Different bisphenols induce non-monotonous changes in miRNA expression and LINE-1 methylation in two cell lines

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Abstract

4,4′-Isopropylidenediphenol (bisphenol A, BPA), a chemical substance that is widely used mainly as a monomer in the production of polycarbonates, in epoxy resins, and in thermal papers, is suspected to cause epigenetic modifications with potentially toxic consequences. Due to its negative health effects, BPA is banned in several products and is replaced by other bisphenols such as bisphenol S and bisphenol F. The present study examined the effects of BPA, bisphenol S, bisphenol F, p,p′-oxybisphenol, and the BPA metabolite BPA β-d-glucuronide on the expression of a set of microRNAs (miRNAs) as well as long interspersed nuclear element-1 methylation in human lung fibroblast and Caco-2 cells. The results demonstrated a significant modulation of the expression of different miRNAs in both cell lines including miR-24, miR-155, miR-21, and miR-146a, known for their regulatory functions of cell cycle, metabolism, and inflammation. At concentrations between 0.001 and 10 µg/ml, especially the data of miR-155 and miR-24 displayed non-monotonous and often significant dose–response curves that were U- or bell-shaped for different substances. Additionally, BPA β-d-glucuronide also exerted significant changes in the miRNA expression. miRNA prediction analysis indicated effects on multiple molecular pathways with relevance for toxicity. Besides, long interspersed nuclear element-1 methylation, a marker for the global DNA methylation status, was significantly modulated by two concentrations of BPA and p,p′-oxybisphenol. This pilot study suggests that various bisphenols, including BPA β-d-glucuronide, affect epigenetic mechanisms, especially miRNAs. These results should stimulate extended toxicological studies of multiple bisphenols and a potential use of miRNAs as markers.

Key words: epigenetic effects; miRNAs; LINE-1 methylation; bisphenols; BPA; BPS; BPF; p,p′-oxybisphenol; BPA β-d-glucuronide; HLF; Caco-2 cells

Highlights

1. Bisphenol A (BPA), its major metabolite BPA β-d-glucuronide, BPF, BPS, and p,p′-oxybisphenol are epigenetically active in HLF and Caco-2 cells.
2. Different bisphenols showed changes in the miRNA expression (miR-21, miR-24, miR-146, and miR-155) and LINE-1 methylation at concentrations between 0.001 and 10 µg/ml in the present pilot study.
3. The results often displayed non-monotonous dose–response relationships and were bisphenol, concentration, and cell-type specific.
4. The expression of miR-155 and miR-24 was most affected by the analyzed bisphenols, revealing U- or bell-shaped curves for most substances.
5. miRNAs could be promising markers for the bioactivity and toxicity of bisphenols.

Introduction

As the epigenome constitutes the interface between the environment and the genome, epigenetic dysregulation by environmental stressors is likely to disrupt different cellular processes that can contribute to cancer risk [1]. Many environmental chemicals such as bisphenol A (BPA) can act as endocrine-disrupting chemicals (EDCs), thereby impeding estrogenic activity and disrupting neurobehavioral development while stimulating carcinogenicity [2]. Furthermore, BPA was shown to cause epigenetic changes in various research models [3, 4], including non-monotonous dose–response relationships [5]. Non-monotonic dose–response curves (NMDRCs) are defined mathematically by a response where the slope of the curve changes sign from positive to negative, or vice versa, somewhere along the range of doses examined. In this case, the slope of the curve was not identified, but significant changes between the reference and investigated concentrations were assigned to verify non-monotonocity.

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As a dynamic regulatory network, the epigenome mediates cellular, tissue-dependent, and thus organismal responses to various stressors from the environment [1]. Epigenetic changes are heritable and lead to adjustments in the gene activity and expression without changing the DNA sequence [6]. These mechanisms encompass DNA methylation and histone modifications as well as non-coding RNAs including microRNAs (miRNAs), among others [7]. DNA methylation depicts the addition of a methyl group to a cytosine of a cytosine–guanine dinucleotide (CpG) site. In promoter regions, this reaction generally results in a suppressed binding activity of transcription factors to the related genes [8]. To estimate global DNA methylation levels, repetitive non-coding elements can be examined [9, 10]. Due to their potential to retrotranspose, a loss of methylation at these sites may lead to chromosomal rearrangements that can contribute to genomic instability and cancer development [11]. Long interspersed nuclear element-1 (LINE-1) is a mobile element comprising 17% of the human genome and is thus discussed to indicate global methylation status and DNA stability [12].

