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Camptothecin efficacy to poison Top1 is altered by Bisphenol A in mouse embryonic fibroblasts

Manoj Sonavane[†], Peter Sykora[†], Joel F. Andrews[†], Robert W. Sobol[†], and Natalie R. Gassman^{*,†}

[†]Department of Oncologic Sciences, University of South Alabama Mitchell Cancer Institute, 1660 Spring Hill Avenue, Mobile, Alabama 36604, USA

Abstract

Bisphenol A (BPA) is used heavily in the production of polycarbonate plastics, thermal receipt paper, and epoxies. Ubiquitous exposure to BPA has been linked to obesity, diabetes, breast and reproductive system cancers. Resistance to chemotherapeutic agents has also been shown in cancer cell models. Here, we investigated BPA's ability to confer resistance to camptothecin (CPT) in mouse embryonic fibroblasts (MEFs). MEFs are sensitive to CPT; however, co-exposure of BPA with CPT improved cell survival. Co-exposure significantly reduced Top1-DNA adducts, decreasing chromosomal aberrations and DNA strand break formation. This decrease occurs despite BPA treatment increasing the protein levels of Top1. By examining chromatin structure after BPA exposure, we determined that wide-spread compaction and loss of nuclear volume occurs. Therefore, BPA reduced CPT activity by reducing the accessibility of DNA to Top1, inhibiting DNA adduct formation, the generation of toxic DNA strand breaks, and improving cell survival.

GRAPHICAL ABSTRACT

* nrgassman@health.southalabama.edu. Tel: 251-445-8430; Fax: 251-460-6994.

COMPETING INTERESTS

M.S., P.S., J.F.A., and N.R.G. declare no competing interests. R.W.S. is on the scientific advisory board for Bio-Techne/Trevigen, Inc.

SUPPORTING INFORMATION

Full scans of western blot image showing Top1 antibody specificity in MEFs.

INTRODUCTION

Bisphenol A (BPA) is a high volume industrial chemical used in the production of a wide-range of consumer products, including plastic ware, epoxies, dental composites and sealants, and thermal receipt paper.¹ The utility of BPA as an industrial precursor for polymerization has driven an exponential increase in its production worldwide with resulting levels of increasing BPA contamination in the air, water, and soil.^{2, 3} As a result, circulating levels of BPA in the 10–100 nM range have been measured in human samples, likely through inhalation, ingestion, and/or absorption exposures.^{4–6}

Free circulating BPA in human samples is concerning, since BPA has estrogenic character and has been reported even at low doses to act as a potent endocrine disrupting chemical. The estrogenic character of BPA has been the focus of numerous studies with growing evidence that BPA alters reproduction, development, and metabolism [reviewed in ^{7–11}]. Population studies have also associated BPA exposure with the development and progression of asthma, diabetes, cardiovascular disease, obesity, and cancer.^{11, 12}

While the molecular mechanisms underlying these disease outcomes are still poorly understood, BPA exposure has been significantly associated with increases in oxidative stress, inflammation, and mitochondrial dysfunction.^{13–20} Further, our recent work and that of others have observed a strong pro-survival effect when BPA is co-exposed with cytotoxic agents.^{21–25} Importantly, BPA co-exposure with chemotherapy treatments, like cisplatin, vinblastine and doxorubicin, has been shown to diminish the cytotoxicity of these agents, and these effects were not mediated via estrogen receptors.²⁵ These potential interactions with chemotherapeutic agents have received little attention. In examining co-exposures of BPA with the oxidizing agent potassium bromate, we observed a delay in the onset of DNA damage response and repair.^{23, 24} This suppression of DNA repair was not previously reported in other co-exposure studies and may indicate that the suppression of DNA damage recognition and removal may be a potential mechanism by which BPA promotes resistant to chemotherapeutic agents.

Chemotherapy, alone or in combination, represents the mainstay treatment in metastatic disease. Some tumors can acquire resistance to drugs intrinsically, whereas others develop resistance after drug treatment. Studies have explored the effects of environmental chemicals on metabolic and detoxifying enzymes, as well as on drug transporters^{26–28}; however, little is known about how BPA modulates the responsiveness of anticancer drugs in cancer cells. Given the ubiquity of BPA, understanding its co-exposure effects and the mechanisms by which it modulates chemotherapeutic activity is critical for understanding the impact BPA exposure has on disease development and treatment.

