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ABSTRACT: This paper reports the chemical identity and mechanism of action and formation of a cell growth inhibitory compound leached from some single-use Erlenmeyer polycarbonate shaker flasks under routine cell culture conditions. Single-use cell culture vessels have been increasingly used for the production of biopharmaceuticals; however, they often suffer from issues associated with leachables that may interfere with cell growth and protein stability. Here, high performance liquid chromatography preparations and cell proliferation assays led to identification of a compound from the water extracts of some polycarbonate flasks, which exhibited subline- and seeding density-dependent growth inhibition of CHO cells in suspension culture. Mass spectroscopy, nuclear magnetic resonance spectroscopy, and chemical synthesis confirmed that this compound is 3,5-dinitro-bisphenol A. Cell cycle analysis suggests that 3,5-dinitro-bisphenol A arrests CHO-S cells at the G_1/G_o phase. Dynamic mass redistribution assays showed that 3,5-dinitro-bisphenol A is a weak GPR35 agonist. Analysis of the flask manufacturing process suggests that 3,5-dinitro-bisphenol A is formed via the combination of molding process with γ-sterilization. This is the first report of a cell culture/assay interfering leachable compound that is formed through γ-irradiation-mediated nitric oxide free radical reaction.

KEYWORDS: Leachables, polycarbonate, plastic labware, 3,5-dinitro-bisphenol A, cell cycle arrest

Single-use plasticware is widely used in life sciences research, drug discovery, bioprocessing, and the storage of consumer and medical products. However, when being contacted with solutions, plastic labware can leach out trace amount of contaminant chemicals, some of which may interfere with biological testing results in an assay specific manner,[1-8] compromise the quality of consumer and medical products,[9-13] or cause the growth inhibition of cells during bioprocessing[14,15].

For single-use labware, leachables are substances that migrate spontaneously into solution under routine conditions of use and storage, while extractables are substances generated under exaggerated temperature and time conditions in the presence of an appropriate solvent.[16] To date, two classes of results-interfering leachables have been identified. The first type of bioactive leachables is plastic additives, chemicals that are used to polymerize, process, or modify end-use properties of plastics. Common additives include antioxidants, antiozonants, biocides, slip agents, and stabilizers. Several additives have been found to leach out of specific disposable plastic labware under routine conditions of usage and storage, and contaminate assay results or products. The biocide di(2-hydroxyethyl)methyldodecylammonium and the slip agent oleamide both were identified as leachables from certain brand polypropylene plastic tubes to be selective inhibitors of human monoamine oxidase-B (hMAO-B).[1] Oleamide is also a known endogenous signaling molecule that binds to several receptors and ion channels.[17,18] Erucamide, another slip agent, was found as a leachable from specific lots of polypropylene pipette tips during routine compound management liquid handling processes to be an agonist of a G protein couple receptor (GPCR) for fatty acids.[2] An unknown leachable from specific batches of plastic centrifuge tubes used to store compounds was found to inhibit the tyrosine kinase Abl1.[3] Several Tergitol nonylphenol ethoxylate series surfactants were also identified as leachables from some polypropylene 1-mL pipette tips to inhibit the mitochondrial respiratory chain complex I, causing false patient diagnostic results.[4] Chemical leachables from disposable labware may represent a widespread problem for DNA and protein quantification assays by interference with spectrophotometric[5] or mass spectroscopy (MS) based proteomics analysis[6], or in some cases directly inhibiting polymerase chain reaction[7]. Antimony, a stabilizer and also a catalyst for the production of polyethylene terephthalate (PET), can also leach into drinking water from PET containers, causing potential environmental contamination.[9]

The second type of bioactive leachables is certain degradation products of plastic polymers and additives. Leaching of bisphenol A (BPA) into foods and drinking water from polycarbonate (PC) containers has generated great public attention as BPA is an endocrine disrupter.[10] Long-term, low-level exposure to endocrine-disrupting chemicals such as BPA and polybrominated diphenyl ether flame retardants was estimated to cost the U.S. $340 billion in annual health care spending and lost wages.[11] The antioxidant dodecyl 3-(3-dodecoxy-3-oxopropyl)-sulfanylpropanoate and its two degradation products, dodecan-1-ol and dodecanoic acid, were found as leachables from some plastic microplates to be selective hMAO-B inhibitors.[8] Recently, bis(2,4-di-*tert*-butylphenyl)phosphate, a γ-irradiation-induced degradation product of the antioxidant *tris*(2,4-di-*tert*-butylphenyl)-phosphite (branded as Irgafos 168®), was found to be a leachable from some polyethylene-film based single-use bioreactors (SUBs) that can inhibit the growth of several Chinese hamster ovary (CHO) cell lines in suspension culture.[14,15] A variety of CHO sublines have been widely used as the host to produce recombinant proteins and antibodies for therapeutic applications.[19,20] Pre-sterilized plastic SUBs have been widely used for bioprocessing due to advantages associated with reduced capital expenditure, increasing manufacturing flexibility, and removal of costly clean-sterilization steps between production runs.[21,22]

PC Erlenmeyer flasks are one of the leading vessels widely used for process/media optimization and seed-train scale-up culture in bioprocessing.[23] Given that the sensitivity of cell growth to leachables is strongly dependent on cell types and culture conditions, we speculate that like some SUBs PC flasks may also leach out cell growth inhibitory compound(s). Here, we report a novel type of bioactive leachables from some Erlenmeyer PC shaker flasks that are formed through a γ-irradiation-mediated free radical reaction. Specifically, 3,5-dinitro-bisphenol A (3,5-dinitro-BPA) was identified as a leachable from these flasks, which arrests CHO-S cells at the G_1/G_o phase and is also a weak agonist for the GPR35. We also describe a strategy that combines multiple techniques including bioassays and routine analytical tools to provide orthogonal confirmation of the bioactive leachables identified.