miRNAs regulate post-transcriptional gene expression and play an important role in RNA silencing [13]. Therefore, they have critical functions in fine-tuning a wide array of biological processes including cellular differentiation, metabolism, homeostasis, and apoptosis, and some can seriously influence carcinogenesis [14]. On the one hand, similar to that of protein-coding genes, the miRNA expression is regulated by multiple transcriptional networks as well as the epigenetic machinery. On the other hand, miRNAs can themselves repress key enzymes that drive epigenetic remodeling, generating regulatory circuits that have a significant effect on the transcriptional landscape of the cell [15]. Recent evidence also suggests that miRNAs can directly modulate gene transcription in the nucleus through the recognition of specific target sites in promoter regions [15].

As miRNA profiles are surprisingly informative [16], studies point toward the fact that they may be used as biomarkers for toxicity or human diseases [17, 18]. A recent systematic review reported that miRNA dysregulation by BPA can lead to serious health concerns by impeding numerous cellular signaling pathways even at low levels [19]. Furthermore, they have shown that miR-21 was the most commonly impacted miRNA [19]. miR-21 is known for its oncogenic and antiapoptotic activity and its overexpression in multiple solid tumors [15]. Likewise, the upregulation of miR-24 has been associated with poor cancer survival in a recent meta-analysis [20] and is related to apoptosis [21–23]. Target analyses of the most commonly altered gene expressions upon changes in miR-146a showed genes related to cancer and inflammatory and neural diseases [24]. Environmental chemicals such as air pollutants, heavy metals, and BPA have also been reported to stimulate DNA methyltransferase (LINE-1) hypomethylation [25], and LINE-1 activity is associated with various tumors predominantly found in the lung and colon [26]. Following BPA treatment in Michigan Cancer Foundation-7 (MCF-7) breast cancer cells, a study revealed a reduced methylation level of oncogenes and an increased methylation of tumor-suppressor genes [27]. Consequently, the accurate quantification of epigenetic effects is not only crucial to clinical diagnosis, drug development, and cancer treatment [28], but also to the authorization of chemicals. BPA is an environmentally omnipresent EDC that can enter the body via the skin, the respiratory system, and the gastrointestinal tract [19]. For example, due to different adverse health effects such as effects on reproduction, disruption of the endocrine system and neurobehavioral development, epigenetic effects, or immune disorders [3, 29], BPA is included in the candidate list of substances of very high concern according to Article 59 [10] of the Regulation (EU) No 1907/2006 (REACH) since 2017 [30] and also regulated in products such as baby bottles made of polycarbonate (Regulation (EU) 10/2011, amended by Regulation (EU) 2018/213), thermal paper (Regulation (EU) 2016/2235), and cosmetics (Regulation (EU) 1223/2009, latest amended by Regulation (EU) 2020/1684). Therefore, BPA will be replaced, but the question is: for other bisphenols (BPS, BPF, BPAPF, etc.) with similar properties—do they also have similar effects? A systematic review revealed that the potential of BPS and BPF to interfere with the hormonal system is comparable with the activity of BPA [31]. Accordingly, there is concern about the safety of other bisphenols. However, scarcely any report has focused on their epigenetic effects as well as on BPA’s major metabolite BPA β-d-glucuronide until now. Therefore, this pilot study aimed to examine the impact of various bisphenols on miRNA and methylation patterns in human Caco-2 and human lung fibroblast (HLF) cell lines. To analyze physiologically relevant concentrations to high doses of bisphenols and to consider NMDRCs, concentrations of 1 ng/ml–10 µg/ml were administered. As the various bisphenols have different water solubilities and could be adsorbed by plastic materials [32, 33], the actual concentrations were measured in the cell media before and after incubation.

Materials and Methods

Chemicals and Analytical Standards

Bisphenols were purchased in chemically pure quality from Sigma-Aldrich (BPA, BPS), Tokyo Chemical Industry (BPF), Accela (p,p’-oxybisphenol), and Santa Cruz Biotechnology (BPA β-d-glucuronide). Stock solutions were prepared from solid standards in concentrations of 10 mg/ml bisphenol in ethanol (EtOH) and were dissolved in cell culture medium to the respective concentrations.

