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Cadmium and decabrominated diphenyl ether mixture: *In vitro* evaluation of cytotoxic, prooxidative and genotoxic effects

Marijana Curcic[a,*], *Ksenija Durgo*[b], *Nevenka Kopjar*[c], *Mario Ancic*[b], *Slavica Vucinic*[d], *Biljana Antonijevic*[a]

[a] *Department of Toxicology "Akademik Danilo Soldatovic", University of Belgrade - Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*

[b] *Department for Biochemical Engineering, Faculty of Food Technology and Biotechnology, Zagreb University, Krsnjavoga 25, 10000 Zagreb, Croatia*

[c] *Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10001 Zagreb, Croatia*

[d] *National Poison Control Center, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Serbia*

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ABSTRACT

In order to look into the combined effects of Cd and BDE-209 *in vitro*, this study was aimed at examining cytotoxic and genotoxic effects using the human colon carcinoma cell line (SW 480) as a biological test system as well as to determine if ROS production was one of the possible mechanisms of their mixture action. This cell line was chosen since ingestion of contaminated food/water represents an important route of exposure to both Cd and BDE-209, which is why intestinal cells are a common target for the contaminants present in food and water. Cells were treated with single Cd in concentrations of 2.5, 7.5 or 15 μg Cd/mL (corresponding to 22, 67 or 134 μM), single BDE-209 in concentrations of 2.5, 5 or 10 μg BDE209/mL (corresponding to 2.5, 5 or 10 μM), and their mixtures (design 3 × 3).

Mixture of Cd and BDE-209 has shown clear potential to reduce the viability of SW 480 cells, as evidenced by cytotoxicity associated with ROS generation. Factorial regression models used to identify type of interaction revealed synergism related to mixture citotoxicity and additive interaction for the effect on ROS production. The results from this introductory study could contribute to the issue of possible adverse effects associated with co-exposure and body burden with two persistent environmental pollutants, Cd and BDE-209.

1. Introduction

The toxicology of mixtures has recently undergone a remarkable and productive development as compared to other commonly-used procedures, which rely on examining effects based on single-substance evaluation. In fact, the toxicity of pollutant mixtures, which represents real environmental conditions in a more accurate manner, may assist in the determination of toxicologically relevant effects. Several

* Corresponding author. Tel.: +381 11 3951248; fax: +381 11 3972840.
E-mail addresses: makitox@pharmacy.bg.ac.rs, marijanacurcic@yahoo.com (M. Curcic), kdurgo@pbf.hr (K. Durgo), nkopjar@imi.hr (N. Kopjar), ancicm@gmail.com (M. Ancic), zarkovuc@eunet.rs (S. Vucinic), abiljana@pharmacy.bg.ac.rs (B. Antonijevic).
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well-designed and decisive studies dealing with the mixture toxicology of heavy metals and persistent organic chemicals have been carried out (Bandele et al., 2012; Buha et al., 2013; Dai et al., 2013; EC, 2009; EPA, 2008; Gennings et al., 2005; Leeman et al., 2013; Montaño et al., 2011; Spurgeon et al., 2010; Teuschler et al., 2002; Theobald et al., 2012), including the mixture of the heavy metal cadmium (Cd) and the flame retardant decabrominated diphenylether (BDE-209) (Curcic et al., 2012; Zhang et al., 2012b).

The presence of Cd and BDE-209 in the environment results in combined exposure and possible toxic effects that are difficult to predict solely based on data on single substances. Cd is widely present in the environment, naturally or as a result of anthropogenic activities, while BDE-209 could be released into the environment by different processes, such as emissions from the production of BDE-209-containing products (ATSDR, 2004, 2012; Birnbaum and Staskal, 2004; Darnerud et al., 2001; De Wit, 2002; EFSA, 2011, 2012; Hardy et al., 2008). Humans are exposed to a mixture of Cd and BDE-209 mainly *via* air and food, which is confirmed by their presence in human tissues (ATSDR, 2004, 2012; EFSA, 2011, 2012; Roosens et al., 2010). The average blood Cd concentration for non-smoking populations with non-occupational exposure rarely exceeds 1 μg/L (Fontain et al., 2008). Environmental exposure can elevate median blood Cd levels to above10 μg/L, while workers occupationally exposed to Cd by inhalation may have levels reaching up to 50 μg/L (ATSDR, 2012). The BDE-209 plasma levels among the general population around the world varied from non-detectable to 17 μg/kg lipid weight (Karlsson et al., 2007).

