Bisphenol A and its analogues disrupt centrosome cycle and microtubule dynamics in prostate cancer

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Abstract

Humans are increasingly exposed to structural analogues of bisphenol A (BPA), as BPA is being replaced by these compounds in BPA-free consumer products. We have previously shown that chronic and developmental exposure to BPA is associated with increased prostate cancer (PCa) risk in human and animal models. Here, we examine whether exposure of PCa cells (LNCaP, C4-2) to low-dose BPA and its structural analogues (BPS, BPF, BPAF, TBBPA, DMBPA and TMBPA) affects centrosome amplification (CA), a hallmark of cancer initiation and progression. We found that exposure to BPA, BPS, DMBPA and TBBPA, in descending order, increased the number of cells with CA, in a non-monotonic dose–response manner. Furthermore, cells treated with BPA and their analogues initiated centrosome duplication at 8 h after release from serum starvation, significantly earlier in G-1 phase than control cells. This response was attended by earlier release of nucleophosmin from unduplicated centrosomes. BPA-exposed cells exhibited increased expression of cyclin-dependent kinase *CDK6* and decreased expression of CDK inhibitors (*p21^{Waf1/CIP1}* and *p27^{KIP1}*). Using specific antagonists for estrogen/androgen receptors, CA in the presence of BPA or its analogues was likely to be mediated via ESR1 signaling. Change in microtubule dynamics was observed on exposure to these analogues, which, for BPA, was accompanied by increased expression of centrosome-associated protein *CEP350*. Similar to BPA, chronic treatment of cells with DMBPA, but not other analogues, resulted in the enhancement of anchorage-independent growth. We thus conclude that selected BPA analogues, similar to BPA, disrupt centrosome function and microtubule organization, with DMBPA displaying the broadest spectrum of cancer-promoting effects.

Key Words

- ▶ endocrine-disrupting chemicals
- ▶ bisphenol A analogues
- ▶ BPA
- ▶ BPS
- ▶ BPF
- ▶ TBBPA
- ▶ DMBPA
- ▶ TMBPA
- ▶ centriole duplication
- ▶ prostate cancer

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Introduction

Bisphenol A (BPA), a prototype endocrine-disrupting chemical (EDC), is a mimic of estrogen present in polycarbonate plastic products, thermal paper and carbonless copy paper. Its leakage from these products

results in its ubiquitous presence at significant levels in the environment. Its effects suggest that it can reprogram the developing human and animal tissues, particularly those sensitive to hormones such as the prostate

(Ho *et al.* 2011, Ho *et al.* 2012). As a result, alternatives to BPA are being developed and are increasingly replacing BPA. Bisphenol S (BPS) is used as a chemical additive in thermal paper, pesticides, dyestuffs, color-fast agents, leather tanning agents, dye dispersants and fiber improvers. Considering that BPS is less biodegradable than BPA (Ike *et al.* 2006, Danzl *et al.* 2009), the widespread use of BPS is of concern. The halogenated derivative of BPA, such as tetrabromobisphenol A (2,2-bis(4-hydroxy-3,5-dibromophenyl)propane, (TBBPA)) is widely used in flame-retardants for building material, paints and epoxy resin-containing plastic products such as electronic circuit boards and other electronic equipment (Kitamura *et al.* 2005). Other less well-studied BPA structural analogues such as bisphenol F (BPF), bisphenol AF (BPAF), tetramethyl bisphenol A (TMBPA) and 3,3'-dimethylbisphenol A (DMBPA) are also increasingly used as BPA replacement materials in polycarbonate resin (Kitamura *et al.* 2005).

Alarming, structural analogues of BPA have already been detected in foods (Vinas *et al.* 2010, Gallart-Ayala *et al.* 2011), indoor dust (Wang *et al.* 2015), thermal receipt papers (Becerra & Odermatt 2012, Liao *et al.* 2012b), sewage sludge (Song *et al.* 2014, Ruan *et al.* 2015) and human bodily fluids (Cariou *et al.* 2008, Fernandez *et al.* 2008, Johnson-Restrepo *et al.* 2008, Jimenez-Diaz *et al.* 2010, Yang *et al.* 2014, Zhou *et al.* 2014). For example, BPS (free and conjugated) has been detected in 81% of urine samples from the general population collected from USA and seven Asian countries ($n=315$; (Liao *et al.* 2012a)). It is well documented that higher levels of BPA exposure is associated with increased risk of cardiovascular disease, obesity, diabetes, immune disorders and a host of reproductive dysfunctions (Keri *et al.* 2007, vom Saal *et al.* 2007, Ho *et al.* 2012, Rogers *et al.* 2013, Zawatski and Lee 2013). At this juncture, as we rapidly introduce BPA substitutes in consumer products, the pressing question that remains to be answered is the adverse health effects of these analogues due to their structural similarity to BPA.