In the present study, we investigated high-dose BPA co-exposure effects with the chemotherapeutic agent camptothecin (CPT) in mouse embryonic fibroblasts (MEFs) to examine the molecular mechanisms that promote cell survival despite genotoxic stress. We employed a high dose of BPA over a short exposure period to concentrate the effects of BPA and to monitor DNA damage and repair effects. MEFs have been used extensively in the characterization of DNA damage response and repair pathways, and offer an ideal model system for studying all aspects of DNA repair.^{29, 30} Additionally, BPA exposure effects have been extensively studied in murine models making this system comparable with the wealth of BPA exposure data accumulated by numerous other sources.^{31, 32} Further, we have previously demonstrated that high-dose BPA (150 μ M) is minimally cytotoxic in the MEF model system with no observed increase in cell proliferation.²⁴ Increases in cell proliferation are common at low doses of BPA and may confound analysis of DNA repair machinery.^{25, 33}

CPT is a topoisomerase-I (Top1) poison that penetrates vertebrate cells readily and targets Top1 covalent complexes (Top1ccs) within minutes of exposure, creating DNA-protein crosslinks, that result in both cytotoxic single-strand and double-strand DNA breaks in treated cells.³⁴ Thus, CPT has become routinely used as a research tool to investigate the genetic factors that are implicated in checkpoint regulation and DNA repair in response to Top1-mediated DNA damage in various organisms.^{35, 36} High-dose BPA co-exposure with CPT prevented CPT-induced cell death. Investigation of this pro-survival effect revealed that co-exposure of BPA with CPT reduced the formation Top1ccs, as well as DNA strand breaks in general. Loss of DNA lesions was not due to loss of Top1 protein. BPA increased the expression of Top1 during exposure and co-exposure. However, BPA exposure induced wide-spread compaction of the chromatin, and an overall loss of nuclear volume, which we propose reduces the accessibility of DNA to Top1, preventing CPT-induced cell death. This work reveals a previously unexplored mechanism by which BPA alters DNA repair capacity and induces genomic instability.

MATERIALS AND METHODS

Cell line

Repair competent mouse embryonic fibroblasts (MEFs) (a gift from Dr. Shigemi Matsuyama, Cleveland, OH) were grown at 37 °C in a 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with glutamine, 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% non-essential amino acids (Life Technologies), and 1% sodium pyruvate (Life Technologies).^{24, 37} Cells were routinely tested and found to be free of mycoplasma contamination.

Cytotoxicity assay

Cytotoxicity was determined by growth inhibition assays. MEFs were seeded at a density of 40,000 cells/well in six-well dishes. The following day, cells were exposed to a range of CPT (Sigma Aldrich) concentrations for 24 h or exposed with 150 μ M BPA (Sigma Aldrich) for 1 h then a range of CPT concentrations co-exposed with 150 μ M BPA for 24 h. BPA was prepared in absolute ethanol and diluted to the final working concentrations in medium. CPT was dissolved in dimethyl sulfoxide (DMSO, Corning) at a concentration of 10 mM then diluted into medium at the time of the experiment. After 24 h exposure to CPT or CPT + BPA, cells were washed in Dulbecco's phosphate buffered saline (PBS, Hyclone) and fresh medium was added. Dishes were then incubated for 6–7 days at 37 °C in a 5% CO₂ incubator until untreated control cells were approximately 80–90% confluent. Cells (triplicate wells for each drug concentration) were counted by a cell lysis procedure³⁸, and results were expressed as the number of cells in drug-treated wells relative to cells in control wells (% control growth).

Immunofluorescence

For immunofluorescent staining, cells were seeded in fluorodishes (World Precision) at a density of 1×10^5 per dish. The next day, cells were untreated, treated with 150 μ M BPA, 80 nM CPT, or co-exposed by treating with 150 μ M BPA for 1 h followed by 24 h exposure with 80 nM CPT and 150 μ M BPA. After 24 h, media was aspirated and samples were then fixed with 3.7% formaldehyde (Fisher) in PBS for 5 mins at room temperature (RT) then washed 3 times with PBS. Cells were permeabilized with 1% sodium dodecyl sulfate (SDS, Sigma Aldrich) solution in PBS for 10 mins at RT, followed by 5 times wash with PBS. Cells were blocked with 5% goat serum in PBS for 30 mins at RT. Primary monoclonal anti-phosphoSer139-H2AX antibody (EMD Millipore) at 1:500 (for γ H2AX staining) and anti-Top1 antibody (abcam) at 1:200 (for Top1) was incubated for 2 h at RT. Rinsed samples with PBS and incubated with Alexa 488 goat anti-mouse and Alexa 546 goat anti-rabbit secondary antibodies at 1:1000 (Life Technologies), respectively in dark for 1 h. Nuclear DNA was stained with 10 mg/mL of NucBlue® fixed cell stain (DAPI, Life Technologies) for 5 mins at RT prior to the completion of secondary antibody incubation period. Finally, cells were washed three times with PBS, air-dry the fluorodishes before mounting the cells for imaging using Prolong Gold mounting media (Life Technologies).

