-*n*-octyloxybenzophenone	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$	$> 1 \times 10^{-3}$
19	2-Hydroxy-5-methylbenzophenone	2.1×10^{-5}	3.1×10^{-5}	1.3×10^{-4}
20	4-Hydroxy-4′-chlorobenzophenone	1.9×10^{-6}	8.1×10^{-7}	2.2×10^{-6}

EcoScreen™ and AR-EcoScreen™, have been developed as a sensitive and rapid method to screen hormonally active chemicals.[14–17] They can detect the transcriptional activities mediated with the human estrogen receptor α (hERα) or androgen receptor (hAR). In this study, we investigated the estrogenic and anti-androgenic activities of benzophenone and 19 hydroxylated benzophenones using these cell lines.

MATERIALS AND METHODS

Reagents —— 17β-Estradiol (E_2), bisphenol A (BPA), 5α-dihydrotestosterone (DHT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE), cycloheximide and benzophenone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydroxyflutamide was purchased from Tronto Research Chemicals, Inc. (NY, U.S.A.), 3-hydroxybenzophenone was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan), and the other benzophenone derivatives and 4-nonylphenol (NP) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The benzophenones tested in this study are listed in Table 1 and their structures were shown in the previous paper.[13]

Cell Culture —— The ERα-EcoScreen™ and AR-EcoScreen™ cells (Otsuka Pharmaceutical, Tokyo, Japan) were derived from the Chinese hamster ovary cell line (CHO-K1, American Type Culture Collection) that was stably transfected with the expression vectors pcDNAERα or pZeoSV2AR containing the cDNA of hERα or hAR, respectively.[14–17] To evaluate the cell viability from test chemicals, the cLuc-EcoScreen™ cell was stably transfected luciferase gene (pcDNA-luc) into CHO-K1 cells, which constitutively expressed luciferase without any induction.[16,17]

They are cultured in assay medium composed of D-MEM/F12 (Invitrogen, CA, U.S.A.) without phenol red, and supplemented with 5% charcoal-dextran treated FBS (Hyclone, UT, U.S.A.). The cells were grown at 37°C in an atmosphere containing 5% CO_2/95% air under saturating humidity and passaged every 3–4 days by trypsinization with 0.25%

Table 2. Androgenic and Anti-Androgenic Activities and Cell Viabilities of Positive Chemicals and Benzophenones

Abbr.	Chemical name	Androgenic activity	Anti-androgenic activity	Cell viability (%)	
		EC_{50} (M)	IC_{50} (M)	at IC_{50}	at 10^{-4} M
DHT	5α-Dihydrotestosterone	1.6×10^{-10}	—	—	—
OH-F	Hydroxy fultamide	—	1.0×10^{-7}	101	—
DDE	1,1-Dichloro-2,2- bis(*p*-chlorophenyl) ethylene	—	2.3×10^{-6}	101	—
BP-1	Benzophenone	$> 1 \times 10^{-4}$	7.7×10^{-5}	132	134
2	2-Hydroxybenzophenone	$> 1 \times 10^{-4}$	9.5×10^{-6}	127	128
3	3-Hydroxybenzophenone	$> 1 \times 10^{-4}$	1.5×10^{-5}	116	68
4	4-Hydroxybenzophenone	$> 1 \times 10^{-4}$	3.2×10^{-5}	121	116
5	2,2′-Dihydroxybenzophenone	$> 1 \times 10^{-4}$	1.5×10^{-5}	114	80
6	2,4-Dihydroxybenzophenone	$> 1 \times 10^{-4}$	1.8×10^{-5}	111	71
7	4,4′-Dihydroxybenzophenone	$> 1 \times 10^{-4}$	3.7×10^{-5}	122	125
8	2,3,4-Trihydroxybenzophenone	$> 1 \times 10^{-4}$	2.1×10^{-5}	111	84
9	2,4,4′-Trihydroxybenzophenone	$> 1 \times 10^{-4}$	1.9×10^{-6}	124	112
10	2,2′,4,4′-Tetrahydroxybenzophenone	$> 1 \times 10^{-4}$	2.5×10^{-6}	124	124
11	2,3,4,4′-Tetrahydroxybenzophenone	$> 1 \times 10^{-4}$	3.1×10^{-5}	115	113
12	2,3′,4,4′-Tetrahydroxybenzophenone	$> 1 \times 10^{-4}$	5.6×10^{-6}	105	42
13	2-Hydroxy-4-methoxybenzophenone	$> 1 \times 10^{-4}$	2.9×10^{-5}	121	118
14	2,2′-Dihydroxy-4-methoxybenzophenone	$> 1 \times 10^{-4}$	6.3×10^{-6}	120	34
15	2,2′-Dihydroxy-4,4′-dimethoxybenzophenone	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$	—	133
16	4-Hydroxy-2′,4′-methoxybenzophenone	$> 1 \times 10^{-4}$	2.6×10^{-5}	96	70
17	2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$	—	94
18	2-Hydroxy-4-*n*-octyloxybenzophenone	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$	—	110
19	2-Hydroxy-5-methylbenzophenone	$> 1 \times 10^{-4}$	2.7×10^{-5}	114	107
20	4-Hydroxy-4′-chlorobenzophenone	$> 1 \times 10^{-4}$	8.2×10^{-6}	128	34

trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt solution.

hERα Mediated Reporter Gene Agonist Assay Procedure ——— The stock solutions of the test chemicals (10^{-1} M in dimethyl sulfoxide, DMSO) were 10 times serially diluted with DMSO to provide 7 concentrations in the range of 10^{-1} to 10^{-7} M. This DMSO solutions were further diluted with D-MEM/F-12 with no supplement, so that the final DMSO concentration was 0.1% (v/v). The ERα-EcoScreen™ cells were suspended at a density of 1×10^5/ml in assay medium, and seeded with 90 μl in 96-well plates. After a 24-hr culture, 10 μl of the prepared sample solution was added to the plates and cultured for 16–24 hr. The final concentrations of test samples applied to the cells ranged from 10^{-4} to 10^{-10} M. The solvent control and 10^{-9} M E_2, which served as the positive control, were incubated in each plate. Following the 24-hr culture, the luciferase substrate, Steady-Glo™ (Promega, WI, U.S.A.) was added to all the assay wells. After shaking at room temperature for 5 min, the chemiluminescence was measured using a Wallac 1420 ARVO SX multi-label counter (Perkin-Elmer, MA, U.S.A.).

hAR Mediated Reporter Gene Agonist Assay Procedure ——— The AR-EcoScreen™ cells were prepared with same procedure as the hERα mediated reporter gene agonist assay using 10^{-8} M DHT in stead of 10^{-9} M E_2 as the positive control.

hAR Mediated Reporter Gene Antagonist Assay Procedure ——— The AR-EcoScreen™ cells and cLuc-EcoScreen™ cells were prepared with same procedure as the hERα mediated reporter gene agonist assay with the following exceptions. D-MEM/F-12 containing 5×10^{-10} M of DHT was used for the final sample diluent. This concentration of DHT was about 70% of the plateau level.

The prepared samples were added to the 96-well plates separately seeded with AR-EcoScreen™ or cLuc-EcoScreen™. The positive control (10^{-8} M DHT), solvent control and 1 μg/ml cycloheximide as cell toxicity positive control were included in each plate.

Data and Statistical Analysis ——— Data were presented as the mean and S.D. of at least triplicate assays. Dose–response data were fitted using the

four-parameter logistic equation of Prism 4 for Windows (GraphPad, San Diego, CA, U.S.A.). For the ERα agonist activity, the 50% effective concentration (EC_{50}) value and the 50% positive concentration (PC_{50}) value were calculated. The EC_{50} value was the concentration of the test chemical corresponding to 50% of its maximum luciferase activity and the PC_{50} value was that corresponding to 50% of the maximum luciferase activity of the positive control (10^{-9} M E_2).[17,18] The 50% inhibition concentration (IC_{50}) value for the AR antagonist assay was the concentration of the test chemical corresponding to 50% inhibition of the 5×10^{-10} M DHT-induced luciferase activity. The IC_{50} value was rejected when the cell viability of cLuc-EcoScreen™ at IC_{50} value would be under 80%.