Cell Culture and Treatment

Caco-2 cells and HLFs were kindly provided by Prof. Dr Walter Jäger, Department of Pharmaceutical Sciences, University of Vienna and Prof. Dr Siegfried Knasmüller, Institute of Cancer Research, Medical University of Vienna. These cell lines were chosen to imitate gastrointestinal and lung exposure. Cells were stored at −70°C in liquid nitrogen before thawing and then incubated in 25 or 75 cm² flasks at 37°C in a humidified atmosphere with 5% CO₂. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, Sigma-Aldrich), whereas the medium for HLFs consisted of RPMI 1640 (Thermo Fisher). Both media were supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen) and 10% (v/v) fetal bovine serum (Merck). Cells were passaged at 70–80% confluency after two to three days. Prewarmed phosphate-buffered saline (PBS, HyClone™) and Accutase® (BioWest) solution were used to rinse the cell monolayer and for subsequent cell detachment.

The cells were seeded at a density of 40 K cells/cm² in 24-well plates and were incubated for two to three days at 37°C in an incubator with 5% CO₂. Cell cultures were treated with 0.001, 0.01, 0.1, 1, and 10 µg/ml of BPA, BPA β-d-glucuronide, BPS, BPF, and p,p’-oxybisphenol for 24 h. Untreated cell cultures were used as controls. In addition, EtOH (0.01% (v/v)) was chosen as the respective solvent control (indicated in the graphs (Figs 1–3) and used for normalization) since no significant differences were found between the mean value of the EtOH (0.01% and 0.1% (v/v)) and the untreated controls.
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Figure 1: Effects of BPA, BPA β-α-glucuronide, BPS, BPF, and p,p′-oxybisphenol on miR-24, miR-155, miR-21, and miR-146a expression levels in Caco-2 cells. Cells were exposed to 0.001, 0.01, 0.1, 1, and 10 µg/ml of the respective bisphenol for 24 h, and the miRNA expressions were detected by qRT-PCR. Data are shown as the mean of relative fold changes ± SD of a minimum of three replicates per treatment.

**P < 0.05; ***P < 0.01; ****P < 0.0001 compared to the solvent control.

RNA Extraction and Quantitative RT-PCR Analysis

After treatment, the total RNA of the cells was isolated using miRNeasy Micro Kit (Qiagen) as per the manufacturer’s instructions for cell culture. RNA quantity and quality were assessed with a Nanodrop One spectrophotometer (Thermo Scientific). Subsequent reverse transcription and cDNA amplification were undertaken according to the protocol for TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher) and obtained cDNA was stored at −20°C. Quantitative real-time PCR was performed using TaqMan™ Fast Advanced Master Mix and TaqMan™ Gene Expression Assays (Thermo Fisher) for miR-24-3p, miR-155-5p, miR-21-5p, and miR-146a-5p according to the user guide supplied by Thermo Fisher. To calculate the miRNA relative fold changes, the ΔΔCt method was used with miR-93 as an endogenous control. This stable internal reference has recently been recommended for human cancer cell lines and plasma samples [34, 35].

DNA Extraction, Bisulfite Conversion, and DNA Methylation Analysis

Global DNA was extracted from the cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol for cell culture. DNA samples were stored at −20°C before the bisulfite conversion was performed. To convert unmethylated cytosines to uracil and thus to distinguish between the
Figure 2: Impact of BPA, BPA β-d-glucuronide, BPF, BPS, and p,p’-oxybisphenol on miR-24 and miR-155, miR-21, and miR-146a expression levels in HLFs. Cells were treated with 0.001, 0.01, 0.1, 1, and 10 µg/ml of the respective bisphenols for 24 h, and the miRNA expressions were detected by qRT-PCR. Data are shown as the mean of relative fold changes ± SD of three replicates per treatment *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001 compared to the solvent control. Abbreviations: BP, bisphenol

latter and unaltered 5-methylcytosines, bisulfite treatment was carried out using the Qiagen EpiTect Bisulfite Kit (Qiagen) as explained in the manufacturer’s instructions. DNA concentrations were determined with a Nanodrop One (Thermo Fisher Scientific) spectrophotometer. LINE-1 promoter methylation was assessed by methylation-sensitive high-resolution melting (HRM) curve analysis. Each reaction was performed in technical duplicates using the EpiTect HRM PCR Kit (Qiagen) according to the EpiTect® HRM™ PCR handbook. Methylation standards of 0% and 100% were mixed to obtain 0, 25, 50, 75, and 100% methylated samples and were included in the assay. PCR amplification and HRM analysis were performed with a Rotor-Gene® Q (Qiagen) containing a 72-well rotor. The data were analyzed with Rotor-Gene® Series Software Version 2.3.1 that produced normalized melting curves. After exporting relative fluorescence units to GraphPad Prism (Version 6), the area under the curve was calculated and linear regression was applied to interpolate each sample value from the data of the standard curve.