Immunostained cells were imaged with a Nikon A1r confocal microscope using a 100X C-Apochromat (Numerical aperture (NA) 1.45) oil immersion objective. For each field, an eight-frame image stack was collected through the Z plane. Fluorescence and DIC image channels were acquired at a resolution of 1024×1024 pixels (0.12 μ m/pixel) using the following laser lines: 405 nm (DAPI), 488 nm (Alexa 488 and DIC) and 561 nm (Alexa 546). Common settings were used to acquire all images to facilitate direct comparison. For analysis, maximum intensity projections were generated from each image stack. DAPI staining was used to segment nuclei, and the mean intensity of γ H2AX and Top1 staining per nucleus was calculated. For each treatment at least 50 ± 15 cells were measured using NIS-Elements software, and the values were reported as mean fluorescence intensity $\pm$ standard error of the mean (SEM) of two biological replicates.

CometChip assay

DNA strand breaks were assessed using the CometChip Platform (Trevigen).³⁹ MEF cells were seeded in 96-well plate at a density of 10^4 cells per well. The next day, cells were untreated, treated with 150 μ M BPA, 80 nM CPT, or co-exposed by treating with 150 μ M BPA for 1 h followed by 24 h and 48 h exposure with 80 nM CPT and 150 μ M BPA. After the designated exposure periods, cells were trypsinized and moved into the chilled 30-micron sized CometChip® apparatus. Cells were gravity loaded into the microwells for 30 min with the CometChip® placed in the cold room (4°C). After loading in the CometChip® apparatus, the chip was washed multiple times with PBS and sealed with low melting point agarose (LMPA, ThermoFisher) (7 ml; 0.8% LMPA/ PBS). The CometChip® was then submerged in lysis solution with detergent for 40 min at 4°C. The CometChip® was run under alkaline (pH>13) conditions (200 mM NaOH, 1 mM EDTA, 0.1% Triton X-100). Electrophoresis was conducted at 22 V for 50 min at 4°C. After electrophoresis, the CometChip® was re-equilibrated to neutral pH using Tris buffer (0.4 M Tris-Cl, pH 7.4). Subsequently, the DNA was stained with 1x SYBR® Gold diluted in Tris buffer (20 mM Tris-Cl, pH 7.4) for 30 min and de-stained for 1 h in Tris buffer (20 mM Tris-Cl, pH 7.4).

Image acquisition was conducted on the Celigo S imaging cytometer at a resolution of 1 micron/pixel with whole plate imaging to avoid imaging variability. Image analysis was conducted using the dedicated Trevigen Comet Analysis Software (CAS), with the box size set to 220×180 pixels which represented a box size that would capture comets from heavily damaged cells without box overlap. For all data, % tail DNA was used to measure the amount of DNA damage, which was calculated as the amount of tail DNA to the total amount of DNA multiply by 100. Data of at least four technical replicates, each with 1500 ± 300 comets were acquired and exported to Excel and subsequently to Graphpad for statistical analysis. Detailed analysis of the CometChip Platform has been described³⁹.

Rapid approach to DNA adduct recovery (RADAR) assay

RADAR assay protocol was adapted from Kiianitsa and Maizels⁴⁰ and optimized for the MEF cells. In brief, MEFs were cultured in 65 mm petri dish at a density of 1×10^6 cells per dish and incubated overnight for cells to adhere. The following day, cells were untreated, treated with 150 μ M BPA, 80 nM CPT, or co-exposed by treating with 150 μ M BPA alone for 1 h followed by 24 h co-exposure with 80 nM CPT and 150 μ M BPA. After designated period, the culture medium was aspirated and cells were immediately lysed on the plate with 1 ml of lysis solution (DNAzol® (Life Technologies), in combination with 1% Sarkosyl (Fisher)). Nucleic acid and DNA-protein covalent complexes (DPCC) were recovered by addition of 0.5 volumes of 100% ethanol, gentle mixing and incubation at -20°C for 5 min, followed by centrifugation for 15 min at $15,000 \times g$ at 4°C. Supernatant was aspirated and the pellet was washed twice by vortexing in 1 ml of 75% ethanol (VWR) followed by 10 min centrifugation. Supernatant was removed and the pellet was immediately resuspended in 200 μ l of freshly prepared 8 mM NaOH (Sigma Aldrich).

Recovered DNA was quantified using AccuBlue™ Broad Range dsDNA Quantification Kits with 9 DNA Standards, as recommended by the manufacturer's instructions (Biotium). DNA

recovery was relatively uniform, and 400–1000 ng of DNA per treatment condition was used for DPCC immunodetection.

