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Research paper

Toxicity assessment of three emerging bisphenols (BPA, BPAP and BPC) and their binary mixtures in an advanced *in vitro* 3D HepG2 cell model

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ABSTRACT

Bisphenols (BPs) are industrial chemicals extensively used in polycarbonate plastics and epoxy resins for everyday products, including food and beverage containers, toys, and thermal paper, representing major sources of human exposure. Bisphenol A (BPA) is the most prevalent; however, due to its endocrine-disrupting, reproductive, and genotoxic effects, it is classified as a substance of high concern by the European Chemicals Agency. Regulatory restrictions have prompted the use of structural analogues, including bisphenol AP (BPAP) and bisphenol C (BPC); however, emerging evidence suggests they may pose similar or greater health risks. Comprehensive data on their toxicity and human exposure, especially in mixtures, remain limited. This study assessed the cytotoxic and genotoxic effects of BPA, and less studied analogues BPAP, BPC, and their binary mixtures using a human-relevant 3D HepG2 spheroid model, representing an advanced *in vitro* system. Spheroids were exposed for 24 and 96 h, and effects were evaluated by ATP-based viability assays, comet assay, and targeted transcriptomics. None of the bisphenols induced significant cytotoxicity, although slight reductions in viability were observed for BPAP, BPC, and mixtures. DNA strand breaks were detected after exposure to BPA, BPC, and mixtures. Transcriptomic analysis revealed modest stress responses and strong upregulation of xenobiotic metabolism genes (*CYP1A1, CYP1A2, CYP3A4, UGT1A1, NAT2*), indicating cellular recognition of the tested bisphenols as bioactive xenobiotics. Antioxidant and DNA repair genes were largely unchanged, except for upregulation of oxidative stress markers *HMOX1* and *SRXN1* and a slight increase in *OGG1*, indicating oxidative DNA damage. Importantly, mixture effects were predominantly additive, with no evidence of synergistic interactions under the tested conditions. Overall, bisphenol exposure primarily triggered oxidative stress and metabolic responses rather than robust DNA damage signalling, suggesting that genotoxicity is largely mediated by reactive oxygen species. By integrating multiple endpoints in a 3D liver model, this study provides a more comprehensive assessment of bisphenol toxicity and highlights potential risks associated with BPA analogues and their mixtures, supporting the need for a comprehensive safety assessment to evaluate their suitability as replacements in consumer products.

1. Introduction

Bisphenol A (BPA) and its structural analogues, such as bisphenol AP (BPAP) and bisphenol C (BPC), are synthetic chemicals originally developed for industrial applications, primarily in the production of polycarbonate plastics, epoxy resins, and other polymer-based materials [1–4]. Due to their widespread use in consumer products, food packaging, and industrial processes, these compounds can migrate from materials into food and the environment, resulting in continuous human and environmental exposure [5–8]. Among bisphenols, BPA is the most extensively studied, largely due to its well-documented endocrine-disrupting activity [8–10] and a broad spectrum of adverse health effects. Numerous studies have linked BPA exposure to reproductive, cardiovascular, immune, and respiratory dysfunctions [11–14] as well as cognitive and behavioural impairments [15]. In addition to endocrine-mediated effects, BPA has been shown to induce genotoxicity, including DNA strand breaks, chromosomal alterations, and dysregulation of genes involved in cell cycle control and DNA damage response in HepG2 models [16–19]. These concerns have led to regulatory actions worldwide, including restrictions or bans on BPA use by the European

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Union, the U.S. Food and Drug Administration, and other regulatory authorities [20–23]. Most recently, the European Commission adopted Regulation (EU) 2024/3190, restricting the use of BPA in various food-contact materials [24].

As regulatory pressure on BPA has increased, the production and use of BPA analogues have expanded considerably. Compounds such as BPAP, BPC, bisphenol S (BPS), bisphenol F (BPF), and numerous other analogues are now widely used as functional substitutes [4,25–27]. To date, more than 200 BPA analogues have been identified, and their production continues to increase annually [7,25,26], with many detected in environmental samples and human biological matrices, reflecting widespread exposure across populations, including Europe [28–31]. Due to their structural similarity and comparable physicochemical properties to BPA, these analogues raise significant toxicological concerns [3,5,16,19,25]. However, toxicity data for most BPA analogues remain limited, particularly regarding their combined effects in mixtures. Several analogues have been reported to exhibit endocrine-disrupting activity comparable to, or even exceeding, that of BPA, along with reproductive toxicity and genotoxic effects [7,14,16–19,32–34]. In 2021, the European Chemicals Agency (ECHA) classified multiple BPA analogues, including BPAP and BPC, under the category “Hazards cannot be clarified due to lack of data,” highlighting substantial data gaps and regulatory uncertainty [35,36]. Consequently, European initiatives such as the Horizon Europe–funded Partnership for the Assessment of Risks from Chemicals (PARC) have emphasized the urgent need for comprehensive hazard and risk assessment of BPA substitutes [37].

A key mechanism contributing to BPA-induced toxicity and genotoxicity is oxidative stress. BPA exposure can disrupt cellular redox homeostasis by increasing reactive oxygen species (ROS) production and impairing antioxidant defenses, leading to oxidative damage of cellular macromolecules, including DNA [23]. In hepatocytes, including HepG2 and HepaRG cell lines as well as primary human hepatocytes, BPA has been shown to increase ROS levels, induce DNA strand breaks, and cause cytotoxicity even at micromolar concentrations [38–41]. Moreover, BPA may interfere with the recognition and repair of oxidative DNA lesions by altering chromatin structure and modulating the activity of DNA repair proteins, thereby enhancing genotoxic outcomes [39,42,43]. However, whether similar oxidative stress–driven mechanisms apply to BPA analogues, particularly in mixture scenarios, remains insufficiently understood.

Importantly, exposure to bisphenols rarely involves a single compound; rather, humans are typically exposed to complex mixtures of BPA and its structural analogues [18,44–46]. Co-exposure may result in additive, synergistic, or potentiating effects, particularly for genotoxic endpoints, which are central to chemical risk assessment [47–49]. However, data on the combined genotoxic effects of bisphenol mixtures remain scarce, and current regulatory frameworks for » unintentional « chemical mixtures, such as environmental contaminants, are still insufficiently developed [50].