Chemical Analysis
The supernatant of the cell cultures (approximately 800 µl) was transferred into 1.5 ml centrifuge tubes and stored at −18°C until analysis at the Environmental Agency Austria. For sample preparation, the supernatants were centrifuged (10 min, 17 000 rpm) to separate the solid matrix from the liquid phase. In total, 200 µl of the supernatant were spiked with an isotope labeled standard
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Figure 3: LINE-1 methylation levels of Caco-2 cells in response to various concentrations of BPA, BPA β-D-glucuronide, BPF, BPS, and p,p′-oxybisphenol. Cells were treated with 0.001, 0.01, 0.1, 1, and 10 µg/ml of the respective bisphenol for 24 h, and the methylation analysis was performed by HRM. Data are shown as the mean ± SD of a minimum of three replicates per treatment *P < 0.05, and **P < 0.01 compared to the solvent control. Abbreviations: BP, bisphenol (BPA Ring d8) and analyzed using a Ultra High Performance Liquid Chromatography system coupled to triple quadrupole tandem mass spectrometry (Waters Aquity UPLC and Waters Xevo TQ-S) in negative electrospray ionization mode. Chromatographic separation was performed on a reversed-phase column. Detection of the different bisphenols was conducted with typical fragment ions ([M–H]± for each compound in the multiple reaction mode. Quantification was performed using internal (for bisphenol A) and external calibration curves ranging from 0.5 ng/ml to 100 ng/ml. Higher concentrated supernatants were diluted with HPLC water to reach suitable concentrations. To check for matrix effects all dilution levels of the two cell lines were spiked with the bisphenol and recovery rates of this spiking-trial were considered in the calculation of the results.

Statistical Analysis
All experiments were performed at least in triplicates, and the results are presented as the mean of each treatment with standard deviation (SD) of the mean. Subsequent data analysis and presentation were done using GraphPad Prism software (Version 6). Data were subjected to analysis of variance, and the Sidak test for multiple comparisons was used to separate the means that were considered statistically significant. Statistical significance was set to a P-value of ≤ 0.05.

MiRNA Target Prediction Analysis
The application miRNet (www.mirnet.ca/) was used to predict target genes and networks of miR-24-3p, miR-155-5p, miR-21-5p, and miR-146a-5p. The obtained non-tissue-specific gene targets were classified into functional groups with an integrated device in miRNet that makes use of miRTarBase Version 8.0 database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The most frequent cellular functions and/or signaling pathways of all four miRNAs as well as specific genes that are regulated by three of the four examined miRNAs were selected for the Results section of this paper.

Results
MiRNA Expression in Bisphenol-treated Caco-2 Cells
Caco-2 cells and the HLF cell line were used to analyze the epigenetic effects of bisphenols. After incubation of Caco-2 cells with bisphenols for 24 h, miRNA levels of BPA-responsive miRNAs were evaluated. Based on preliminary experiments with BPA and estrogen analyzing various miRNAs (including miR-19b, miR-21, miR-27b, miR-122, miR146a, miR-155, and miR-205), miR-24, miR-155, miR-21, and miR-146a were selected for further analysis (Fig. 1A–T), as they showed the most promising effects. A significant upregulation of miR-146a (Fig. 1D) was observed after exposure of 0.1 and 1 µg/ml BPA (P < 0.001, P < 0.01, respectively) versus the value of the EtOH control. The bar graph suggests a concentration-dependent bell-shaped curve. BPA β-D-glucuronide significantly downregulated the expression of miR-21 (Fig. 1H) at a concentration of 1 µg/ml (P < 0.05) as compared with the control value. In addition, a significantly lower miR-21 level (Fig. 1K) was observed with 0.1 and 10 µg/ml BPS (P < 0.05 and P < 0.001, respectively), whereas BPS attenuated miR-155 expression (Fig. 1J) at a concentration of 1 µg/ml (P < 0.05). BPF treatment points towards a U-formed dose–response curve for miR-24 (Fig. 1M) with a significantly reduced miRNA level at 0.1 µg/ml (P < 0.05). Incubation with 0.1 µg/ml BPF also lowered the levels of miR-21 (Fig. 1O). Likewise, miR-155 expression (Fig. 1N) was generally diminished after BPF.
exposure leading to significant downregulations at concentrations of 0.1 and 10 µg/ml BPF (P < 0.05 and P < 0.01, respectively) compared with the solvent control. p,p’-Oxybisphenol significantly reduced miR-24 (Fig. 1T) levels at low doses (0.001 and 0.1 µg/ml; P < 0.0001 and P < 0.01, respectively) in comparison with the ETOH control.

