MAJOR RESULTS FROM FRENCH RESEARCH PROGRAMS ON BROMINATED FLAME RETARDANTS

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Abstract
Research programs on BFR, ending in 2007, are carried out in France, with the aim to (1) provide first data about human exposure levels in France in pregnant women, (2) determine the metabolism of the main BFR (deca-BDE, TBBPA) in rat during gestation, (3) achieve *in vitro* comparative human/rat metabolic studies for these molecules, and (4) determine the biological activity of BFR residues (parent molecules + major metabolites). Our results clearly demonstrate that in the French population studied, deca-BDE as well as other PBDE of high molecular weight (mainly octa and nona-BDE) account for a large part of the PBDE present in adipose tissue, mother's milk, maternal and umbilical cord serum, which is fully consistent with *in vivo* experiments carried out in pregnant rats. This result confirm the relevance of monitoring these highly brominated compounds, which is rarely achieved due to analytical difficulties. An unexpectedly high contribution of TBBPA to the overall BFR contamination of the latter two matrices was found. *In vitro*, oxidative metabolism and metabolites biological activity strongly suggest that additional studies should be conducted in the field of TBBPA's potential toxicity.

Introduction
Brominated Flame Retardants (BFR) are organo-halogen contaminants of the environment and the food-web, which have attracted a growing interest of scientists questioning their possible toxicity towards human beings[1,2]. Over the last decade, most of the attention has been focused on polybrominated diphenyl ethers (PBDE). There is a general consensus on the fact that PBDE bearing 4 to 6 bromine atoms (namely congeners BDE-47, 99, 100, 153 and 154) account for the major part of BFR residues found in animal and human tissues, which is consistent with most of the data published so far. Deca-bromodiphenyl ether (BDE-209) is the only BDE commercial mix which use remains authorised within the European Union, and by far the most frequently used PBDE world-wide. Deca-BDE, as well as other PBDE of high molecular weight, have been identified in human tissues only occasionally[3,4], because the presence of these congeners is generally not monitored, due to analytical difficulties. Likewise, very little data concerning the human exposure to tetra-bromo bisphenol A (TBBPA) is available[5]. Though TBBPA accounts for more than 50% of the BFR market, very little attention has been focused on this compound, compared to PBDE, because this BFR is expected to be readily eliminated due to phase II biotransformation[6], and is obviously not a persistent organic pollutant.

Materials and Methods
All Human samples analysed in the study were collected by the gynaecology-obstetric unit of the Centre Hospitalier Universitaire (CHU) Toulouse, France. These samples (including maternal and cord serum, maternal adipose tissue and breast milk) were obtained from 142 volunteer women hospitalised for caesarean deliveries from April 2003 to September 2006. The first 49 sample batches were used for methodological development and validation[7]. Thus, the present exposure assessment study concerns 93 mother/new-born pairs included in the study from November 2004 to September 2006. The included volunteer women were between 20 and 46 years old, with a mean value observed at 32.5 years old. The experimental protocol was approved by a local ethical committee in accordance with French regulation, and the informed consent of all participating subjects was

obtained. The analytical procedure used for sample preparation and GC-HRMS measurement of BFR (tri- to deca-BDE and TBBPA) was described elsewhere[7].
Radio-labelled compounds used for metabolic studies in rat: [^{14}C]-BDE-209 (di-pentabromophenyl ether) was synthesised, from ring-[^{14}C]-diphenyl ether and was purified by SPE on C18 Macherey Nagel glass cartridges. Its purity was checked by radio-HPLC (>99.8%)[8]. [^{14}C]-TBBPA was synthesised from ring-[^{14}C]-bisphenol A and was purified using SPE and glass cartridges. Its purity was checked by radio-HPLC (>99.8%)[9].
Metabolism studies : for each molecule, three conventional pregnant Wistar rats were individually housed in stainless steel metabolism cages. Animals were daily dosed by oral route from gestation days 16 to 19, with either 1.95 mg/kg b.w./day [^{14}C]-BDE-209 or 191.5 µg/kg b.w./day [^{14}C]-TBBPA. Urine and feces were collected daily over 4 days. Animals were killed by exsanguination on day 20 of gestation, 24h after the last BFR dosage. Quantities of [^{14}C] in urine, plasma and amniotic fluid were determined by direct counting in a Packard liquid scintillation counter. Radioactivity in rat carcasses, homogenised faeces, fresh tissues and extraction pellets was determined by complete combustion using a Packard 307 Oxidizer, followed by $^{14}CO_2$ quantification using a liquid scintillation counter. Following solvent extraction, samples were analysed by radio-HPLC. Detailed protocols and analytical conditions were as described elsewhere[8,9]. Metabolites were quantified by ^{14}C-monitoring and were isolated by HPLC separation and C18 glass cartridges SPE. The identification of BDE-209 and its metabolites was achieved by APPI-ITMS or LC-APPI-ITMS. The biological activity of TBBPA and selected metabolites was tested using hPPAR alpha, delta and gamma reporter cell lines[10] at 10^{-5}M in at least 3 independent experiments. For each experiment, tests were performed in quadruplicate.