EXPERIMENTAL SECTION

Materials. BPA, 3,3'-dinitro-BPA, 3,3',5,5'-tetra-nitro-BPA, 4-tert-butyl-2,6-dinitrophenol, 3,5-dinitrophenol, 4-chloro-2,6-dinitrophenol, 4-tert-butyl-2,6-dinitroanisole, and 2,6-dinitrohydroquinone were obtained from Sigma (St. Louis, MO, USA). ML-145 and zaprinast were obtained from Tocris Chemical Co. (St. Louis, MO, USA). CHO-5/9α cell line was obtained from American Type Cell Culture (ATCC; Manassas, VA, USA). FreeStyle™ CHO-S cell line, CD OptiCHO medium, FxCycle™ PI/RNase staining solution, and USB® Corp 4% para formaldehyde dolution were purchased from Thermo Fisher (Boston, MA, USA). BD Pharmigen™ staining buffer was obtained from BD Biosciences (San Jose, CA, USA).

Water extraction protocol. 2L Erlenmeyer PC shaker flasks from Corning or ThermoFisher were incubated with 400 mL deionized water at 37 °C on an orbital shaker at 90 revolutions per minute (rpm) for 3 days. The water extraction solution was then concentrated down to ~2 mL using a Rota-Vapor at 50 °C. For PC resins, 20 g raw resin was placed inside a glass vial and γ-irradiated. Both raw and γ-irradiated resins were put in a 250 ml glass beaker, followed by extraction with 100 mL water at 37 °C on an orbital shaker at 90 rpm for 3 days. The collected water solution was then concentrated down to 1 mL for further analysis. For pure BPA monomer coated glass flasks, 5 mL of 0.5 mg/ml BPA in methanol was used to coat a 250 mL glass flask by rotating it for 10 min. After removal of excess solution, the coated bottle was dried in air and γ-irradiated. After γ-irradiation, 5 mL acetonitrile was added. The resultant acetonitrile solution was then analyzed directly with high performance liquid

chromatography (HPLC)/MS. All γ-irradiation treatments were performed at 18–36 KiloGray (kGy).

Cell culture. Suspension culture was routinely maintained using an Infors Multitron Pro 25 mm orbital shaker at 37 °C, 8 % CO_2, and 85 % humidity in 1X CD OptiCHO™ medium supplemented with 8 mM Corning glutagro™ (Corning) and 1X hypoxanthine and thymidine using a protocol recommended by the medium manufacturer. The shaking speed was set to be 130 rpm for flasks of various sizes from different vendors, or 225 rpm for 24 well deepwell plates (24 DW, Corning). The orbital diameter of the shaking platform was 15 mm and 50 mm for the flasks and the 24 DW plates, respectively. The culture volume was 2.5 mL for 24 DW plates. To study the effects of the water extracts of the flasks on cell growth, aliquots of the 200x concentrated water extracts from the flasks were added to the culture media in 24DW cell culture plates. To determine the IC_{50} values of different chemicals, aliquots of serial dilutions of testing chemicals were added to CHO 24DW cell culture. The seeding density was 0.15 x 10^6 cells/mL for culture in the deepwell plate, but was variable for flasks of different size. The relative percentage of cell growth under a specific condition was calculated by normalizing to the dimethyl sulfoxide (DMSO) control. The IC_{50} values were analyzed with a non-linear regression method using GraphPad Prim 5 software.

Cell viability analysis. The number and viability of cells were monitored daily using trypan blue exclusion assay and a Vi-Cell™ cell viability analyzer (Beckman Coulter).

Cell cycle analysis. CHO-S cells were cultured in 125 mL shaker flasks with an initial seeding density of 0.15 x 10^6 cells/mL in the presence or absence of 3 μM 3,5-dinitro-BPA. The number and viability of cells were monitored daily. At different time points, 10^6 cells were collected and then pelleted by centrifugation at 300x g for 5 min. The cells were washed once with Dulbecco's phosphate buffered solution, and then fixed by incubating with 2% para formaldehyde solution for 10 min at room temperature. The cells were then pelleted again and the fixative solution was removed. The cells were re-suspended in the staining buffer from FxCycle™ PI/RNase staining kit according to the manufacturer instruction. The stained cells were then analyzed using a BD FACS Calibur Flow Cytometer (BD Biosciences). 30,000 events were collected for each sample at FL2 channel for propidium iodide fluorescence (linear, 532 nm excitation and 584 nm emission). The percentages of cells at G_1/G_0, S, and G_2/M phases were analyzed using the BD Cell Quest Pro software.

Dynamic mass redistribution (DMR) assays. Human colorectal adenocarcinoma HT-29 cells, obtained from ATCC, were cultured in the complete medium (McCoy's 5a medium modified supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 2 mM glutamine, and penicillin-streptomycin antibiotics) at 37°C under 5% CO_2. For DMR assays HT-29 cells were seeded in a 384well biosensor microplate at 30,000 cells per well suspended in 50 μL of complete medium and cultured at 37 °C under 5% CO_2 overnight. The plates were washed twice using a plate washer (Bio-Tek Microplate Washers ELx405), then maintained in 30 μL of the 1x HBSS (Hank's balanced salt solution containing 20 mM HEPES) assay buffer, and further incubated inside an Epic BT system (Corning) for 1 hr. For agonist profiling, a 2 min baseline was first established. After compound addition, the cellular responses were recorded immediately. For antagonist inhibition assays, cells were initially treated with ML145 for 1 hr, followed by stimulation with the agonist at a fixed dose. The data represent mean ± std from two independent measurements, each with four replicates (n = 8).

