IN VITRO STUDY TOWARDS THE ENDOCRINE ACTIVITY AND THE GENOTOXIC POTENTIAL OF MIGRATION PRODUCTS FROM PLASTIC BABY BOTTLES

C. Simon[1], M. Onghena[2], A. Covaci[2], E. Van Hoeck[3], J. Van Loco[3], M. Elskens[4], K. Van Langenhove[4], T. Vandermarken[4], H. Demaegdt[5], B. Mertens[3], K. Vandermeiren[5], M.L. Scippo[1]

[1]Laboratory of Food Analysis, FARAH – Veterinary Public Health, Department of Food Sciences, CART, University of Liège, Bd de Colonster, 20, BatB43b, Liège, BELGIUM; [2]Toxicology center, Faculty of sciences, University of Antwerp, Universiteitplein 1, Wilrijk, BELGIUM, [3]WIV-ISP, Scientific Institute of Public Health, R. Juliette Wystman, 14, Brussels, BELGIUM ; [4]Analytical and Environmental Chemistry, Vrije Universiteit Brussel, Bd de la plaine 2, Ixelles, BELGIUM ; [5]CODA-CERVA, Leuvensesteenweg, 17, Tervuren, BELGIUM

Introduction

Bisphenol A (BPA) is documented in several studies to be a well-known source of food contamination [1]. The measurements of BPA in human fluids and tissues highlighted that its presence in food constitutes the primary route of human exposure [2]. Some studies showed that BPA, which can act as a hormone and disrupt normal endocrine function, may be associated to several health problems and diseases. Recently, the European food safety authority conducted a risk assessment on BPA and concluded that though studies concerning the potential health hazards associated with BPA are still uncertain, they are suggesting a potential negative effect on the human body. Following that assessment, the European Union took a series of measures, including a ban for the import and sale of polycarbonate (PC) baby bottles to reduce BPA exposure of infants [3].
Plastic alternatives to PC, which have massively appeared on the Belgian market, include polypropylene (PP), silicone, polyamide (PA) and polyethersulfone (PES). Although there are several studies on BPA migration from PC into foodstuff under a variety of conditions [4,5,6], there is almost no information on the consequences for human health of the migration of chemicals from PC alternatives, including baby bottles commonly labelled "BPA free". In a recent opinion (No. 8697, 11.03.2010), the Belgian Superior Health Council issued its concern regarding the alternatives to PC currently used. Furthermore, they asked to investigate the possible risks associated with the use of these alternatives.
In this study, a screening towards the endocrine activity of chemicals migrating from PC alternatives, identified by the group of Simoneau [7], was performed by using different reporter gene assays. Furthermore, the genotoxic potential of these compounds was also assessed with the Vitotox assay, an indicator test for DNA damage. The aim of the screening was to select the substances that may present a risk for human health and thus require further characterization.

Materials and methods

Cell line. The stably transfected MCF7-ER cell line, provided by ULg [8], was used to assess effects on the human oestrogen receptor (hER2). The CALUX® cell lines (Chemically Actived LUciferase eXpression) containing the human peroxisome proliferator-activated receptor (hPPARy) and the human Thyroïd ß receptor (hTR ß), were provided by BioDetection System (Amsterdam, The Netherlands). The CALUX bioassays for the study of the activation (or inhibition) of the mouse aryl hydrocarbon receptor (mAhR) and the human estrogen receptor (hER1) were kindly provided by Michael Denison (University of Davis, CaliforniaUSA).
Luciferase expression assay. Cells were seeded in a 96 –well plate in 100 µl of DMEM medium, without phenol red for hER transactivation luciferase gene expression assay, containing 10 % dextran-coated charcoal-stripped foetal calf serum. Following 24 h of incubation, medium was removed and replaced by fresh medium containing the appropriate standard or test compound dissolved in DMSO (the final concentration of DMSO in culture medium was 1 %). A standard curve and solvent control wells were included in each assay. Each test compound was tested at concentrations of 0.001, 0.01, 0.1 and 1 mM and within each assay, all data were related to the maximal response of the standard. Following 24 - 48 h of treatment (depending of the cell line), the cells were observed under the microscope to reveal morphological changes indicating potential cytotoxicity, and a cytotoxicity assay was performed on PPARy and TRß cell line. In order to lyse the cells, medium was removed, cells were rinsed and lysing solution was added to each well. Plates were incubated 5 min at room temperature before luciferin addition and luminescence measurement using a microplate luminometer. To assess for possible inhibitory activities of the test compounds, a test was run in parallel in the presence of an appropriate standard at a concentration close to the EC50. All experiments were performed in triplicate.
VitoTox assay. The VITOTOX test uses two different bacterial reporter strains of Salmonella typhymurium (TA 104) based on an SOS-response. One has a luciferase gene under the control of the recN promoter. Consequently, light is produced when DNA is damaged (TA 104-recN2-4 strain or genox strain). The second bacterial strain contains lux-genes under the control of a constitutive promoter. Light production is thus not influenced by genotoxic compounds (pr1 or cytox strain). However, this strain serves as an internal control

