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Estrogenic Potency of Food-Packaging-Associated Plasticizers and Antioxidants As Detected in ERα and ERβ Reporter Gene Cell Lines

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This study presents the estrogenic potency of 21 food-packaging-associated compounds determined for the first time, using two transfected U2-OS (human osteoblasts devoid of endogenous estrogen receptors) estrogen receptor (ER) alpha and beta cell lines. Six plasticizers and three antioxidants were slightly estrogenic in the ERα cells. The model compounds bisphenol A and nonylphenol, one plasticizer [tris(2-ethylhexyl)trimellitate (TEHTM)], and two antioxidants (propyl gallate and butylated hydroxyanisole) were estrogenic in both ERα and ERβ cells. Compared to estradiol (E_2), these compounds appeared to be relatively more estrogenic in the ERβ cells than in the ERα cells. Three sorbitol-based plasticizers activated neither ERα nor ERβ and may be good replacements of existing plasticizers. All responses were additive with the response of E_2. This indicates that they may contribute to the total effects of the pool of estrogenic compounds humans are exposed to. The estrogenic potencies of these compounds, together with the suggested beneficial effect of ERβ-mediated responses and adverse ERα-mediated effects, support the importance of detecting characteristics for ERα and ERβ response separately in independent models, as done in the present study.

KEYWORDS: Antioxidant; endocrine disrupter; ERα; ERβ; estrogen; food packaging; plasticizer; pseudo-estrogen

INTRODUCTION

Incidences of hormone-related cancers, especially breast cancer, are increasing worldwide (*1*). In The Netherlands, age-specific breast cancer incidence increased by 23% in the period from 1989 to 2000 (*2*). In addition to some known risk factors such as radiation exposure (*3*), genetic predisposition (*4*), alcohol intake (*5*), and a higher fat intake (in animal studies) (*6*), estrogenic compounds, such as natural estrogens and pseudo-estrogens, are suspected of increasing a woman's risk of developing breast cancer (*7*). Pseudo-estrogens are all estrogenic active compounds that are not naturally present in the female body. In vitro, some of these pseudo-estrogens (genistein and quercetin) cause cell proliferation of breast cancer cells via interaction with the estrogen receptor (*8*).

At least two different estrogen receptors (ER) exist, namely, ERα and ERβ (*9*). Besides differences in binding capacities for specific ligands there are differences in tissue distribution of the two receptors. In the mammary gland, uterus, and testes ERα is the predominant receptor (*10*). The ovary contains both receptor subtypes in large amounts, and in the prostate ERβ is the most abundant receptor (*10*). Upon ligand binding homo- and heterodimers of the ERα and ERβ can be formed in vivo and in vitro, and these dimers can activate the estrogen responsive elements initiating gene expression. It has been suggested that the ERβ might modulate the estrogenic effects of the ERα by forming heterodimers with ERα, thereby reducing the ERα-mediated response, resulting in, for example, inhibition of ERα-mediated proliferation of breast cancer cells in vitro (*11*−*14*). The inability to obtain a full-length cDNA of the ERβ without mutation (*15*) from mRNA isolated from the human T47D breast cancer and Caco-2 colon cancer cells further supports the idea that ERβ activation may be beneficial and modulate possible adverse ERα-mediated estrogenic effects. Given the differential biological responses upon ERα or ERβ activation, it is of importance in the study of endocrine-disrupting compounds to characterize their interaction with both receptors separately. Therefore, the objective of the present study was to characterize the ERα- and ERβ-mediated estrogen

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response of a wide range of food contaminants and existing and newly developed direct and indirect food additives for their ERα- and ERβ-type estrogenic potential.

Some endocrine-disrupting compounds are known for their estrogenic activity, such as phthalates (*16*−*20*), nonylphenol (NP) (*20*−*24*), and bisphenol A (BPA) (*20*, *25*−*29*). Human intake of these and other pseudo-estrogens is mainly via food and originates from natural food chain constituents, containing, for example, pseudo-estrogens, from a contaminated environment (for example, DDT), from food packages (BPA) (*26*), and/or from additives such as antioxidants or plasticizers used in packaging material (*30*).

In the present study we determined the ER-type specific estrogenic potency of food-associated compounds by using two different human osteoblastic (U2-OS) reporter gene cell lines. U2-OS cell lines are devoid of endogenous estrogen receptors. The two transfected cell lines stably express either ERα or ERβ in addition to 3xERE-tata-Luc as a reporter gene (*31*).

The compounds tested are estradiol (E_2, as a reference), BPA, NP, included in the studies to allow comparison with existing literature data (*23*, *32*−*34*), eight existing plasticizers, three newly developed plasticizers based on sorbitol, and nine existing antioxidants. All plasticizers and antioxidants are generally used in food-packaging material and were therefore included in the present study to investigate their estrogenicity, of which little is known so far. **Table 1** summarizes and presents all compounds studied.

