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Scientific paper

MUTAGENICITY AND DNA DAMAGE OF BISPHENOL A AND ITS STRUCTURAL ANALOGUES IN HEPG2 CELLS

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Environmental oestrogen bisphenol A (BPA) and its analogues are widespread in our living environment. Because their production and use are increasing, exposure of humans to bisphenols is becoming a significant issue. We evaluated the mutagenic and genotoxic potential of eight BPA structural analogues (BPF, BPAF, BPZ, BPS, DMBPA, DMBPS, BP-1, and BP-2) using the Ames and comet assay, respectively. None of the tested bisphenols showed a mutagenic effect in *Salmonella typhimurium* strains TA98 and TA100 in either the presence or absence of external S9-mediated metabolic activation (Aroclor 1254-induced male rat liver). Potential genotoxicity of bisphenols was determined in the human hepatoma cell line (HepG2) at non-cytotoxic concentrations (0.1 μmol L^{-1} to 10 μmol L^{-1}) after 4-hour and 24-hour exposure. In the comet assay, BPA and its analogue BPS induced significant DNA damage only after the 24-hour exposure, while analogues DMBPS, BP-1, and BP-2 induced a transient increase in DNA strand breaks observed only after the 4-hour exposure. BPF, BPAF, BPZ, and DMBPA did not induce DNA damage.

KEY WORDS: *Ames test, bisphenols, comet assay, genotoxicity*

Bisphenol A (BPA; 4,4'-propane-2,2-diyldiphenol, CAS no. 80-05-7) is used in the production of food contact materials, such as baby bottles, food containers, and protective coatings for canned food and beverages, and for metal lids on glass jars and bottles. At higher temperatures, longer contact with, and higher pH of the contact medium, BPA monomer can hydrolyse and leach into food and beverages. Average exposure concentrations range from 10 μg kg^{-1} to 70 μg kg^{-1} in solid canned food and from 1 μg L^{-1} to 23 μg L^{-1} in liquid canned food (1). BPA has been under scrutiny due to concerns over potential adverse health effects related to its endocrine-disrupting activity (2-3). The first regulatory risk assessment report on BPA published by the Canadian government (4) has resulted in the ban of BPA in baby bottles in Canada. In January 2010, the US Food and Drug Administration expressed concern about potential adverse health effects of BPA in infants and children. The use of BPA in food contact materials has been banned in Japan, Canada, and many US states, and the US Congress is considering federal ban on BPA in all food and beverage containers (5). In 2011, the European Union prohibited the manufacture, marketing, and import of baby bottles containing BPA (6).

Current efforts are focused on replacing BPA with safer food contact materials. All of these alternative materials need to be assessed for appropriate functionality and safety using state-of-the-art methodology and scientific knowledge (1).

Bisphenols (Table 1) are a class of chemicals known as diphenylmethanes, which contain two benzene rings separated by one central carbon atom, usually with a 4-OH substituent on both benzene rings (e.g. BPA, BPF, BPAF, BPZ, and DMBPA). In some bisphenols, the central carbon atom is replaced by a sulphone group (e.g. BPS, DMBPS, or BP-1) or sulphide moiety (e.g. BP-2). Some BPA analogues seem to be safer alternatives to BPA in industrial applications (7). For example, the production of bisphenol S (BPS), which is stable at high temperatures and resistant to sunlight, is increasing from year to year (7-8). The largest US manufacturer of thermal paper has been using BPS as a replacement for BPA since 2006 (9). However, insufficient data are available to tell whether these BPS-containing papers are safer than BPA-containing papers. While BPA is moderately susceptible to environmental breakdown, BPS may be more persistent (10-11). Another example is BP-1, whose use as polymer bottle component was first reported more than 30 years ago (12). Lotti et al. (13) reported that poly(butylene terephthalate) modified with BP-1 showed improved glass transition temperature and thermal stability. Bisphenol F (BPF) and bisphenol AF (BPAF) are also used for polycarbonate resin production (14). From the viewpoint of biodegradability in the aquatic environment, BPF is more biodegradable under aerobic and anaerobic conditions than BPA, and may replace BPA to lower environmental risks (11). BPF also occurs as a monomer of phenol-formaldehyde resin. BPAF is a component of certain plasters and is used as a rubber bridging material, while DMBPA is a monomer of polycarbonate, epoxy, and polyester resins (15). Due insufficient toxicity data and structural similarity to BPA, BPAF has been nominated for a comprehensive toxicological characterisation by the US National Institute of Environmental Health Sciences (16).