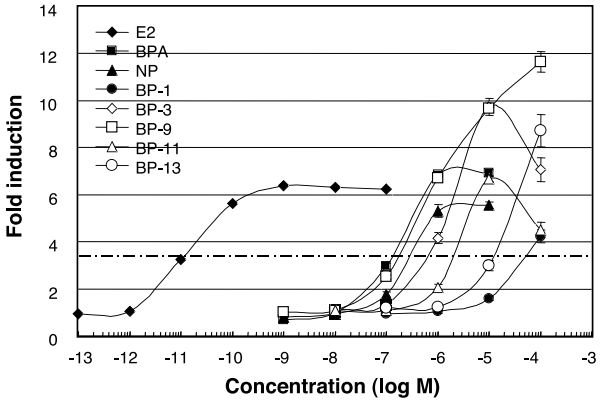

Fig. 1. Estrogenic Activities of Typical Chemicals in hERα Mediated Mammalian Reporter Gene Assay

Abbreviations are listed in Table 1. Values are presented as fold induction compared to vehicle controls and represent the mean ± S.D. of triplicate assays. The dashed line shows 50% activity of 10^{-9} M E_2 (PC_{50}).

RESULTS

Estrogenic Activity of Benzophenones in the hERα Mediated Reporter Gene Agonist Assay

The estrogenic activity of E_2, BPA, NP and the benzophenones, as measured by the dose response curves (Fig. 1) and corresponding EC_{50} and PC_{50} values (Table 1) were determined using ERα-EcoScreen™.

The estrogenic activity of E_2 was detectable at levels greater than 10^{-12} M. The maximum induction was about 6-fold greater than the control at concentrations over 10^{-10} M. BPA and NP, which are well-known endocrine disrupting chemicals, showed approximately 1/10000 and 1/20000 of the E_2 activity, respectively.

The eighteen benzophenones showed estrogenic activities between 10^{-8} and 10^{-4} M. Among of them, eight hydroxylated benzophenones, such as 3-hydroxybenzophenone, 2,4,4′-trihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone and 4-hydroxy-4′-chlorobenzophenone, elicited luciferase induction much higher than E_2, which is referred to as super-agonism.[19] The mechanism of this phenomenon is not clear, but it may be due to the stimulated receptor and/or co-activation factor renewal by the pseudo-estrogens or the increased stability of luciferase. Due to the super-agonism, these chemicals were shown high EC_{50} values, though their induction was detectable at low concentrations. Therefore, the PC_{50} values were used for evaluating the estrogenic activity in substitution for the EC_{50} values.

The PC_{50} values of 2,4,4′-trihydroxybenzophenone and 2,2′,4,4′-tetrahydroxybenzophenone were about 10^{-7} M, which were comparable to BPA and NP. Benzophenone, 2,2′-dihydroxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone showed weak estrogenic activities at 10^{-4} M. Only two benzophenones, 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid and 2-hydroxy-4-*n*-octyloxybenzophenone displayed no activity.

Androgenic Activity of Benzophenones in the hAR Mediated Reporter Gene Agonist Assay

The AR agonist activities of the benzophenones were examined (Table 2). Four chemcals, 4,4′-trihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4,4′-dimethoxybenzophenone, showed a 1.7–2.0 fold weak induction in AR activity only at 10^{-4} M. By this means, all of the benzophenones could not be detected their EC_{50} values. In other words, they had no or very weak androgenic activity.

Anti-Androgenic Activity of Benzophenones in the hAR Mediated Reporter Gene Antagonist Assay

The anti-androgenic activities of hydroxyflutamide, DDE and the benzophenones were determined using the AR-EcoScreen™ in the presence of 5×10^{-10} M DHT. In addition, the cell toxicity was measured using the cLuc-EcoScreen™. The anti-androgenic activity (IC_{50}) and the cell viability are shown in Table 2, and the dose–response inhibitory curves of typical chemicals are shown in Fig. 2.