Given the widespread use of BPA and its analogues, combined with significant gaps in toxicity data, especially regarding mixture effects, there is an urgent need for human-relevant testing strategies. To address this, there has been a global shift from traditional animal testing toward New Approach Methodologies (NAMs), including advanced *in vitro* models with improved human relevance [51,52]. These approaches aim to generate more mechanistically informative data while reducing animal use, in line with EU legislation (Directive 2010/63/EU) and the mission of the EU Reference Laboratory for alternatives to animal testing [51,53,54]. In particular, three-dimensional (3D) hepatic *in vitro* models better recapitulate the structural and functional complexity of human liver tissue than conventional 2D cultures and offer improved predictive capacity for assessing oxidative stress, genotoxicity, and mixture effects [55–59].

In this study, we address critical knowledge gaps regarding the genotoxic and oxidative effects of BPA and its less studied analogues, BPAP and BPC, both individually and in binary mixtures, using HepG2-derived 3D hepatic spheroids. BPAP is widely used in the production of polymers such as epoxy resins, polycarbonates, and polyesters, primarily serving as a plasticiser and flame retardant [3,60,61], and has been identified as an endocrine-disrupting compound [62]. Similarly, BPC is present in a variety of consumer products, including flooring, textiles, toys, and food packaging [6,62] and, due to concerns over potential reproductive toxicity and endocrine-disrupting activity, has been evaluated by ECHA under the Community Rolling Action Plan (CoRAP) [63]. HepG2-derived spheroids were exposed to BPA, BPAP, BPC, and their binary mixtures for 24 and 96 h. The effects of bisphenol exposure on cell viability, DNA damage, oxidative stress, and gene expression were assessed using ATP-based viability assays, the comet assay, and targeted transcriptomics analyses. While oxidative stress and genotoxic effects of BPA and its analogues have been previously reported, the present study advances current understanding by integrating these endpoints within a 3D HepG2 spheroid model and by examining binary mixtures of less-studied analogues (BPAP and BPC). This multi-endpoint approach in a human-relevant 3D model enables a more comprehensive evaluation of cellular responses to bisphenol exposure and contributes to a better understanding of the potential health risks associated with BPA analogues and their mixtures, thereby supporting the need for more robust regulatory evaluation and risk assessment.

2. Materials and methods

2.1. Chemicals

Bisphenol A (4,4’-(propane-2,2-diyl)diphenol; BPA), Bisphenol AP (4,4′-(1-Phenylethylidene)bisphenol; BPAP), and Bisphenol C (4,4’-(2,2-dichloroethene-1,1-diyl)diphenol; BPC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions of BPA, BPAP, BPC [16 mM each] and benzo[a]pyrene [B(a)P, 9.908 mM] were prepared in DMSO and stored at −20 °C. Working solutions for cell treatment were freshly prepared in cell medium immediately prior to use. The final DMSO concentration did not exceed 0.3% and was adjusted to 0.3% and 0.06% for 24 h and 96 h exposures, respectively.

The following chemicals were used in the study: Penicillin/streptomycin, Na-pyruvate, L-glutamine, Dimethylsulphoxide (DMSO), Methylcellulose, benzo[a]pyrene (B(a)P CAS-No. 50-32-8), and DEPC-treated water (w4502) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Minimum Essential Medium Eagle (MEME-10370-047), Trypsin-EDTA (0.25%), TripLE Express (12604-013), Foetal Bovine Serum (FBS), and Trypan Blue (15250-061) were obtained from Gibco (Paisley, Scotland, UK). Normal melting point (NMP; 16500-100) and Low melting point (LMP; 16520-050) agaroses were from Invitrogen (USA). Phosphate-buffered saline (PBS) and Methanol were purchased from PAA Laboratories (Dartmouth, NH, USA). GelRed solution was from Biotium (Fremont, CA, USA). Triton X-100 and Collagenase (Type I - 17018029) were obtained from Fisher Sciences (New Jersey, USA). CellTiter-Glo® Luminescent Cell Viability Assay (G7570) was obtained from Promega (Madison, WI, USA). The high-capacity cDNA kit, TaqMan Gene Expression Assays, and TaqMan Universal PCR Master Mix (4440038) were purchased from Applied Biosystems (New Jersey, USA). PreAmp GrandMaster Mix (TA05-50) was from TATAA Biocenter AB (Göteborg, Sweden). GE 48.48 Dynamic Array Sample & Assay loading Reagent Kit – 10 IFCs (85000821), and 48.48 Dynamic Array Gene expression chips were obtained from Fluidigm (South San Francisco, CA, USA). RNeasy Mini Kit (74104) was obtained from Qiagen Life Science (Hilden, Germany).

2.2. Spheroid formation

The human hepatocellular carcinoma cell line, HepG2 (ATCC-HB-8065™, Manassas, VA, USA), was cultured in MEME medium enriched with non-essential amino acids (NEAA) and supplemented with 10%

FBS, 2.2 g/L sodium bicarbonate, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin, and 1 mM sodium pyruvate. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For experimental purposes, 3D cell models (spheroids) were formed from a single-cell suspension employing the forced floating method (85 × g, 1.5 h, 28 °C), as outlined by Štampar et al. (2019) [64]. Each spheroid was initiated with 3000 cells, seeded into 96 U-bottom well plates (Falcon™ 96-Well, Non-Treated, U-Shaped-Bottom Microplate), and incubated for three days prior to chemical treatment.

2.3. Treatment conditions

After three days of initial growth, the culture medium was discarded, and spheroids were treated with BPA, BPAP, and BPC, either individually or in binary combinations at a 1:1 ratio (BPA + BPAP; BPA + BPC) (Table 1). Exposures were conducted for 24 and 96 h in 96-U-bottom well plates at different concentration ranges selected to avoid cytotoxicity during prolonged exposure. For the 96 h treatments, the medium containing BPs was replaced after 48 h (re-treatment). Solvent controls (SC) and appropriate positive controls (PC) were included in all experiments. All experiments were independently replicated three times, with assay-specific technical replicates. For the cytotoxicity assay, five spheroids were used per condition. For the comet assay, three spheroids per condition were analysed, with 50 nuclei scored per sample. For qPCR analyses, samples consisted of pooled material from 24 spheroids, with two technical replicates (duplicate pipetting) performed for each sample.

2.4. Cytotoxicity

The cytotoxicity of the tested bisphenols and their binary mixtures was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacturer's instructions (Promega), with minor modifications as previously described by Štampar et al. (2021) [56]. Three-day-old spheroids were exposed to individual bisphenols BPA, BPAP, and BPC or to their binary mixtures for 24 and 96 h at the applied concentrations stated in Table 1. Luminescence was measured using a Cytation 5 plate reader (BioTek, Winooski, VT, USA). Each experiment included a contro (media), a solvent control (cells exposed to 0.3% and 0.06% DMSO for 24 and 96 h, respectively) and a positive control (15% DMSO). The obtained data were normalized to the solvent control. Statistical analysis was done using One-way ANOVA (*$p < 0.05$) (GraphPad Prism V10 Software, San Diego, CA, USA).