While the toxicity of BPA has received a lot of attention, BPA analogues have not, despite the fact that they are threatening to become dominant environmental pollutants in the near future and that their impact on the environment and human health requires urgent attention (7). The main mechanism underlying BPA-induced adverse effects is endocrine disruption that may lead to developmental and/or reproductive disorders (2-3). Moreover, endocrine disruptors may induce carcinogenic effects due to epigenetic events or due to genotoxic effects. One potent endocrine disruptor and evidenced carcinogen, diethylstilbestrol (DES), is structurally related to BPA (17-18). Both DES and BPA have a hydrophobic core with two OH groups at each end of the backbone whose distance is similar (9.0 Å to 9.2 Å for BPA and 9 Å to 11.5 Å for DES at different conformations) (19). Studies of BPA genotoxicity have yielded conflicting results. BPA is considered non-genotoxic because it was negative to a set of basic genotoxicity tests. It was not mutagenic in the *Salmonella*/microsome assay (20-21), did not induce gene mutations (21-22) or chromosomal aberrations (23) in mammalian cells *in vitro*, and failed to induce chromosomal aberration and micronucleus formation *in vivo* in mice bone marrow (24). In contrast, BPA induced numerical chromosomal aberrations and morphological changes in cultured SHE cells (22) and in mice it induced achromatic lesions and c-mitotic effects in bone marrow cells (24). In addition, BPA metabolite(s) were shown to bind to DNA in a cellular system (25-26), in cultured SHE cells (22), and in rodent liver *in vivo* (27-28). Moreover, in oestrogen receptor (ER)-positive MCF-7 cells, BPA caused DNA strand breaks that were ER-dependent (29). In turn, BPF has been reported to induce DNA strand breaks, but not micronuclei, in human hepatoma HepG2 cells (30). Audebert et al. (31) have recently found that BPF genotoxicity depended on the metabolic capabilities of cells. In human HepG2 cells it induced histone H2AX phosphorylation, an indicator of DNA double-strand breaks. BPAF induced metaphase arrest and micronucleus formation in V79 cells (32). In SHE cells, BPAF did not induce gene mutation or chromosomal aberrations, but induced aneuploidy and morphological changes (15, 33).

The aim of this *in vitro* study was to investigate the mutagenic and genotoxic potential of a series of BPA analogues selected from three structural groups based on the bridging moiety between two phenolic rings and substitution pattern on phenolic rings. The first group has a central carbon atom (BPA, BPF, BPAF, BPZ, and DMBPA), the second group contains sulphones (BPS, DMBPS and BP-1), and the third group a sulphide analogue (BP-2). BP-1 and BP-2 were denoted by numbers to avoid confusion between abbreviations found in literature.

MATERIALS AND METHODS

Chemicals

Bisphenol A (BPA, >99 % pure; CAS # 80-05-7), bisphenol F (BPF, 98 % pure; CAS # 620-92-8), bisphenol AF (BPAF, 97 % pure; CAS # 1478-61-1),

bisphenol Z (BPZ, 98 % pure; CAS # 843-55-0), 2,2-bis(4-hydroxy-3-methylphenyl)propane (DMBPA, 97 % pure; CAS # 79-97-0), bisphenol S (BPS, 98 % pure; CAS # 80-09-1), 4,4'-sulfonylbis(2-methylphenol) (DMBPS, 97 % pure; CAS # 16346-97-7), [sulphonylbis(benzene-4,1-diyloxy)]diethanol (BP-1, 95 % pure; CAS # 27205-03-4), 4,4'-sulphanediyldiphenol (BP-2, 99 % pure; CAS # 2664-63-3), William's medium E, ampicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glucose-6-phosphate (disodium salt), benzo[a]pyrene (B[a]P; CAS # 50-32-8), ethidium bromide solution (CAS # 1239-45-8), dimethyl sulphoxide (DMSO; CAS # 67-68-5), EDTA (CAS # 6381-92-6), and Triton X-100 were obtained from Sigma (St. Louis, MO, USA). Normal melting point (NMP) agarose and low melting point agarose (LMP) were from Invitrogen (Carlsbad, CA, USA). Lyophilised Aroclor 1254-induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox (Boone, NC, USA). Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine, and phosphate buffered saline (PBS) were sourced from PAA Laboratories (Dartmouth, MA, USA) and trypsin from BD-Difco (Le Pont-De-Claix Cedex, France). All other reagents were of the purest grade available and all solutions were made using distilled water.

Bacterial strains

Salmonella typhimurium strains TA98 (frame shift mutations) and TA100 (base pair substitutions) were obtained from Professor B. N. Ames (University of California, Berkeley, USA) and were regularly checked for their phenotypic characteristics [histidine/biotin dependence, rfa marker (crystal violet), uvrB deletion (UV sensitivity), and the presence of plasmid pKM101 (ampicillin resistance)]. The working bacterial cultures were prepared from frozen permanents by overnight incubation (37 °C) in the Oxoid nutrient broth no. 2 (Oxoid, Basingstoke, UK) in the presence of 25 μg mL^{-1} ampicillin.