Amplification of centrosomes is one of the early hallmarks of cancer initiation and progression (Godinho & Pellman 2014). During interphase, microtubules (MTs) arising from the centrosome maintain the tissue architecture, polarity and organelle transport. The centrosome also directs the formation of bipolar mitotic spindles, which is essential for accurate chromosome segregation to daughter cells (reviewed in Tarapore and Fukasawa 2000). Upon cytokinesis, each daughter cell inherits only one centrosome from a mother cell, and thus, the centrosome must duplicate once in each cell cycle, in synchrony with other cell cycle events including

DNA synthesis. In mammalian cells, the centriole, the core component of the centrosome, initiates duplication at the G1/S boundary (reviewed in Mazia 1987). Regulation of centrosome duplication is very tightly controlled, and any disturbance in this process can lead to abnormalities in the centrosome cycle and function.

We recently found that higher urinary BPA was found in PCa patients when compared to non-PCa subjects (Tarapore *et al.* 2014). Additionally, exposure of immortalized normal prostate epithelial cell lines (NPrEC and RWPE-1) and four PCa cell lines (LNCaP, C4-2, 22Rv1 and PC-3) to low-dose BPA (p mole range) increased centrosome amplification (CA), promoted MT nucleation and regrowth at centrosomes and enhanced anchorage-independent growth (Tarapore *et al.* 2014); all these cellular processes are well-accepted markers of cancer initiation and progression (Pihan *et al.* 2001, Lingle *et al.* 2002, Pihan *et al.* 2003, Mori *et al.* 2009, Ogden *et al.* 2013). Extending on our BPA study, here we examined whether exposure to low-dose BPA analogues (BPS, BPF, BPAF, TBBPA, TMBPA and DMBPA) has comparable effects as BPA on these cancer-related processes. We found that most of them exert similar, but not completely comparable action, as BPA. However, it appears that DMBPA, like BPA, has an effect on both CA and anchorage-independent growth.

Materials and methods

Cell lines

The prostate cancer (PCa) cell lines LNCaP and C4-2 (between passages 19 and 35) were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Corning) with 10% FBS and supplements recommended under standard conditions. RWPE-1 cell lines were purchased from ATCC and cultured in Defined Keratinocyte-SFM medium (ThermoFisher Scientific) with growth-promoting supplement. Cells were maintained at 37°C in a humidified incubator with a 5% CO₂ atmosphere. These cell lines have been authenticated by ATCC using STRS analysis.

BPA and analogue treatments

LNCaP and C4-2 cell lines were seeded in 6-well plates with coverslips at 20,000 cells/mL. After 24 h, cells were rinsed with PBS and maintained in phenol red-free RPMI-1640 with 10% charcoal-stripped serum (CSS) for another 24 h. After this incubation period, media was replaced with fresh 10% CSS media and cells were treated with BPA,

BPF, BPAF, BPS, TBBPA, DMBPA and TMBPA (Sigma) to a final concentration of 0, 0.01, 0.1, 1, 10 or 100 nM. For the time-point experiment, cells grown in 35 mm wells with coverslips were serum starved using phenol red-free RPMI-1640 with 0.05% CSS for 48 h. At time-points 0, 8, 16, 24, 32 and 40 h, media was changed to phenol red-free RPMI-1640 with 20% CSS ± BPA analogues at concentrations which gave the maximum effects.

To investigate whether CA by BPA, BPS, TBBPA and DMBPA was dependent on nuclear receptors estrogen receptor α (ER α /ESR1), ER β /ESR2, androgen receptor (AR) or G protein-coupled estrogen receptor (GPER1/GRP30), cells were seeded and placed in phenol red-free RPMI-1640 with 10% CSS as described above. After 24–48 h, media was replaced with fresh 10% CSS medium with 1 μ M final concentrations of inhibitors (from Tocris, Minneapolis, MN, USA), Fulvestrant/ICI-182,780, MPP (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride), PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) bicalutamide, G15 ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone) in the presence and absence of 0.1 nM BPA, 0.1 nM BPS, 0.01 nM TBBPA and 0.01 nM DMBPA for 72 h.

Indirect immunofluorescence

Cells were fixed using 100% methanol at -20°C for 5 min and processed as described previously (Tarapore et al. 2012) with γ -tubulin antibody (clone GTU88 antibody, Sigma), anti-pericentrin (Anti-PCNT Rabbit polyclonal, Sigma Prestige Antibodies HPA016820) along with anti-centrin antibody (clone 20H5 antibody, Millipore and Santa Cruz sc-50452). Cells were immunostained for centrioles as well as for centrosomes in the same slide. When the number of centrosome=2, number of centrioles is 3 or 4. For NPM analysis, cells were fixed using 10% formalin:acetic acid at 25°C for 20 min. Cells were permeabilized with 0.7% Triton-X at 25°C for 2 min, double-stained for gamma-tubulin (clone GTU88) or centrin and nucleophosmin (Abcam ab15440 rabbit polyclonal). Antibodies were detected with Alexa Fluor conjugated fluorescent antibodies. Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher).