The anti-androgenic activity of hydroxy-

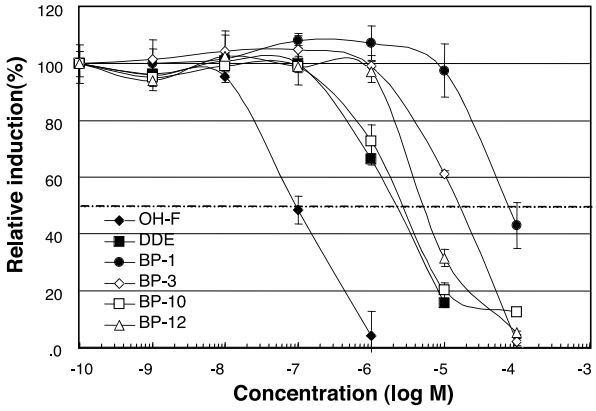

Fig. 2. Anti-Androgenic Activities of Typical Chemicals in hAR Mediated Mammalian Reporter Gene Assay with 5.0×10^{-10} M DHT

Abbreviations are listed in Table 2. Values are presented as percentage induction with 100% activity defined as the activity achieved with 5.0×10^{-10} M DHT, and represent the mean ± S.D. of triplicate assays . The dashed line shows 50% inhibition of 5.0×10^{-10} M DHT (IC_{50}).

flutamide and DDE, which are known anti-androgenic compounds, became detectable at levels greater than 10^{-8} and 10^{-7} M, and their IC_{50} values were 1.0×10^{-7} and 2.3×10^{-6} M, respectively. Seventeen benzophenones had anti-androgenic activities between 10^{-4} and 10^{-7} M. The activity of 2,4,4′-trihydroxybenzophenone and 2,2′,4,4′-tetrahydroxybenzophenone were expressed at levels greater than 10^{-7} M. The corresponding IC_{50} values were 1.9×10^{-6} and 2.5×10^{-6} M, respectively. Their potencies were similar to DDE. Benzophenone showed activity only at 10^{-4} M, and its IC_{50} was 7.7×10^{-5} M. While, three hydroxylated benzophenones, 2,2′-dihydroxy-4,4′-dimethoxybenzophenone, 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid and 2-hydroxy-4-*n*-octyloxybenzophenone did not show anti-androgenic activities.

Some benzophenones produced cytotoxicity. For example, 2,3′,4,4′-tetrahydroxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone and 4-hydroxy-4′-chlorobenzophenone killed about 60% of the cells at 10^{-4} M. However, all of the cell viabilities at the concentration of their IC_{50} were over 96%. Therefore, it was evaluated that the cytotoxicity did not affect the IC_{50} values of these benzophenones.

DISCUSSION

This hERα mediated reporter gene assay showed a higher sensitivity for the detecting estrogenic activity of the benzophenones than the other *in vitro* assays, such as the MCF-7 cell assay,[2,3] the competitive binding assay[4,5] and the recombinant yeast assay.[6] Compared with our previous results using the yeast two-hybrid assay,[13] this assay detected the activity of 18 benzophenones, while the yeast two-hybrid assay detected the activity in 15 benzophenones. These three benzophenones including benzophenone could not detected 10% relative effective concentration (REC_{10}) in the yeast two-hybrid assay though they showed weak induction, and this reporter gene assay could detect their activities because of its sensitivity. The other 15 positive compounds were in common with both assays, and the estrogenic activities tested by this method (PC_{50}) and the yeast two-hybrid assay (REC_{10}) were correlated ($r = 0.79$).

Both assays showed a similar structure-activity relationship as follows. Benzophenones hydroxylated at the 3 or 4 position had more estrogenic activity, while the benzophenones without them had no or lower activity. Furthermore, when a hydroxyl group was added to the 2-position in the 4-hydroxylated benzophenones, the activity was enhanced, for instance, 4-hydroxybenzophenone < 2,4-dihydroxybenzophenone, and 4,4′-dihydroxybenzophenone < 2,4,4′-trihydroxybenzophenone. We hypothesized that the 2-hydroxyl group interacted with the carbonyl group, the benzophenone structure became fixed, and the molecule fit the hERα receptor. However, a hydroxyl group added to the 3-position in 4-hydroxylated benzophenones reduced the activity in the order 2,4-dihydroxybenzophenone > 2,3,4-trihydroxybenzophenone, and 2,4,4′-trihydroxybenzophenone > 2,3,4,4′-tetrahydroxybenzophenone. The 3-hydroxyl group probably interfered with the role of the 4-hydroxyl group.