Determination of mutagenicity with the Ames test

Bisphenol mutagenicity was tested with the Salmonella/microsomal reverse mutation assay (34-35). Prior to the testing, BPs were dissolved and diluted in 100 % DMSO to give final concentrations of (0.004, 0.02, 0.1, and 0.5) mg per plate. Overnight cultures of *S. typhimurium* strains TA98 and TA100 (100 μL) as well as corresponding BP dilutions (100 μL) were added to 2 mL of molten top agar containing a limited amount of histidine/biotin (42 °C), gently mixed, and poured onto minimal agar plates. For the assay with metabolic activation, 500 μL of S9 mix (containing 4 % S9 – Aroclor-induced rat liver microsomal fraction) was also added to 2 mL of molten soft agar. Benzo[a]pyrene (B[a]P; final concentration 10 μg per plate) and 4-nitroquinoline-*N*-oxide (4-NQNO; final concentration 0.5 μg per plate) were used as positive controls for testing in the presence and absence of S9 mix, respectively. 100 % DMSO was used as a solvent control. The number of His+ revertants was counted after 48 h (TA100) and 72 h (TA98) of incubation at 37 °C. Three plates were used per treatment point. The mutagenic potential of the samples was expressed as an induction factor (IF), where IF = (number of revertants in the presence of the sample)/(number of revertants in solvent control).

For the purposes of this study, a non-statistical procedure was used to evaluate the results of the Ames test. A compound was considered a mutagen if it produced a reproducible, dose-related increase in the number of revertant colonies in one or more strains and induced at least a twofold increase in the number of revertants in respect to solvent control (IF≥2) (34).

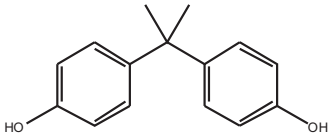
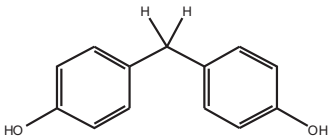
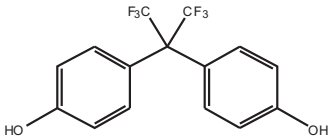
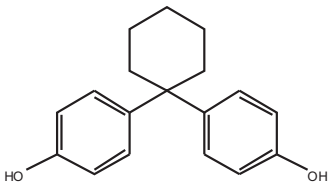
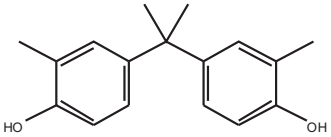
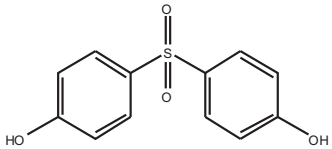
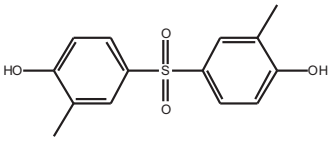
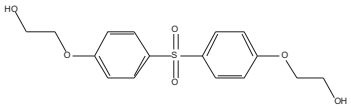
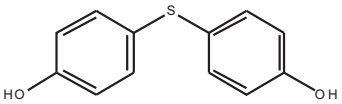
Human HepG2 cells

These cells were selected as they express a range of xenobiotic-metabolising enzymes (36-40) as well as ERα and ERβ receptors (41). The HepG2 cells were provided by Professor Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). They were grown in William's medium E containing 15 % FBS, 2 mmol L^{-1} L-glutamine, and 100 U mL^{-1} penicillin/streptomycin in 5 % CO_2 at 37 °C. The cells were used at passages between 3 and 10. For sub-cultivation, the cells were trypsinised, washed with phosphate-buffered saline (PBS, pH 7.4), centrifuged at 100 *g* for 5 min, and separated by pressing the suspensions through a syringe (needle 0.9x40 mm, Becton Dickinson, S.A., Fraga, Spain).

Cell viability

Bisphenol cytotoxicity was determined with the MTT assay in accordance with the procedure used by Mosmann (42) with minor modifications (43). HepG2 cells were seeded into 96-well microtitre plates at a density of 8,000 cells per well. After a 24-hour

Table 1 *BPA and its structural analogues*

Compound	Structure	Chemical name
BPA		4,4'-(propane-2,2-diyl)diphenol
BPF		4,4'-methylenediphenol
BPAF		4,4'-(perfluoropropane-2,2-diyl)diphenol
BPZ		4,4'-(cyclohexane-1,1-diyl)diphenol
DMBPA		2,2-bis(4-hydroxy-3-methylphenyl)propane
BPS		4,4'-sulfonyldiphenol
DMBPS		4,4'-sulfonylbis(2-methylphenol)
BP-1		((sulfonylbis(4,1-phenylene))bis(oxy))dimethanol
BP-2		4,4-thiodiphenol

