Data Article

Cytotoxic and apoptotic data of BPA and BPA alternatives TMBPF, BPAF, and BPS in female adult rat and human stem cells

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A B S T R A C T

Here, we used female adult rat adipose-derived stem cells (rASCs) and human adipose-derived stem cells (hASCs) to compare the toxicities and potencies of several widespread environmental toxins that may be endocrine-disrupting chemicals, including bisphenol A (BPA), and the newer BPA alternatives bisphenol S (BPS), bisphenol AF (BPAF), and tetramethyl bisphenol F (TMBPF). Adult stem cells were cultured to 80% confluency in vitro and then exposed to BPA (1 and 10 μM), 17β-estradiol (E2; 10 μM), BPS (1 and 10 μM), BPAF (3 × 10⁻³–30 μM), TMBPF (0.01–50 μM), or control media alone (with 0.01% ethanol) for varying time intervals from 20 min to 5 hrs. Using several cellular assays, the levels of cell death, apoptosis, caspase-6 activation, and potencies were compared across chemical treatments and vehicle-treated controls. There was significantly decreased cell viability and increased apoptosis in rat and human stem cells treated with each BPA analog, as early as 20 min of exposure, and at low doses. With higher magnification, higher resolution imaging it was evident that in many of the BPA analog-treated cells, the Apopxin Deep Red dye indicative of apoptosis was localized to the cytoplasmic compartments of cells, while the nuclear green DCS1 dye indicative of late-stage apoptosis and necrosis was localized to the nuclei of cells. Notably, BPAF and TMBPF showed cytotoxicity...
Specifications Table

| Subject                          | Biology                              |
|---------------------------------|--------------------------------------|
| Specific subject area           | Developmental toxicology and endocrine disrupting chemicals |
| Type of data                    | Images (microscopy), figures, videos |
| How data were acquired          | EVOS M5000 inverted microscope (Thermo Fisher Scientific, Waltham, MA, USA) |
| Data format                     | Raw                                  |
| Parameters for data collection  | Analyzed                              |
| Description of data collection  | Exposure to BPA (1 and 10 μM), 17β-estradiol (E2; 10 μM), BPS (1 and 10 μM), BPAF (1 and 10 μM), BPAF (3 × 10^-5–30 μM) or TMBPF (0.01–50 μM) |
|                                 | Rat and human stem cells were exposed to BPA, E2, BPS, BPAF, TMBPF, or control media alone (with 0.01% ethanol) for various intervals from 20 min to 5 hours. The effects and potencies were compared across chemical treatments and controls. |
| Data source location            | Moraga, California, United States    |
| Data accessibility              | Data is with this article            |
| Related research article        | K.G. Harnett, A. Chin, S.M. Schuh, BPA and BPA alternatives BPS, BPAF, and TMBPF, induce cytotoxicity and apoptosis in rat and human stem cells, Ecotox. Environ. Safety. 216 (2021) 112210. DOI: 10.1016/j.ecoenv.2021.112210. |

Value of the Data

- This data is useful as these BPA alternatives are mass-produced in plastic and consumer products and are prevalent in our environment, yet few studies have analyzed their cytotoxicity, effects, and potencies in mammalian or human stem cells.
- This is the first report on the cytotoxicity and apoptotic effects of the newest BPA alternative TMBPF, used across industries from epoxies to food-contact coatings for metal beverage and food cans.
- Those working in the toxicology and developmental biology fields, as well as the general scientific community and public, would benefit from learning about these results, as there are direct consequences and implications for human health and disease.
- These data can also inform future public policy and the development of better regulations, alternative practices, and safer, more sustainable chemicals to reduce hazardous human exposures.
- These data highlight the need for better toxicological characterization of all BPA alternatives and encourage future collaborative studies comparing effects across various species and cell types.
1. Data Description

We exposed rat and human ASCs to various doses of BPA, BPS, BPAF, or TMBPF from intervals of 20 min to 5 hrs and compared them to cells treated with E2 or control media alone (with 0.01% ethanol). The cells were treated with the various chemicals and then examined with the respective cellular assays. The numbers of live/dead cells, apoptosis, and caspase-6 activation were quantified and analyzed by fluorescent microscopy imaging. We discovered that rat and human stem cells exposed to higher concentrations of the chemicals showed more cell death, as indicated by the increased red fluorescence in cell nuclei, which occurs with the breakdown and permeability of cell membranes allowing the red dye to bind to fragmented DNA (Fig. 1). Increasing cell death with higher concentrations of the BPA analogs was observed in both rat ASCs (Fig. 1A) and human ASCs (Fig. 1B; see red cells). At higher magnification, we also observed punctate nuclear localization of the red ethidium homodimer dye indicative of DNA fragmentation and cell death (Fig. 1; see white arrows). Nearly 100% of the controls and E2-treated cells showed high levels of green fluorescence, indicating esterase activity of live viable cells (Fig. 1).