Pyrolysis sample preparation. Commercial 2L PC flasks were cut into several pieces, which were then placed into a 250 mL glass flask. 100 mL dichloromethane was added to the glass flask to completely dissolve the PC pieces. The resulted solution was poured into approximately 400 mL methanol (used as an anti-solvent) to precipitate out the polymers. After filtration, the remaining solution was concentrated to dryness and then re-dissolved in methanol. The final pyrolysis sample solution was used for subsequent HPLC/MS analysis.

HPLC/MS analysis. HPLC was performed on an Acquity UPLC H class system (Waters Corp.) with PDA and QDA detectors using a Waters BEH C8, 1.7 μm, 2.1 X 100 mm column and eluted with an acetonitrile/water reverse phase system. Mobile phase A was 30-80% (v) acetonitrile/0.1% (v) TFA; while B was 80% -100% (v) acetonitrile/0.1% (v) TFA, C was 100% (v) acetonitrile/0.1% (v) TFA. The gradient of separations was generally A (0-4 min), B (4-5.5 min), and C (5.5-9 min), unless specifically mentioned. Flow rate was 0.5 mL/min. UV detector at 254, 280, or 360 nm was used to separate leachable molecules in the extract samples. Individual components were given structural assignments based on both retention time and molecular weight data obtained from the negative ion mass spectrum of the peak, in comparison to standard compounds when available.

High-resolution MS analysis. High-resolution MS was carried out at the Chemical Core facility of Cornell University. In addition, direct infusion electrospray ionization mass spectrometry on a Thermo LCQ Deca XP mass spectrometer operating in both positive and negative ion modes was also used. Relative concentrations were determined by the ion current value in nA of a given species and comparing to another reference peak from the same sample. For quantification, a calibration curve for 3,5-dinitro-BPA was established in the negative mode of ionization on the synthesized compound of known concentration over a range of 3 orders of magnitude. Furthermore, the tandem MS was performed using an Orbitrap Elite mass spectrometer under high duty cycle mode at 180 volts (ThermoFisher).

Nuclear magnetic resonance (NMR) analysis. Multiple NMR instruments were used. For the synthesized 3,5-dinitro-BPA, ^{1}H-NMR data were collected on a Bruker AVANCE III 400MHz NMR spectrometer. For the peak #18 sample collected from 60 2L PC flasks, ^{1}H-NMR spectra were obtained using an Agilent DD2 700MHz NMR in conjunction with a 16.4 T superconducting magnet. The ^{1}H spectra were acquired with a delay time of 1 s, a 45 degree pulse width and as a composite of 32 to 252 free induction decays. ^{1}H,^{1}H-homonuclear correlation spectroscopy (COSY) data were acquired by collecting 16 transients over 128 increments of t1 with a d1 of 1 s and a d2 of 20 ms. Data in both direct and indirect dimensions were apodized with squared sinebell weighting functions. Nuclear Overhauser effect spectroscopy (1D NOESY) data were acquired with a delay time of 1 s, a 90

degree pulse width, a mixing time of 500 ms and as a composite of 800 free induction decays. Samples were dissolved in $CDCl_3$ (Cambridge Isotope Laboratories, Andover, MA, USA). A 5 mm Shigemi NMR tube (Sigma), which allowed the solvent volume to be reduced to ~1/3 of that required by conventional 5 mm NMR tubes, was used in order to achieve high sensitivity. For commercial compounds, solution ^{1}H-NMR spectra were obtained using a Varian UnityInova 300MHz NMR in conjunction with a 7 T superconducting magnet. The ^{1}H spectra were acquired with a delay time of 1 s, a 45 degree pulse width and as a composite of 32 free induction decays. Theoretical spectra were obtained using Advanced Chemistry Development ACD/Labs software. Chemical shifts (δ) were reported in parts per million (ppm), and determined relative to the internal standard tetramethylsilane at δ 0.0 ppm. Spin multiplicities were assigned as s (singlet), d (doublet), and br (broad).

Synthesis of 3,5-dinitro-bisphenol A. All reagents were received from commercial sources and used without any further purification or drying. All solvents were purified and dried by standard methods. The reactions were monitored by thin layer chromatography. The target products were determined by NMR, and ESI-mass spectra obtained on an LC-MS instrument (Waters Acquity UPLC, Waters Micromass ZQ 2000). The purity of the final compounds was ≥ 95%. Detailed synthesis procedure with characterization data was provided in Supplementary Materials.

Statistical analysis. The statistical significance reported was obtained using One-way ANOVA with the Prism 6.0.

RESULTS AND DISCUSSION

Identification of a cell growth inhibitory compound from the water extracts of PC flasks. Cell culture conditions, such as seeding density, cell passage number and media type can affect the sensitivity of cells to growth inhibitory leachables. We first assessed the growth of suspension CHO-S cells in 2L PC and polyethylene terephthalate (PETG) Erlenmeyer flasks using 2 different seeding densities. Results showed a cell density and flask dependent growth pattern (**Fig. 1**). Compared to PETG flasks under the same culture conditions, for the high seeding density (500K cells/ml) viable cell density obtained was 15% less at Day 3 but similar at Day 4 and 5 in PC flasks; however, for the low seeding density (100k cells/ml) viable cell density obtained was 39% less at Day 3, 23% less at Day 4, but similar at Day 5 in PC flasks. On the other hand, the cell viability was greater than 98% throughout the culture under all conditions. This result indicates the presence of cell growth inhibitory, but not cell toxic, leachable(s) in the PC flasks.

To identify cell growth inhibitory leachable(s), water extracts were obtained using the water extraction protocol described above. This water extraction protocol was chosen to mimic the routine condition of use for suspension cell culture, and at the same time to facilitate follow-up analysis. HPLC profiling of the 200x concentrated water extracts revealed a complicated leachable profile (**Fig.2a**), indicating that many different compounds can be released into the media during cell culture.