incubation at 37 °C, the growth medium was replaced with fresh medium containing from 12.5 μmol L^{-1} to 100 μmol L^{-1} of BPs, and the cells were incubated for additional 24 h. The final concentration of DMSO in solvent control and dilutions was 0.1 %. MTT was then added to a final concentration 0.5 mg mL^{-1}, and the cells further incubated at 37 °C for 3 h. The medium was removed and formazan crystals dissolved in DMSO. The optical density (OD) of the solution in each well was measured against a blank (a well with DMSO) at 570 nm (the formazan absorption peak) and at 690 nm (measurement of the medium turbidity caused by cell debris) with a GENios™ microplate spectrofluorometer (Tecan, Trappes, France). The viability of cells was determined by comparing relative formazan concentrations (OD570-OD690) of the treated cells with those of untreated solvent control cells. Five individual wells were measured per treatment point. The experiment was repeated twice. Statistical significance between the treated groups and

control was determined using a two-tailed Student's *t*-test, where P<0.01 was considered significant.

Determination of genotoxicity with the comet assay

Stock solutions of BPs (100 mmol L^{-1}) were prepared in DMSO, and dilutions were prepared in the culture medium. The final concentration of DMSO in dilutions did not exceed 0.1 %. Solvent control (cell growth medium containing 0.1 % DMSO) and positive control (30 μmol L^{-1} B[a]P) were included in each experiment. HepG2 cells were seeded at a density of 40,000 cells/well into 12-well microtitre plates (Corning Costar Corporation, Corning, NY, USA) and left overnight at 37°C in 5 % CO_2 to attach to the plates. The growth medium was then replaced with fresh medium containing BPs in the following concentrations: 0.1 μmol L^{-1}, 1 μmol L^{-1}, and 10 μmol L^{-1}. The cells were incubated for 4 h and 24 h. At the end of exposure, the cells were washed, trypsinised, and resuspended in fresh medium for the comet assay.

The comet assay was performed as described by Singh et al. (44) with minor modifications (45). Briefly, 30 μL of cell suspension was mixed with 70 μL of 1 % LMP agarose and added to fully frosted slides coated with 80 μL of 1 % NMP agarose. The cells were then incubated in a lysis solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris, 1 % Triton X-100, pH 10) at 4 °C for at least 1 h, at which point the slides were placed into an alkaline solution (300 mmol L^{-1} NaOH, 1 mmol L^{-1} EDTA, pH 13) at 4 °C for 20 min so as to allow DNA unwinding, and electrophoresed at 25 V (300 mA) for 20 min. Finally, the slides were neutralised in a 400 mmol L^{-1} Tris buffer (pH 7.5) for 15 min and stained with EtBr (5 μg mL^{-1}). Images of 50 randomly selected nuclei per experimental point were captured using a fluorescence microscope (Eclipse 800, Nikon, Tokyo, Japan) and analysed with image analysis software (Comet Assay IV, Perceptive Instruments, Suffolk, UK).

Statistical analysis

The statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). A one-way analysis of variance (one-way ANOVA) was used to analyse the differences in tail intensity between treatments within each experiment. Dunnett's multiple comparison test was used to compare sample groups with control. Fifty cells were analysed per experimental point in each of at least two independent experimental cultures. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, a line in the box presents the median, and the bars represent 95 % confidence intervals. $P<0.05$ was considered to be statistically significant. In all experiments, the results of BP-treated cells are compared with those of solvent control cells.

RESULTS AND DISCUSSION

Ames test

Table 2 shows that neither BPA nor any of its analogues were mutagenic. Four of the bisphenols tested in our study, namely BPA, BPF, BPZ, and BPS, have already tested non-mutagenic in *S. typhimurium* TA98 and TA100 either with or without S9 metabolic activation, and our results have confirmed these findings (20-21, 30, 46-48). As the remaining five bisphenols (BPAF, DMBPA, DMBPS, BP-1 and BP-2) have not been tested for mutagenicity using the Ames test, our findings are the first to show that BPAF, DMBPA, DMBPS, BP-1, and BP-2 are not mutagenic in *S. typhimurium* TA98 and TA100 with and without S9 metabolic activation at the tested concentrations.

However, judging by the density of the background lawn, analogues DMBPA and BP-2 at 0.5 mg per plate, and BPZ and BPAF at 0.1 mg and 0.5 mg per plate were toxic to both *S. typhimurium* strains in the presence and in the absence of metabolic activation.

In order to detect cross-linking and/or oxidative properties of BPA and its analogues, other bacterial strains like *Salmonella typhimurium TA102* or *Escherichia coli WP2* or *WP2 (pKM101)* should be used.

Bisphenol cytotoxicity

At the concentrations of up to 100 μmol L^{-1}, BPA, BPF, BPZ, BPS, DMBPS, BP-1, and BP-2 did not affect cell viability after 24 h. DMBPA reduced cell viability by 35 % at the highest tested concentration, whereas BPAF reduced cell viability by 50 % at 50 μmol L^{-1} and by 70 % at 100 μmol L^{-1} (Table 3).