2.5. Genotoxicity – DNA strand breaks

The genotoxicity of BPA, BPC, BPAP, and their binary mixtures was assessed using the comet assay as described by Štampar et al. (2019) [64] in accordance with the MIRCA guidelines [65]. Three-day-old spheroids were exposed to the tested bisphenols and their combinations at concentrations listed in Table 1. After exposure, five spheroids per condition were collected, dissociated into a single-cell suspension using a combination of mechanical disruption and enzymatic digestion (5 mg/mL collagenase in MEME without supplements, diluted with TrypLE at a 1:2 ratio) [66]. Cell viability was evaluated by trypan blue staining prior to further processing. Comet images were captured using a fluorescence microscope (*Ti* Eclipse inverted microscope, Nikon, Japan) and analysed using Comet IV image analysis software (Instem, UK). Benzo[a]pyrene B(a)P (30 μM for 24 h and 1 μM for 96 h) was used as a PC. Data were deposited in the DiRROS repository [67]. The results are presented as % of tail DNA. Statistical analysis was performed using a mixed effects model (*$p < 0.05$).

Table 1
Treatment conditions and applied concentrations of BPA, BPAP, BPC, and their binary mixtures in HepG2 spheroids.

Time of Exposure	Single Compound or Binary Mixture	Concentrations (μM)
24 h	BPA, BPAP, BPC	10, 20, 40, 80[a]
	BPA + BPAP/BPC	10 + 10, 20 + 20, 40 + 40[a]
96 h	BPA, BPAP, BPC	1, 2, 4, 8[a]
	BPA + BPAP/BPC	1 + 1, 2 + 2, 4 + 4[a]

[a] Concentrations used only for viability testing.

2.6. Targeted transcriptomics

The expression of selected targeted genes involved in xenobiotic metabolism (*CYP1A1, CYP1A2, CYP3A4, UGT1A1, UGT2B7, NAT2*), nuclear receptors (*PXR, CAR*), transcription factor (*AHR*), cell proliferation (*KI67*), apoptosis (*BBC3, BCL2, BAX*), DNA damage response (*TP53, GADD45α, CDKN1A*), and oxidative stress (*OGG1, GCLC, CAT, HMOX1, SRXN1*) was analysed by quantitative real-time PCR (qPCR) using a 48.48 Dynamic Array™ IFC for gene expression (Fluidigm, US) [17]. Three-day-old spheroids were exposed to individual BPA, BPC, and BPAP and their binary mixtures, at concentrations listed in Table 1. Benzo[a]pyrene (20 μM) was used as a positive control. Total mRNA was isolated from a pool of 24 spheroids for each experimental point, using the RNeasy Mini Kit. The purity and concentration of the isolated mRNA were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, USA), while RNA integrity was verified by agarose gel electrophoresis using the PowerPac 3000 (BioRad, Hercules, CA, USA) (Supplementary Material (SM1: Gel electrophoresis)). For reverse transcription, 1 μg of total mRNA from each sample was converted to cDNA using the cDNA High-Capacity reverse transcription Kit (Applied Biosystems, USA). The qPCR assays were conducted using 48.48 Dynamic Array™ IFC chips on the BioMark HD system. All selected target genes [Supplementary Material (SM2: List of selected Genes)] were preamplified prior to qPCR analysis. Data analysis was performed using the open web program QuantGenious, applying relative quantification methods referenced against the solvent control [68]. Changes in gene expression greater than 1.5-fold were considered biologically relevant, with upregulation defined as relative expression >1.5 and downregulation as <0.66, respectively. Data were deposited in the DiRROS repository (Štampar et al., 2026). The statistical analysis was performed by one sample *t*-test (*$p < 0.05$).

3. Results and discussion

Growing concerns about the potential adverse effects of BPA and its structural analogues on human and environmental health have prompted increasing research into their biological impacts. Humans and animals are commonly exposed to multiple bisphenols simultaneously, through ingestion, inhalation, or dermal contact, yet toxicological data on co-exposures remain limited. Understanding the combined effects of BPA and its analogues is therefore essential for evaluating their risks. Co-exposure may result in additive, synergistic, or potentiating effects that are not apparent when chemicals are studied individually, highlighting the need for experimental approaches that cannot be predicted from single-compound studies. Importantly, such combined exposures better reflect real-world conditions, where multiple bisphenols co-occur.

To investigate the adverse effects of bisphenols, metabolically competent HepG2-derived 3D spheroids were used to assess the cytotoxic and genotoxic potential of BPA, BPAP, and BPC, both individually and in binary mixtures (BPA/BPAP and BPA/BPC). These advanced 3D models support physiologically relevant responses by maintaining extracellular matrix components, enabling direct cell–cell interactions, and allowing repeated-dose exposure under conditions that better mimic human liver tissue than conventional 2D cultures [58]. The effects of BPA, BPAP, BPC, and their binary combinations on cell viability in 3-day-old HepG2 spheroids were evaluated using the ATP assay after 24 h (short-term) and 96 h (prolonged) exposures (Table 1). None of the

individual BPs or their binary mixtures, at concentrations up to 80 μM (24 h) and 8 μM (96 h), induced biologically relevant cytotoxic effects in HepG2 spheroids (Fig. 1). A slight, dose-dependent decrease in cell viability was observed for BPAP, BPC, and the BPA/BPAP combination after 24 and 96 h of exposure; however, viability remained above 80%, indicating that all tested concentrations were suitable for further genotoxicity analyses. The obtained results for individual-compound exposures are consistent with previous studies in HepG2 cells [18,19], and the findings on co-exposure align with the limited available data on binary mixtures of bisphenol analogues. Previous research has shown that BPA does not affect cell viability in a monolayer [16] and 3D [17] HepG2 cell cultures at concentrations up to 80 μM after 24 and 96 h of exposure. Several studies have reported that BPA analogues may exhibit greater cytotoxic potential than BPA in mammalian cell lines [17, 69–71], with Padberg et al. demonstrating that BPC is more toxic than BPA in the HepG2 cell line [72]. In addition, co-exposure of BPA with its analogues, BPAP and BPC, did not significantly impact cell viability after 24 and 96 h in the hepatic 3D cell model, as assessed by the MTT assay [18].