MATERIALS AND METHODS

Chemicals and Reagents. E_2 (>98%), BPA (>99%), and tricine (>99%) were obtained from Sigma (Zwijndrecht, The Netherlands). $NaHCO_3$ (>99.5%), NaOH (>99%), EDTA·$2H_2O$ (>99%), $MgSO_4$·$7H_2O$ (>99.5%), 1,4-dithiothreitol (>99%), (L+) ascorbic acid (>99.7%), and diethyl ether (>99,7%) were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO, >99.9%) was obtained from Acros (Geel, Belgium). Tris(hydroxymethyl)aminomethane (>99.9%) was obtained from Invitrogen (Carlsbad, CA). D-Luciferin (>99.5%) was obtained from Duchefa (Haarlem, The Netherlands). *trans*-1,2-Diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid monohydrate (CDTA, >99%) and NP (~85%; technical mixture) were obtained from Fluka (Buchs, Germany). $(MgCO_3)_4Mg(OH)_2$·$5H_2O$ was obtained from Aldrich (Milwaukee, WI). Di(2-ethylhexyl) phthalate (DEHP) was obtained from TCI (Tokyo, Japan). ATP was obtained from Roche (Mannheim, Germany). All plasticizers and antioxidants (**Table 1**) are of technical grade. To enable the reader to search for the compounds in **Table 1**, all compounds are numbered in sequence. Sorbitol-based plasticizers were produced by Agrotechnology and Food Innovations, Wageningen UR, in the project sugar polyol based plasticizers, aiming at the development of efficient, undisputed, renewable esters as plasticizers and solvents. CAS Registry Numbers are given in **Table 1**. All chemicals were dissolved and diluted in DMSO. Food-packaging-associated compounds were tested at least two times in five concentrations ranging from 0 to 5×10^{-5} M. As in vivo exposure to pseudo-estrogens always occurs in combination with the presence of endogenous hormones, the compounds were also tested with E_2 present at its EC_{50} value in both cell lines. To this end the cells were incubated with the tested compounds at doses ranging from 0 to 5×10^{-5} M in the presence of E_2 at its EC_{50} value for agonist action.

Cell Culture. U2-OS ERα and ERβ cells (*31*) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (31331-028, Gibco), buffered with 1260 mg/L $NaHCO_3$, supplemented with 7.5% fetal calf serum (FCS, Australian origin, 10099, Invitrogen) and 0.5% nonessential amino acids (minimal essential medium 100×, 11140-035, Gibco). ERα medium was supplemented with geneticin (200 μg/mL, Invitrogen) and hygromycin (50 μg/mL, Duchefa) as selection markers. ERβ medium was supplemented with geneticin (200 μg/mL) only. Cells were cultured at 37 °C at 7.5% CO_2 in a humidified atmosphere.

Luciferase Assay. U2-OS cells were washed twice with phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}; Gibco), trypsinized (Gibco) and diluted in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red (21041-025, Gibco), buffered with 1260 mg/L $NaHCO_3$, supplemented with 5% dextran-coated charcoal (DCC)-stripped FCS (*36*) and 0.5% nonessential amino acids. ERα cells were seeded at a density of 10000 cells/well and ERβ cells were seeded at a density of 7500 cells/well in 96-well Nunclon plates (167008, NUNC). After 24 h, assay medium was renewed, and 24 h later, assay medium was removed and replaced by 100 μL of exposure medium, with test compounds dissolved in DMSO concentrations of 0.1%. For combination experiments DMSO concentrations were 0.2%. All experiments with antioxidants used 700 μM ascorbic acid in the exposure medium to prevent autoxidation. Compounds were tested in triplicate; outer rows were filled with 200 μL of sterile PBS to prevent effects of evaporation on the outer side of the plates. After 24 h of exposure, medium was removed and cells were washed with 0.5× PBS. Cells were lysed with 30 μL of hypotonic low-salt buffer (10 mM Tris, 2 mM DTT, and 2 mM CDTA; pH 7.8). Plates were put on ice for 15 min to allow swelling of the cells and subsequently frozen at −80 °C for at least 30 min to lyse the cells. Plates were thawed on ice and shaken for 2 min at room temperature. Luciferase activity was measured at room temperature in a luminometer (Labsystems, Luminoskan RS). First, background light emission was measured for 2 s, then 100 μL of "flashmix" [20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$·$5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA·$2H_2O$, 2 mM DTT, 0.47 mM d-luciferin, and 5 mM ATP; pH 7.8] was automatically injected, and light emission was immediately measured during 2 s and extinguished with 50 μL of 0.2 M NaOH to stop the reaction.

Data Analysis. Relative light units (RLUs) in every well were corrected for the corresponding background signal, measured before luciferin addition. To be able to compare all data, the response of the solvent control (0.1% DMSO for single and 0.2% for combination experiments) was set at 0%. The maximum induction of luciferase obtained at 10 pM E_2 for ERα cells and at 1250 pM E_2 for ERβ cells was set at 100%. In every experiment an E_2 calibration curve was included using 0, 0.3, 1, 2, and 10 pM E_2 for ERα and 0, 10, 60, 100, and 1250 pM E_2 for ERβ. Dose−response curves were fitted using Slidewrite 6.10 for Windows.

Estradiol equivalency factors ($EEF_{10} = EC_{10\ estradiol}/EC_{10\ compound}$) were calculated for the responses in ERα and ERβ cell lines. EC_{10} values were interpolated from the E_2 calibration curves; the EC_{10} values were used instead of EC_{50} values because for less potent compounds factors such as insolubility hamper measurements at higher concentrations. For the more potent compounds, such as BPA and NP, also EEF_{50} values were calculated as $EC_{50\ estradiol}/EC_{50\ compound}$ (**Table 2**).

Responses were taken into account only when they were above the limit of quantification (LOQ), defined as the DMSO response plus 3 times its standard deviation (SD). The limit of detection (LOD) was defined as the DMSO response plus 2 times its SD. The LOQ interpolated in the E_2 curve gave the lowest effect concentration (LEC).