In their recent study, Audebert et al. (31) reported that 24-hour exposure to BPA (50 μmol L^{-1} and 100 μmol L^{-1}) and BPF (100 μmol L^{-1}) reduced the viability of several cell lines, including HepG2 cells.

Table 2 *Mutagenic effects of nine bisphenols, determined by the Ames test with* S. typhimurium *strains TA98 and TA100 in the presence and absence of an S9 mix. Revertants are presented as means of triplicate plates ± standard deviation.*

		TA98 + S9 mix		TA98 - S9 mix		TA100 + S9 mix		TA100 - S9 mix	
Sample	**Added / µg per plate**	**Revertants**	**IF[a]**	**Revertants**	**IF**	**Revertants**	**IF**	**Revertants**	**IF**
C[b] (DMSO)		32.3±1.5	1.0	26.7±5.1	1.0	97.3±9.6	1.0	103.0±6.6	1.0
PC[c] (B[a]P)	10	228±42.3	7.1			784.0±215.3	8.1		
PC[d] (4-NQNO)	0.5			408.0±69.4	15.3			2725.3±193.5	26.5
BPA	4	42.3±9.5	1.3	27.3±2.1	1.0	92.3±17.2	1.0	97.3±7.2	0.9
	20	42.7±7.0	1.3	24.3±2.5	0.9	79.7±7.2	0.8	105.3±22.0	1.0
	100	48.3±4.2	1.5	26.0±4.4	1.0	105.3±18.4	1.1	97.7±1.5	1.0
	500	32.3±3.2	1.0	22.0±2.8	0.8	91.7±5.5	1.0	T[e]	
BPF	4	41.3±7.8	1.3	27.0±7.0	1.0	108.0±16.7	1.1	102.7±9.5	1.0
	20	40.0±6.1	1.2	31.0±8.0	1.1	100.3±19.4	1.0	102.3±19.9	1.0
	100	38.3±2.1	1.2	29.5±6.4	1.1	109.3±5.9	1.1	100.7±9.9	1.0
	500	33.7±0.6	1.0	23.3±2.1	0.9	103.7±11.7	1.1	112.7±16.7	1.1
BPAF	4	34.3±8.1	1.1	33.0±2.6	1.2	94.7±7.6	1.0	92.0±10.4	0.9
	20	41.3±5.9	1.3	33.7±7.6	1.3	114.3±5.7	1.2	107.7±11.6	1.1
	100	T		T		T		T	
	500	T		T		T		T	
BPZ	4	37.0±7.9	1.1	31.3±6.4	1.2	98.3±8.6	1.0	98.0±4.6	1.0
	20	50.3±4.5	1.6	32.7±3.2	1.2	89.0±15.1	0.9	99.7±14.3	1.0
	100	43.7±5.1	1.4	T		T		107.7±10.5	1.1
	500	T + P[f]		T + P		T + P		T + P	
DMBPA	4	32.3±10.6	1.0	29.0±3.5	1.1	95.7±7.8	1.0	114.3±13.6	1.1
	20	44.3±2.1	1.4	21.7±0.6	0.8	107.7±6.4	1.1	115.3±5.7	1.1
	100	43.0±4.6	1.3	24.0±7.5	0.9	113.3±15.3	1.1	114.7±15.0	1.1
	500	T		T		T		T	
BPS	4	38.7±4.0	1.2	23.0±3.6	0.9	83.7±10.0	0.9	98.7±7.0	1.0
	20	37.3±5.1	1.1	25.7±1.2	1.0	107.0±11.3	1.1	93.0±13.0	0.9
	100	33.7±1.5	1.0	27.3±3.8	1.0	101.3±13.9	1.0	90.0±6.6	0.9
	500	36.3±5.0	1.1	21.7±4.6	0.8	97.3±7.2	1.0	78.0±6.2	0.8
DMBPS	4	35.0±7.2	1.1	26.3±4.6	1.0	108.0±20.0	1.1	105.7±4.7	1.0
	20	42.7±3.1	1.3	33.7±4.0	1.3	114.3±6.5	1.2	110.3±6.7	1.1
	100	38.7±8.3	1.2	28.0±3.0	1.1	105.0±20.0	1.1	115.7±2.1	1.1
	500	35.7±0.6	1.1	30.5±0.7	1.1	111.0±4.0	1.1	112.7±9.1	1.1
BP-1	4	34.0±6.0	1.1	27.0±4.6	1.0	112.3±9.0	1.2	125.3±14.5	1.2
	20	32.7±5.5	1.0	22.7±5.0	0.9	105.3±19.9	1.1	110.7±13.1	1.1
	100	30.0±1.7	0.9	26.0±2.0	1.0	92.7±17.2	1.0	111.0±18.3	1.1
	500	39.0±7.0	1.2	28.5±0.7	1.1	100.0±1.0	1.0	108.0±13.5	1.1
BP-2	4	40.3±1.5	1.3	31.0±4.2	1.1	101.0±4.0	1.0	104.0±10.8	1.0
	20	39.7±4.5	1.2	27.7±3.5	1.0	103.0±9.5	1.1	114.0±5.2	1.1
	100	42.7±3.1	1.3	24.3±1.2	0.9	97.7±8.5	1.0	113.0±6.9	1.1
	500	T		T		T		T	