These effects on cell viability were quantified and analyzed over many time points, replicates, and trials. Almost all of the compounds showed clear dose-responses and decreased numbers of live viable cells with increasing doses and exposure times (Fig. 2). Although due to many cells lifting off of the glass and undergoing cell death at variable rates, cell counts and percent live viable cells were variable, although the differences were statistically significant and repeatable across trials (as shown with the scatter plots of the individual data points in Fig. 2). BPA was cytotoxic at doses of 1 and 10 μM (Fig. 2A and C), while TMBPF was much more cytotoxic and at lower doses (Fig. 2B and D). TMBPF caused significant cell death at doses as low as 0.01 μM and was 100-fold more potent than BPA. It is of note, the human ASCs were more sensitive to the BPA analogs than rat ASCs (Figs. 2 and 3). At 20 min and 5 h of exposure many hASC wells had almost complete loss of all cells (likely dying and lifting off the plate) with very few

![Image](https://example.com/image1.png)

**Fig. 1.** Progression of stem cell death with increasing doses of BPA and BPA analogs. Representative fluorescent microscopy images of (A) rat ASCs and (B) human ASCs exposed to BPA, TMBPF, and BPAF at 2 hrs of exposure. Higher levels of cell death were observed at increasing doses of BPA, TMBPF, and BPAF, indicated by the increase in red fluorescence and loss of green fluorescence/esterase activity (magnification 200X, scale bar = 150 μm; righthand images magnification 400X, scale bar = 20 μm; arrows indicate nuclear fragmented DNA).
Fig. 2. Dose-dependent effects of BPA and BPA analogs on cell viability. Scatter plots showing the percentages of live viable cells, their variance, and the means, after exposure to control media, or increasing concentrations of BPA and TMBPF at (A) 20 min, 2 hrs, and 5 hrs in rat ASCs (n=13–45 images/treatment; 3–7 trials); and (B) 20 min and 2 hrs in human ASCs (n=15–28 images/treatment; 2–4 trials) (*P < 0.005).

Fig. 3. BPA Analog LC₅₀s. Regressions of the percent of live viable cells at various doses of TMBPF in rat ASCs (A), and human ASCs (B), and BPAF in rat ASCs (C), and human ASCs (D) at 20 mins of exposure. Exponential equations were fit to the data (R² values = 0.84 (rASCs) and 0.89 (hASCs), and 0.99 (rASCs) and 0.42 (hASCs)). From these regressions LC₅₀ values of 0.88 μM (rASCs) and 0.06 μM (hASCs) were calculated for TMBPF (A, B), and 0.0049 μM (rASCs) and 0.0012 μM (hASCs) for BPAF (C, D).
cells to image. This was also seen at later time points. Because of the hypersensitivity, the time points of 20 min and 2 h were used. Based on regressions of the percent of live viable cells at all concentrations at 20 min of exposure, the calculated LC$_{50}$ of TMBPF in rat ASCs was 880 nM and in human ASCs was 60 nM (Fig. 3A and B). BPAF was the most cytotoxic to both rat and human stem cells, inducing significant effects at doses as low as 3 nM (Fig. 3C and D). BPAF was 1,000-fold more potent than BPA, yielding an LC$_{50}$ of 4.9 nM in rat ASCs and 1.2 nM in human ASCs (Fig. 3C and D), which is very similar to previous findings in embryonic development of *Xenopus laevis* [3].