Anchorage-independent growth assay

Anchorage-independent growth was measured by assessing the efficiency of colony formation in a semi-

solid medium. Cells were first chronically exposed to BPA analogues for ~10 passages. Concentrations used were as follows: For LNCaP BPA 0.1 nM, BPS 0.1 nM, TBBPA 0.01 nM, DMBPA 0.1 nM, TMBPA 0.1 nM; for C4-2 BPA 0.1 nM, BPS 0.1 nM, TBBPA 0.01 nM, DMBPA 0.01 nM and TMBPA 0.1 nM. Approximately 2500 cells/35 mm well were then embedded in soft agar. Medium was refreshed twice a week with and without BPA analogues. After 2–3 weeks, colonies were counted under a microscope. All conditions were performed in triplicate and repeated twice. Colony-forming efficiency was calculated as the number of colonies counted divided by the total number of cells plated, multiplied by 100.

Quantitative real-time RT-PCR (qPCR) for gene expression

Total RNA (1 μ g) from LNCaP and C4-2 cells treated with BPA as described above, was reverse-transcribed using SMARTscribe Reverse Transcriptase (Clontech) and

Table 1 Details of qRT-PCR primers.

Transcript	Primer name	Sequence (5'–3' direction)
p53	hu-p53-F	CGTCTGGGCTTCTTGCACTCT
	hu-p53-R	AGCTGCACAGGGCAGGTCTT
CDK4	hu-CDK4-F	CTTCTGCAGTCCACATATGCAACA
	hu-CDK4-R	CAACTGGTCGGCTTCAGAGTTTC
CDK6	hu-CDK6-F	TGGTGACCAGCAGCGGACAA
	hu-CDK6-R	ACCACAGCGTGACGACCACT
CDK2	hu-CDK2-F	CCAGGAGTTACTTCTATGCCTGA
	hu-CDK2-R	TTCATCCAGGGGAGGTACAAC
p21 ^{Waf1} (CDKN1A)	hu-p21-F	GAGGAAGACCATGTGGACCTGT
	hu-p21-R	GCGGATTAGGGCTTCCTCTTG
p27 ^{KIP1} (CDKN1B)	hu-p27-F	GCAACCGACGATTCTTCTACTCAA
	hu-p27-R	GCTTCATCAAGCAGTGATGTATCT
cyclin A2	hu-CCNA2-F	GGGACAAAGCTGGCCTGAATC
	hu-CCNA2-R	AGTGTCTCTGGTGGGTTGAGGA
cyclin E1	hu-CCNE1-F	AGTGTCTCTGGTGGGTTGAGGA
	hu-CCNE1-R	GCTTGCACGTTGAGTTTGAGTA
cyclin D1	hu-CCND1-F	CCGTCCATGCGGAAGATC
	hu-CCND1-R	CCTCCTCCTCGCACTTCTGT
p150 ^{glued} (DCTN1)	hu-DCTN-F	CACCTGTGATGAAGGGCATGG
	hu-DCTN-R	ATCAGTTCCTCTCTTTTGAGGAC
EB1 (MAPRE1)	hu-MAPRE-F	CAGAGGCCCATCTCAACACA
	hu-MAPRE-R	CAATACGTTGACCTGCTGCAT
Ninein (NIN)	hu-NIN-F	ACTATATCCGGGACCGCTT
	hu-NIN-R	CGAGGTACCAAACTTTTCTGC
CEP150	hu-CEP350-F	TAGCAGCCGCCAAGAAAGTC
	hu-CEP350-R	GACGTTGAGTCTTTTCATCAGGA
RPS3	hu-RPS3-F	AGCCACCAGAACACAGAATG
	hu-RPS3-R	CTAGTGGCCACCTTTTCAGC

Forward and reverse primers were designed to overlap at least one exon–exon boundary of the target gene. The primer positions are based on the reference sequence of the target genes in the GenBank. Expression of ribosomal protein 3 gene (RPS3) or GAPDH (for CCNA2) was used as control for sample normalization in the quantification of the gene of interest expression by real-time RT-PCR analysis.

random hexamers (Promega). Amplification was detected using PerfeCTa SYBR Green FastMix, Low ROX reagent (Quanta Biosciences, Gaithersburg, MD, USA) using the ViiA 7 Real-Time PCR System (ThermoFisher). Primer sequences are listed in Table 1. The target gene expression was normalized against the individual endogenous RPS3, and the relative change in transcript level was calculated with the delta-delta CT method.