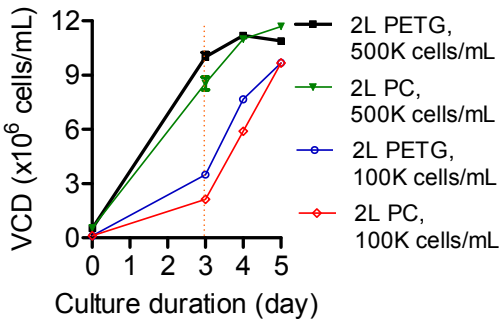


Figure 1. The growth pattern of CHO-S cells in 2L PC *vs.* PETG flasks. Cells from the mid-logarithmic growth stage were seeded at 1 x 10^5 cells/mL or 5 x 10^5 cells/mL seeding density into Corning plain bottom 2L PETG (Cat. No. 431280) or 2L PC flasks with 400 mL working volume. The flasks were incubated in an orbital shaker at an agitation rate of 130 rpm until the maximum viable cell density was achieved (5 days). The cells were enumerated and assessed for viability using the trypan blue exclusion assay. Data represents mean ± std (n=3).

Eighteen peaks in total were collected and tested individually for their effects on the proliferation of CHO-S cells in 24-well deepwell plates for 3 days. Results showed that only peak #18 greatly suppressed cell *growth*, while all peaks had little impact on cell *viability* (**Fig.2b**). Peak #18 alone was found to contribute to almost 100% of the overall cell growth inhibition produced by the total extract obtained (**Fig.2c**). These results indicate that the peak #18 leachable is a primary contributor to the inhibition of CHO-S cell growth.

Identification of peak #18 as 3,5-dinitro-BPA. To ascertain the chemical identity of peak #18, we purified and obtained about 100 μg in total of peak #18 from the water extracts of 60 2L flasks. Although the peak #18 obtained was not highly pure (see below), high resolution orbitrap negative ion mode MS showed that peak #18 has a main m/z of 317.07688 (**Fig.S1**). This result, together with the isotopic distribution and the abundance of the A+1 peak, indicates that $C_{15}H_{14}N_2O_6$ represents the most probable formula. Chemical mining through several public databases including ChemBL, PubChem, and ChemSpider led us to speculate one of seven possible dinitro-BPA isoforms as a candidate for peak #18, given the identical formula, the possible presence of BPA derivatives in the extract, as well as documented toxicity of certain dinitrophenol compounds[24].

The possibility that peak #18 is a dinitro-BPA was further supported by evidence from tandem MS. Negative ion tandem MS under high duty cycle mode at 180 volts showed that peak #18 gave rise to m/z values of 317.32 (-H), 302.35 (-CH_3), 300.22 (-OH), 287.20 (-NO), 285.25 (-OH, -CH_3), and 272.28 (-NO, -CH_3). This fragment pattern is similar to, although its ion abundance pattern is different from, commercial 3,3'-dinitro-BPA (**Fig.S2**). Furthermore, derivatization of peak #18 with N,O-bis(trimethyl-silyl)trifluoracetamide to form trimethylsilyl derivatives revealed that the peak #18 obtained is a mixture of dinitro-BPA (~80%) and a possible chloro-dinitro-BPA (~20%) (**Fig.S3**). This result was further confirmed by re-analyzing the collected peak #18 sample using UPLC/MS (**Fig.S3**).

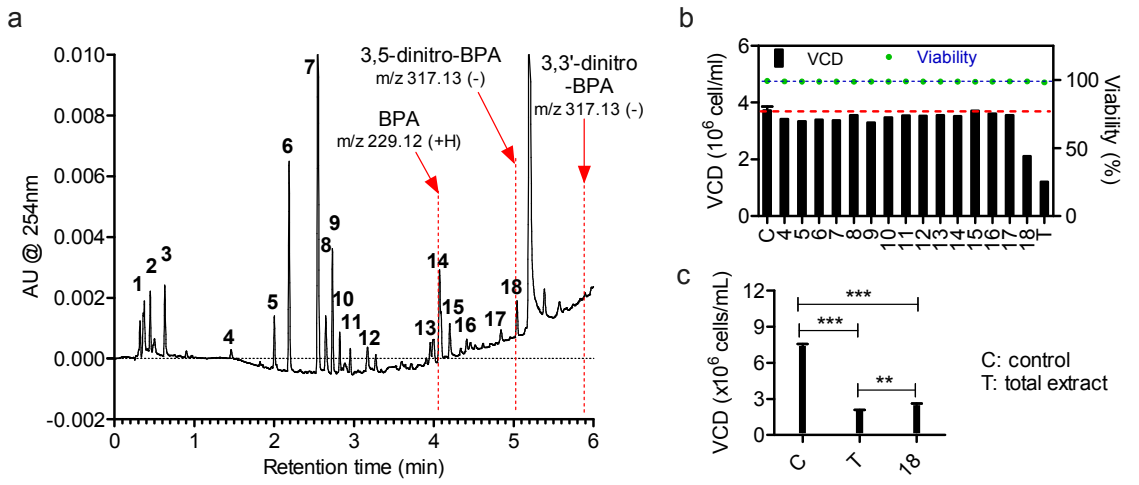
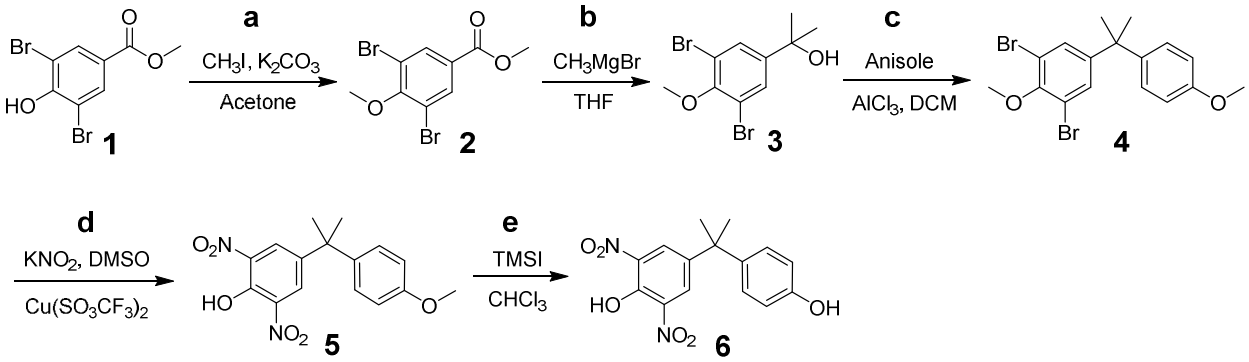