RESULTS

Estradiol (1, E_2) Calibration Curve. Exposure of the ERα and ERβ cell lines to increasing concentrations of E_2 (**1**) resulted in sigmoidal dose−response curves (**Figure 1**). The LEC for ERα cells was 0.3 pM (± 0.3), and the LEC for ERβ cells was 6.6 pM (± 3.9). EC_{50} values differ almost 20-fold, being 6.6 (± 1.6) pM for ERα cells and 124.2 (±29.6) pM for ERβ cells. The maximum RLU value in the ERα cell line was 5 times higher than the maximum RLU value in the ERβ cell line. When expressed as induction relative to DMSO, however, the maximum induction of the ERβ cell line was 12 times higher than that in the ERα cell line, because of the relatively high background signal in the ERα. To be able to compare dose−response curves, results are presented on scales from 0 to 100% induction by E_2 (**Figure 1**). From **Figure 1** it can be derived that at 30 pM E_2 luciferase induction in the ERα cells declines,

Table 1. Food-Packaging-Associated Compounds Tested for Their Estrogenicity in the ERα and ERβ Reporter Gene U2-OS Assays, Including Their Molecular Structures and CAS Registry Numbers[a]

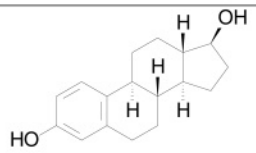
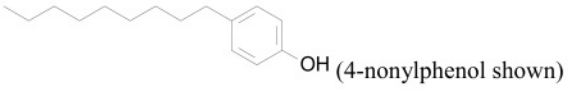
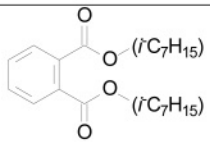
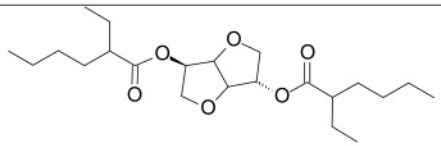
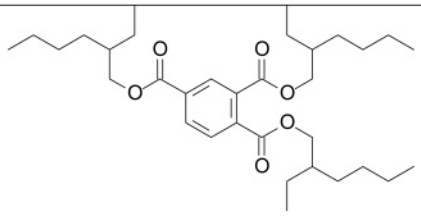
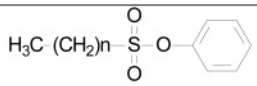
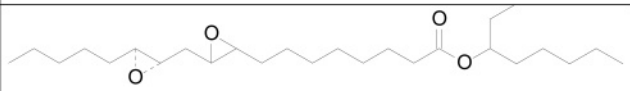
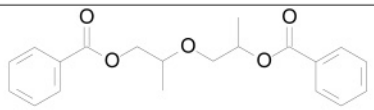
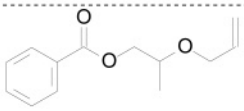
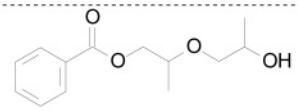
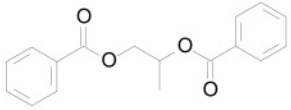
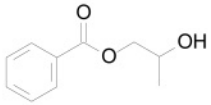
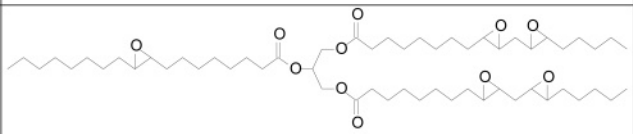
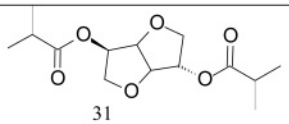
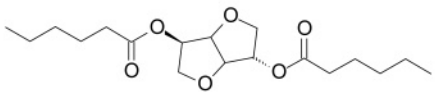
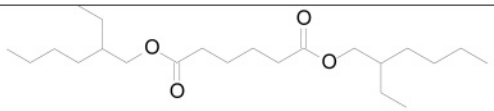
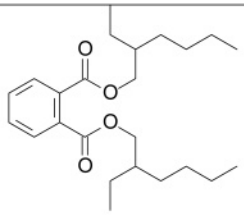
Compound		Structure	CAS no.
1	17β-estradiol (E_2)		[50-28-2]
2	Bisphenol A (BPA)		[80-05-7]
3	Nonylphenol (NP); 85%, technical mixture	 (4-nonylphenol shown)	[104-40-5]
Plasticizers			
4	Diisoheptyl phthalate (DIHP)		[41451-28-9]
5	Isosorbide di 2-ethylhexanoate (IsDEH)		[94593-67-6]
6	Tris (2-ethylhexyl) trimellitate (>99%) (TEHTM)		[3319-31-1]
7	Phenyl alkylsulfonate	 n= 14-16	[91082-17-6]
8	Octyl epoxy stearate		[128514-16-9]
9	Benzoate mixture (5 compounds)		
9a	Dipropylene glycol dibenzoate (89.4%)		[27138-31-4]
9b	(2-Propenyloxy) propyl benzoate (2.35%)	 D1- Me	[197178-94-2]

Table 1. (Continued)

9c	Dipropylene glycol monobenzoate (4.98%)	 2(D1- Me)	[32686-95-6]
9d	Propylene glycol dibenzoate (2.29%)		[19224-26-1]
9e	Propylene glycol monobenzoate (0.28%)		[37086-84-3]
10	Epoxidized soy bean oil (ESO)		[8013-07-8]
11	Isosorbide diiso-butyrate (IsDiB)	 31	[125344-74-3]
12	Isosorbide di-*n*-hexanoate (IsDH)		[64896-69-1]
13	Di (2-ethylhexyl) adipate (DEHA)		[103-23-1]
14	Di (2-ethylhexyl) phthalate (DEHP)		[117-81-7]