[a] *IF: induction factor;*
[b] *solvent control (100 µL per plate);*
[c] *positive control benzo[a]pyrene (+S9);*
[d] *positive control 4-nitroquinoline (-S9);*
[e] *T: toxic effect (reduced background lawn);*
[f] *P: precipitation;*

However, they determined cell viability by measuring DNA content with in-cell western (ICW) assays, while we used an MTT assay that is based on the measurement of the metabolic activity of living cells. This may explain the differences in the observed cytotoxicity of BPA and BPF between the two studies.

Bisphenol-induced DNA strand breaks in HepG2 cells

HepG2 cells showed a significant increase in DNA strand breaks after four hours of exposure to DMBPS, BP-1, and BP-2. This increase was observed even at the lowest tested concentration of 0.1 μmol L^{-1}, but no dose-response relationship was observed. In contrast, BPA, BPF, BPAF, BPZ, DMBPA, and BPS did not induce a significant increase in DNA strand breaks (Figure 1).

After 24 h of exposure, a significant increase in DNA strand breaks was observed only in the cells exposed to BPA in all concentrations and to BPS at 0.1 μmol L^{-1} and 10 μmol L^{-1}. Again, no dose-response relationship was observed (Figure 1).

The selected concentrations of BPs are below cytotoxic but are still relevant for or even higher than human exposure (1). The issues surrounding low-dose effects of endocrine disrupting chemicals have been discussed at the National Program (NTP) Workshop on Low Dose Effects of Endocrine Disrupting Chemicals (49-50). Types of DNA damage detected with the alkaline comet assay include single- and double-strand breaks, alkali-labile sites, DNA-DNA/DNA-protein cross-links, and single-strand breaks associated with incomplete excision repair (51). However, in most cases the detected DNA strand breaks and alkali-labile sites are the intermediates formed during the repair of different types of DNA lesions by base or nucleotide excision repair (52). As DNA lesions detected with the comet assay may be transient due to the effective DNA repair, we observed short-term (4-hour) and long-term (24-hour) exposure to BPs.

Our BPA findings are in agreement with several recent studies. Iso et al. (53) reported that BPA induced DNA strand breaks in ER-positive MCF-7 cells (at 1 μmol L^{-1} and 100 μmol L^{-1}) and that its genotoxicity was ER-dependent, as evidenced by much lower effect in ER-negative MDA-MB-231 cells. The increase in DNA strand breaks was significant as soon as after three hours of exposure and increased even further up to hour 24, which is in agreement with our study. When applied *in vivo* in rats, BPA induced micronucleus formation and structural chromosome aberrations in bone marrow as well as DNA damage in lymphocytes (46). Tiwari et al. (46) also observed increased plasma levels of 8-hydroxydeoxyguanosine, an increase in lipid peroxidation, and a decrease in glutathione activity in the liver, suggesting that oxidative stress could be one of the mechanisms of BPA genotoxicity. In a study by Audebert et al. (31) on the other hand, BPA failed to induce H2AX histone phosphorylation in HepG2 cells, but induced it in human renal adenocarcinoma (ACHN) cells. The authors explained this difference between the two cell lines with differences in the biotransformation of BPA.

In our study, BPF at concentrations up to 10 μmol L^{-1} did not induce DNA damage. This result is in line with the study by Cabaton et al. (30), who did not detect DNA strand breaks in HepG2 cells exposed to BPF at concentrations lower than 50 μmol L^{-1}. At higher concentrations, however, they observed a significant increase in DNA strand breaks. BPF also induced a significant increase in H2AX histone phosphorylation in HepG2, ACHN, and human epithelial colorectal adenocarcinoma (LS174T) cells, a phenomenon that was observed only at the two highest concentrations tested (50 μmol L^{-1} and 100 μmol L^{-1}) (31).

In HepG2 cells exposed to low concentrations of DMBPS, BP-1, and BP-2, the increase in DNA strand breaks was significant after four, but not after 24 h of exposure, whereas in cells exposed to BPA and BPS, DNA strand breaks were observed only after the 24-hour exposure. However, these changes were small and not dose-related. Even so, our results do not undermine the genotoxic potential of BPA analogues and call for further research. Scientific evidence supports the hypothesis that natural oestrogens, synthetic oestrogen diethylstilbestrol, as well as BPA generate reactive oxygen species (ROS) during biotransformation and that certain reactive species, predominantly quinones, can react with DNA and cause DNA damage (54). One pathway of BPA metabolism is the hydroxylation of one of its symmetric phenyl rings to form its catechol, o-OH BPA, which can oxidise to o-quinone BPA (55) and, in turn, react with DNA. o-Quinone BPA forms predominantly depurinating adducts o-OH-BPA-6-N3Ade and o-OH-BPA-6-N7Gua (56-58). Sakuma et al. (59) found that o-quinone BPA could increase ROS formation and oxidise the guanine moiety of deoxyguanosine in the DNA of primary rat hepatocyte cultures. Adducts such as these, formed during BPA metabolism as well as oxidative DNA damage are readily detected by the alkaline comet assay.