Figure 2. 2L Erlenmeyer PC shaker flask leachable profile and impact on CHO-S cell growth. (**a**) HPLC/UV chromatogram at 254 nm of the 200x concentrated water extract from a flask. (**b**) The effect of different leachable peaks on CHO-S cells. (**c**) Viable cell density (VCD) in the presence of total peak #18 only *versus* total water extracts from a single flask. Control: water extract from glass bottle. (**b,c**) Cells were cultured in 24-well deepwell plates for 3 days at 225 rpm and 37 °C. The seeding density was 0.15 x 10^6 cells/mL. The final concentration of water extracts added was 8x. Data represents mean ± std (n=3). ***, p-value <0.001; **, p-value <0.05.



Scheme 1. Synthesis of 3,5-dinitrobisphenol A (6). Reagents and Conditions: (a) **1**, anhydrous K_2CO_3, acetone; CH_3I, acetone, room temperature (rt); yield, 95%; (b) **2**, THF; CH_3MgBr, diethyl ester, 0°C; rt, 84%; (c) **3**, anisole, CH_2Cl_2; $AlCl_3$, diethyl ester, 0°C; rt, 60%; (d) **4**, $Cu(SO_3CF_3)_2$, KNO_2, DMSO; 130°C, 54%; (e) **5**, $CHCl_3$; trimethylsilyl iodide (TMSI); 50°C, 10%.

Given the small quantity and impurity of the peak #18 obtained, we performed ^{1}H-NMR with 2000 scans using the Pre-SAT technique, an effective approach to suppress residual water and solvents in NMR.[25] Results showed that there are clearly three major aromatic proton environments (8.15, 7.16 and 6.88 ppm), and peaks at 7.16 and 6.88 ppm are doublets with J coupling of 8 MHz, indicating protons in the ortho position (**Fig.S4**). 1D NOESY data did not indicate any interactions between protons, while COSY data did not indicate any correlation of the protons at 8.15 ppm with the protons at 7.06 or 6.80 ppm (**Fig.S5**). These results suggest that the prominent component of peak #18 is highly likely 3,5-dinitro-BPA (**Fig.3a**).

To further confirm this, we attempted to synthesize 3,5-dinitro-BPA. Although it has never been reported in the literature, we successfully synthesized 3,5-dinitro-BPA based on nitrite replacement of the bromo groups of 3,5-dibromo-BPA (**scheme 1**). The UPLC retention time, MS (m/z of 317.15), and ^{1}H-NMR (400 MHz, CDCl3: δ 11.32 (s, 1H), 8.16 (s, 2H), 7.08 (d, J = 8.0 Hz, 2H), 6.81 (d, J = 8.0 Hz, 2H), 4.96 (br, 1H), 1.71 (s, 6H)) of the synthesized 3,5-dinitro-BPA almost completely matched peak #18 (**Fig.S6**). Together, these analyses confirmed that peak #18 is 3,5-dinitro-BPA.

3,5-dinitro-BPA is a cell cycle arrest agent. To elucidate the mechanism of action of 3,5-dinitro-BPA in CHO-S cell growth inhibition, we first performed a structure-activity relationship analysis of a small set of nitrophenol compounds, except for 3-nitro-BPA which was not available (**Fig.3a**). Results obtained using 24-well deepwell suspension culture showed that among the nine compounds tested, 4-chloro-2,6-dinitrophenol, 2,6-dinitro-hydroquinone, and 4-tert-butyl-2,6-dinitro-anisole all had little impact on the proliferation of CHO-S cells, while the other six compounds gave rise to dose-dependent inhibition of CHO-S cells after 3 days culture (**Fig.3b,c**). Interestingly, the six active compounds appear to fall into two categories. The first group, comprised of 4-tert-butyl-2,6-dinitrophenol, 3,3'-dinitro-BPA, and 3,5-dinitro-phenol, almost completely inhibited cell