The presence of both Cd and BDE-209 in the human body may induce either individual or combined toxicity. Cd is classified by the International Agency for Research on Cancer (IARC) as a "human carcinogen", while BDE-209 is classified as a "possible human carcinogen" (IARC, 2013).

This study examines the combined effects of Cd and BDE-209 as a continuation of the interesting results obtained regarding their *in vivo* toxicity on thyroid hormones in rats in a previous study (Curcic et al., 2012). Cd + BDE-209 mixtures lowered thyroid hormone levels more potently than Cd or BDE-209 alone. The combined effect on the levels of T3, FT3, T4, and FT4 were interpreted as additive. Furthermore, Zhang et al. (2012a) studied the ecotoxicological joint effects of BDE-209 and Cd contamination on soil microbes and enzymes. The interaction between BDE-209 and Cd was reported as synergistic, antagonistic or additive, depending on the effect, exposure dose, and time.

Cyto- and/or genotoxicity of single Cd and single BDE-209 have been observed in different cell lines (Al-Assaf et al., 2013; Barrouillet et al., 2001; Djokic et al., 2014; He et al., 2008; Hu et al., 2007; Liu et al., 2009; Pellacani et al., 2012; Riva et al., 2007; Reistad and Mariussen, 2005; Zhang et al., 2012b). DNA damage in acute Cd toxicity is related with liberated reactive oxygen species (ROS) while the roles of ROS in chronic Cd toxicity and carcinogenesis have been controversial depending on experimental conditions (Liu et al., 2009). The mentioned effects are solely documented for single chemicals, and there are no such data on Cd and BDE-209 mixtures. In order to look into the combined effects of Cd and BDE-209 *in vitro*, this study was aimed at examining cytotoxic and genotoxic effects using the human colon carcinoma cell line (SW 480) as a biological test system as well as to determine if ROS production was one of the possible mechanisms of their mixture action. This cell line was chosen since ingestion of contaminated food/water represents an important route of exposure to both Cd and BDE-209, which is why intestinal cells are a common target for the contaminants present in food and water.

2. Materials and methods

2.1. Human cell lines

The human colon carcinoma cell line (SW 480), as a model for the intestinal system, was provided as a donation by the Rudjer Boskovic Institute, Zagreb, Croatia. Cells were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (GIBCO, USA), supplemented with 10% foetal bovine serum (GIBCO, USA), and 1% of penicillin/streptomycin solution (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Chemicals

Cadmium-chloride ($CdCl_2 \times H_2O$) was purchased from Merck (Darmstadt, Germany), while BDE-209 (98% pure) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from commercial sources.

The concentration ranges used in the experiment were: 2.5, 7.5 or 15 μg Cd/mL (corresponding to 22, 67 or 134 μM), 2.5, 5 or 10 μg BDE-209/mL (corresponding to 2.5, 5 or 10 μM, respectively), and their mixtures (design 3×3). Concentrations of Cd were chosen based on different exposure levels including low, moderate and high exposure (Brzóska and Moniuszko-Jakoniuk, 2005). Stock solutions of $CdCl_2$ and BDE-209 were obtained by dissolving pure chemicals in phosphate-buffered saline (PBS; pH 7.4) solution and dimethyl sulphoxide (DMSO) respectively. Working concentrations were prepared by dissolving stock solutions in Dulbecos Modified Eagle Medium (DMEM) medium. The highest concentration of DMSO did not exceed 0.2%. Potential toxic effect of 0.2% DMSO was measured and compared to negative controls in order to eliminate false results. Because of the high photo degradability of BDE-209, all analyses were done under dark conditions.

2.3. Neutral red assay

Cytotoxicity of Cd and/or BDE-209 was determined by neutral red (NR) assay (Babich and Borenfreund, 1990). Briefly, 200 μL of SW 480 cells suspension was seeded in dark 96-plate wells at a concentration of 3×10^4 cells/mL. After a 24-h incubation, cells were treated with appropriate concentrations of Cd and/or BDE-209 solutions for 72 h during the exponential phase of growth to determine cytotoxic effect and/or cell cycle arrest caused by Cd/BDE-209. A negative control with 0.2% DMSO and PBS solution was maintained in the same conditions. After treatment, the medium with Cd, BDE-209, and Cd/BDE-209 was removed, cells were washed twice with 10 mM PBS, and 100 μL of 50 μg/mL NR solution was added to each well. NR dye accumulates in lysosomes of live cells because it is