wherein, if the light production goes up, the test compound affect the lux gene in a different way than damaging the DNA. On the other hand, a decrease in light production indicates a toxic response. For each test compound, in a first run, a serial dilution was prepared starting from a 100 mM stock solution in DMSO. The final concentrations of the test compound in the plate were 10 µM, 100 µM and 1 mM (in 1% DMSO). For compounds with a low solubility in DMSO, ethanol was used as solvent. 4-Nitroquinoline oxide (4-NQO) was used as a positive control in the absence of S9 metabolizing fraction and benzo(a)pyrene (BaP) was used as a positive control in the presence of S9 metabolizing fraction. The final concentrations of 4-NQO and BaP in the measuring plate amounted to 4 pbb and 8 ppm, respectively. After the addition of the test compound to the bacteria, the light emission was recorded every 5 min by a luminometer, and this for a period of four hours. The signal to noise ratio (S/N) S/N was calculated for both strains separately or, specifically, the light production of exposed bacteria divided by the light production of non-exposed bacteria, was automatically calculated for each measurement. The maximum S/N between 120 and 240 minutes of the genox strain and the minimum S/N ratio between 120 and 240 minutes of the cytox strain were determined automatically. Afterwards, the maximum and minimum S/N ratios of the genox and cytox strains respectively for the different dilutions were plotted graphically to assess the dose-effect relationship. The ratio of the maximum Genox and Cytox strain was also calculated. A substance was considered genotoxic when (i) max S/N (genox) / max S/N (cytox) >1.5; (ii) max S/N in genox showed a good dose-effect relationship and (ii) max S/N (genox/cytox) showed a good dose-effect relationship. The test substances were not considered genotoxic if S/N (genox) increased rapidly within the first 30 min or immediately showed a high value as SOS induction is not yet possible within this short period of time. Test substances were considered cytotoxic when S/N (cytox) ratios were consistently below 0.8.
Statistical analysis. The values in the table 1 are scores representing the strength of the response on each receptor for the "agonist" and the "antagonist" assay, the cytotoxicity and genotoxicity. For the strength of the response, the score varies between 0 and 2, for genotoxicity and cytotoxicity, the score is 0 or 1. With these scores, a principal component analysis (PCA) was realised in order to investigate the relation between the agonist and antagonist effects on the different hormone receptors and to compare the cytotoxic effect of the compounds observed in the different cell lines and the Vitotox test. PCA was performed using the software Unscrambler X, version 10.3 (64 bit) of the CAMO Software AS company. The extraction procedure was based on a normalised PCA using the correlation matrix with Varimax rotation and a Kaiser normalization [9]. The statistical tests were performed at a significance level of 5 %.