The genotoxic potential of BPA, BPAP, BPC, and their binary mixtures was further evaluated using the comet assay after 24- and 96 h exposures (Fig. 2). The comet assay is a sensitive technique for detecting a broad range of DNA lesions, including DNA single- and double-strand breaks (SSBs and DSBs), alkali-labile sites such as apurinic/apyrimidinic sites, and SSBs resulting from incomplete excision repair [65]. After 24 h, BPA and BPAP induced significant DNA damage at concentrations ≥20 μM, whereas BPC exhibited significant effects only at 40 μM. In the binary mixture experiments, the combination of BPA and BPAP did not induce significant DNA damage when each compound was present at 10 μM or 20 μM. In contrast, the combination of BPA and BPC caused significant DNA damage at both 10 + 10 μM and 20 + 20 μM per compound. These results indicate that, at the tested concentrations, the effects of BPA/BPAP were not enhanced in combination, while the BPA/BPC mixture showed additive effects consistent with the individual compound responses (Fig. 2A). Prolonged exposure for 96 h resulted in genotoxic effects at substantially lower concentrations compared to 24 h (Fig. 2B). BPA and BPAP induced significant DNA damage at 2 μM and 4 μM, whereas BPC elicited significant effects at concentrations ≥1 μM. In the binary mixtures, BPA combined with BPAP induced significant DNA damage at 1 + 1 μM and 2 + 2 μM, while the combination of BPA and BPC caused significant effects only at 2 + 2 μM. For example, the combination of BPA/BPAP at 1 μM each (total 2 μM) induced DNA damage similar to that caused by 2 μM of either compound alone, indicating that the mixtures produce additive effects rather than synergistic interactions under the tested conditions. Previous *in vitro* studies have shown that BPA and its analogues can induce both DNA and chromosomal damage in 2D and 3D cellular models, particularly in liver-derived human cell lines [16,17,19,44,73], as well as in estrogen receptor-positive MCF-7 breast cancer cells [74]. Furthermore, BPA has been consistently reported to increase γH2AX foci, a marker of DNA double-strand breaks, in HepG2 [17,19,38,75], HepaRG [76], human peripheral blood mononuclear cells [77], MCF-7, and BEAS-2B bronchial epithelial cells [78], with lower levels of DNA damage observed in MDA-MB-231 breast adenocarcinoma cells [74]. However, a subset of studies has reported no detectable genotoxic effects, with several investigations finding no mutagenic or clastogenic potential of BPA in various *in vitro* test systems [16,38]. Based on the available evidence, the European Food Safety Authority [52] concluded that BPA is unlikely to pose a genotoxic hazard via a direct mechanism of action.

To complement the assessment of DNA damage, we employed a targeted transcriptomics approach to investigate how BPA, BPAP, BPC, and their binary mixtures modulate the expression of targeted genes involved in key cellular pathways. Targeted transcriptomics provides insight into how chemical exposures affect the expression of genes in critical molecular pathways, helping to elucidate mechanisms underlying adverse cellular outcomes. In this study, transcriptional changes in genes involved in key cellular pathways, including xenobiotic metabolism (encoding phase I and II enzymes: *CYP3A4, CYP1A2, CYP1A1, UGT2B7, UGT1A1, NAT2*), nuclear and cytoplasmic receptors (*PXR, CAR, AHR*), DNA damage response (*TP53, MDM2, GADD45a, CDKN1a*), oxidative stress response (*OGG1, GCLC, CAT, HMOX1, SRXN1*), proliferation (*Ki67*) and apoptosis (*BBC3, BCL2, BAX*) were analysed in HepG2 spheroids after 24- and 96-h exposures to BPA, BPAP, BPC, and their binary mixtures. Among the nuclear receptor genes analysed, *PXR, CAR*, and *AHR*, key regulators of xenobiotic sensing and metabolic activation, exhibited distinct, time-dependent transcriptional responses, however, these differences should be interpreted with caution due to the use of lower concentrations at 96 h (Fig. 3, Table SM3). After 24 h of exposure, *CAR* was the most strongly upregulated, particularly by BPA Upregulation was also detected following exposure to binary mixtures of BPA/BPAP (10 + 10 μM), and BPA/BPC (10 + 10 μM), (20 + 20 μM). In contrast, *PXR* and *AHR* expression levels remained largely unaltered at 24 h, suggesting limited involvement of these receptors in the early transcriptional response. Following prolonged exposure (96 h) to lower concentrations, a shift in nuclear receptor activation was observed, characterized by selective induction of *AHR*, whereas *CAR* and *PXR* expressions remained largely unaffected. The upregulation of *CAR* may represent an early initiating event that precedes the broader activation of xenobiotic metabolism observed after 24 h of exposure. *CAR* is a well-established regulator of both phase I (e.g., CYP enzymes) and phase II (e.g., UGT enzymes) detoxification pathways [79]. Consistent with this role, *CAR* induction at 24 h, particularly following BPA exposure and in

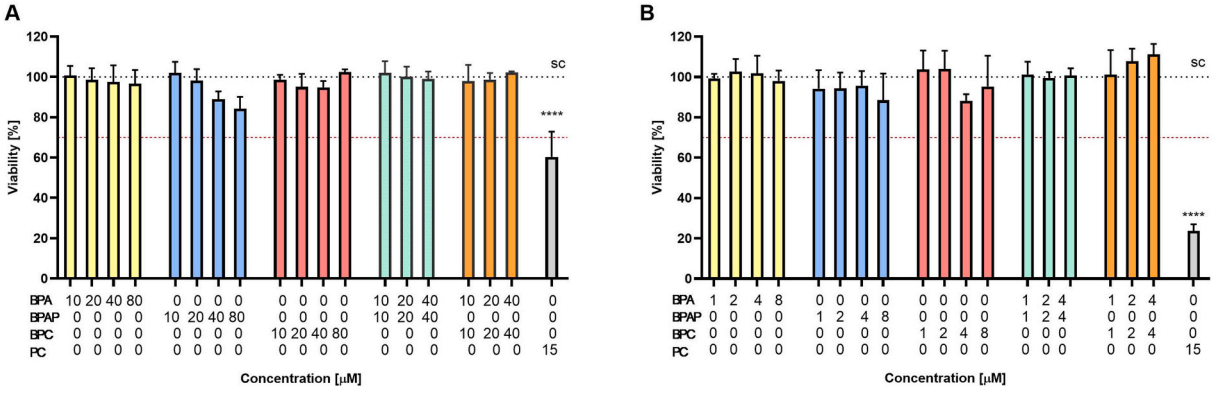

Fig. 1. Cell viability in HepG2 spheroids (CellTiter-Glo3D assay) after 24 h (A) and 96 h (B) exposure to BPA (yellow), BPAP (blue), BPC (red), and their binary mixtures (green and orange). PC - positive control (15% DMSO). Results are presented as mean ± SD. *Significantly different from solvent control, p**** <0.0001 (one-way ANOVA; Dunnett's multiple comparison test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