MiRNA Expression in Bisphenol-treated HLFs

After analyzing the effects of bisphenol compounds on miRNA profiles in Caco-2 cells, the same 24 h experiment was carried out using HLFs (Fig. 2A–T). Following BPA exposure, we observed a significant upregulation of miR-24 (Fig. 2A), miR-155 (Fig. 2B), miR-21 (Fig. 2C), and miR-146 (Fig. 2D) levels with 1 µg/ml BPA (P < 0.05) compared with the value of the ETOH control. Metabolite BPA β-D-glucuronide significantly reduced the expressions of miR-155 (Fig. 2F) at 0.1 µg/ml (P < 0.05) and of miR-21 at 0.001, 0.01, 0.1, and 10 µg/ml (P < 0.05) as compared with the solvent control. Regarding BPS, a concentration of 1 µg/ml (Fig. 2I) led to a significantly elevated miR-24 expression (P < 0.05) and enhanced miR-146 levels (Fig. 2L) at 10 µg/ml (P < 0.01) versus the ETOH control. In contrast to this, significantly reduced levels of miR-21 (Fig. 2O) were observed with 0.01, 1, and 10 µg/ml BPF (P < 0.01) compared with the ETOH solvent control. p,p’-Oxybisphenol triggered an enhanced expression of miR-24 (Fig. 2Q) at a concentration of 1 µg/ml (P < 0.01) and reduced the levels of miR-21 (Fig. 2T) at 0.001 µg/ml (P < 0.05) and miR-146 (Fig. 2T) and 10 µg/ml (P < 0.05).

LINE-1 Methylation Level in Bisphenol-treated Caco-2 Cells

To evaluate potential modulations in LINE-1 promoter methylation, Caco-2 cells were exposed to increasing concentrations of bisphenols for 24 h. BPA (Fig. 3A) decreased the methylation level at the lowest and the highest concentration observed (0.001 and 10 µg/ml, P < 0.05) suggesting a bell-shaped dose-response relationship. Likewise, p,p’-oxybisphenol (Fig. 3E) reduced the methylation level of the LINE-1 promotor region at a concentration of 0.01 µg/ml (P < 0.05), while 10 µg/ml led to a small increase (P < 0.01) contra the solvent control, thus revealing an NMDR.

Analysis of the Bisphenol Recovery Rates in the Cell Supernatants

The measurement of BPA in the cell media supernatants showed good recovery rates related to the theoretically estimated concentrations (97.8% ± 5% for Caco-2 cells; 104% ± 9% for HLFs). Preliminary results showed higher deviation for other bisphenols (e.g. p,p’-oxybisphenol) (not lower than a recovery of 80%). To check matrix effects all dilution levels of the two cell lines were spiked with the bisphenols and recovery rates were calculated. Some bisphenols (e.g. BPS or p,p’-oxybisphenol) showed a higher influence of matrix effects during the measurement, other bisphenols (e.g. BP) were not influenced in a significant way during the measurement. Recovery rates determined from the spiking trial were considered in the calculation of the final concentrations. Other effects that may have influences such as adhesive effects of bisphenols to cell-particles could not be investigated during this study. The determination of the real concentrations of BPA β-D-glucuronide was not possible due to strong matrix effects caused by the cell media.

Potential Gene Targets of Selected miRNAs: Networks Analysis and Functional Pathways

In order to elucidate possible molecular mechanisms of bisphenol-induced toxicity, the miRNA network tool miRNet was used to explore miRNA target genes and the related signaling pathways and cellular functions. Pathway analysis of miR-24, miR-155, miR-21, and miR-146a classified their target genes and the most enriched and significantly associated biological functions can be seen in Table 1. Cancer- and immune system-related cell signaling pathways, other signal transduction pathways, and genes associated with focal adhesion, the cell cycle, RNA transport, and apoptosis were among the top-ranked pathways. Moreover, Table 2 shows the predicted target genes of the selected miRNAs that were identified to be regulated by at least three out of these four miRNAs.