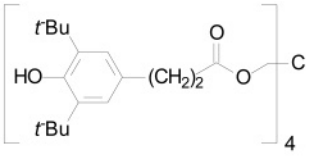
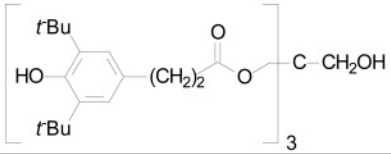
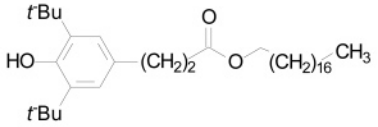
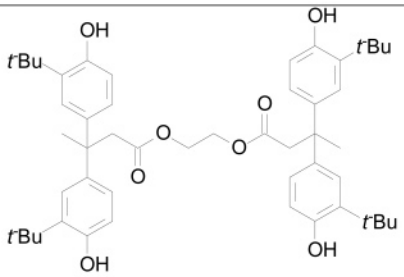
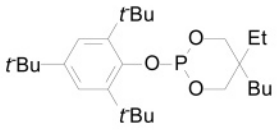
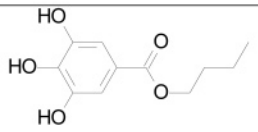
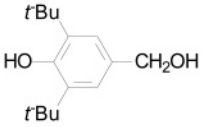
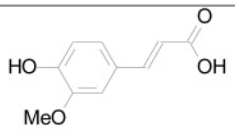
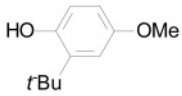
Antioxidants		**Structure**	**CAS no.**
15	Antioxidant mixture consisting of two compounds		
15a	Pentaerythritol tetrakis[3-(3,5-di-tert-butyl-4-hydroxy phenyl)] propionate (>96%)		[6683-19-8]
15b	Pentaerythritol tris [3-(3, 5-di-tert-butyl-4-hydroxy-phenyl)] propionate (<4%)		[84633-54-5]

Table 1. (Continued)

16	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-propionate		[2082-79-3]
17	Ethylene bis[3,3-bis (3-tert-butyl-4-hydroxy phenyl)butyrate]		[32509-66-3]
18	Amines, bis(hydrogenated rape-oil alkyl)methyl, N-oxides	Unknown	[204933-93-7]
19	2,4,6-Tris (tert-butyl) phenyl-2-butyl-ethyl-1,3-propanediol phosphite		[161717-32-4]
20	Propyl gallate		[121-79-9]
21	3,5-Di-tert-butyl-4-hydroxybenzyl alcohol >97%		[88-26-6]
22	4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)		[1135-24-6]
23	Butylated hydroxyl anisole (BHA)		[25013-16-5]

[a] Consecutive numbers for compounds are used to facilitate searching for the compounds in the text.

and therefore this data point was not included in defining the dose−response curve.

Food-Packaging-Associated Compounds. Figure 2 presents the results from a representative experiment showing the luciferase induction in ERα and ERβ cells upon exposure to different food-packaging-associated compounds. The maximum luciferase induction by BPA (**2**) and NP (**3**) is higher than the maximum luciferase induction by E_2, especially in the ERα cells (**Figure 2**). **Table 2** presents the EC_{50} and EEF_{50} values obtained for E_2, NP, and BPA from a series of four similar independent experiments. To compare results obtained in the ERα and ERβ cell line **Table 2** also presents the ratio $EEF_{10}\alpha/EEF_{10}\beta$, which, by definition, equals 1 for E_2. If the ratio $EEF_{10}\alpha/EEF_{10}\beta$ of a compound is <1, this compound is relatively more estrogenic in the ERβ cell line, showing, compared to E_2, a relatively higher EEF_{10} in the ERβ than in the ERα cell line. The $EEF_{10}\alpha/EEF_{10}\beta$ ratios of BPA and NP are ≈0.1, meaning they are relatively more estrogenic as compared to E_2 in the ERβ cell line than in the ERα cell line.

The results of tested compounds that show activity in the ERα and ERβ cell lines are summarized in **Table 3**. All sorbitol-based plasticizers [isosorbide-di-2-ethylhexanoate (**5**), isosorbide diiso-butyrate (**11**), and isosorbide-di-*n*-hexanoate (**12**)], the phenyl alkylsulfonate (**7**), and epoxidized soybean oil (ESO)

Table 2. EC_{50} and EEF_{50} Values of Nonylphenol (NP) and Bisphenol A (BPA) Compared to Estradiol (E_2), As Determined in the U2-OS ERα and U2-OS ERβ Cell Lines

compound	assay	EC_{50} (nM)	EEF_{50}	$EEF_{50}\alpha/EEF_{50}\beta$	max effect as % relative to E_2-max
E_2	ERα	0.006 ± 0.002	1	1	100
	ERβ	0.124 ± 0.03	1		100
NP	ERα	124 ± 63	5.3×10^{-5}	0.054	114 ± 26
	ERβ	149 ± 57	8.3×10^{-4}		122 ± 10
BPA	ERα	216 ± 35	3.1×10^{-5}	0.094	170 ± 17
	ERβ	234 ± 110	5.3×10^{-4}		116 ± 11