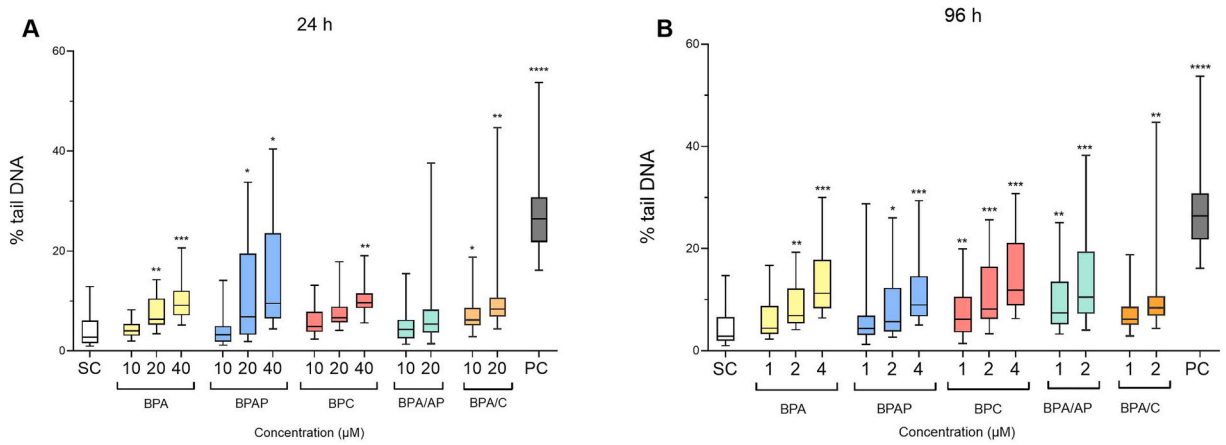

Fig. 2. Genotoxic effects of individual bisphenols (BPA, BPAP, BPC) and their binary mixtures (BPA/BPAP or BPA/BPC) in 3-day-old HepG2 spheroids, assessed by the alkaline comet assay after 24 h (A) and 96 h (B) exposures. Results are presented as % of tail DNA in the form of boxplots with a 95% confidence interval. The box represents the interquartile range (Q1–Q3) with the median shown by a line, and whiskers indicate the minimum and maximum values. SC = solvent control (0.1% DMSO); PC = positive control (30 μM BaP). *p < 0.001 vs. SC (mixed effects analysis) *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

binary mixtures, coincided with increased expression of multiple metabolic genes, including *CYP3A4, CYP1A1, CYP1A2, UGT1A1*, and *NAT2*. This coordinated transcriptional pattern suggests that bisphenol exposure engages CAR-driven regulatory networks to enhance cellular xenobiotic biotransformation capacity. In contrast, the lack of substantial transcriptional activation of *PXR* and *AHR* at 24 h suggests a limited contribution of these receptors to the early response under experimental conditions. This may reflect compound-specific receptor selectivity, insufficient ligand affinity at the tested concentrations, or transient activation occurring outside the selected sampling window. Notably, a shift in nuclear receptor involvement was observed after prolonged exposure (96 h), characterised by selective induction of *AHR,* while *CAR* and *PXR* remained largely unchanged. The delayed upregulation of *AHR* suggests its involvement in a later phase of the cellular response, potentially associated with sustained exposure, low-level oxidative stress, or the accumulation of bioactivated metabolites. *AHR* signalling is known to be responsive not only to parent xenobiotics but also to endogenous and xenobiotic-derived ligands generated during prolonged metabolic processing [80,81]. Together, these findings suggest a potential time-dependent shift from CAR-dominated early metabolic sensing toward AHR-mediated transcriptional responses; however, concentration-dependent effects cannot be excluded, underscoring the dynamic nature of nuclear receptor signalling following exposure to bisphenols and their mixtures.

Among all tested biomarker genes, the strongest and statistically significant deregulations following bisphenol exposure were observed in genes involved in xenobiotic metabolism. After 24 h, the most prominent transcriptional changes were observed for *CYP1A1, CYP1A2, CYP3A4, UGT1A1,* and *NAT2*, indicating an early activation of both phase I and phase II metabolism pathways (Fig. 3A–Table SM3). Among these, *CYP1A1* showed the strongest induction, with expression levels triggered by 40 μM BPA (2.6-fold) and 20 μM and 40 μM BPC (2.5-fold and 3.8-fold, respectively), followed by the BPA/BPAP and BPA/BPC mixtures at 10 + 10 μM and 20 + 20 μM (1.6-fold and 1.7-fold and 2.1-fold, 4.5-fold, respectively). Similarly, *CYP1A2* was upregulated, with BPC at 20 μM and 40 μM producing 2.8-fold and 4.8-fold increases, respectively, and BPA/BPC mixtures at 10 + 10 μM and 20 + 20 μM reaching approximately 2-fold and 6.5-fold, respectively. *CYP3A4*, a key target of both *CAR* and *PXR*, was significantly induced by BPA at 20 μM (3.1-fold), BPAP at 10 μM (7-fold), and BPC at 20 μM (2.7-fold). Binary mixtures also enhanced *CYP3A4* expression, with BPA/BPAP (10 + 10 μM) increasing it 3.7-fold and BPA/BPC (20 + 20 μM) inducing a 2.1-fold increase. *UGT1A1*, a phase II conjugating enzyme primarily regulated by *CAR*, was upregulated by BPA at 10 (1.8-fold) at 20 μM (1.3-fold) and at 40 μM (3.7-fold), BPAP at 10 μM (5.1-fold), BPAP at 20 μM (1.5-fold), BPC at 20 μM (2.5-fold), BPC at 40 μM (2.3-fold) and BPA/BPAP binary mixtures at 10 + 10 μM (3.1-fold), BPA/BPC binary mixtures at 10 + 10 μM and 20 + 20 μM (1.8-fold and 2.4-fold, respectively). Similarly, *NAT2*, another important enzyme involved in xenobiotic biotransformation, showed the highest upregulation at the mRNA level in response to BPA at 10 μM (1.6-fold), BPA at 20 μM (2.3-fold) and BPA at 40 μM (3.9-fold), BPAP at 10 μM (7-fold), BPC at 40 μM (3.2-fold), and in binary mixtures BPA/APAP at 10 + 10 μM (4.0-fold) and BPA/BPC at 10 + 10 μM and 20 + 20 μM (2.5-fold and 4.3-fold, respectively). After 96 h of exposure to lower concentrations, a similar pattern of metabolic gene expression was observed; however, the level of induction was generally reduced compared to the 24-h time point. This decrease was particularly evident for *CYP1A1, UGT1A1*, and *NAT2*, suggesting possible feedback regulation, adaptive downregulation, or a decline in receptor-mediated transcriptional activity over time. Overall, the upregulation of *CYP*s, *UGT*s, and *NAT*s indicates activation of oxidative metabolism and detoxification pathways as a response to the cellular recognition of the tested bisphenols and their binary mixtures as bioactive xenobiotics. Notably, BPA, BPAP, and BPC elicited broadly similar transcriptional responses, suggesting comparable activation of xenobiotic metabolism among these compounds.