In order to confirm programmed cell death or apoptosis, we used multi-color enzyme apoptosis/necrosis assays and found that rat ASCs (Fig. 4A) and human ASCs (Fig. 4B) at 60 min of exposure to the BPA analogs exhibited clear signs of apoptosis (see red/pink cells) and late-stage apoptosis and necrosis (see green cells). Upon higher magnification, higher resolution imaging it was evident that the Apopxin Deep Red dye indicative of apoptosis was localized to the cytoplasmic compartments, while the nuclear green DCS1 dye indicative of late-stage apoptosis and necrosis was localized to the nuclei of many BPA analog-treated cells (see inset images in Fig. 4). At the population level, the greatest amounts of apoptosis were observed in human ASCs treated with TMBPF and BPAF (Fig. 4B; see red and green cells). To confirm apoptosis-mediated pathways underlying BPA analog action, we assayed cells for the upregulation of caspase-6 activity, a common proteinase mediator of apoptosis. Nearly 100% of rat and human stem cells treated with the BPA analogs showed high caspase-6 activity after 60 min of exposure, as indicated by the high levels of green cellular fluorescence, which was quantified across trials (Fig. 5A and B). At the cellular level, high magnification images show strong cytoplasmic green fluorescence of the enzyme-linked dyes, indicative of high caspase-6 activity (Fig. 5; see inset images). All raw data for all figures can be found in our supplementary material.

Fig. 4. Stem cells exposed to BPA analogs undergo apoptosis. Rat ASCs (A) and human ASCs (B) exposed to various doses of BPA analogs exhibit clear signs of apoptosis (red/pink) and late-stage apoptosis and necrosis (green), with the greatest amounts of apoptosis seen in human ASCs treated with TMBPF and BPAF (red = Apopxin Deep Red, indicates apoptosis; green = DNA Nuclear Green DCS1, indicates late-stage apoptosis and necrosis; blue = CytoCalcien Violet 450, indicates normal cells) (magnification 200X, scale bar = 150 μm). Inset images show magnified cells undergoing apoptosis with pink/red cytoplasmic localization and green nuclear localization (magnification 400X).
2. Experimental Design, Materials and Methods

2.1. Isolation and culture of rat ASCs

Rat adipose-derived stem cells were harvested via surgery and enzymatically digested from the inguinal fat pads of female Lewis rats as previously described [4,5]. Cells were cultured in flasks in a humidified 37 °C, 5% CO₂ incubator. Rat ASCs were then isolated, and cells from passages 1 through 4 were frozen, stored at −80 °C, and used for all experiments (kindly provided by Dr. David Sahar, U.C. Davis, CA). Rat ASCs were cultured in Modified Minimal Essential Medium Alpha Modification (SH30265.01, GE Healthcare Life Sciences, Chicago, IL, USA) supplemented with 10% fetal bovine serum (FBS) (10437-010, Gibco, Carlsbad, CA, USA) and 1% Penicillin Streptomycin (Penstrep) (10378-016, Gibco, Carlsbad, CA, USA). Cells were maintained with aseptic cell culture and split and passaged enzymatically every 5 to 7 days. Two to four days before experiments, the cells were enzymatically passaged with 0.5% trypsin (T3924, Sigma-Aldrich, St Louis, MO, USA) and replated in 6-well plates for ideal growth conditions and a confluency of 60-80%.

2.2. Isolation and culture of human ASCs

Adipose-derived stem cells (ASCs; Pre-Adipocytes), a type of mesenchymal stem cell (MSC), were purchased from Lifeline Cell Technology and cultured and used in all experiments (FC-0062, Lifeline Cell Technology, Frederick, MD, USA). Human ASCs were originally harvested via liposuction surgery from two consented adult female donors. Human ASCs were cultured in Fibro-Life basal medium containing FGF (Fibroblast Growth Factor), insulin, ascorbic acid, L-glutamine, hydrocortisone hemisuccinate, 10% FBS, and the anti-microbial supplement gentamicin and amphotericin B (LL-0011, Lifeline Cell Technology). Cells were maintained with aseptic cell culture and cultured in a humidified 37 °C, 5% CO₂ incubator at a density of approximately 5,000 cells per cm². Every 5–7 days, cells were split enzymatically with 0.05% trypsin/0.02% EDTA (Ethylenediaminetetraacetic acid) (CM-0017, Lifeline Cell Technology) and gentle agitation for 5 min followed by trypsin neutralizing solution (CM-0018, Lifeline Cell Technology). Cells were then replated in 6-well plates for ideal growth conditions, maintaining a confluency of 75–80%.