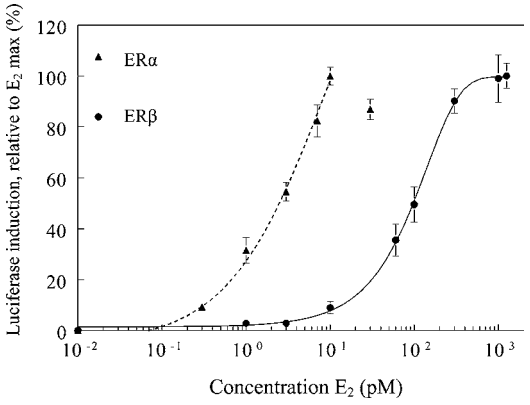


Figure 1. Dose–response curves of U2-OS ERα and U2-OS ERβ cells exposed for 24 h to estradiol (E_2). The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

(**10**) appeared to be nonestrogenic in both cell types. Nonestrogenic antioxidants were the antioxidant mixture [96% pentaerythritol tetrakris[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)]propionate (**15a**) and 4% pentaerythritol tris[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)]propionate (**15b**)], octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (**16**), ethylene bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)butyrate] (**17**), bis(hydrogenated rape-oil alkyl)-methyl *N*-oxides (**18**), 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol (**21**), and *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid) (**22**).

Five plasticizers appeared to be estrogenic in the ERα cell line and not in the ERβ cell line; these include di-isoheptyl phthalate (DIHP, **4**), di(2-ethylhexyl) phthalate (DEHP, **14**), di-(2-ethylhexyl)adipate (DEHA, **13**), octylepoxy stearate (**8**), and the benzoate mixture [89.4% dipropyleneglycol dibenzoate (**9a**), 2.35% (2-propenyloxy) propyl benzoate (**9b**), 4.98% propyleneglycol dibenzoate (**9c**), 2.29% propyleneglycol benzoate (**9d**), and 0.28% propyleneglycol monobenzoate (**9e**)]. Tris(2-ethylhexyl) trimellitate (TEHTM, **6**) was estrogenic in both cell lines with an $EEF_{10}\alpha/EEF_{10}\beta$ ratio of 0.075. The estrogenic potencies of positive compounds as compared to E_2 can all be found in **Table 3**.

The antioxidants propyl gallate (**20**) and butylated hydroxyanisole (BHA, **23**) were estrogenic in the ERα cells as well as in the ERβ cells with $EEF_{10}\alpha/EEF_{10}\beta$ ratios of 0.17 and 0.07, respectively. The antioxidant 2,4,6-tri-*tert*-butylphenyl-2-butyl-ethyl-1,3-propanediolphosphite (propanediolphosphite) (**19**) was estrogenic only in the ERα cells (**Table 3**).

Effects of Combined Exposures. The compounds shown to be active in the ERα and ERβ cells were tested in the presence of E_2 at its EC_{50} level to test whether the responses of these combined exposures are additive. Panels **a** and **b** of **Figure 3** show the results obtained for TEHTM (**6**) in the ERα and ERβ cell lines, respectively. Additive effects were observed and similar data were obtained for the other compounds shown to be active in the ERα or ERβ cell line when tested alone.

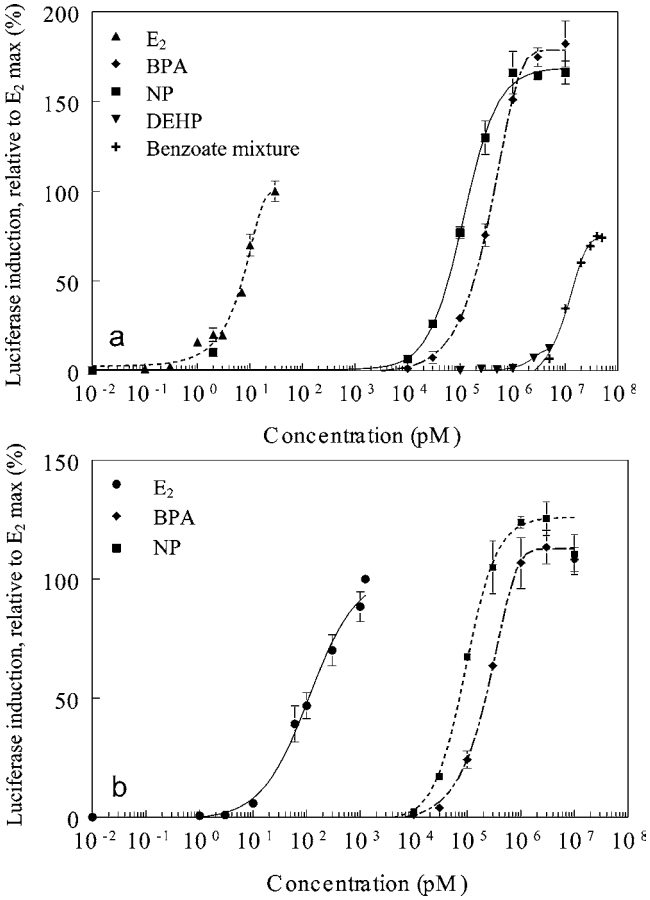


Figure 2. Dose-dependent induction of luciferase activity (**a**) in the ERα cell line and (**b**) in the ERβ cell line by bisphenol A (BPA), nonylphenol (NP), di(2-ethylhexyl)phthalate (DEHP), and a benzoate mixture. DEHP and the benzoate mixture did not induce luciferase activity in the ERβ cell line. The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