Cytochrome P450 (CYP) enzymes play a crucial role in the oxidation of both endogenous and exogenous compounds, including steroids, fatty acids, and xenobiotics [81]. Bisphenols are known to undergo oxidative metabolism mediated by CYP enzymes, forming reactive intermediates that are subsequently detoxified through glucuronidation, a phase II reaction catalysed by UDP-glucuronosyltransferases (UGTs) [82]. The transcriptional upregulation of *CYP1A1*, *CYP1A2*, *CYP3A4*, and *UGT1A1* observed in our study, particularly after 24 h exposure to BPA, BPAP, BPC, and their binary mixtures, is consistent with these pathways, suggesting activation of both phase I and phase II metabolism. Notably, BPA, BPS, BPF, and BPAF have been shown to be oxidised into reactive intermediates [83] capable of forming DNA adducts, an important mechanism potentially underlying their genotoxic effects [84–86]. This suggests that bisphenol analogues such as BPAP and BPC may contribute to genotoxicity via CYP-mediated formation of reactive intermediates that interact with DNA. Hercog et al. (2019) highlighted the central role of CYPs and UGTs in bisphenol metabolism, showing that HepG2 cells exposed to BPs for 24 h strongly induced *CYP1A1* but only moderately upregulated *UGT1A1* [17]. Furthermore, the expression of *CYP1A1* and *UGT1A1* is regulated by the ligand-activated transcription factor AhR [87,88], and the observed upregulation of these genes in our study likely involves an AhR-dependent mechanism, consistent with previous

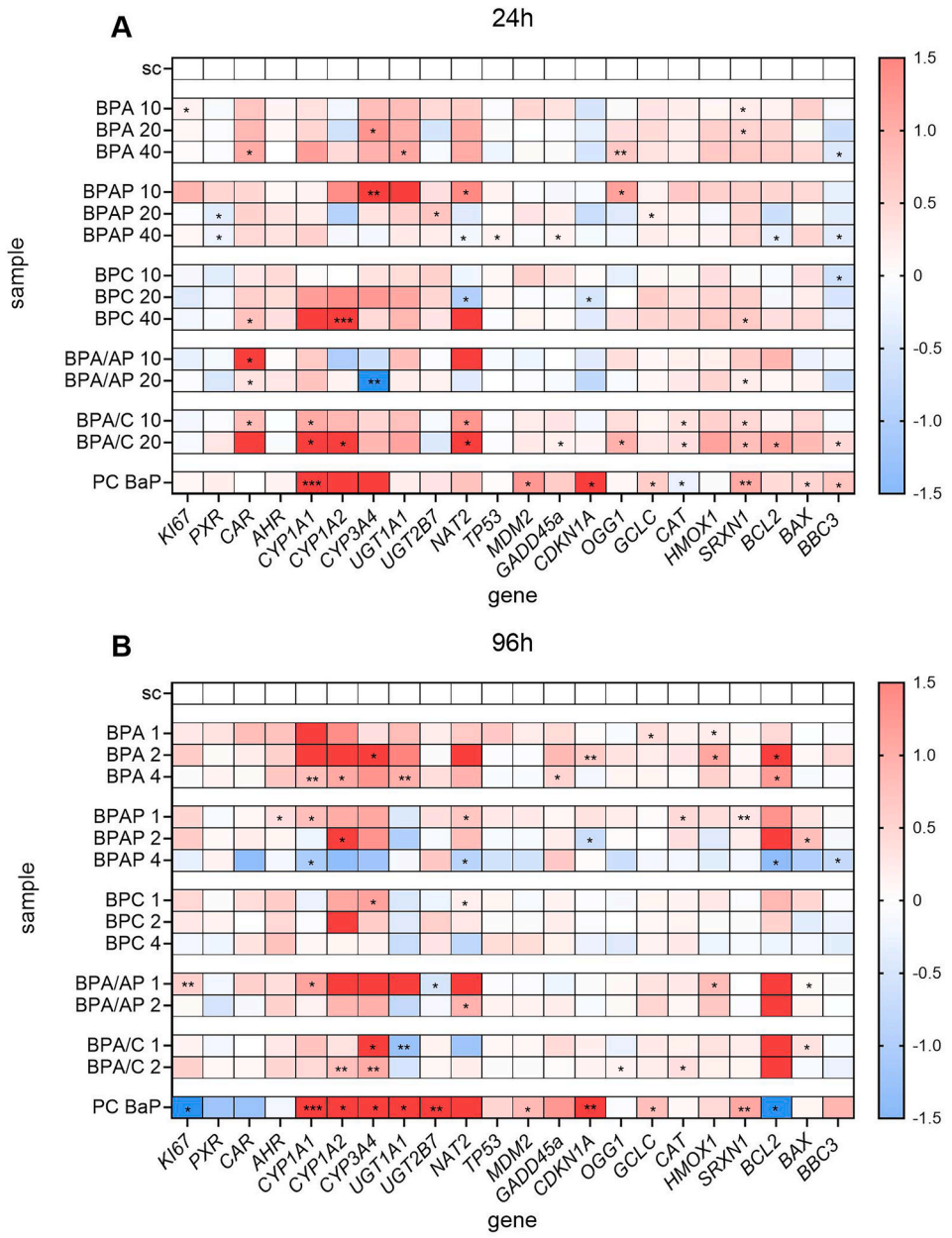

Fig. 3. Heat map showing gene expression profiles of nuclear receptors, metabolic, DNA damage response, oxidative stress, and apoptosis-related genes after 24 h (A) and 96 h (B) exposure, assessed by qPCR using the Fluidigm 48.48 IFC system. Data were analysed with QuantGenious (relative quantification to housekeeping genes). Results are expressed as fold-changes relative to the solvent control. Genes with >1.5-fold (red) or <0.66-fold (blue) changes, corresponding to approximately +0.6 and −0.6 in log2 scale, respectively, were considered up- or downregulated. For visualization, fold changes were log2-transformed. Statistical significance was performed separately by one-sample *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) in Graph Pad Prism (v10.0). Quantitative fold-change values with SD are provided in the Supplementary Materials (SM3: Quantitative gene expression results). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