A BioDot® SF Microfiltration Apparatus (BioRad) was used for isolation and immunodetection of the DPCCs. Briefly, equal volumes of solubilized DPCC isolates were diluted in Tris-buffered saline (TBS, Amresco) to achieve final volume of 200 µl. The entire sample was applied to nitrocellulose membrane using the BioDot. The membrane was blocked for 1 h in blocking buffer (5% skim milk in TBST (TBS containing 0.1% Tween 20, TBST)), followed by 4°C overnight incubation with rabbit polyclonal anti-Topoisomerase I (anti-Top1) and anti-double strand DNA (dsDNA, abcam) antibodies (1:1000 dilution in blocking buffer). The membrane was washed with TBST, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the membrane was washed three times in TBST and DPCC signal was detected using enhanced chemiluminescence (ECL, Advantisa). Top1 was detected with anti-Top1 antibody and the amount of DNA loaded for each sample was monitored using ds DNA antibody. DPCC signals were quantified using the Image Lab software, which was determined as the ratio of the percentage of band intensity of Top1 to that of the percentage of band intensity of ds DNA. The observed values for the treated samples were compared with the control signals and Top1-DNA adduct signal was expressed as the average of the three biological replicates that were performed on different days ± SEM.

Western blot

MEF cells were seeded in 100 mm dishes at a density of 0.5×10^6 cells per dish and incubated overnight. The next day, cells were exposed with culture medium (no treatment), 150 µM BPA, 80 nM CPT, or co-exposed by treating with 150 µM BPA for 1 h followed by 24 h exposure with 80 nM CPT and 150 µM BPA. After 24 h, cells were scraped and stored overnight at –80°C. Frozen pellets were lysed in 150 µl of ice-cold lysis buffer (25 mM β-glycerolphosphate, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, and 0.3% NP-40) plus 1X Halt protease and phosphatase inhibitor and incubated for 30 min on ice. Lysates were centrifuged at 15,000 x g, for 15 min, 4°C and supernatant fraction was collected to determine protein concentrations using Bradford Quick Start protein assay. Proteins (30 µg/well) were resolved on 4–20% SDS-PAGE gel, and transferred to PVDF membrane using Bio-Rad Trans Blot system. Membranes were blocked in blocking buffer (5% skim milk in TBST (TBS containing 0.1% Tween 20, TBS-T)), and then incubated with the indicated primary antibodies at 4°C overnight. Overnight membrane was washed with TBST and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Signals of the bound antibody were detected using ECL. The membrane was stripped by incubating with Review Western Blot Stripping Buffer for 30 mins at 50°C, followed by three times 5 min wash with TBST at RT.

Chromosome Aberration (CA) assay

MEFs were seeded in a 65 mm dish at a density of 1.5×10^5 cells per dish. The following day, cells were untreated, treated with 150 µM BPA, 80 nM CPT, or co-exposed by treating with 150 µM BPA for 1 h followed by 24 h exposure with 80 nM CPT and 150 µM BPA. Colcemid with 0.1 µg/ml was added for 4 h at the end of the drug treatment. After desired

exposure period, cells were trypsinized and wash once with 5 ml of PBS. Cells suspension was centrifuged and pellet was resuspended in 5 ml of hypotonic solution (0.075 M KCl) for 30 min at RT, followed by resuspension in 5 ml of ice cold Carnoy's fixative solution (methanol: acetic acid, 3:1), adding drop by drop while vortexing. Store the samples at -20°C overnight. The next day, centrifuged the samples, aspirate the Carnoy's fixative solution and pellets were resuspended in 750 μl of freshly prepared ice cold Carnoy's fixative solution.

Metaphase spreads were performed by putting 3–5 drops of the cell suspension from pipette (one by one) directly onto the slide that is inclined at 45° angle. Immediately place the slide on the preheated hot plate (60°C) to dry the slide and observe the quality of spreads under phase contrast microscope. The slides were stained with giemsa staining solution (giemsa stain diluted in Gurr's buffer, Fisher), and were air dried. At least 25 metaphase spread images were collected using bright field microscope and analyzed for CA and their mean were calculated as frequency of $\text{CA} \pm \text{SEM}$.

Nuclear condensation assay

For nuclear condensation assay, 1×10^5 cells were seeded per well in a 6-well plate containing coverslip. The next day, cells were untreated and exposed to 150 μM BPA for 24 h. After 24 h, culture media was aspirated and cells were fixed with 3.7% formaldehyde for 10 min at RT, followed by cell permeabilization with 1% SDS for 5 min at RT. Cells were blocked with PBS containing 5% goat serum for 30 min at RT, and then incubated with 5 $\mu\text{g}/\text{ml}$ of Hoechst (Life Technologies) stain diluted in PBS for 5 min at RT. Finally, cells were washed with PBS and air-dried coverslips containing stained cells was mounted on coverslips for imaging using Prolong Gold mounting media.