MT-Aster formation

To examine the effect of BPA analogues on MT-aster formation, cells were treated for 3 days with concentrations as follows: For LNCaP BPA 0.1 nM, BPS 0.1 nM, TBBPA 0.01 nM, DMBPA 0.1 nM, TMBPA 0.1 nM; for C4-2 BPA 0.1 nM, BPS 0.1 nM, TBBPA 0.01 nM, DMBPA 0.01 nM, TMBPA 0.1 nM. Cells were then treated with nocodazole (Sigma, 1.5 µg/mL) for 40 min on ice to depolymerize the interphase MTs. Cells were rinsed to remove the nocodazole, and then cells were incubated in fresh warm medium for 7 min at 37°C to allow for MT regrowth. Cells were fixed with methanol at room temperature and stained for centrosomes (anti-γ-tubulin Sigma GTU88 T6557 mouse monoclonal; anti-γ-tubulin Santa Cruz, CA C-20 sc7396 goat polyclonal) and MTs (anti-α-tubulin Santa Cruz DM1A sc32296 mouse monoclonal). Cells were scored (~300 cells counted) for MT-aster formation.

Statistical analysis

All *in vitro* experiments were performed in triplicate and repeated 3 times. Values are reported as fold change ± s.e.m. Statistical significance was tested by two-tailed Student's *t*-test. *P*-values ≤ 0.05 were considered significant (**P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001).

Results

Exposure to low-dose BPA, BPS, TBBPA and DMBPA promotes centrosome amplification (CA) in a non-linear manner

Androgen-dependent (AD; LNCaP) and -independent (AI; C4-2) prostate cells were treated with low-dose

Figure 1

Low doses of BPA, BPS, TBBPA and DMBPA promote CA: LNCaP (A) and C4-2 (B) cell lines were treated with various doses of BPA, BPF, BPAF, BPS, TBBPA, DMBPA or TMBPA for 72 h in 10% CSS media. Cells were fixed and immunostained for centrosomes and DNA. Using fluorescence microscopy, cells with amplified centrosomes were scored and plotted for each dose of 0, 0.01, 0.1, 1, 10 and 100 nM. Significance was determined using the Student's *t*-test (**P* < 0.05). Bars, s.e. of the three independent experiments.

levels of BPA, BPF, BPAF, BPS, TBBPA, DMBPA or TMBPA (0.01–100 nM) and examined for centrosome numbers per cell (Figs 1 and 2). These concentrations are at environmentally relevant levels detected in adults and children (Zhou *et al.* 2014). Untreated cells that serve as controls showed the expected normal centrosome profile, in which most of the cells (>90%) contain either one or two centrosomes. The cell lines treated with BPA, BPS, TBBPA and DMBPA have a significant increase (two- to six-fold) in the number of cells with three or more centrosomes (Figs 1 and 2) at either 0.01 or 0.1 nM concentrations.

Also, the dose–response is found to be non-monotonic, as is commonly reported for the action of xenoestrogens and endocrine disruptors (Vandenberg *et al.* 2012, Vandenberg 2014). Moreover, CA is also observed in LNCaP cells in response to BPF and BPAF albeit at higher concentrations (100 and 10 nM, respectively), but not in C4-2 cells. Both cell lines were insensitive to treatment with TMBPA, implying that TMBPA does not affect the centrosome cycle. In summary, BPA, BPS, DMBPA and TBBPA, in descending order, exert the strongest effects on CA in a non-linear manner for both the AD and AI PCa cell lines.

Figure 2

Representative staining showing cells with one, two and >2 centrosomes. LNCaP and C4-2 PCa cells were treated with (A) BPA, (B) BPS, (C) TBBPA and (D) DMBPA as indicated. Cells were fixed and immunostained with rabbit anti-γ-tubulin (red). DNA was stained using DAPI (blue). Arrows point to centrosomes (n=number of centrosomes). Pericentrin staining gave similar results. Scale bar, 20 μm. Area in square is magnified and offset to the main panel.

Figure 3

BPA promotes the initiation of centrosome duplication at an earlier time-point during G1 phase: LNCaP and C4-2 cells were serum starved in the presence of 0.5% CSS, media changed to 20% CSS±BPA, BPS, TBBPA and DMBPA, and cells fixed at the time-points indicated in graph.

Potential mechanism of CA dysregulation is related to an earlier timing of initiation of centrosome duplication