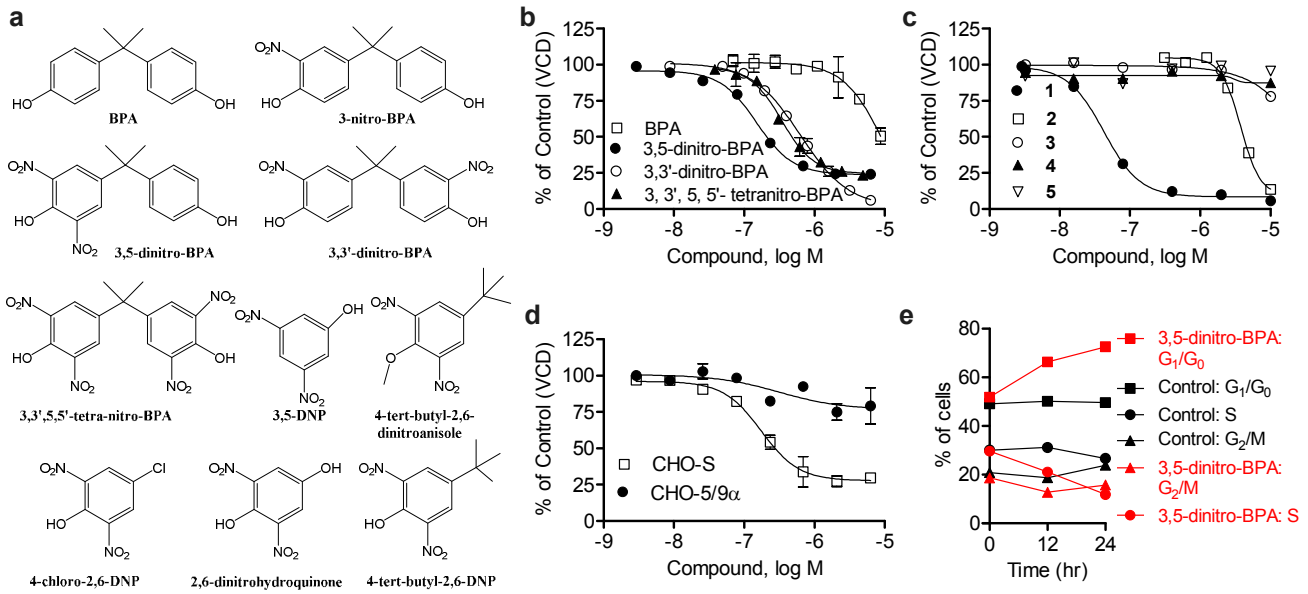


Figure 3. Compounds and their effects on cell growth inhibition and cell cycle. (a) Chemical structures of bisphenol A (BPA) and dinitrophenol (DNP) compounds tested. (b,c) Dose-dependent inhibition of CHO-S cells by different compounds, including BPA compounds (b), DNP compounds including **1**: 4-tert-butyl-2,6-DNP; **2**: 3,5-DNP; **3**: 2,6-dinitro-hydroquinone; **4**: 4-chloro-2,6-DNP; **5**: 4-tert-butyl-2,6-dinitroanisole (c). (d) The sensitivity of two CHO sublines to 3,5-dinitro-BPA. For (b-d) Cells were cultured in a 24-well deepwell plate for 3 days. Cell seeding density: 0.15 x 10^6 cells/mL. Data represents mean ± std (n=3). (e) CHO-S cell cycle analysis as a function of treatment time with 3 μM 3,5-dinitro-BPA. Control: equal amount of dimethyl sulfoxide (0.3%).

growth, indicating cytotoxicity. In contrast, the second group, comprised of 3,5-dinitro-BPA and 3,3',5,5'-tetranitro-BPA, only suppressed cell growth, indicating that both compounds may act as cell cycle arrest agents with little cytotoxicity. BPA at high doses (~10 μM) only partially suppressed cell growth. The potency rank order was found to be 4-tert-butyl-2,6-dinitrophenol ($logIC_{50}$: -7.37±0.04) > 3,5-dinitro-BPA (-6.82±0.04) > 3,3',5,5'-tetra-nitro-BPA (-6.46±0.03) > 3,3'-dinitro-BPA (-6.20±0.02) > 3,5-dinitrophenol (-5.42±0.02) > BPA (~10 μM) (n=3 for all). The lack of effect by 4-tert-2,6-dinitroanisole suggests that the OH group of 2,6-dinitrophenol compounds is essential for the inhibition of cell growth. These data also suggest the importance of the 4-position modification of 2,6-dinitrophenol compounds for cell growth inhibition.

Next, we compared the sensitivity of two different CHO sublines, CHO-S and CHO-5/9α, to 3,5-dinitro-BPA. Several sublines derived from CHO-K1 cells have been adapted to suspension culture and used as the host for the bioproduction of recombinant proteins and antibodies; because each subline has its unique genome and metabolic properties,[26-28] each would be expected to have different sensitivity to cell growth inhibitory compounds.[14,15] Our results showed that CHO-5/9α cells were less sensitive to 3,5-dinitro-BPA than CHO-S cells (**Fig.3d**).

Lastly, we examined the cell cycle distribution of CHO-S cells in the presence of 3 μM 3,5-dinitro-BPA using flow cytometery. Our results suggest that this compound arrests the cells predominately at the G_1/G_0 phase of the cell cycle with no effect on cell viability (**Fig.3e**).

Cell cycle arrest can be induced through multiple molecular mechanisms.[29,30] To elucidate possible mechanisms of 3,5-dinitro-BPA, we performed a chemical similarity search within the ChemBL database.[31] Chemical similarity analysis is a useful approach to predict novel compound-target interactions, based on the principle that similar compounds will likely have similar properties and bind to same proteins.[32-36] Our results showed that in the ChemBL database only 3,3'-dinitro-BPA and 3,3',5,5'-tetra-nitro-BPA are two known compounds similar to 3,5-dinitro-BPA. Examining the annotated biological activity data in the database showed that 3,3'-dinitro-BPA is known to inhibit lysine-specific demethylase 4A (KDM4A), geminin, and chromobox protein homolog 1 (CBX1), all with low micromolar potency. Gene knockdown or chemical inhibition of KDM4A[37], or geminin[38], or CBX1[39,40] has been shown to cause cell cycle arrest and even cell death. 3,3'-dinitro-BPA was found to display no estrogenic activity, but high genotoxicity.[41] These results led us to hypothesize that the three compounds may cause cell growth inhibition at least partially through these molecular targets. Further study is warranted to test this hypothesis.