Cells were imaged with a Nikon A1r confocal microscope using a 60X C- Apochromat (NA 1.4) oil immersion objective to image stained cells. For each field, images were collected as fifteen-frame image stacks through the Z plane to sample the entire nucleus. Images were acquired at a resolution of 512×512 pixels ($0.41 \mu\text{m}/\text{pixel}$) using the 405 nm laser line to image DAPI staining, and the 488nm laser line to generate a brightfield Differential Interference Contrast (DIC) image. For analysis, maximum intensity projections of each image stack were generated. DAPI staining was used to segment individual nuclei, and mean DAPI intensity was calculated for each nucleus. 150 cells per treatment were measured using NIS-Elements software, and the values were reported as mean fluorescence intensity $\pm$ SEM.

Statistical Analysis

Unless stated otherwise, data are represented as $\pm$ SEM of three biological replicates. Statistical tests were performed by one-way analysis of variance (ANOVA) and Student's t test. Statistical significant * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS

BPA co-exposure reduces cytotoxic effect of camptothecin in MEFs

We first examined the sensitivity of wild type, repair competent MEF cell line to camptothecin and determined whether co-exposure with high-dose BPA altered the cytotoxicity of BPA. We previously established that 150 μ M BPA showed minimal cytotoxic effect in the wild type MEF cell line and that this dose did not increase cellular proliferation.²⁴ As shown in Figure 1, camptothecin alone induced a dose dependent decrease in cell viability that was antagonized by a 24 h co-exposure treatment with a high-dose of BPA (150 μ M). MEFs were highly sensitive to CPT, with the highest tested concentration (100 nM) decreasing the number of viable cells to approximately 10%. BPA co-exposure was effective against all the tested CPT concentrations that resulted in drug-induced cytotoxicity. The maximal BPA protective effect was found to be at the two highest CPT concentrations (80 nM and 100 nM), promoting cell survival ranging from 38% to 42%.

CPT-mediated strand breaks reduction by BPA

CPT cytotoxic effects are mediated by its stabilization of the Top1-DNA cleavable complex.^{41, 42} The stabilization inhibits the religation step, creating a DNA-protein covalent complex (DPCC) and a single strand break in the DNA. If unrepaired, this DPCC is encountered by the replication fork or transcription machinery causing stalling and inducing additional DNA strand breaks.^{43–45} To assess if BPA co-exposure altered the formation or signaling of CPT-induced strand breaks, we measured strand break formation using the CometChip assay³⁹ and examined the signaling by the appearance of phosphorylated H2AX (γ H2AX) (Figure 2).

The CometChip assay correlates the amount of DNA strand breaks in individual cells relative to the percentage of tail DNA, under alkali conditions.³⁹ A small fraction of DNA strand breaks are observed 24 h after exposure to CPT alone, with a more significant increase in strand breaks occurring 48 h after exposure to CPT alone (Figure 2A). Co-exposure with BPA reduced the formation of these DNA breaks at both time points, with 48 h showing the more significant decrease in the % tail DNA.

We also examined DNA strand break signaling by γ H2AX 24 h after treatment with BPA, CPT, or co-exposure to both agents. Figure 2B shows the formation of γ H2AX in the MEFs, with CPT inducing a significant increase in foci formation. Quantification of the mean nuclear fluorescence intensity showed an increase in γ H2AX signal 24 h after CPT exposure alone, but this increase was significantly reduced when co-exposed with BPA (Figure 2C). A small percentage of γ H2AX-positive nuclei were observed with BPA treatment alone, consistent with our previous data demonstrating high doses of BPA induce low levels of base lesions and strand breaks.^{23, 24}

Reduction of CPT induced Top1-DNA adducts by BPA co-exposure

The reduction in CPT-induced DNA strand breaks suggests that BPA co-exposure interferes with the CPT-mediated stabilization of Top1-DNA complex. To assess this mechanism, cells were treated with BPA, CPT or co-exposed with BPA and CPT for 24 h as previously

described, and the covalently bound Top1-DNA covalent complex was immuno-detected using the RADAR assay (Figure 3A). RADAR is a rapid and sensitive assay that enables quantitative immuno-detection of protein-DNA covalent complexes using specific antibody. The amount of DNA loaded for each sample was monitored using dsDNA as a loading control (Figure 3A). Quantification of three independent RADAR assays is shown in Figure 3B, with the Top1-DNA covalent complex normalized to its loading control. As expected, the Top1-DNA adduct signal was increased in CPT treated samples (Figure 3B). Co-exposure with BPA significantly reduced the CPT-induced Top1-DNA adduct signal, consistent with the loss of DNA strand breaks. Interestingly, BPA alone showed a slight decrease in Top1-DNA adducts as compared to control, though the mechanism for the reduced number of adducts is unclear.