We next determined the temporal pattern of centrosome amplification with the relevant BPA analogues. Cells were serum starved for 48 h. Cells were then serum-stimulated and at every 8 h for a period of 40 h, the number of amplified centrosomes per cell was scored. We found that the number of amplified centrosomes increased with time such that at 32 h, >10% cells had amplified centrosomes (Fig. 3A). To maintain tight regulation of centrosome duplication, there are mechanisms in place to (1) inhibit the re-duplication of centrosomes in G1-phase of the cell cycle once centrosome duplication is initiated and (2) inhibit re-duplication of centrosomes in S-phase. First, we arrested cells in S-phase using DNA synthesis inhibitor aphidicolin, in the presence and absence of the various BPA analogues, but did not observe any significant centrosome number defects compared to vehicle control. This indicated that the observed CA is not due to defects in inhibition of centrosome re-duplication during S-phase. Next, to determine whether the timing for centrosome duplication is disrupted, we examined whether cells duplicated their centrosomes earlier in G1-phase, in the presence of BPA and its analogues. In vehicle control cells, less than 10% of centrosomes start duplicating at the 16-h time-point, in both LNCaP and C4-2 cells (Fig. 3B, white bar). However, in the presence of BPA and its analogues at the 8- and 16-h time-point, a 15–30% decrease in the number of unduplicated centrosomes (and a corresponding increase in the number of duplicated centrosomes) is observed indicating that centrosomes duplicated at an earlier time-point in the cell cycle in the presence of BPA. Each centrosome consists of a pair of centrioles and surrounding pericentriolar matrix. Immunostaining of centrin was performed to delineate whether PCa cells exposed to BPA were undergoing centriole duplication or separation. The percent cells undergoing centriole separation did not significantly change with time for both cell lines. At the 16-h time-point, on BPA treatment, 20–25% of the

centrin spots increased from 2 (1 centrosome) to 3 or 4 (2 centrosomes) for both LNCaP and C4-2 cells, indicating that centriole duplication has occurred and not centriole separation (Fig. 3C).

To obtain a better understanding of the mechanism responsible for the earlier initiation of centrosome duplication, we examined for nucleophosmin (NPM) expression on single non-duplicated centrosomes. NPM localizes between the paired centrioles, and loss of NPM from the non-duplicated centrosome signals the first step in centrosome duplication. Cells were serum starved for 48 h, and then stimulated with serum. At various time-points (Fig. 3D and E), cells were stained for NPM and centrosomes and examined for localization of NPM on unduplicated centrosomes. We found that in the presence of BPA, BPS, TBBPA and DMBPA, NPM was released from the single (non-duplicated) centrosome at an earlier time-point than that in control untreated cells. That is, for example, at the 16-h time-point, in the untreated cells, 42% (LNCaP, Fig. 3E) and 22% (C4-2, Fig. 3E) of cells have NPM on single centrosomes, versus 10% (LNCaP, Fig. 3E) and 4% (C4-2, Fig. 3E) in BPA-treated cells. Moreover, in the TBBPA-treated cells, we see earlier release of NPM in LNCaP cells, but not in C4-2 cells, similar to the CA profile (Fig. 1A and B, TBBPA). An 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) incorporation assay did not indicate a significant difference in cell proliferation between cells treated with vehicle or BPA, after release from serum starvation. Moreover, the total NPM protein levels in the BPA-treated cells did not change on 24-h treatment. Our data thus indicate that there is dysregulation in the timing of initiation of centrosome duplication and an earlier release of NPM from centrioles in cells treated with BPA and the respective analogues.

We then looked at the expression of G1-phase genes that are involved in the process of initiation of centrosome duplication. We found that expression of *CDK2*, *CDK4*, *cyclin E1* and *cyclin A2* did not change significantly in long-term BPA-treated cells (LNCaP/BPA and C4-2/BPA, Fig. 3F). *p27^{kip1}* and *p53*

At each time-point, cells were scored for centriole and centrosome numbers in LNCaP and C4-2 cell lines. (A) Scoring of number of cells with amplified centrosomes. (B) Scoring of number of cells with single centrosome. (C) Scoring of number of cells with duplicated centrioles. Around 5–10% of cells had separated centrioles at 0, 8 and 16-h time-points. (D) Representative staining showing cells with NPM on single centrosomes. Unduplicated (a–c, g–i) and duplicated (d–f, j–l) LNCaP (a–f) and C4-2 (g–l) cells were immunostained for gamma-tubulin (green, a, d, g, j), NPM (red, b, e, h, k) and DAPI (blue). DAPI images (blue) were merged with gamma-tubulin (green) and NPM (red). Arrows indicate the positions of the centrosome (*n*=number of centrosomes). Scale bar, 20 μ m. (E) Cells were scored for the presence or absence of NPM on non-duplicated centrosomes at various time-points in LNCaP and C4-2 cell lines. (F) Relative expression as determined by qRT-PCR for *p53*, *CDK4*, *CDK6*, *CDK2*, *p21^{Waf1}*, *p27^{kip1}*, *Cyclin D1 (CCND1)*, *Cyclin A2 (CCNA2)* and *Cyclin E1 (CCNE1)* in LNCaP (upper panel) and C4-2 (lower panel) cells in the absence (white bar) and presence (gray bar) of BPA. For the previously mentioned experiments, significance was determined using the Student's *t*-test compared to 0 h or vehicle treatment (**P*<0.05, ***P*≤0.01, ****P*≤0.001). Bars, s.e. of three independent experiments.