However, different results were obtained when a hydroxyl group was added to anather benzene ring. The added hydroxyl group enhanced the activity in this reporter gene assay, for instance, 4-hydroxybenzophenone < 4,4′-dihydroxybenzophenone, 2,4-dihydroxybenzophenone < 2,4,4′-trihydroxybenzophenone, and 2,3,4-trihydroxybenzophenone < 2,3,4,4′-tetrahydroxybenzophenone, but it reduced the activity in the yeast two-hybrid assay.

The *in vivo* uterotrophic assay of benzophenones

Table 3. Comparison of Estrogenic Activities of Typical Chemicals with Reporter Gene Assay, Yeast Two-Hybrid Assay and Uterotrophic Assays

Abbr.	Chemical name	Mammalian reporter gene assay	Yeast two-hybrid assay	Uterotrophic assay Lowest positive dose (mg/kg/day)		
		PC_{50} (M)	REC_{10} (M)	Nakagawa[5]	Yamazaki[8,18]	Koda[20]
BPA	Bisphenol A	1.4×10^{-7}	1.1×10^{-5}	—	20	100
NP	4-Nonylphenol	3.2×10^{-7}	4.6×10^{-7}	—	200	—
BP-1	Benzophenone	6.5×10^{-5}	$> 1 \times 10^{-3}$	> 400	> 200	—
4	4-Hydroxybenzophenone	1.2×10^{-6}	4.5×10^{-6}	100	200	—
6	2,4-Dihydroxybenzophenone	7.7×10^{-7}	1.8×10^{-6}	—	—	625
7	4,4′-Dihydroxybenzophenone	4.6×10^{-7}	3.8×10^{-5}	—	200	—
9	2,4,4′-Trihydroxybenzophenone	1.6×10^{-7}	1.8×10^{-5}	—	40	100
10	2,2′,4,4′-Tetrahydroxybenzophenone	1.2×10^{-7}	1.4×10^{-5}	—	200	—

were reported by Nakagawa,[5] Yamazaki[8] and Koda[20] (Table 3). They showed a good correlation with their PC_{50} values in this reporter gene assay. The reporter gene assay provided results more similar to the *in vivo* uterotrophic assay than the yeast two-hybrid assay did. This was provably caused by the steric exclusion of the coactivator situated next to the estrogen receptor in the yeast two-hybrid assay.

An anti-androgenic activity was displayed by 17 benzophenones. All of them also showed an estrogenic activity, and 2,4,4′-trihydroxybenzophenone and 2,2′,4,4′-tetrahydroxybenzophenone expressed the strongest activities for both the estrogenic and anti-androgenic activities. The hydroxyl group in 2-position of benzophenones had a tendency to enhance this activity. However, the effect of the hydroxyl group in other positions on the anti-androgenic activities was less clear than that on the estrogenic activities. Since the correlation between the estrogenic and anti-androgenic activities was 0.68, the hydroxylated benzophenones could easily bind with both receptors.

In contrast, these benzophenones showed no or very weak androgenic activities. Yamazaki[8] reported the Hershberger assay of 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone and 2,4,4′-trihydroxybenzophenone, in which they did not show the androgen agonistic effect.

In fact, the most of hydroxylated benzophenones could easily bound with the estrogen and androgen receptors. When the molecule was bound to the receptors, the receptor-molecule complex would initiate an estrogenic activity in estrogen receptor, though they inhibit the androgen-like molecules from initiating the androgen activity in androgen receptor. In other words, hydroxylated benzophenones would act as endocrine disrupters based on both their estrogenic activity and anti-androgenic activities.

This investigation revealed that hydroxylated benzophenones possessed both estrogenic activity and anti-androgenic activities at high frequency. These chemicals are used in commercial products such as UV stabilizers in plastics, sunscreens, and cosmetics. In addition, they are also produced in the body as metabolites of benzophenone and its derivatives. Because of their wide exposure and ability to disrupt endocrine activity, more work is needed to evaluate the potential health effects of hydroxylated benzophenones.

Acknowledgements This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports and Technology, Japan.