BPA alters Top1 expression and localization in MEFs

To further understand the mechanism by which BPA co-exposure decreased the CPT-induced Top1-DNA covalent complex signal, we examined Top1 expression and localization in BPA, CPT or co-exposed MEFs 24 h after treatment using immunoblotting and immunofluorescent staining (Figure 4). Unexpectedly, immunoblotting showed increased Top1 protein expression in BPA alone or BPA co-exposed with CPT (Figure 4A and B), despite the reduction in Top1-DNA covalent complexes. We did note faint, potentially non-specific, band in the immunoblotting results that remained unchanged after treatment. This band may be due to species specificity issues between human and mouse, and testing of other antibodies resulted in more non-specific bands for the mouse cells than was observed for the human (data not shown). Increased levels of Top1 were observed for the 91 kDa band, which corresponds to the mouse *Top1* gene product.

This increase in Top1 protein expression in BPA exposed samples was also confirmed by immunofluorescent staining (Figure 4C). We also observed low protein expression of Top1 in untreated control cells, which increased slightly after CPT treatment. Cellular distribution of Top1 was altered by BPA exposure, relocating a large amount of the Top1 into the cytoplasm (Figure 4C, inset).

BPA causes nuclear condensation in MEFs

Immunofluorescence of Top1 protein distribution in the MEFs also revealed that BPA exposure decreased the nuclear size (Figure 4C). To verify that nuclear area was reduced in the presence of BPA, we performed 3-D imaging of untreated MEFs and BPA-treated MEFs using the nucleic acid stain Hoechst 33342 (Figure 5A). Hoechst emits blue fluorescence when bound to dsDNA and the intensity is higher in heterochromatin regions than in euchromatin regions, indicative of the degree of chromatin compaction across nuclei. BPA exposed cells showed a significant reduction in nuclear volume, mapped by the total Hoechst of the nucleus, when compared to control cells (Figure 5B). Additionally, the mean fluorescence intensity of the nucleus was higher (Figure 5C), with large, bright foci apparent (Figure 5A, inset) in cells exposed to BPA. The observed compaction of DNA along with the nuclear condensation indicates BPA alters chromatin structure.

Co-exposure with BPA reduces CPT-induced genomic instability

Alterations in chromatin structure along with DNA damage can induce genome instability, chromosomal aberrations and aneuploidy.⁴⁶ It has been proposed that CPT-induced DSBs are considered a lesion that produces chromosomal damage.^{47, 48} To assess whether BPA reduces the frequency of CPT-induced chromosomal aberrations (CA), we examined untreated MEFs (control) and MEFs treated with BPA, CPT or BPA co-exposure with CPT for 24 h and metaphase spreads were scored for CA frequency. Examples of the types of CA produced by different treatments are shown in Figure 6A. As expected, CPT treated cells showed a significant increase in the frequency of CA when compared to control. Upon BPA co-exposure, we observed a statistically significant decrease in the CA frequency compared to CPT only treated cells. However, BPA alone did not show a significant increase in the CA frequency, showing similar magnitude of CA frequency to that of control samples (Figure 6B), which is consistent with this dose of BPA being minimally cytotoxic and only inducing low levels of DNA damage.^{23, 24}

DISCUSSION

Our work has uncovered a previously unexplored mechanism by which high-dose BPA alters the effectiveness of CPT as a chemotherapeutic agent: by altering nuclear structure and inducing chromatin compaction, thus inhibiting Top1-DNA adduct formation and reducing the formation of toxic DNA strand breaks and promoting cell survival.

BPA is a focus of public concern because of its estrogenic character.⁴⁹ However, a number of recent studies have demonstrated that BPA induces DNA damage via epigenetic alterations^{50–53} and has protective effects against genotoxic stress^{21–24}, independent of estrogen receptors and in the presence of competing hormones. Additionally, these effects have been observed at both environmentally relevant low nanomolar doses³³ and at high micromolar doses.^{24, 54, 55} Here we chose 150 μ M BPA co-exposure with chemotherapeutic agent over short exposure duration (24 h) to concentrate BPA effects and closely monitor DNA damage and repair effects. DNA repair processes are highly efficient and high-dose/short-duration exposures are often employed to better examine DNA repair proteins and mechanisms, so we selected the BPA dose with these considerations in mind. Our results showed that high-dose BPA co-exposure reduces the cytotoxic effect of CPT in MEFs, and we have also observed similar improvements in survival with the CPT analogue topotecan (Fig S2). The observed protective effect was specific to BPA co-exposure and not due to BPA alone, as the high-dose BPA selected in this study showed no increase in cell proliferation in MEFs²⁴ and other mammalian cell models.^{14, 56} Similar antagonizing effects were also observed following BPA co-exposure with an oxidizing agent in MEFs²⁴ and with multiple chemotherapeutic agents in breast cancer cells²⁵, and after doxorubicin exposure in colorectal cancer cells.²¹ Considering that BPA could be a weak carcinogen⁵⁷ and that humans are experiencing chronic BPA exposure each day in the presence of other genotoxic stressors¹, it is equally important to examine and characterize co-exposure effects to understand their health implications.