BPA analogues included in our study differ in the bridging atom between the two phenyl rings [sulfone (BPS) and sulfide (BP-2) moieties instead of a carbon atom], in the functional groups on the bridging carbon

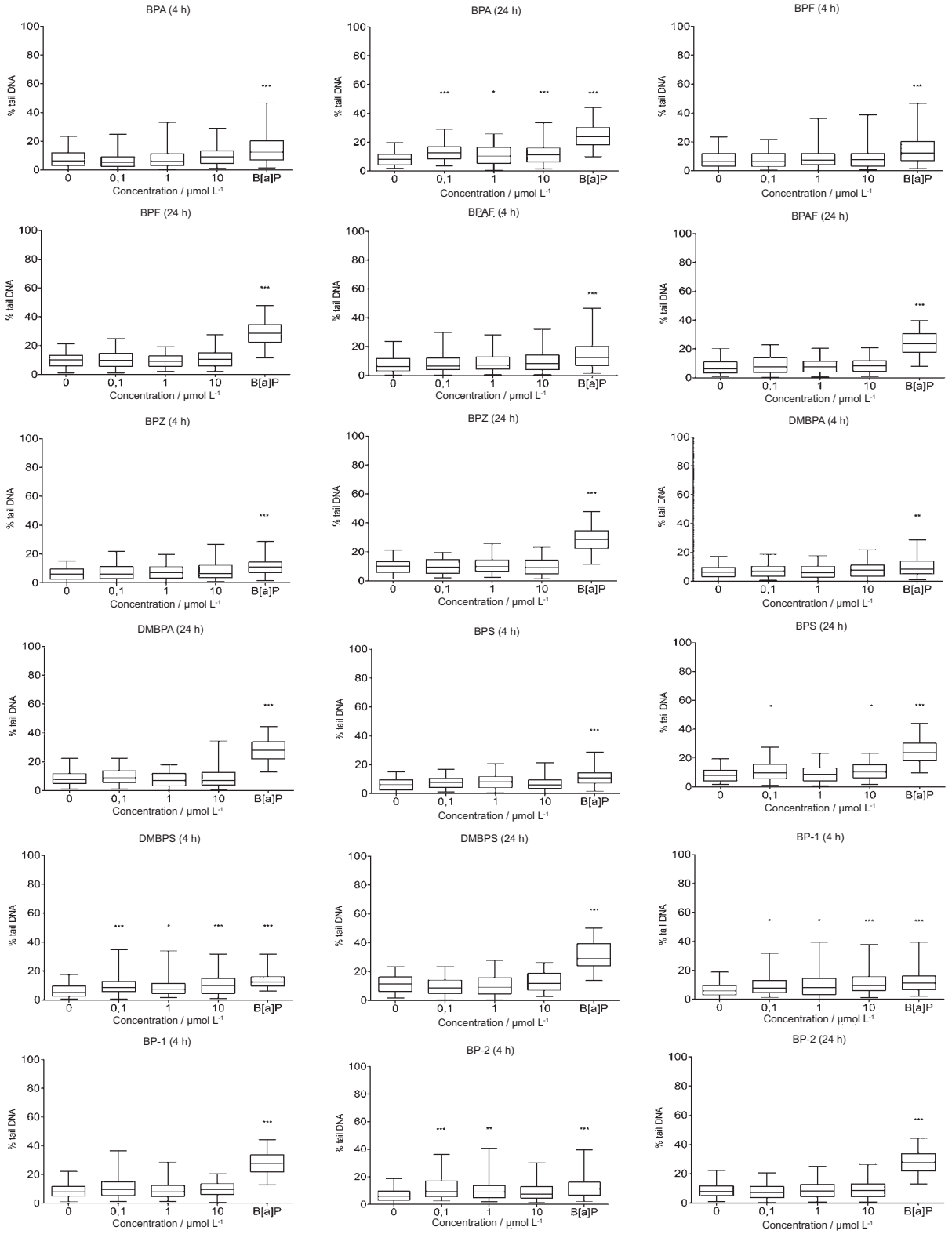


Figure 1 *DNA damage (comet assay) induced by bisphenols in HepG2 cells after 4 h and 24 h of exposure to concentrations of 0.1 μmol L^{-1}, 1 μmol L^{-1}, and 10 μmol L^{-1}.*
B[a]P (30 μmol L^{-1}) was used as the positive control. The level of DNA strand breaks is expressed as tail intensity. Fifty cells were analysed per experimental point in each of at least two independent experimental cultures. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentile, a line in the box represents the median value, and the bars represent 95 % confidence intervals.
** significant difference (one-way ANOVA; Dunnett's multiple comparison test) between BPs-treated cells and solvent control (0),*
*** (P<0.01) and *** (P<0.001)*