Figure 4

CA in the presence of BPA and its analogues may involve ER- α (ESR1). LNCaP and C4-2 Cells were treated with inhibitors for ER (ICI), ESR1 (MPP), ESR2 (PHTPP), AR (bicalutamide) and GPR30 (G15) for 72h in the presence and absence of BPA and its analogues as indicated. Cells were fixed and scored for number of cells with centrosome numbers ≥ 3 . Significance was calculated relative to no inhibitor group.

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Bars, s.e. of the three independent experiments.

expression was decreased (0.5- and 0.4-fold, respectively) and *CDK6* (13-fold) and *cyclin D1* (1.4 fold) increased in LNCaP/BPA cells compared to that in vehicle control (Fig. 3F). This implies that *p53*, *p27^{kip1}*, *CDK6* and *cyclin D1* could be the main drivers for initiation of centrosome

cycle in AD LNCaP/BPA cells. For the AI C4-2/BPA cells, there was decreased expression of *p21^{Waf1}* (0.4-fold). Hence, in the BPA-treated AD and AI cells, the expression of different CDK inhibitors (*p27^{kip1}* and *p21^{Waf1}*, respectively) is downregulated. These results indicate that the G1-phase

Figure 5

BPA, BPS, DMBPA and TBBPA enhancement of centrosomal MT-aster formation is accompanied with increased expression of CEP350. LNCaP (A), C4-2 (B) cells and (C) RWPE1 cells were treated with vehicle (veh), BPA, BPS, TBBPA, DMBPA and TMBPA as indicated. Cells were fixed and immunostained for centrosomes (anti- γ -tubulin) and MTs (anti- α -tubulin). Centrosomal aster formation was counted as positive if centrosomes nucleated greater than 15 asters. (A, B, C) The percent cells positive for MT-asters were scored (A', B', C') Representative staining showing MT-aster formation. (D) Relative expression as determined by qRT-PCR for *p150^{glued}*, *EB1*, *Ninein* and *CEP350* in LNCaP and C4-2 cells in the absence (white bar) and presence (gray bar) of BPA. For above experiments, significance was determined using a Student's *t*-test compared to vehicle treatment (* $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Bars, S.E.M. of the three independent experiments.

CDKs (*CDK4*, *CDK6* and *CDK2*) are affected by BPA, albeit by downregulating the expression of different CDK inhibitors.

CA in the presence of BPA and some of its analogues is dependent on estrogen receptor alpha (ESR1)

The goal of this experiment was to identify which and whether estrogen and androgen receptors mediate CA

induced by exposure to BPA and its analogues. Five inhibitors were used: (a) Fulvestrant (ICI) an antagonist for ESR1 and ESR2 (Fitts *et al.* 2011), which degrades receptor proteins; (b) MPP, an antagonist for ESR1; (c) PHTPP, an antagonist for ESR2; (d) bicalutamide an antagonist for AR; and (e) G15, an antagonist for GPER1. Cells were treated with 0.1 nM BPA, BPS and DMBPA or 0.01 nM TBBPA in the presence and absence

of inhibitors for the respective nuclear receptors (Fig. 4). Treatment of cells with BPA and its analogues in the presence of ICI, but not bicalutamide or G15, inhibited CA, suggesting that ESR1 or ESR2 is involved in this process. Furthermore, treatment of cells with BPA and its analogues along with MPP (ESR1 antagonist), and not with PHTPP, inhibited CA suggesting that the CA induced by BPA and its analogues is dependent on ESR1.

Low-dose BPA, BPS, TBBPA and DMBPA enhance MT-aster formation, a measure of tumor progression

Centrosomes are the MT-organizing center in cells. During interphase, changes in MT dynamics usually associate with loss of tumor architecture, increased motility and invasiveness, decreased cell adherence and thus, tumor aggressiveness (Lingle et al. 2002). To perform MT-aster formation assay, LNCaP, C4-2 and RWPE1 cells were exposed to BPA and its analogues for 3 days. Cells were treated with nocodazole to depolymerize the interphase MTs; nocodazole was then removed, and cells were incubated in fresh warm medium for MT regrowth. The centrosomes' ability to nucleate, anchor and elongate MTs was determined by co-immunostaining for centrosomes (anti- γ -tubulin) and MTs (anti- α -tubulin). The aster-forming activity of centrosomes was assessed according to the previously established protocol (Tarapore et al. 2012). 20–30% of untreated LNCaP, C4-2 and RWPE-1 cells (Fig. 5A, B and C, respectively) showed MT-aster formation, whereas ~48–60% cells treated with BPA, BPS, TBBPA and DMBPA showed the presence of MT-asters (Fig. 5A, A', B, B', C and C'). Our data thus indicate that BPA and some of its analogues enhance MT-aster formation.