findings showing BPA stimulation of the AhR-DNA-binding complex [89]. Previous studies demonstrated that BPA and BPAF, at human exposure-relevant concentrations (ng/mL range), and their mixtures, significantly upregulated *CYP1A1* and *UGT1A1* expression [17]. In addition, Schmidt et al. (2017) investigated the biotransformation of several bisphenol analogues, including BPF, BPAF, BPZ, and DMBPA in human liver microsomes (HLM) across multiple CYP isoforms (CYP3A4, CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2E1) [88]. Complementing these findings, Nakamura et al. (2011) reported that BPA reactions involving substitution at the same position are primarily catalysed by CYP3A4 and CYP3A5, highlighting the collective role of these CYP enzymes as key players in bisphenol metabolism [90]. Although our results indicate early activation of xenobiotic metabolism, following exposure to the tested BPs, it should be noted that HepG2 cells, even when cultured in a 3D configuration, still exhibit a lower overall metabolic capacity than primary hepatocytes. Consequently, it remains unclear to what extent the observed changes in metabolic gene expression reflect meaningful metabolic processing of bisphenols or result in the formation of reactive intermediates that could have caused or contributed to the observed genotoxic effects in the present study.

Further, we evaluated whether bisphenol exposure affected cellular proliferation in HepG2 spheroids by quantifying *Ki67* gene expression, a widely used marker of proliferative activity [91] (Fig. 3). Exposure to BPC for 24 h led to a downregulation of *Ki67* mRNA level (0.62-fold),

whereas BPA and BPAP alone did not significantly alter proliferation. By 96 h, *Ki67* expression returned to control levels for all treatments, suggesting that the initial BPC-induced inhibitory effect was transient. Co-exposure of BPA with either BPAP or BPC did not produce any measurable changes in *Ki67* expression at either time point, indicating that interactions between BPs did not exacerbate effects on cellular proliferation under the tested conditions. These findings align with previous studies reporting that BPA did not significantly affect proliferation in MCF-12A or HepG2 cells at concentrations up to 40 μM, while BPC at 40 μM decreased proliferation after 24 h [19,92]. Collectively, these data indicate that, under the tested conditions, both individual and combined BPs exposures have minimal impact on HepG2 spheroid proliferative activity.

To further explore the cellular response to BPs exposure, the expression of DNA damage response genes was evaluated, as alterations of these genes are recognised molecular markers of genotoxic and carcinogenic stress [93–96]. Specifically, the transcriptional response of *TP53, MDM2, CDKN1A, and GADD45A* was assessed following exposure to BPs and their mixtures. Among these genes, transcriptional changes were generally weak or absent suggesting that under the applied conditions, BPs did not strongly activate DNA repair and genotoxic signalling pathways, despite the presence of DNA strand breaks. This may suggest that cells rely on alternative protective mechanisms, including antioxidant defences and cell cycle control, to cope with genotoxic insults [97]. Given the apparent involvement of antioxidant defences in mitigating BPs-induced stress, we next assessed genes associated with oxidative stress and redox homeostasis to evaluate the cellular adaptive responses (Fig. 3). Redox balance is essential for maintaining cellular integrity, as normal metabolism continuously generates reactive oxygen species (ROS), such as superoxide, peroxides, and hydroxyl radicals, as well as reactive nitrogen species (RNS), including nitric oxide and peroxynitrite [98,99]. When this balance is disrupted, excessive ROS and RNS can overwhelm antioxidant defences, leading to oxidative damage of cellular macromolecules and genotoxic outcomes characterized by oxidative DNA lesions, mutations, and impaired DNA repair [99,100]. In this context, the DNA damage detected by the comet assay is likely attributable to oxidative stress-driven DNA lesions [39]. In the present study, exposure of HepG2 3D spheroids to BPs resulted in differential regulation of oxidative stress–related genes, with selective activation of cellular defence mechanisms observed after 24 h, but no induction evident after 96 h, which may reflect both adaptive responses and lower exposure concentrations (Fig. 3B). Genes involved in antioxidant defence and base excision repair (BER), including *GCLC, CAT,* and *OGG1,* were only weakly induced or remained unchanged, suggesting that under the tested conditions BPs do not strongly stimulate glutathione synthesis, catalase-mediated detoxification, or BER pathways in response to oxidative stress [101]. These findings are in agreement with those of Hercog et al. (2019), who showed that genes encoding key antioxidant enzyme, including *SOD, CAT,* and *GPX1,* which are essential for ROS scavenging, were not significantly affected by BPs exposure, while *GCLC* was upregulated in response to BPA [17]. Similarly, Maćczak et al. (2017) reported that exposure of human erythrocytes to BPA and its analogues (BPS, BPF, and BPAF) did not significantly affect antioxidant enzyme activities at comparable concentrations, with measurable effects observed only at concentrations more than 10-fold higher than those applied in the present study [102]. Notably, the mRNA level of *OGG1*, a key enzyme in the BER pathway responsible for excising oxidised DNA bases such as 8-oxo-7,8-dihydroguanine (8-oxoG), was moderately upregulated at 24 h following exposure to BPA at 40 μM, BPAP at 10 μM, and the BPA/BPC at 20 + 20 μM binary mixture, reflecting the presence of oxidative DNA lesions [23,103]. Unlike the oxidative stress-related genes described above, *HMOX1* and *SRXN1*, canonical targets of the Nrf2–ARE pathway, were consistently upregulated after 24 h, reflecting adaptive responses in HepG2 spheroids. *HMOX1*, which catalyses heme degradation and produces cytoprotective metabolites [104–106], showed the most sustained response, supporting its role in protection against oxidative insults, while SRXN1, which is involved in the repair of hyperoxidized peroxiredoxins and the restoration of peroxiredoxin function [107], displayed a more moderate pattern of induction. The most pronounced induction of *XMOX1* was observed after exposure to BPA at 40 μM (1.7-fold), BPAP at 10 μM (1.6-fold), BPC at 40 μM (1.6-fold), and in binary mixtures such as BPA/BPAP at 20 + 20 μM (1.5-fold), BPA/BPC at 10 + 10 μM (1.5-fold), and BPA/BPC at 20 + 20 μM (2.4-fold). *HMOX1* remained moderately elevated after 96 h at lower concentrations, including BPA at 2 and 4 μM, and the BPA/BPAP mixture at 1 μM, emphasizing its role in long-term adaptation to oxidative insults. *SRXN1*, involved in the repair of hyperoxidized peroxiredoxins and the restoration of peroxiredoxin function [107], was moderately upregulated only after 24 h of exposure to BPA at 40 μM (1.6-fold), BPAP at 10 μM (1.5-fold), BPC at 40 μM (1.6-fold), and mixtures of BPA/BPAP at 10 + 10 μM (1.6-fold), and BPA/BPC at 20 + 20 μM (1.8-fold). Overall, the observed deregulations of oxidative stress–related genes were moderate, with no strong up- or down-regulation detected under the tested conditions.