Results and discussion

Study of the activation or inhibition of hormone receptors. The activity of chemicals migrating from plastic baby bottle on the hER, hPPARγ hTR ß, and mAhR was investigated. Among the 25 migrating chemicals tested, some substances clearly show an activity on one or several receptors, while others require further testing to clarify their activities. To achieve the statistical analysis, a scoring was attributed to each substance depending on the response strength. For the assay with cells exposed to the pure compound only, herein referred to as "agonist assay", (i) a score of zero was assigned to substances not acting on the receptor, (ii) a score of 0.1 to substances whose activity was unclear and required further investigations, (iii) a score of 1 to substances whose response exceeded 10 % of the maximal standard induction, and (iv) a score of 2 to substances whose response exceeded 50 % of the maximal standard induction or whose response was clearly dose related (full dose-response curve). For the assays with cells exposed to the test compound in the presence of an appropriate standard at a concentration close to the EC50, called hereafter "antagonist assay", (i) a score of zero was assigned to substances not acting on the receptor, (ii) a score of 0.1 to substances whose activity was unclear and required further investigations, (iii) a score of 1 to substances whose response was decreasing for two consecutive points by about 10 % of the standard maximal induction without cell death, and (iv) a score of 2 to substances inducing a complete inhibition without cell death. Table 1 provides an overview of the results obtained for the 25 test compounds.
Vitotox assays. None of the compounds tested fulfilled all criteria for a positive result in the Vitotox test. Despite the fact that the Vitotox assay is a screening assay for DNA damage and thus not a mutagenicity assay, it exhibits a good correlation with the Ames test (± 90%). More tests are required to complete the full genotoxic profile of the compounds, but nevertheless, these results already provide a first indication of the lack of genotoxicity associated with the different migration products.
Statistical analysis. To highlight relationships between the results of the CALUX tests and the genotoxicity assay, a PCA was carried out. The set of data consisted of a matrix of 25 lines (25 substances) and 18 columns (18 variables). For the panel of luciferase expression assays, including the effects on oestrogen receptors (ER1 and ER2), thyroid (TR), unsaturated fatty acid (PPAR) and aryl hydrocarbon (AhR), 3 variables have been studied. They corresponded to the agonist (AGon), antagonist (ANta) and toxic effects (CATox). It is important to note that the variable CATox was estimated either from the visual estimation of cell viability (ER and AhR receptors) or by using the resazurin assay (PPAR and TR receptor). For the Vitotox, the 3 variables selected correspond to genotoxic (GENTox) and cytotoxic effects with (CyTox+) or without the metabolizing fraction

(CyTox-). Since these 18 variables are categorical or qualitative variables, they were recoded to numeric variables using the score calculations defined above.

Table 1 : Overview of the activation or inhibition of hormone receptors by 25 chemicals

Name	SLM [3] (mg/kg)	ER1 AGon	ER1 ANta	ER2 AGon	ER2 ANta	AhR AGon	AhR ANta	PPARϒ AGon	PPARϒ ANta	TRβ AGon	TRβ ANta
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB)	5	++	-	+	-	-	-	-	-	-	-
Bisphenol A	0.6	++	-	/	/	-	-	+	?	-	?
2,4,6-Trimethyl benzaldehyde	ni	-	?	-	?	-	-	-	+	-	-
2,4-Di-tert-butyl phenol	60	-	+	+	-	-	-	-	-	-	-
2,6-Di-tert-butyl-p-benzoquinone	60	-	-	-	?	-	-	-	?	-	-
2-Butoxyethyl acetate	60	-	-	-	-	-	-	-	-	-	-
2-Methylnaphthalene	60	+	-	+	-	-	-	-	-	-	-
2-Propenoic acid,3-(4-methoxyphenyl)-2-ethylhexyl ester	ni	++	-	+	-	+	-	-	++	-	++
2-Propenoic acid-2-ethylhexyl ester	0.05	-	-	-	?	-	-	+	-	?	?
3,5,5-Trimethyl-2-cyclohexen-1-one	60	-	?	?	-	-	-	-	+	-	-
4-Tert-butylcyclohexyl acetate	ni	-	-	+	-	-	-	-	+	-	-
Alpha-methylstyrène	0.05	++	-	/	/	-	-	-	+	-	-
Benzaldehyde-4-methylthio	ni	-	?	+	-	-	-	-	-	-	-
Benzophenone	0.6	++	-	++	-	-	?	-	+	-	-
Butylated hydroxytoluene (BHT)	3	-	?	-	?	-	?	-	-	-	?
Camphor	60	++	-	++	-	-	-	-	++	-	-
Cyclododecene	ni	++	-	?	-	-	-	-	-	-	-
Cyclohexanone	60	-	-	-	?	-	-	-	-	-	-
Cyclohexanone-5-methyl-2-(1-methylethyl)	60	-	-	-	?	-	?	-	+	-	-
Dodecanoic acid 1-methylethyl ester	60	++	-	+	-	-	?	-	++	-	+
Erucamide	60	-	?	/	/	-	-	-	-	-	-
Hexadecanoic acid	60	-	?	+	-	-	-	-	?	-	-
Hexadecanoic acid methyl ester	60	+	-	?	?	-	-	-	?	-	-
Napthalene	60	-	?	?	-	-	-	-	-	-	-
N-butyl benzenesulfonamide	ni	-	?	?	-	-	-	-	+	-	-
Octadecanoic acid	60	+	-	?	-	-	-	-	-	-	-
Bisphenol S	ni	++	-	++	-	-	?	+	-	-	-
Diisobutyl phthalate	ni	/	/	+	-	/	/	-	+	-	+
Dibutyl phthalate	0.3	/	/	/	/	/	/	-	+	-	+
Diethylhexylphthalate	1.5	/	/	/	/	/	/	-	++	-	+