DISCUSSION

The present study investigated the ER-type specific estrogenic potency of some foodborne compounds using two different U2-OS reporter gene cell lines stably expressing either ERα or ERβ in addition to 3xERE-tata-Luc (*31*). The results obtained reveal differences in estrogen activity toward both receptor types of different estrogenic compounds as measured with the U2-OS ERα and U2-OS ERβ cell lines. The EC_{50} of E_2 (**1**) in the ERα cell line appeared to be 20 times lower than the EC_{50} of E_2 in the ERβ cell line. E_2 tested in an ERα- and ERβ-transfected human embryonic kidney (HEK) cell line showed a comparable difference in activation (*37*). As can be seen in **Table 4**, presenting an overview of EC_{50} values for E_2, BPA (**2**) and NP (**3**) reported in the literature based on other mammalian reporter- and estrogen-dependent cell proliferation models, the EC_{50} values reported in these literature studies are more in line with the EC_{50} obtained in the present study in the ERα cells than with the EC_{50} for the ERβ cells (**Table 2**). This suggests that the T47D reporter cell line (ER-CALUX) (*23*), E-screen (*32*, *33*), and MVLN cell assays (*33*, *34*) detect primarily ERα-type

Table 3. Lowest Effect Concentration (LEC, Concentration That Equals the Effects of DMSO + 3 × SD), Estradiol Equivalence Factors (EEF_{10}), and Maximum Effect of All Estrogenic Compounds Tested Positive within the Present Study Compared to E_2

	ERα			ERβ			
compounds	LEC (μM)	EEF_{10}	max effect (%)	LEC (μM)	EEF_{10}	max effect (%)	$EEF_{10}\alpha/EEF_{10}\beta$
E_2	0.3×10^{-6}	1	100	6.6×10^{-6}	1	100	1
NP	0.25	4.6×10^{-5}	114	0.01	5.2×10^{-4}	122	0.09
BPA	0.18	2.5×10^{-5}	170	0.02	2.3×10^{-4}	116	0.11
plasticizers							
DIHP	2.3	3.1×10^{-7}	33	*a*	*a*	*a*	
DEHP	0.97	2.2×10^{-7}	14	*a*	*a*	*a*	
DEHA	25.4	4.9×10^{-8}	16	1.7	*b*	3.1	
TEHTM	8.1	1.2×10^{-7}	113	4.9	1.6×10^{-6}	76	0.075
octyl-epoxystearate	7.9	1.3×10^{-8}	26	*a*	*a*	*a*	
benzoate mixture	3.7	2×10^{-7}	72	*a*	*a*	*a*	
antioxidants							
propyl gallate	2.1	6.5×10^{-7}	109	2.5	3.9×10^{-6}	60	0.17
propanediolphosphite	31.7	1.2×10^{-8}	7.4	*a*	*a*	*a*	
BHA	5.9	5.2×10^{-8}	18.3	8.4	7.7×10^{-7}	15.6	0.07

[a] Activity below limit of quantification (DMSO + 3 × SD). [b] Activity too low to calculate an EEF_{10}.

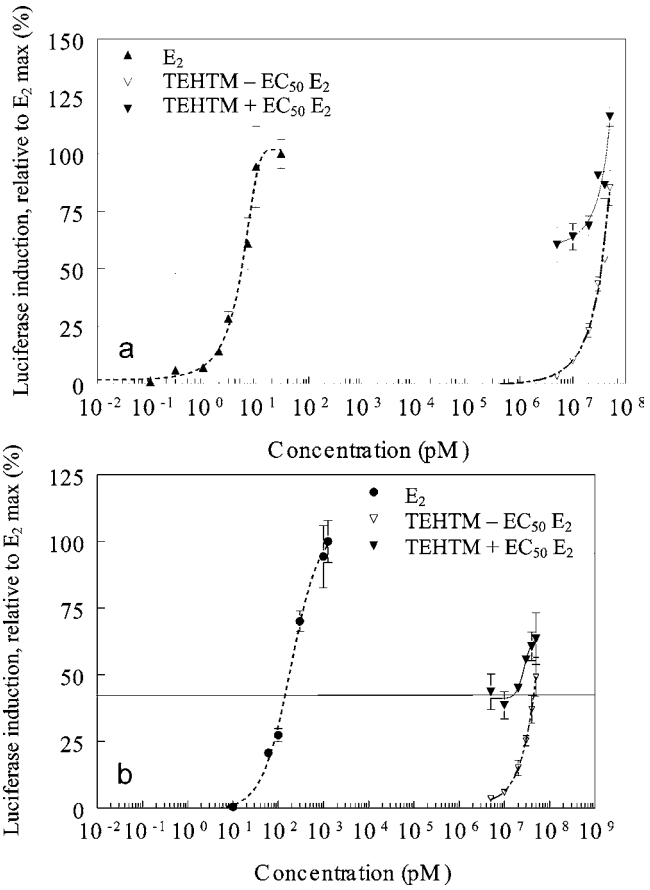


Figure 3. Dose–response curve of (**a**) ERα cells exposed for 24 h to estradiol (E_2), tri-2-ethylhexyltrimellitate (TETHM) alone, and TEHTM + 5 pM E_2 (straight line indicates the EC_{50} level of E_2) and (**b**) ERβ cells exposed for 24 h to estradiol (E_2), TETHM alone, and TEHTM + 100 pM E_2 (straight line indicates the EC_{50} level of E_2). The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

responses. However, as these cell lines are not specifically free of other endogenous steroid receptors, interactions between the ER, the androgen receptor, and even the progesterone receptor cannot be excluded. This further supports the importance of detecting characteristics for ERβ response in an independent cell model such as the U2-OS ERβ model used in the present study. This ERα-type response in the ER-CALUX, E-screen, and MVLN cell assays could be due to the fact that all of these cell systems mainly express endogenous ERα and estrogenicity is dependent on the most abundant receptor (*14*, *32*). The luciferase induction by BPA of more than the maximum induction by E_2 (set at 100%) in the U2-OS ERα cells is in accordance with the induction previously shown in the T47D reporter cell line (ER-CALUX) (*23*) (**Table 4**). Legler et al. suggested that this "superagonism", that is, a maximal response being higher than that induced by E_2, could be due to stimulated receptor and/or cofactor renewal (*23*). As the U2-OS cell line is devoid of endogenous receptors, it can be excluded that there is cross-reactivity of different receptors. Another explanation could be that BPA modulates the activity of several kinases involved in ERE activation. To gain more insight into the mechanism underlying this overstimulation, molecular studies are needed, which are, however, beyond the scope of the present study.