Table 3 *Viability of HepG2 cells exposed to bisphenols, as determined by the MTT assay.*[a]

	Viability / %								
Concentration μmol L^{-1}	**BPA**	**BPF**	**BPAF**	**BPZ**	**DMBPA**	**BPS**	**DMBPS**	**BP-1**	**BP-2**
12.5	110±6	114±5	107±16	99±7	121±8	99 ±2	101±7	108±8	101±9
25	114±19	112±6	100±21	105±12	123±10	100±7	108±8	106±5	103±9
50	116±9	118±12	50±4*	113±9	100±13	98±8	118±10	109±3	113±5
100	90±15	123±13	31±4*	125±14	67±12*	94±4	129±12	113±13	117±10

[a] *Independent experiments were performed in 5 replicates and repeated two times. Data show one out of two independent experiments, which showed the same trend. The survival was normalized to the solvent control (100 % viability). Values are expressed as mean ± SD.*
** Significant difference between the solvent control and BP-treated cells (Student's t-test,* P<*0.001).*

atom [without methyl groups (BPF), with two trifluoromethyl groups (BPAF), and with a cyclohexyl ring (BPZ)], and in substitutions on both phenol rings [methyl groups on positions 3 of phenyl rings (DMBPA and DMBPS) and substituted both phenol groups (BP-1)] (Table 1). These structural differences may explain the differences in their metabolism in HepG2 cells and the extent of formation of reactive quinone intermediates and ROS. However, structural differences alone cannot clearly explain the structure-activity relationship in this series of BPA analogues or why BPF, BPAF, BPZ, and DMBPA did not induce DNA damage, and why BPA and BPS did induce significant DNA damage only after 24 h of exposure. It has recently been demonstrated that BPA and BPF are metabolised in HepG2 cells predominantly to conjugated sulphate metabolites (31, 60). However, we still do not know to what extent BPA and its analogues are biotransformed into reactive quinone intermediates in HepG2 cells, and how stable these intermediates are. Less stable intermediates are probably less harmful, due to their rapid reaction with water molecules, while more stable intermediates can also react with biological molecules and be more harmful. It has been hypothesised that those natural products that undergo oxidation to quinones and are then rapidly hydrated are unlikely to damage important biological macromolecules (61).

In conclusion, neither BPA nor its analogues induced bacterial mutations, and the minor and transient DNA damage induced by BPA, DMBPS, BP-1, and BP-2 in HepG2 cells was observed at concentrations that are higher than human exposure. Our data suggest that the toxic potential of BPA lies in the formation of reactive quinone metabolites and oxidative stress and warrant further investigation of genotoxic and mutagenic effects of other BPA analogues.

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Povzetek

MUTAGENOST IN POŠKODBE DNA POVZROČENE Z BISFENOLOM A IN NJEGOVIMI STRUKTURNIMI ANALOGI V CELIČNI LINIJI HEPG2

Okoljski estrogen, bisfenol A (BPA), in njegovi strukturni analogi so v veliki meri prisotni v našem okolju. Ker njihova proizvodnja in uporaba naraščata, je vse pomembneje ovrednotiti njihovo toksičnost zaradi izpostavljenosti ljudem. Z Amesovim in kometnim testom smo ovrednotili mutagenost in genotoksičnost osmih strukturnih analogov BPA (BPF, BPAF, BPZ, BPS, DMBPA, DMBPS, BP-1 in BP-2). Nobeden od testiranih bisfenolov ni izkazoval mutagenega delovanja na sevih TA98 in TA100 *Salmonelle tryhimurium* v prisotnosti in odsotnosti metabolne aktivacije (z Aroklorom 1254 inducirani encimi podganjih jeter). Potencialno genotoksičnost pa smo določali s kometnim testom na celični liniji humanega hepatoma (HepG2) pri necitotoksičnih koncentracijah (0.1 µmol L^{-1} do 10 µmol L^{-1}) po 4-urni in 24-urni izpostavljenosti. BPA in njegov analog BPS sta pri kometnem testu povzročila poškodbe DNA samo po 24-urni izpostavljenosti, medtem ko so analogi DMBPS, BP-1 in BP-2 povzročili prehodne poškodbe DNA (samo po 4-urni izpostavljenosti). BPF, BPAF, BPZ in DMBPA niso povzročili poškodb DNA.

KLJUČNE BESEDE: *Amesov test, bisfenoli, kometni test*

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