The initial processing of the Top1-DNA covalent complex is clearly of critical importance in assessing the cellular responses to Top1 poisons, and it has been well demonstrated that

CPT-induced cell death results from the collision of the stabilized Top1-DNA cleavage complex with the transcription machinery and replication fork, creating single strand and double-strand DNA breaks.⁵⁸ In this study, BPA and CPT co-exposed cells revealed a clear reduction in the formation of DNA strand breaks observed by γ H2AX foci formation and as measured by the CometChip assay (Figure 2A-C). The CometChip assay revealed a significant reduction in DNA strand breaks after 48 h of BPA co-exposure. A similar overall reduction in DNA strand breaks was also observed after BPA co-exposure with potassium bromate (KBrO₃), which also showed a co-exposure pro-survival effect.²⁴ The observed reduction in DNA strand breaks further correlates with the reduction in CPT-induced Top1-DNA adducts by BPA co-exposure (Figure 3B). Given that BPA can mediate its effects by acting on various cellular and molecular mechanisms⁵⁹ and that CPT fails to form the Top1-DNA adducts after BPA co-exposure, multiple endpoints are needed to precisely address the mechanism by which BPA co-exposure promotes cell survival.

The molecular target Top1 is clearly required to manifest cytotoxic effects of CPT and it is reasonable to predict that cell sensitivity to this drug can be modulated by total Top1 levels. It has been reported that Top1 protein levels are frequently upregulated in cancer.^{60, 61} Our study revealed that Top1 is significantly overexpressed when exposed to BPA (Figure 4A, B and C), despite the fact that we did not see any alteration in the gene expression profile of Top1 in whole genome analysis of mRNA isolated after 24 h BPA exposure.²³ Interestingly, we also observed Top1 localization into the cytoplasm in BPA exposed cells. Top1 enzyme activity is regulated by post translational modifications, and Top1 poisoning promotes sumoylation and ubiquitylation of Top1 for processing and/or degradation in response to DNA damage^{62, 63}, which is in accordance with our observed Top1 expression in cells exposed to CPT. On the other hand, upregulation and further cytoplasmic localization of Top1 with BPA exposure suggests that BPA is altering the Top1 enzyme activity and potentially the repair of CPT-induced Top1-DNA adducts. Since decreased drug-target interaction is a possible mechanism of resistance to CPT⁶⁴, we examined chromatin structure to determine if BPA reduces the ability of Top1, and therefore CPT, to access the DNA.

Chromatin relaxation plays an important role in the detection and signaling of DNA damage, and studies have shown that DNA lesions are not detected or repaired efficiently within densely packed chromatin regions.⁶⁵⁻⁶⁷ Such reduced accessibility of sites of DNA damage to repair proteins can have an impact on genomic stability.^{68, 69} A recent study demonstrated that a BPA derivative induced nuclear chromatin condensation in gingival fibroblasts.⁷⁰ Our study reveals the impact of BPA exposure on chromatin structure with nuclear volume loss and aggregation of DNA into higher-order structures (Figure 5). Studies have also shown links between BPA exposure and enzymes (EZH2 and DNMT1) that can alter the chromatin structure.^{71, 72} Thus, the observed chromatin compaction could be associated with the reported epigenetic changes, such as histone modification and global methylation changes induced by BPA exposure^{9, 51, 73}, reducing the accessibility of DNA to Top1 and DNA repair proteins. Binding of CPT to DNA alters transcription and replication activities that lead to DNA strand breaks. Studies have shown that the formation of a DSB is dependent on RNA transcription and that chromatin condensation can possibly play a role in converting CPT-induced transcription mediated SSBs into a DSB to create chromosomal

aberrations^{74, 75}. The reduction in CPT-induced CA frequency in BPA co-exposed cells (Figure 6A and B) could possibly occur due to chromatin condensation induced by BPA.

In conclusion, this study, together with our recent findings, suggests that BPA exposure induces a number of cellular events in MEFs resulting in a protective effect against genotoxic stressors, highlighting a role for BPA in nuclear chromatin compaction. Here, we proposed a model by which BPA confers resistance to CPT. BPA causes nuclear shrinkage and DNA compaction, and thus prevents Top1 from accessing and binding to the DNA to form the Top1-DNA cleavable complex. It is well established that CPT interacts with the Top1-DNA complex^{58, 76}, and the reduction of the Top1-DNA complex formation by BPA exposure might reduce the efficacy of CPT as a chemotherapeutic agent. The loss of nuclear volume by BPA may have profound consequences for transcription and replication and may affect cell division if chromosomes are not correctly spatially oriented. Given that BPA alters remodeling of chromatin structure, this model provides a direction for further studies to understand the carcinogenic potential and the mechanism by which BPA confers resistance to chemotherapeutic agents, and suggests that further investigation is warranted to examine the effects of BPA exposure in both drug development and in disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