Consistent with these results, BPA has been shown to generate ROS both *in vivo*, in the rat liver and testis [108], and *in vitro* across multiple cell models [39,109–111], with ROS contributing to BPA-mediated DNA strand breaks [112]. BPs analogues, including BPS, BPF, and BPAF, have also been reported to induce ROS formation in human blood cells [102, 113]. Extending these observations, Štampar et al. (2023) demonstrated that exposure to BPA, BPAP, BPC, and their mixtures elevated oxidative stress in HepG2 spheroids, as evidenced by increased malondialdehyde (MDA) levels, ultimately leading to DNA damage [18].

Given the moderate oxidative stress and limited activation of DNA damage response genes observed in HepG2 spheroids, we next evaluated whether these cellular stress signals affected apoptosis, or programmed cell death, a highly regulated process triggered by severe DNA damage or impaired survival signals [114] [115]. We therefore examined the expression of apoptosis-related genes within the BCL-2 family, including *BBC3 (PUMA)*, *BAX,* and *BCL2*, which play critical roles in determining cell fate under stress conditions. *BBC3 (PUMA)* encodes a pro-apoptotic regulator that responds to cellular stress, particularly DNA damage, and as a direct target of p53, promotes apoptosis by neutralising anti-apoptotic proteins such as BCL-2 [116,117]. BAX, a pro-apoptotic protein, facilitates apoptosis by inducing mitochondrial outer membrane permeabilization (MOMP), which triggers cytochrome *c* release and subsequent caspase activation, ultimately leading to programmed cell death. In contrast, *BCL2* encodes an anti-apoptotic protein that inhibits BAX and BAK, preventing mitochondrial permeabilization and promoting cell survival. The dynamic balance between these pro- and anti-apoptotic proteins determines whether a cell undergoes apoptosis or shifts towards cell survival through activation of anti-apoptotic pathways [118]. The observed expression pattern was characterised by limited activation of pro-apoptotic signalling and a more pronounced induction of anti-apoptotic pathways, suggesting that HepG2 spheroids predominantly shifted towards cell survival rather than apoptosis under the tested conditions. After 24 h of exposure to BPA, BPAP, and BPC, HepG2 spheroids exhibited an early stress response marked by a modest upregulation of both *BAX* (pro-apoptotic) and *BCL2* (pro-survival), while *BBC3* (PUMA) expression remained unchanged, suggesting that apoptosis was either weak or regulated through alternative pathways (Fig. 3A–Table SM3). Following 96 h of exposure, *BAX* expression decreased while *BCL2* remained upregulated, suggesting that cells resisted apoptosis and shifted toward survival through activation of anti-apoptotic pathways. In particular, *BCL2* was upregulated after BPA exposure (2 and 4 μM for 5.2-fold and 2.4-fold, respectively), BPAP (1 and 2 μM for 3-fold and 3.8-fold, respectively), BPC (1 μM for 2.2-fold), and by the binary combinations BPA/BPAP (1 and 2 μM, for 5-fold and 4-fold, respectively) and BPA/BPC (1 and 2 μM, for 4.2-fold and 3.6-fold, respectively), reflecting the activation of anti-apoptotic pathways to counteract stress-induced cytotoxicity.

Previous studies have shown that BPA, BPAP, and BPC increase the

proportion of apoptotic cells after 96 h of exposure to low concentrations (0.01–10 μM), as indicated by Annexin V staining [19]. BPA-induced cytotoxicity has been linked to oxidative stress, which plays a central role in apoptosis by disrupting homeostasis and damaging macromolecules. Excess ROS can activate *BAX*, leading to mitochondrial dysfunction, cytochrome *c* release, and caspase-dependent cell death [119–122]. Recent research by Abdulhameed et al. (2022) has outlined multiple mechanisms through which BPA-induced oxidative stress triggers apoptosis in liver cells [123]. Similarly, Padberg et al. reported that BPC induces cytochrome *c* release in monolayer HepG2 cultures, further supporting the role of oxidative stress in apoptosis induction [72]. These findings emphasize the interplay between oxidative stress and programmed cell death, suggesting that the balance between apoptotic signalling and survival pathways determines cellular fate upon prolonged BPs exposure.

4. Conclusion

This study provides mechanistic insights into the cellular effects of BPA, BPAP, BPC, and their binary mixtures in an advanced *in vitro* 3D hepatic model - HepG2 spheroids. Exposure to the studied BPs induced measurable DNA damage, as detected by the comet assay, despite limited activation of well-characterized DNA repair and damage signalling pathways, indicating that BPs-induced genotoxicity occurs primarily through oxidative stress rather than direct DNA interaction. Metabolic processing of BPs might contribute to oxidative stress and the possible formation of reactive intermediates. Our findings underscore the utility of HepG2 spheroids as a human-relevant model for detecting metabolism-associated cellular responses, even though quantitative extrapolation to primary hepatocyte metabolism remains limited. Importantly, co-exposure of BPA with BPAP or BPC produced additive effects on DNA damage and cellular stress, reflecting the cumulative impact of the individual compounds rather than synergistic enhancement. These findings highlight the relevance of assessing mixture effects in toxicological studies, as human exposure typically involves simultaneous contact with multiple BPs rather than single compounds. From a regulatory perspective, the results indicate that BPA analogues, often marketed as safer alternatives, may themselves pose genotoxic risks. Comprehensive safety assessment should therefore account for indirect mechanisms, including oxidative stress, metabolic activation, and cumulative exposures. Overall, the study supports realistic, mixture-focused testing strategies to better protect human health from BPs-related hazards.

Ethics approval

The authors declare no involvement of Human Participants and/or Animals. In the study, human cell line was used, HepG2 cells (HB-8065™) obtained from the ATCC-Cell bank.

CRediT authorship contribution statement

Martina Štampar: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft. **Tim Ravnjak:** Data curation, Formal analysis, Methodology, Software. **Alja Štern:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Bojana Žegura:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2026.112232.

Data availability

Generated raw data of this study will be available in the DiRROS repository http://hdl.handle.net/20.500.12556/DiRROS-27240, upon acceptance for publication.

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