**Discussion**

Human studies have reported that even nanomolar exposure concentrations of BPA are associated with an increased risk of...
metabolic, cardiovascular, and neurological diseases as well as cancers [36, 37]. As a result of these health concerns and a subsequent ban of BPA in baby products and receipts, industries have started to replace BPA with other bisphenol-group compounds such as BPS and to a smaller extent also BPF, although studies have already questioned their safety [31]. Moreover, our research showed that BPA is epigenetically highly active, which possibly links bisphenol exposure to the occurrence of various diseases [4, 38]. Therefore, the goal of our study was to investigate epigenetic effects of BPA, its metabolite BPA β-d-glucuronide, and other bisphenols such as BPS, BPF, and p.p′-oxybisphenol using miRNA profiling and LINE-1 methylation analysis. Due to the number of selected bisphenols, a limitation of this study is the small number of replications and tested concentrations. Therefore, we consider the present project as a pilot study. However, to our knowledge, this is one of the first reports analyzing epigenetic responses to different bisphenols and taking into consideration the metabolite BPA β-d-glucuronide in Caco-2 as well as in HLF cells.

In the current study, different bisphenols altered miRNA expressions and LINE-1 methylation in distinct ways. As a dynamic regulatory network, the epigenome mediates cellular, tissue-dependent, and organismal responses to various stressors from the environment. It is interesting to note that the bar charts of most bisphenol-group compounds revealed NMDRCs that often showed a reduced miRNA expression at the mean concentration of 0.1 µg/ml in Caco-2 cells. NMDRCs can be found in the literature for EDGs and BPA in a variety of biological systems including cultured cells, whole organ cultures, laboratory animals, and humans [5, 39]. Following BPA treatment in Caco-2 cells, the bar charts of not only miR-146, but also of miR-155 (Fig. 1B) tend to display non-monotonic dose–response relationships. Consistently, the impact of BPA β-d-glucuronide on miR-24 (Fig. 1E) and miR-155 levels (Fig. 1F) suggests NMDRCs showing the lowest expression at concentrations of 0.1 and 0.01 µg/ml, respectively. p.p′-Oxybisphenol treatment also resulted in non-significant NMDRCs regarding miR-155 (Fig. 1R); the lowest miR-155 expression was detected at 0.1 µg/ml, whereas 0.001 µg/ml p.p′-oxybisphenol tended to elevate miR-155 (Fig. 1R). In HLF experiments, observed dose–response curves seemed less pronounced (e.g. BPF regarding miR-24, miR-155, and miR-146; Fig. 2M, N, and P). Comparable non-monotonic effects could be seen after incubation with BPA as well as BPS (here only regarding miR-24; see Fig. 2I). Moreover, our results indicated that miR-155 was generally most affected by different bisphenols as can be observed by a consistent downregulation in Caco-2 cells.

Regarding LINE-1 methylation, the data suggest a bell-shaped curve after BPA exposure and a partly U-shaped dose–response curve after treatment with p.p′-oxybisphenol. Similarly, although non-significant, metabolite BPA β-d-glucuronide (Fig. 3B) tended to attenuate LINE-1 methylation at the lower concentrations (0.001–0.1 µg/ml) but led to an elevation in the µg/ml range (1 and 10 µg/ml).

Therefore, our data demonstrated that other bisphenols can affect miRNA and methylation levels and that the BPA metabolite BPA β-d-glucuronide is also biologically active with regard to miRNA modulation. BPA is metabolized to BPA β-d-glucuronide in the liver, its predominant metabolite, and excreted via the kidneys [40]. For a long time, research suggested that BPA β-d-glucuronide was inactive due to its inability to bind to estrogen receptors [41]. Nonetheless, it could exert influence via non-estrogenic activity, which was revealed for BPA before (e.g. glucocorticoid receptor) [42]. In human serum and urine samples, BPA β-d-glucuronide was shown to be present at higher concentrations than free BPA (up to 11.9 and 18.7 ng/ml, respectively) [43] and could also be found in human blood [40]. Boucher et al. demonstrated that BPA β-d-glucuronide was able to induce adipogenesis in 3T3-L1 cells [44].

It could be observed that the results showed differences between the two cell lines. Higher concentrations of bisphenols in HLFs often resulted in an increased expression of miRNAs. The differentially expressed miRNAs suggest that they were altered individually and not via changes in the miRNA processing machineries. This is supported by a study showing that neither DROSHA nor DICER was modulated upon BPA treatment [45].