Together, these results suggest that 3,5-dinitro-BPA is a potent cell cycle arrest agent, thus inhibiting the growth of CHO-S cells; and different CHO sublines have distinct sensitivity to this leachable.

Agonistic activity of 3,5-dinitro-BPA at the GPR35.

Given that certain nitrophenol compounds are GPR35 agonists,[42] we examined the activity of 3,5-dinitro-BPA at the GPR35, which is endogenously expressed in HT29[43]. Profiling using DMR assays showed that 3,5-dinitro-BPA triggered a dose dependent DMR signal (Fig.4), but with a potency well below 3,3',5,5'-tetra-nitro-BPA (EC_{50} of 2.35 μM)[42]. Of note, 3,5-dinitro-BPA at 100 μM did not reach the maximal

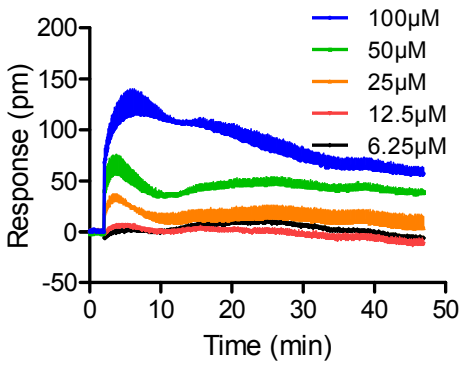


Figure 4. The dose dependent dynamic mass redistribution signal (wavelength shift in picometer, pm) of 3,5-dinitro-BPA in HT29 cells**.** Data represents mean + std (n = 8).

response (~250 pm in amplitude) arising from the activation of GPR35 as determined by the known GPR35 agonist zaprinast.[42,43] However, ML-145 at 1 μM was found to completely block the DMR of 100 μM 3,5-dinitro-BPA (data not shown). ML-145 is a known GPR35 antagonist.[44] Together, these results suggest that 3,5-dinitro-BPA is a weak GPR35 agonist.

Quantification of 3,5-dinitro-BPA per flask. Next, we quantified the amount of 3,5-dinitro-BPA per flask using UPLC. Our results showed that the water extraction protocol extracted 1.52±0.14 μg (n=3) 3,5-dinitro-BPA from each flask, suggesting that its effective concentration could reach as high as ~5 μM when the 2L flasks are used in 1L culture, which is sufficient to cause the maximal inhibition of CHO-S growth. However, compared to the 2L PETG flasks after 3 days suspension culture, we only observed 15% or 39% less cells in the PC flask when the seeding density was 5x or 1x 10^5 cells/mL, respectively (**Fig.1**), suggesting that less 3,5-dinitro-BPA is released into cell culture media during 3 days culture compared to the water extraction.

Mechanism of formation of 3,5-dinitro-BPA. We next examined how 3,5-dinitro-BPA is formed in 2L PC flasks. First, we performed MS analysis of the extracts using a negative ion scan. Results showed that besides 3,5-dinitro-BPA the water extracts also contain 3,3'-dinitro-BPA (m/z of 317.13; retention time of 5.88), which was at trace amounts (Fig.2) as confirmed with the commercial compound. This result suggests a nitric oxide free radical reaction is a common mechanism to form nitro-BPAs.

Second, we examined the effect of γ-irradiation. UPLC/MS didn't detect any nitro-BPA compounds in the 200x concentrated water extracts from flasks that had not been treated with γ-irradiation (**Fig.5a**), suggesting that this treatment is necessary to form 3,5-dinitro-BPA.

Third, we examined the effect of molding process on 3,5-dinitro-BPA formation. UPLC/MS analysis of the water extracts of γ-irradiated PC resin showed no 3,5-dinitro-BPA (**Fig.5a**). This result suggests that the molding process also contributes to the formation of 3,5-dinitro-BPA, presumably via degradation of PC during the manufacturing process.

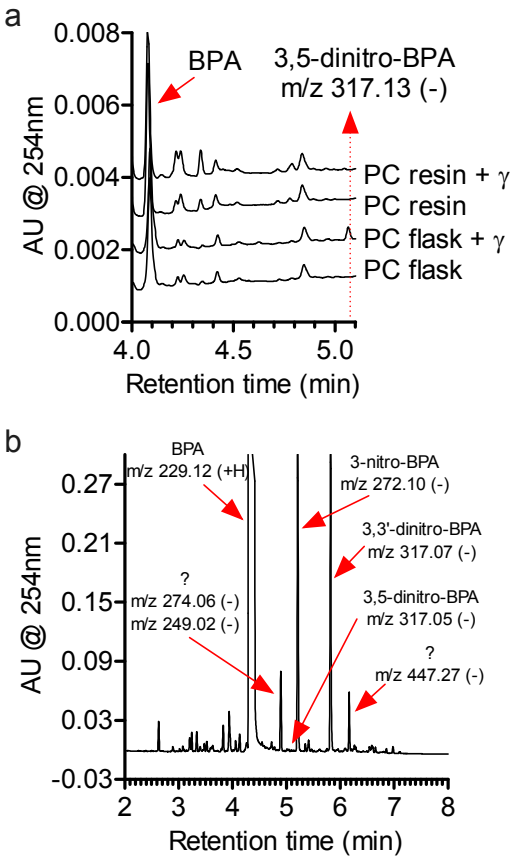


Figure 5. (a) HPLC/UV chromatogram at 254 nm of the water extracts from molded shaker flasks and PC resin before and after gamma-irradiation. (b) HPLC/UV chromatogram at 254 nm of a water extract from a γ-irradiated thin film of pure BPA monomer coated on a glass flask.