Recently Gustafsson and co-workers demonstrated, using the breast cancer cell line T47D with tetracyclin-dependent inducible

Table 4. Overview of EC_{50} and EEF_{50} Values of Nonylphenol (NP) and Bisphenol A (BPA) Compared to Estradiol (E_2), As Reported in the Literature for Reporter Gene and Proliferation Mammalian Cell Models

compounds	assay	EC_{50} (nM)	EEF	% max effect rel to E_2	ref
E_2	ERα	0.02	1		*35*
	ERβ	0.06	1		*31*
	ER-CALUX	0.006	1	100	*23*
	E-screen	0.0045	1	*a*	*32*
	E-screen	0.005	1	*a*	*33*
	MVLN cells	0.005	1	*a*	*33*
	MVLN cells	0.015	1	*a*	*34*
	HGELN cells	0.040	1	*a*	*33*
NP	ER-CALUX	260	2.3×10^{-5}	135	*23*
	E-screen	400	1.3×10^{-5}	*a*	*33*
	MVLN cells	400	1.3×10^{-5}	*a*	*33*
	MVLN cells	463	3.2×10^{-5}	*a*	*34*
	HGELN cells	500	8×10^{-5}	*a*	*33*
BPA	ER-CALUX	770	7.7×10^{-6}	153	*23*
	E-screen	410	1.1×10^{-5}	*a*	*32*
	E-screen	200	2.5×10^{-5}	*a*	*33*
	MVLN cells	200	2.5×10^{-5}	*a*	*33*
	HGELN cells	210	1.9×10^{-4}	*a*	*33*

[a] Not given in the literature.

ERβ expression in addition to the ERα, that ERβ expression inhibits proliferation induced by estradiol at 10 nM and by the drugs 4-hydroxytamoxifen and raloxifene at 1 μM (*14*). This suggests that ERβ can influence the cell proliferation induced by ERα. In the present study, BPA and NP are 10 times more estrogenic in the ERβ cell line, compared to E_2, than in the ERα cell line. This is reflected by an $EEF_{10}\alpha/EEF_{10}\beta$ ratio of 0.1, which indicates that in theory BPA and NP might to some extent modulate the negative estrogenic effects via the ERα, because of their relatively high-affinity binding to the ERβ. For BPA a specific migration limit of 0.05 mg/kg of food and a tolerable daily intake (TDI) of 0.01 mg/kg of body weight (bw)/day have been established. This TDI is above the average consumer intake, which is estimated by the SCF to range from 0.00048 mg/kg of bw/day for adults to 0.0016 mg/kg of bw/day for infants in a realistic estimate of exposure via food (*38*). Average intake of nonylphenol was estimated by Geunther et al. to be 7.5 μg/person/day, which would be equal to 125 ng/kg of bw/day for a 60 kg person (*39*).

Various plasticizers, including DIHP (**4**), DEHP (**14**), DEHA (**13**), TEHTM (**6**), octyl-epoxystearate (**8**), and the benzoate mixture (**9**), showed estrogenic activity in vitro, activating the ERα with EEF_{10} values between 10^{-8} and 10^{-7}. Of all of these compounds, only TEHTM was estrogenic in the ERβ cells, revealing an $EEF_{10}\alpha/EEF_{10}\beta$ comparable to those of NP and BPA. For DEHP human exposure is estimated to range from 30 μg/kg of bw/day in the regular population to 457 μg/kg of bw/day in hemodialysis patients (*40*). For DEHP the TDI of the general population is set at 0.05 mg/kg of bw/day (*41*). The specific migration limit of DEHA is 18 mg/kg of food, and the TDI is 0.3 mg/kg of bw/day. The median intake in The Netherlands and other western European countries is ≈1 mg/day; this means 16.6 μg/kg of bw/day for a 60 kg person (*42*).

The compounds showing estrogenic activity in our study have been tested in several other models for their estrogenic activity, showing variable results. DIHP was not found to be estrogenic in vitro in an estrogen receptor competitive ligand binding and mammalian- and yeast-based gene expression assays (*43*). On the other hand, a small estrogenic in vivo effect of DIHP on uterine wet weight in ovariectomized Sprague−Dawley rats was found (*43*). Takeuchi et al. (*44*) reported DIHP and DEHP to be active in an ERα-transfected CHO cell line with comparable EEF_{20} values of, respectively, 6.3×10^{-6} and 5.5×10^{-6}. The difference with our EEF_{10} values can be due to the different cell types used or to differences in the interpolation of EC_{10} or EC_{20} values. DEHP was not estrogenic in the T47D ER-CALUX cell line (*45*); this discrepancy with our results in the ERα cells could be due to a relative overexpression of the ERα receptor in our U2-OS transfected cell line compared to the T47D cell line with endogenous ERα expression used in the ER-CALUX.