MiRNAs play a role in mRNA translation by silencing or degrading mRNA products thereby regulating multiple cellular processes. MiR-155 is enhanced in B-cell lymphomas, breast, lung, colon, and pancreatic tumors and hematopoietic malignancies [15] and is associated with inflammation [46]. Not many studies have evaluated the impact of BPA and its substitutes on miR-155 expression so far. In HA cells (hemangiomia: HDEC and CRL-2686 cells), nanomolar concentrations of BPS reduced miR-155 levels and thus triggered an increase of basic fibroblast growth factor expression, which is related to HA development [47]. Similarly, our data displayed a downregulating effect after addition of moderate concentrations of all analyzed bisphenols in Caco-2 cells, whereas higher doses of BPA (1 µg/ml) and BPS (10 µg/ml) in HLFs tended to increase miR-155 expression, likely indicating cell type- and concentration-related differences. An upregulation of miR-155 was also observed in an in vitro model of bovine cumulus-oocyte complexes with 50 µg/ml BPA [48] and in a study applying 0.025 µg/ml BPA in human placental cells, but for a period of six days [49].

As a key miRNA of numerous physiological processes such as proliferation and apoptosis, miR-24 was also shown to be overexpressed in pathological conditions such as cancer, multiple sclerosis, hyperglycemia, and Alzheimer’s disease [50]. In our study, the mean concentration (0.1 µg/ml) of BPF and p.p′-oxybisphenol and other bisphenols significantly decreased, and BPA β-d-glucuronide tended to attenuate miR-24 in Caco-2 cells. In line with these results, Cho et al. used microarray analysis to detect differently regulated miRNAs in male testes of mouse Sertoli cell line TM4 and found two-fold lower miR-24 levels after 20 µg/ml BPA exposure for 24 h [51]. In contrast to this, we detected a significant upregulation with BPA, BPS, and p.p′-oxybisphenol in HLFs, although only at one concentration (1 µg/ml). Again, these differences could possibly be attributed to different doses of BPA and distinct cell types.

BPA was shown to increase miR-146a involved in endocrine regulation and immune homeostasis [52]. Consistent with our BPA results, De Felice et al. found a significantly higher expression of miR-146a in placental samples of pregnant women living in areas with a high BPA pollution [24]. As fetuses are undergoing rapid growth and development, prenatal BPA exposure is specifically critical [53]. In human placental cell lines, BPA led to an attenuated proliferation and an elevated sensitivity to the DNA damaging agent bleomycin [49]. Similarly, BPA triggered enhanced miR-146a expression in testicular Leydig cells of mice thereby impeding steroidogenesis via Metastasis Associated 1 Family Member 3 mRNA repression [52]. Nonetheless, except for an upregulation with 10 µg/ml BPS in HLFs, we did not find significantly induced miR-146a expression levels in response to the other bisphenol compounds, suggesting that this dysregulation might be restricted to BPA. However, the highest concentrations of p.p′-oxybisphenol decreased miR-146a, although most BPA substitutes did not result in any changes. A stable profile of miR-146a was also found after
BPA or BPS addition to bovine oocytes at the LOAEL of 50 μg/ml [48], revealing possible tissue or species-dependent differences. As a miRNA controlling antiapoptotic regulation, miR-21 is crucial for cell survival by suppressing apoptotic gene expression [54]. Thus, it can be speculated that a partial downregulation observed after BPF, BPA β-D-glucuronide (HLF), and BPS (Caco-2 cells) in the current study could enhance apoptosis-related transcription. Similar to our results following exposure to certain bisphenols, BPA also reduced the expression level of miR-21 in 3T3-L1 preadipocytes (80 μM) [55] and MCF-7 cells mediated by ER (estrogen receptor)-α [56]. On the other hand, miR-21 is also called an onco-miR due to its inhibiting effect on the tumor-suppressor gene PTEN [57], which is supposed to be associated with BPA's cancer-promoting impact in reproductive tissues [48]. In Caco-2 cells, we saw a slight upregulation following treatment with p,p′-oxybisphenol. Tilghman et al. explained differential effects of BPA on miR-21 in MCF-7 breast cancer cells by ER-positive or -negative signaling pathways [56].