A number of proteins involved in MT anchorage and elongation at the centrosome have been identified. These include p150^{glued}, EB1, Ninein (NIN) and CEP350 (Mogensen et al. 2000, Yan et al. 2006, Azimzadeh & Bornens 2007). As BPA, BPS, TMBPA and DMBPA had similar effects on MT-aster formation, we used the prototype bisphenol BPA for further analyses. We examined the expression of these genes in LNCaP and C4-2 cells. Although no significant changes were observed in the expression of p150^{glued}, EB1 and NIN, CEP350 was found to be upregulated 2.5- and 13-fold in LNCaP and C4-2 cells treated with BPA, respectively (Fig. 5D).

Table 2 Anchorage-independent growth (%average $\pm$ s.e. of colonies).

	LNCaP	C4-2
Vehicle	0.64	2.00
BPA	0.46 $\pm$ 0.14	5.30 $\pm$ 0.79**
BPS	0.69 $\pm$ 0.34	2.94 $\pm$ 0.4
TBBPA	0.93 $\pm$ 0.5	2.22 $\pm$ 0.62
DMBPA	1.90 $\pm$ 0.25**	3.66 $\pm$ 0.36**
TMBPA	0.66	1.92

**P value <0.006, computed using t-test.

Chronic exposure to DMBPA promotes anchorage-independent growth

Cancer progression can also be measured *in vitro* by anchorage-independent growth (Mori et al. 2009). The ability of chronic low-dose BPA, BPS, TBBPA and DMBPA exposure to promote anchorage-independent growth of LNCaP and C4-2 cells was assessed by a soft agar colony formation assay. The cells were grown in medium with or without BPA or its analogues for 10–14 passages before they were seeded on soft agar in the presence of same analogues. As previously observed (Tarapore et al. 2014), only the C4-2 cells showed significant increase in number of colonies on treatment with BPA (Table 2). The percent efficiency of colony formation (mean $\pm$ s.e.) increased to 5.3 $\pm$ 0.8% with BPA treatment compared with 2.0% in unexposed controls (P < 0.006). However, on chronic treatment with DMBPA, both LNCaP and C4-2 cells showed an increase in efficiency of colony formation of ~2.4- and 1.83-fold compared with unexposed controls (P < 0.006).

Discussion

In this study, we examined the effects of 6 BPA analogues along with BPA at nanomolar range on CA to ascertain their endocrine-disrupting effects with reference to BPA. We found that exposure to BPS, DMBPA and TBBPA resulted in a non-monotonic increase in number of cells with CA, maybe primarily via ESR1 signaling. In addition to promoting CA, exposure to BPS, TBBPA and DMBPA also resulted in increased stability of MT-asters at centrosomes. Finally, treatment of cells with DMBPA but not with the other analogues resulted in an enhancement of anchorage-independent growth, similar to BPA exposure. Thus, DMBPA appears to be the most potent analogue when compared to BPA in its action to promote cancer-related features.

BPA has now been in commercial use for 57 years, and numerous health effects including increasing

cancer risk have been reported to be associated with its usage (Keri et al. 2007, Ho et al. 2012, Rogers et al. 2013, Zawatski & Lee 2013). We and others have demonstrated the involvement of BPA in increasing PCa risk through early life reprogramming (Ho et al. 2006, Tang et al. 2012, Prins et al. 2014) as well as chronic life exposure (Lam et al. 2015). These observations are in agreement with a limited amount of human evidence including a small retrospective study, which found BPA levels in the urine of PCa patients were higher than those in non-PCa patients (Tarapore et al. 2014). Multiple mechanisms have been proposed for BPA action, including epigenetic reprogramming of gene expression (Ho et al. 2006, Tang et al. 2012), increased expansion of stem-like progenitor cells in the prostate (Prins et al. 2014), altering biosynthesis pathways such as the SNORDs (Ho et al. 2015), disrupting the MTs (Pfeiffer et al. 1997, Ochi 1999), causing adduct formation and DNA damage (Fic et al. 2013) and increasing CA (Tarapore et al. 2014). Centrosomes are the MT-organizing center in cells, and by this process regulate cell orientation, polarity and migration. They are involved in mitosis as well as organelle transport. Increase in centrosomal MT anchoring and regrowth is a marker for tumor cell progression, and changes in MT dynamics usually associate with loss of tumor architecture and hence promote loss of cell adhesion, tumor motility and invasiveness (Lingle et al. 2002). Growth on soft agar is indicative of a transformed phenotype. We found that although BPA, BPS, TBBPA and DMBPA increased CA and MT dynamics, only BPA and DMBPA significantly transformed the cells. Going forward, investigating whether DMBPA increases risk to PCa is of paramount importance. Whether BPA and DMBPA are allowing for expansion of a stem cell population or allowing the establishment of a more metastatic phenotype remains to be determined and is an area of on-going research.