Fig. 5. Stem cells exposed to BPA analogs undergo upregulation of Caspase-6 activation. Representative fluorescent microscopy images of (A) rat ASCs and (B) human ASCs exposed to TMBPF, BPAF, and BPA. Exposed cells fluoresce green due to active Caspase-6 (blue indicates cell nuclei; magnification 200X, scale bar = 150 μm). Inset images show magnified cells expressing high cytoplasmic Caspase-6 activity (magnification 400X).
2.3. Preparation of BPA analogs and reagents

Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA; BPA, E2, BPS, BPAF) and Tokyo Chemical Industry (Tokyo, Japan; TMBPF). Stock solutions of E2 (E8875; >98% purity), BPA (133027; >97% purity), BPS (103039; >98% purity), BPAF (90477; >99% purity), and TMBPF (M1099; >98% purity), at 10 mM were prepared in 95% ethanol in glass bottles, to avoid any additional leaching of plasticizer chemicals typically found in plastic bottles. A working stock of 10X was made for E2, BPA, BPS, BPAF, and TMBPF, and diluted to the final treatment concentrations on the day of the experiments. The final concentration of ethanol in all treatment and control solutions was 0.01%, a concentration known to have no negative effects on cell viability and growth [2].

2.4. BPA analog treatments

All experiments were carried out in 6-well plates with glass coverslips (22 × 22 mm, size 1.5) placed in the bottom of the wells. Preliminary rangefinder assays were conducted with each chemical to find the range of concentrations over which cellular and systemic toxicity might occur. Additionally, previous studies on embryo cell cleavage division and development also provided initial ranges to test in these preliminary studies [3]. In the final experiments not all chemicals were used at the same concentrations and exposure times. In preliminary assays we also found that BPS was the least cytotoxic and required quite high, non-environmentally-relevant concentrations to cause toxicity, and was therefore not used in all rASC and hASC experiments. At the desired cell confluency of ~75–80%, final experiments were performed in which rat ASCs were exposed to concentrations of: BPA at 0, 1 and 10 μM; E2 at 0 and 10 μM; BPS at 0, 1, and 10 μM; BPAF at 0, 3 × 10⁻³, 0.03, 0.3, and 3 μM; and TMBPF at 0, 0.01, 0.1, 1, 10, and 50 μM. Rat ASCs were exposed to each BPA analog for the following time intervals: BPA: 0, 20, 120, 300 min; E2: 0, 20, 60, 120, and 300 min; BPS: 0, 20, 60, 120, and 300 min; BPAF: 0, 10, 20, 60, and 300 min; and TMBPF: 0, 20, 60, and 300 min. Human ASCs were exposed to concentrations of: BPA at 0, 1 and 10 μM; E2 at 0 and 10 μM; BPAF at 3 × 10⁻³, 0.03, 0.3, and 3 μM; and TMBPF at 0, 0.01, 0.1, 1, 10, and 50 μM. Human ASCs were exposed to each BPA analog for 0, 20, 60, and 120 min. Each condition was run in duplicate or triplicate per trial, with at least 2–7 trials.

2.5. Determination of cell viability

Cell viability and the percent of live viable cells was determined using the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (L3224; Thermo Fisher Scientific, Waltham, MA, USA). After cells were exposed to varying concentrations of BPA alternative chemicals for specific time intervals, cells were washed with PBS (phosphate buffered saline), and the coverslips were removed from the 6-well plates via fine tip forcesps. Cells were then treated with PBS containing 2 μM calcein-AM and 1 μM ethidium homodimer, protected from light for 30 min. Coverslips were then mounted on slides and analyzed immediately using fluorescence microscopy.

2.6. Determination of apoptosis and necrosis

To determine if cell death was occurring via apoptosis we used the Apoptosis/Necrosis Assay Kit for mammalian cells (ab176749; Abcam, Cambridge, UK). Dimethyl sulfoxide (DMSO; D2650, Sigma-Aldrich) was used to make a stock of the CytoCalcein Violet 450 (at 200X). Based on the live/dead assay results and time course experiments, we selected 60 min as a good intermediate time point that might best capture peaks in apoptosis. Following initial exposure to BPA analogs
for 60 min, or 70% ethanol as a positive control, and following the manufacturer’s instructions, the cells were washed with the assay buffer and then treated with a master mix of Apoptxin Deep Red Indicator (from a 100X stock), Nuclear Green DCS1 (from a 200X stock), and CytoCalcein Violet 450 (from a 200X stock). The cells were incubated at room temperature protected from light for 60 min, and then mounted on slides. The cells were analyzed immediately using fluorescent microscopy.