With respect to the safety assessments of the various plasticizers tested, the following findings of the present study are of interest. DEHA appeared to be less estrogenic ($EEF_{10}\alpha$ of 4.9×10^{-8}) than DEHP ($EEF_{10}\alpha$ of 2.2×10^{-7}) (**Table 3**); therefore, DEHA would be preferable over DEHP to use in food packaging with regard to the estrogenic effect only. However, given the current concern about the migration of adipates (*46*, *47*), substances such as DEHA cannot be considered as viable alternatives to DEHP in food-contact applications, despite the result with our estrogenicity assay. Of course, other mechanisms for endocrine disruption, such as (anti)androgenicity, have to be taken into account as well.

Octyl-epoxystearate (**8**) is used as a secondary plasticizer in PVC, as is epoxidized soybean oil (ESO, **10**). As ESO is not estrogenic at all and octyl-epoxystearate very slightly estrogenic in our ERα cells, ESO seems to be a better choice to use as a plasticizer (**Table 3**). Our results, obtained with all plasticizers used in food packaging that are currently in use, reveal that several of these plasticizers are slightly estrogenic. Three newly developed plasticizers based on sorbitol, isosorbide di 2-ethylhexanoate (**5**), isosorbide-diiso-butyrate (**11**), and isosorbide-di-*n*-hexanoate (**12**), activated neither the ERα nor the ERβ. Therefore, as far as estrogenicity is concerned, these plasticizers may be good replacements of existing plasticizers. However, additional data on these newly developed plasticizers regarding persistence, migration, toxicity, and accumulation in food products are necessary to be able to fully judge the added value of replacement.

Of the antioxidants, propanediolphosphite (**19**) was slightly estrogenic with an EEF_{10} of 1.2×10^{-8} in the ERα cell line. Of all antioxidants tested, propyl gallate (**20**) and BHA (**23**) were the only ones revealed to be estrogenic in both the ERα and ERβ cells. As can be noted again, their potencies in the ERα were lower compared to the ERβ-mediated activity, especially for BHA (**Table 3**). For these compounds, BHA exposure is estimated to be 0.11 mg/person/day (*48*) and the ADI ranges from 0.3 to 0.5 mg/kg of bw/day (*30*). Propyl gallate's ADI is 1.4 mg/kg of bw/day (*30*).

The response of the test compounds in the ERα and ERβ cell lines in the presence of the EC_{50} value (5 and 100 pM for the ERα and ERβ cell lines, respectively) revealed that all combination effects were additive. This indicates that estrogenic effects of compounds in vitro are additive, a finding which should be studied in vivo as well. The concentrations of E_2 used for these combined exposures (5 and 100 pM) are commonly found in female blood, and they are even low compared to E_2 levels during pregnancy (*49*).

All together the results of the present study reveal that plasticizers have estrogenic activity in the ERα U2-OS reporter cell line, although at ≈100 times higher concentrations than those of the known pseudo-estrogens BPA and NP. However, because additivity seems to occur and all of these compounds are regularly used in food packages, together they may contribute significantly to the estrogenic effects of the total pool of compounds with estrogenic activity originating from the food chain. Most compounds are estrogenic in the ERα cell line, indicating that the effects predominantly will occur in tissues expressing ERα, such as mammary gland, uterus, and testes (*10*). When compared to the effects of E_2 (**1**), for NP (**3**), BPA (**2**), TEHTM (**6**), and propyl gallate (**20**), the EEF_{10} is relatively higher in the ERβ than in the ERα, resulting in an $EEF_{10}\alpha/EEF_{10}\beta$ ratio lower than 1. This indicates that these compounds might give rise to relatively larger formation of ERα/ERβ heterodimers than upon exposure to E_2, thereby possibly diminishing the negative effects of the ERα as seen in vitro (*12*, *13*). The plasticizers DIHP (**4**), DEHP (**14**), DEHA (**13**), octyl epoxystearate (**8**), and the benzoate mixture (**9**) and the antioxidant propanediolphosphite (**19**) are all exclusively estrogenic via the ERα and might therefore only have adverse endocrine-disrupting effects on human health.

Finally, comparison of the EC_{50} values obtained in the ERα and ERβ U2-OS cells to the EC_{50} values obtained in other models testing estrogenic activity indicates that the EC_{50} values in most of these other cell systems are more in line with the EC_{50} obtained in the ERα cells than with the EC_{50} for the ERβ cells. This observation supports the importance of detecting characteristics for ERα and ERβ response separately in independent models such as the U2-OS ERα and ERβ cells, as done

in the present study. Food-packaging-associated compounds are estrogenic at concentrations 100 times higher than the known compounds NP and BPA. The results obtained in this study are important in determining the different reactions of the same compounds on two different types of ER. Both ERs are present in different tissues and able to modulate each other's activity. For most compounds daily intake estimates are in the range from 0 to 0.457 μg/kg of bw/day, and therefore the concentrations tested in vitro were high compared to the average human intake; however, they were important in determining the intrinsic potential of these compounds to act as estrogenic agonists. To be able to translate the in vitro findings to the in vivo situation, currently studies are being performed with ER reporter gene mice to establish the estrogenicity of some of the estrogenic food-packaging-associated compounds in vivo.

ABBREVIATIONS USED

U2-OS, human osteoblastic cell line; ERα/β, estrogen receptor alpha/beta; ERE, estrogen responsive elements; Luc, luciferase gene; EC_{50}, concentration at which 50% of the effect is reached; pM, picomolar; DDT, dichlorodiphenyltrichloroethylene; RLU, relative light units; DCC, dextran-coated charcoal.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of May 20, 2006, has been corrected. Some values in the max effect (%) columns of Table 3 have been corrected in the revised ASAP posting of May 26, 2006.

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