γH2AX	phosphorylated H2AX
BPA	Bisphenol A
CA	chromosomal aberration
CPT	Camptothecin
DAPI	4,6-Diamidino-2-phenylindole
DIC	differential Interference Contrast
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPCC	DNA protein covalent complexes
DSB	double strand break
dsDNA	double strand DNA

ECL	enhanced chemiluminescence
FBS	Fetal bovine serum
KCl	potassium chloride
LMPA	low melting point agarose
NA	Numerical aperture
MEFs	mouse embryonic fibroblasts
PBS	phosphate buffered saline
RADAR	Rapid Approach to DNA adduct recovery
RT	room temperature
SSB	single strand break
Top1	Topoisomerase-I

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Figure 1.

Camptothecin induced-cytotoxicity is antagonized by BPA in MEFs. MEFs were treated with increasing concentrations of CPT for 24 h (solid circles) or pre-treated with 150 μ M BPA for 1 h, followed by co-exposure with BPA and increasing concentrations of CPT for 24 h (open circles) (See Material and Methods). Treatment was removed after 24 h, and cells were allowed to grow for 6–7 days before counting. Results are expressed as the average number of cells remaining to that of the control (% control growth) $\pm$ the standard error of mean (SEM).

Figure 2.

BPA co-exposure reduces CPT-induced strand breaks.

(A) The mean percentage of comet tail DNA $\pm$ SEM observed in untreated or treated cells 24 and 48 h after exposure. Statistical significant: * $P < 0.05$, ** $P < 0.01$.

(B) Representative images of endogenous strand break signaling marker γ H2AX (green) in control untreated cells and cells treated with BPA, CPT, or co-exposed to both BPA and CPT. DAPI (blue) was used to visualize the nuclei. Scale bar = 10 μ m.

(C) Mean fluorescence intensity of the γ H2AX per nuclei (arbitrary units [AU]). Each bar represents the mean $\pm$ SEM of two biological replicates.

Figure 3.

Reduction in CPT-induced Top1-DNA adducts by BPA co-exposure.

(A) RADAR assay for specific detection of Top1-DNA adducts isolated from cells at different exposure conditions. Double strand DNA (dsDNA) was used as a loading control.

(B) Quantification of Top1-DNA adduct signals for each treatment condition. Results are expressed as the induction of Top1-DNA adduct signals normalized to the control with the mean signal $\pm$ SEM calculated from three biological replicates. Statistical significant: * $P < 0.05$.

Figure 4.

BPA exposure alters Top1 expression and cellular localization in MEFs.

(A) Whole cell lysates from each treatment were analyzed for Top1 expression by immunoblotting. α -tubulin antibody was used as a loading control.

(B) Measurements of relative Top1 protein levels for each sample determined by the densitometry results of western blots. Each bar represents the mean $\pm$ SEM calculated from three biological replicates. Statistical significant: *P < 0.05 ** P < 0.01.

(C) Representative images of Top1 detected in untreated or BPA, CPT, or co-exposed cells. DAPI (blue) was used to visualize the nuclei. Merge images show the localization of Top1 in the nucleus for control and CPT treated cells, and also in cytoplasm for cells treated with BPA only and BPA co-exposed with CPT (Inset: magnified image showing Top1 expression in the cytoplasm). Scale bar = 10 μ m. See also Figure S1.

Figure 5.

BPA induces nuclear shrinkage and DNA compaction in MEFs.

(A) Representative images of Hoechst (blue) stained MEF cells untreated (control) or treated with 150 μ M BPA. (Inset: magnified image of the nuclear region showing increased Hoechst stained intensity in cells treated with BPA. Scale bar = 10 μ m.

(B) Quantified mean area per nucleus of the Hoechst stained control and BPA treated cells.

(C) Mean fluorescence intensity per nucleus in control and BPA treated MEF cells. Each bar represents the mean $\pm$ SEM calculated from two biological replicates. Statistical significant: *** $P < 0.001$.

Figure 6.

MEFs showed reduced CPT-induced chromosomal aberrations (CA) in presence of BPA.

(A) Representative illustrations of the metaphase spreads showing chromosomal changes after each treatment. Arrows indicate different chromosomal aberrations (RC = radial chromosomes, TD = terminal deletion, B = break, G = gap). Inset magnified observed CA in CPT treated cells and BPA co-exposed with CPT.

(B) Quantification of CA frequency expressed as mean $\pm$ SEM, for 25 metaphases per treatment. Statistical significant: *** $P < 0.001$.