Using miRNet, a miRNA-centered pathway database, we identified numerous gene targets that are connected to modulations in miR-24, miR-155, miR-21, and miR-146a. Pathway enrichment of these miRNAs demonstrated that the most common targets are related to cancer, the mitogen-activated protein kinase signaling pathway, and focal adhesion. Furthermore, changes in the analyzed miRNAs could cause dysregulations in cell cycle progression (e.g. E2F2), RNA transport, and apoptosis (e.g. APAF1). Likewise, alterations in various signaling pathways were predicted by miRNet including Toll-like receptor (merging into NF-κB pathway). Likewise, alterations in various signaling pathways were predicted by miRNet including Toll-like receptor (merging into NF-κB pathway), T-cell receptor (e.g. NFAT), TGF-β, p53, and Wnt signaling pathway. These cascades are mostly linked to general cellular functions such as cell proliferation and differentiation as well as immune responses. Several studies have shown that dysfunctions of these signaling pathways are associated with tumorigenesis [58–60]. In our target analysis, we found genes that were potentially modulated by three out of the four investigated miRNAs (e.g. TGFB1, MYC, ErbB, BRCA1, EGFR). Various studies confirm changes in the expression of these genes or proteins upon bisphenol exposure: in breast cancer cell lines, low concentrations of BPFA [61] and tetrachloro-BPA [62] enhanced protein expression of c-Myc and cyclin D1, important for cell cycle progression. In mice, this led to an activation of epidermal growth factor receptor (EGFR) and subsequent increases in growth factor expression related to mammary carcinoma [63]. EGFR, linked to the erythroblastic leukemia viral oncogene signaling pathway, is the receptor for epidermal growth factor and TGF-α, among others, and thus stimulates cell growth while inhibiting apoptosis, which is why it is also considered as an oncogene [64]. In a murine and intestinal epithelial cell model, the transcriptional activity of NF-κB, production of inflammatory cytokines, as well as apoptotic processes were promoted by BPA, explaining the risk for BPA-induced gut injury [65].

Aberrant DNA methylation due to BPA exposure has been reported in various studies [4, 38, 66]. We therefore assessed LINE-1 methylation in Caco-2 cells, which is a marker of global methylation status [12]. As a common element of the human genome, its hypomethylation is linked to genomic instability and a higher risk of cancer development [26]. Our analysis of cytosine-guanine dinucleotide shores in the LINE-1 promoter revealed significantly lower methylation following the lowest and highest concentration of BPA and thus showing an NMDRC. Likewise, treatment with low-dose p,p′-oxybisphenol resulted in diminished methylation, whereas a rise was observed after the addition of the highest concentration. We found a similar trend towards a non-monotonous dose–response effect with BPA β-D-glucuronide, indicating that both—low and high—concentrations can affect DNA methylation. Most other bisphenols resulted in a diminished methylation level, which can result in an enhanced gene expression similar to changes in the miRNA expression. Our results are mostly consistent with Miao et al. who found lower methylation levels at LINE-1 sites in the sperm, but not in the peripheral blood, of occupationally BPA-exposed men in China [25]. Nahar et al. showed organ-dependent methylation-modifying effects: hypermethylation of LINE-1 with BPA was only detected in fetal human placenta [67]. The only study that also examined BPA as well as BPS and BPF found a tendency of decreased LINE-1 methylation in MCF-7 breast cancer cells [66]. Thus, BPA methylation aberrations may only be visible in certain cell lines or tissues. We thus recommend to further validate the effects and possible mechanisms for differences in the results. Likewise, LINE-1 methylation is an indicator of global DNA methylation, so that the cytosine-guanine dinucleotide islands of more specific DNA promoters should be explored in future studies.

As can be seen in our results mostly describing NMDRCs, the application of several concentrations is important to show the whole spectrum of effects and to not limit the comparability with other studies. We therefore highly recommend making use of various concentrations in future bisphenol research. Therefore, a strength of our study is not only the application of concentrations that are physiologically and toxicologically relevant but also the use of different bisphenols. Likewise, we investigated the actual BPA concentrations in the cell suspensions by UHPLC and found good recovery rates.

Conclusion

Aiming to study epigenetic effects of BPA in comparison with other bisphenols, we explored miRNA profiles and LINE-1 methylation after treatment with various concentrations of BPA, its major metabolite BPA β-D-glucuronide, BPF, BPS, and p,p′-oxybisphenol. Bisphenols are discussed to affect cell growth, cell invasion, apoptosis, and other cellular processes and miRNAs contribute to their regulation. Therefore, miRNAs might belong to biomarkers for the bioactivity of bisphenols. Although being preliminary, the results showed that miRNA and methylation patterns were deregulated by different bisphenols, which often displayed non-monotonous dose–response curves. These effects were not only cell-type specific but also highly individual concerning the selected bisphenols. Changes in the expression of essential miRNAs could also be a new approach to study possible toxic epigenetic consequences of bisphenols and the metabolite. Furthermore, not only the effects on the molecular targets of miRNAs need to be further investigated, but also the epigenetic activity of BPA β-D-glucuronide should be discussed for its toxicological relevance in future studies.

Supplementary data

Supplementary data is available at EnuEpig online.

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