Fourth, we performed γ-irradiation treatment of a thin film of pure BPA monomer coated on a glass flask. Results showed that γ-irradiation induced a high amount of 3,3'-dinitro-BPA and 3-nitro-BPA, but a much smaller amount of 3,5-dinitro-BPA (**Fig.5b**), also suggesting that nitro-BPA compounds may form upon γ-irradiation after polymer degradation.

Fifth, we examined the possibility that the formation of 3,5-dinitro-BPA is a flask surface event, as indicated by the low amount of 3,5-dinitro-BPA formed in the bulk BPA monomer film. Assuming that the 3,5-dinitro-BPA formed in a flask can reach as high as a monolayer surface coverage and has a molecular size similar to 3,3'-dinitro-BPA, we would expect to obtain ~20 μg 3,5-dinitro-BPA per flask. This is much higher than what was actually extracted (1.25 μg per flask, see above), suggesting less than monolayer coverage. 3,3'-dinitro-BPA has a maximal planar area of 1.95 nm^2, and a molecular volume of 1.44 nm^3.[45] The surface area of a flask exposed to water extraction is about 800 cm^2. Furthermore, our HPLC/MS analysis showed that a second round of repeated water extraction of the same flask only led to <5% of the amount of 3,5-dinitro-BPA obtained in the first round water extraction. This analysis suggests that the formation of

3,5-dintro-BPA is highly likely a surface event upon γ-irradiation of PC flasks.

Finally, we prepared and analyzed the bulk pyrolysis sample of γ-irradiated 2L PC flasks by first dissolving several flask pieces and then precipitating out the polymers. Results showed that the pyrolysis sample contained little 3,5-dinitro-BPA (**Fig.S7**). Of note, our analysis of the pyrolysis sample (**Fig.S7**) also confirmed the Fries type free radical reactions, leading to the destruction of the carbonate linkages and the formation of a number of phenol coupling products and salicylate esters, as reported previously[46,47].

Together, these results suggest that 3,5-dinitro-BPA is only formed on the surface of PC flasks and via a combination of high temperature molding process-induced polymer degradation with a γ-irradiation-mediated nitric oxide free radical reaction.

Conclusion

The study described here exemplifies our strategy to identify results-interfering leachables from disposable plastic labware/vessels, and our commitment as a labware tool provider to address the increasing demand from academic and industrial labs for better quality single-use products.

Here, we applied multiple analytical tools (such as HPLC, MS, NMR), together with chemical mining and synthesis, to discover 3,5-dinitro-BPA as a bioactive leachable from certain Erlenmeyer PC shake flasks.

We also employed multiple bioassays (e.g., cell proliferation, viability, and functional assays), together with chemical similarity analysis, to examine the mechanism of action of the bioactive leachables identified. The results showed that 3,5-dinitro-BPA acts as a potent cell cycle arrest agent to inhibit the proliferation of CHO-S cells in suspension culture, as well as a weak agonist to activate the GPR35 in HT29 cells. We also found that the sensitivity of cells to 3,5-dinitro-BPA varied between different CHO sublines as well as different cell seeding density, which explains why the majority of end users do not observe growth inhibition with commercially available PC vessels.

Finally, we applied materials science know-how and precision manufacturing process controls to identify the mechanism of formation of the bioactive leachables. As a result, 3,5-dinitro-BPA was identified as a novel results-interfering leachable that is formed through γ-irradiation-mediated nitric oxide free radical reaction. Our results also point to possible solutions, including increasing initial seeding density, pre-washing the flasks, and switching to PETG flasks, when a specific cell line under culture is highly sensitive to this leachable compound.

The presence of bioactive substances that can leach into bioassay solutions may present an underestimated issue for the quality of biological testing results reported in the literature, given the wide-spread use of disposable plasticware in life sciences research and drug discovery. The possibility of certain leachables to diminish the productivity of bioprocessing by inhibiting cell growth,[14,15] or to decrease the quality of biologics by increasing aggregation or triggering *in vivo* responses[16,48] has called for collaborations among polymer resin suppliers, labware manufacturers, and end users to develop a supply chain/quality process to secure high quality products for academic and industrial applications. In this regard, we would like to point out that as PC flasks are available in multiple formats and made using different grad resins or manufacturing processes from different manufacturers, it is highly likely that these commercially available PC flasks may have different leachable profiles including 3,5-dintro-BPA. With the present discovery, together with the approaches to identify, quantify and assay, of 3,5-dinitro-BPA as a cell growth inhibitory leachable from some PC flasks, vendors shall collaborate with polymer resin suppliers to develop high quality products and with end users to develop solution to minimize or eliminate any results-interference effects of specific leachables including 3,5-dinitro-BPA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Supporting UPLC/MS/NMR data, and synthesis procedure and characterization of 3,5-dinitro-BPA (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of Interest Disclosure

Corning is a manufacturer of plastic labware including single use polycarbonate and PETG flasks.

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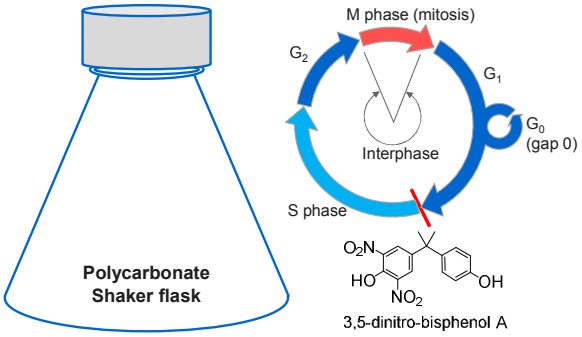
Polycarbonate
Shaker flask
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G1
G0
(gap 0)
Interphase
S phase
O2N
HO
NO2
OH
3,5-dinitro-bisphenol A