As we replace BPA with other BPA analogues in plastic containers, bottles, receipts and toys, it is important to note that the health effects of these analogues are just emerging. For example, BPA analogues such as BPS, BPF and TBBPA were found to disrupt multiple nuclear receptors and may therefore interfere with the endocrine system (Molina-Molina et al. 2013). Moreover, exposure of zebrafish to BPS during their development impaired their subsequent reproduction potential and hormonal balance when adult (Naderi et al. 2014). BPS also disrupted membrane-initiated estradiol-induced cell signaling, leading to altered cell proliferation, cell death and prolactin release (Vinas & Watson 2013) in a rat pituitary cell line. Rapid exposure to low-dose BPS showed

proarrhythmic impact on *ex vivo* female rat hearts and myocytes (Gao et al. 2015). For the first time in this study, we show that like BPA, BPS, TBBPA and DMBPA affect CA, probably via ESR1.

In the context of mechanism, we found that centrosome duplication occurs at an earlier time-point in the presence of BPA, BPS, DMBPA and TBBPA. In AD (LNCaP) and AI (C4-2) cells, the same group of CDKs (CDK4, CDK6 and CDK2) is affected by BPA, albeit through downregulating the expression of different CDK inhibitors *p27^{kip1}* and *p21^{Waf1}*, respectively (Fig. 6). We show that in the presence of BPA, the expression of *CDK6* and *cyclin D1* increases and that of *p21^{Waf1}* or *p27^{kip1}* decreases. Previous studies show that CDK4, CDK6 and CDK2 phosphorylate NPM at Thr199 initiating the separation of centrosomes (Hinchcliffe et al. 1999, Cuomo et al. 2008, Adon et al. 2010), as the first step in centrosome duplication. We and others have shown that centrosomes can duplicate during prolonged exposure to G1-phase, albeit much slower than when arrested at G1/S boundary or S-phase (Hinchcliffe et al. 1999, Tarapore et al. 2002). We can thus speculate that an earlier release of NPM would allow a second round of centrosome duplication to occur later during G1-phase or early S-phase, in BPA-treated cells. Timing of NPM release at non-duplicated centrosomes thus appears to be the key step disrupted in cells treated with BPA and some of its analogues. This does not appear to be loss of regulation of centrosome re-duplication during S-phase as arresting cells using DNA synthesis inhibitors did not result in significant differences in centrosome amplification of BPA and its analogues compared to vehicle control. This suggests that controls are still in place to inhibit re-duplication of centrosomes during S-phase.

Figure 6

NPM in regulation of the initiation of centrosome duplication. Centrosome-bound NPM dissociates from centrosomes upon phosphorylation by CDK2, CDK4 or CDK6, which triggers the initiation of centriole duplication. *p21^{Waf1}* and *p27^{kip1}* inhibit CDK activation, and thus, initiation of centrosome replication.

By nucleating and anchoring MTs, the centrosome influences MT-directed processes including shape, polarity, organelle transport, adhesion, motility and division of cells. We found that CA was observed in only 10–15% cells in a population on exposure to BPA analogues. However, 40% more cells showed the formation of MT-asters compared to vehicle control. Hence, we are probably observing the effects of BPA and its analogues on a function of the centrosome distinct from the CA phenotype. Involved in the process of MT anchoring at the centrosomes, are the proteins NIN, p150^{Glued}, EB1 and CAP350 (Yan et al. 2006, Pihan 2013). We found that expression of *CEP350* is upregulated in BPA-treated LNCaP and C4-2 cells compared to untreated controls. Previous studies have suggested that CAP350, EB1 and FOP (FGFR1 oncogene partner) form a centrosomal complex required for MT anchoring and elongation. Depletion of CAP350, FOP or EB1 by siRNA caused loss of MT anchoring and profound disorganization of the MT network, whereas the overexpression of CAP350 promoted an increase in the stability of the MT network (Hoppeler-Lebel et al. 2007). Our results on the MT-aster assay further support this result and suggest that overexpression of CAP350 could be promoting MT anchoring and stability. Intriguingly, in addition to its function in MT anchoring, CEP350 has also been shown to be involved in regulating procentriole stability and growth/assembly (Le 2008). The implications of overexpression of CEP350 on exposure to BPA and its analogues thus warrant further investigation.

In conclusion, we have found that some BPA analogues (BPS, TBBPA and DMBPA) are more potent than others (BPF, BPAF and TMBPA) for CA, an indicator for cancer severity. BPA affects the expression of two different G1-phase CDK inhibitors, which target the same signaling pathway, resulting in the release of NPM from centrosomes during G1-phase at an earlier time-point. BPA, BPS, TBBPA and DMBPA all equally affect MT anchorage and growth, a proxy for cell aggressiveness. Finally, DMBPA is most comparable to BPA in that it can affect anchorage-independent growth of cells, a measurement of cell transformation. Our results therefore indicate that it is increasingly important to examine the actions of these analogues before their use becomes too widespread as substitutes for BPA in consumer products.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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