REFERENCES

1) Colborn, T., vom Saal, F. S. and Soto, A. M. (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and human. *Environ. Health Perspect.*, **101**, 378–384.
2) Nakagawa, Y., Suzuki, T. and Tayama, S. (2000) Metabolism and toxicity of benzophenone in isolated rat hepatocytes and estrogenic activity of its metabolites in MCF-7 cells. *Toxicology*, **156**, 27–36.
3) Nakagawa, Y. and Suzuki, T. (2002) Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells. *Chem.-Biol. Interact.*, **156**, 27–36.
4) Blair, R. M., Fang, H., Branham, W. S., Hass, B. S.,

Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R. and Sheehan, D. M. (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci.*, **54**, 138–153.
5) Nakagawa, Y. and Tayama, K. (2001) Estrogenic potency of benzophenone and its metabolites in juvenile female rats. *Arch. Toxicol.*, **75**, 74–79.
6) Schultz, T. W., Seward, J. R. and Sinks, G. D. (2000) Estrogenicity of benzophenones evaluated with a recombinant yeast assay: comparison of experimental and rules-based predicted activity. *Environ. Toxicol. Chem.*, **19**, 301–304.
7) Schreurs, R., Lanser, P., Seinen, W. and 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.
8) Yamazaki, K., Takeyoshi, M., Sawaki, M., Imatanaka, N., Shinoda, K. and Takatsuki, M. (2003) Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. *Toxicology*, **183**, 93–115.
9) Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B. and Lichtensteiger, W. (2001) *In vitro* and *in vivo* estrogenicity of UV screens. *Environ. Health Perspect.*, **109**, 239–244.
10) Ma, R., Cotton, B., Lichtensteiger, W. and Schlumpf, M. (2003) UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. *Toxicol. Sci.*, **74**, 43–50.
11) Stocklinski, A. W., Ware, O. B. and Oberst, T. J. (1980) Benzophenone metabolism I. Isolation of *p*-hydroxybenzophenone from rat urine. *Life Sci.*, **26**, 365–369.
12) Okereke, C. S., Abdel-Rhaman, M. S. and Friedman, M. A. (1994) Disposition of benzophenone-3 after dermal administration in male rats. *Toxicol. Lett.*, **73**, 113–122.
13) Kawamura, Y., Ogawa, Y., Nishimura, T., Kikuchi, Y., Nishikawa, J., Nishihara, T. and Tanamoto, K. (2003) Estrogenic activities of UV stabilizers used in food contact plastics and benzophenone derivatives tested by the yeast two-hybrid assay. *J. Health Sci.*, **49**, 205–212.
14) Kojima, H., Iida, M., Katsura, E., Kanetoshi, A., Hori, Y. and Kobayashi, K. (2003) Effects of a diphenyl ether-type herbicide, chloronitrofen, and its amino derivative on androgen and estrogen receptor activities. *Environ. Health Perspect.*, **111**, 497–502.
15) Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K. and Kobayashi, K. (2004) Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environ. Health Perspect.*, **112**, 524–531.
16) Satoh, K., Ohyama, K., Aoki, N., Iida, M. and Nagai, F. (2004) Study on anti-androgenic effects of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. *Food Chem. Toxicol.*, **42**, 983–993.
17) Araki, N., Ohno, K., Takeyoshi, M. and Iida, M. (2005) Evaluation of a rapid *in vitro* androgen receptor transcriptional activation assay using AR-EcoScreen™ cells. *Toxicol. In Vitro*, **19**, in press.
18) Yamazaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Imatanaka, N. and Takatsuki, M. (2002) Comparison of reporter gene assay and immature rat uterotrophic assay of twenty-three chemicals. *Toxicology*, **170**, 21–30.
19) Legler, J., van den Brink, C. E., Brouwer, A., Murk, A. J., van der Saag, P. T., Vethaak, A. D. and van der Burg, B. (1999) Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol. Sci.*, **48**, 55–66.
20) Koda, T., Umezu, T., Kamata, R., Morohoshi, K. and Morita, M. (2003) Evaluation of estrogen like effects of chemicals using OECD uterotrophic assay protocols (1). In *The 6th Annual Meeting of Japan Society of Endocrine Disrupters Research Abstracts*, Sendai, p. 90.