SML, specific limit of migration; Ago, agonist assay; Anta, antagonist assay; ni, not included in annex 1; /, not tested yet; -, no effect; ?, reaction not clear; +, (for induction) response is more than 10 % of the maximal standard induction or (for inhibition) response is decreasing, without cell death, for two consecutive points, more than 10 % of the maximal standard induction; ++, (for induction) response is more than 50 % of the maximal standard induction or (for inhibition) a complete inhibition, without cell death.

The main conclusions that can be drawn so far from this study are the following: three groups of variables with few correlations amongst them, but with significant correlations within their groups were identified. These are “CYTox”, “CATox” and “AGon & ANta”. For the CALUX panel tests, the low correlation between the variable groups "Catox" and "Agon & Anta" suggests that substances that interact with different receptors are not necessarily those inducing deleterious effects (Figure1). Regarding the 25 tested substances, the oestrogen hormones (ER) and the unsaturated fatty acid receptors (PPAR) are the most reactive for both the agonist and the antagonist effects (Figure 2). Among these 25 substances, several of them can interfere simultaneously on several receptors as agonist and / or antagonist. Moreover several substances, inducing agonist and antagonist effects on different receptors have been tested positive for cytotoxicity in the VitoTox and resazurin assay.

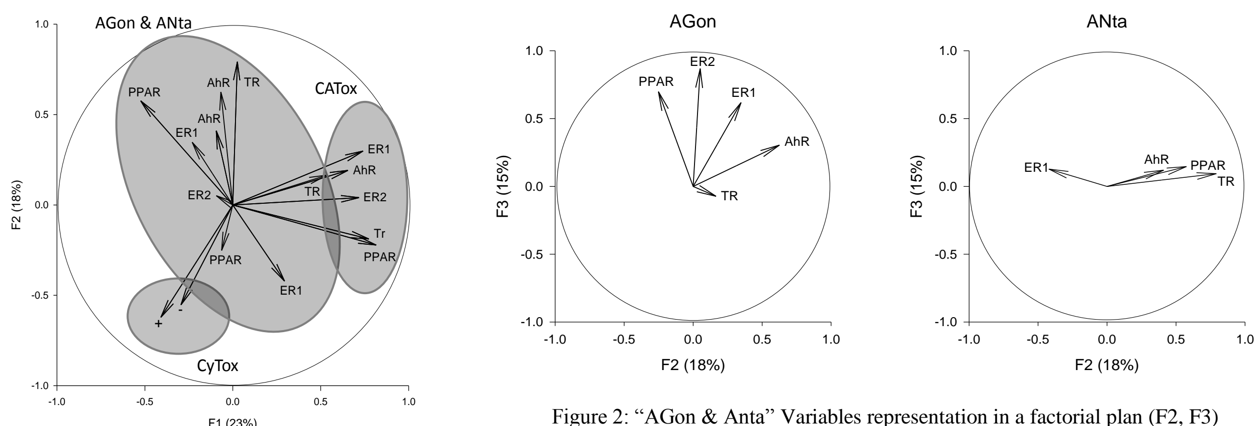

Figure 2: “AGon & Anta” Variables representation in a factorial plan (F2, F3)

Figure 1: PCA loading plots obtained after Varimax rotation for the whole dataset. The factor loadings represent the correlations between the original variables and the principal components

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