2.7. Determination of caspase-6 activity

Caspase-6 activity was measured using the Caspase-6 (Active) Staining Kit for mammalian cells (ab219936, Abcam) following the manufacturer’s instructions. The enzyme-linked dye indicators FAM-VEID-FMK were diluted in DMSO to make a 150X stock solution. Based on previous time course and exposure data, the cells were exposed to BPA analogs for 60 min, and then washed with washing buffer. The cells were then treated with the master mix of FAM-VEID-FMK (from the stock) and Hoeschst (from a 500X stock), and incubated at 37 °C with 5% CO₂, protected from light for 60 min. The cells were analyzed immediately using fluorescent microscopy.

2.8. Fluorescence imaging and cell quantification

After the cells were treated with the respective assays, the slides were imaged at 100 to 400X magnification with fluorescent microscopy on an EVOS M5000 inverted microscope (Thermo Fisher Scientific, Waltham, MA, USA). From the captured images, live and dead cells were quantified based on the presence of green and red cellular fluorescence due to the calcine-AM and ethidium homodimer-1 dye, respectively, for those treated with the Live/Dead Cytotoxicity assay. The percentages of live viable cells were calculated based on the total number of live (green) and dead (red) cells, and were compared across treatments, trials, and time points. For the Apoptosis-Necrosis assay, the cells were assessed for the overall presence of red/pink (apoptosis) and green (late-stage apoptosis/necrosis), and the overlapping orange-yellow cells compared to the overall expression of blue (live, normal) cells. Cells treated with the Active Caspase-6 assay were quantified based on blue fluorescent nuclei (Hoeschst) yielding the total number of cells, and green fluorescence (FAM-VEID-FMK) yielding caspase-6 activity. The percentage of cells expressing caspase-6 activity, a hallmark of apoptosis, was quantified across trials.

2.9. Data analysis

At least 10–20 images of each slide of each treatment were captured by raster scanning. All images captured were analyzed and the cells expressing the different colors indicating live/esterase activity, apoptosis, necrosis, or caspase-6 activity, were quantified across trials. Percentages were compared across all BPA analog treatments and trials. All results are expressed as the percent of live viable cells or mean ± standard deviation of the mean for at least 2–7 independent trials. All image captures and cell counts were performed individually, with a subset quantified and compared among at least two independent observers. Quantification of about 80% of all images were counted/scored by two different independent observers, at the beginning of the experiments. The observers knew the bisphenols, doses, and time points when quantifying images. The observers worked very closely together for all initial experiments and training, and went through many counts and scoring together, following the same precise method for image quantification, in which cells that were green were counted as alive and cells that were red (had red nuclei) were counted as dead. We compared the counts of the two observers and found that they had a cross-observer correlation of better than 95%. Therefore, all subsequent counts were
performed by either of the same two independent observers throughout the study, for consistency and sharing of the high workload. The LC$_{50}$ was calculated for TMBPF and BPAF by plotting the mean percentage of live viable cells for various doses at 20 min, and solving for the concentration at $y = 50$ (\% survival), from the exponential regression equations. To define the rank order of potency, the mean percent of cell survival (mean percent of live viable cells) at 20 min of exposure for all chemicals was quantified and the corresponding LC$_{50}$s were determined and compared. The Student’s t-test (two-tailed) was performed on all test groups versus controls to determine statistically significant differences. A One-Way ANOVA with repeated measures and Tukey’s Multiple Comparison test were then performed to compare significant differences between time points and treatments, using Microsoft Excel and the statistical program R. $P$ values of less than 0.005 ($^*$) were considered statistically significant.

**Ethics Statement**

All animal experimental procedures were approved and performed in accordance with U.C. Davis School of Medicine and Saint Mary’s College of California Institutional Animal Care and Use Committees (IACUC) and comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

**CRediT Author Statement**

Kristen G. Harnett: Conceptualization, Methodology, Investigation, Software, Validation, Formal Analysis, Writing - Original Draft, Writing - review & Editing; Ashley Chin: Investigation, Validation, Formal Analysis, Writing - Review and Editing; Sonya M. Schuh: Conceptualization, Methodology, Resources, Funding Acquisition, Investigation, Formal Analysis, Supervision, Project Administration, Writing - Original Draft, Review, and Editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi: 10.1016/j.dib.2021.107183.

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