Results and Discussion

BFR residues in human samples:

Fig.1

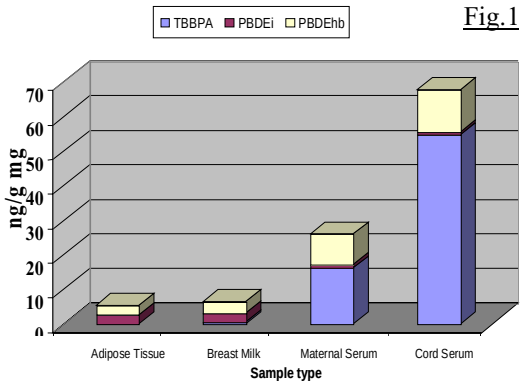
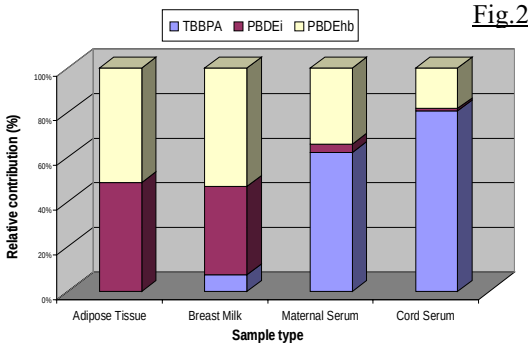


Fig.2



The global median levels of BFR (ng/g l.w.) measured in maternal adipose tissue, breast milk and serum, as well as in umbilical cord serum are shown in Fig. 1. Median levels of tri- to hepta-BDE (PBDEi) were found to be 2.6 ng/g l.w. in adipose tissue, and 2.5 ng/g l.w. in breast milk. These results (which will be fully detailed in the very next future), indicate that residual levels of the 7 more commonly measured PBDE congeners (*e.g.* BDE-28, 47, 99, 100, 153, 154 and 183) are relatively similar in this study compared to other published European data. However, a major and non commonly reported observation was the high contribution (around 50 %) of highly brominated PBDE congeners (PBDEhb : octa- to deca-BDE) to the total PBDE load measured in these samples. PBDEhb measured in the analysed adipose tissue and breast milk samples mainly consisted of deca-BDE-209 (26% and 45%, respectively), Octa-BDE-#3 (27% and 11%, resp.) and nona-BDE-207 (28% and 21 %, resp.). Interestingly, it was found that the major part of BFR residues detected in mother's blood and in the umbilical cord blood was not associated with tri- to hepta- PBDEs, but with higher brominated PBDEs as well as TBBPA. The Contribution of TBBPA, tri- to hepta-PBDE (PBDEi) and octa- to deca-PBDE (PBDEhb) to the total BFR load measured in the different biological matrices is further detailed in Fig. 2.

Deca-BDE and TBBPA metabolic fate in pregnant rats:

Metabolic balance results for deca-BDE and for TBBPA are summarised in table 1. As extensively discussed elsewhere[8], our studies demonstrate that deca-BDE, administered orally to rats at a late gestation stage (days 16-20) is efficiently absorbed (*ca.* 20% of the administered dose) and distributed in tissues. Liver is the target tissue (>6% of the administered dose). This corresponds to 11.1 ppm (µg/g) of deca-BDE equivalents. Even higher

residual concentrations are found in the ovaries and the adrenals (16 and 33 ppm, respectively) and nearly 1% of the administered dose is recovered in placenta and foetuses, demonstrating the trans-placental passage of deca-BDE residues. Radio-HPLC profiling showed that, depending on the tissue, between 10 to 30% of the radioactivity were associated with metabolites (Fig. 3). Main deca-BDE metabolites were identified as nona-BDEs (BDE-206, 207 and 208), as well as an octa-BDE congener and hydroxylated octa-BDE[8]. Traces of hepta-BDE were also detected, but no PBDE congeners bearing less than 7 bromine atoms could be detected in this 4-days study. These *in vivo* results clearly demonstrate that in rat, deca-BDE is efficiently absorbed and metabolised and that both the parent compound and high molecular weight PBDE (octa and nona-BDEs) are found in tissues as well as foetuses.

% of the administered dose	Deca-BDE	TBBPA
Faeces	66,29 ± 1,35	87,00 ± 1,44
Digestive tract content	5,33 ± 1,50	5,29 ± 1,54
Urine	0,11 ± 0,02	0,52 ± 0,07
Adrenals	0,16 ± 0,05	< 0,01
Ovaries	0,13 ± 0,02	< 0,01
Liver	6,48 ± 1,03	0,04 ± 0,01
Kidneys	0,29 ± 0,05	< 0,01
Stomach	0,20 ± 0,07	< 0,01
Small intestine	0,64 ± 0,15	0,10 ± 0,05
Heart	0,08 ± 0,01	< 0,01
Lung	0,13 ± 0,02	< 0,01
Spleen	0,06 ± 0,01	< 0,01
Large intestine	0,16 ± 0,04	0,07 ± 0,06
Uterus	0,24 ± 0,09	< 0,01
Brain	0,01 ± 0,00	< 0,01
Placenta	0,47 ± 0,05	< 0,01
Foetuses (whole litter)	0,43 ± 0,03	< 0,01
Amniotic fluid	0,02 ± 0,00	< 0,01
Carcass	9,23 ± 1,21	0,05 ± 0,02
Cages	< 0,02	0,06 ± 0,03
TOTAL	**91,1 ± 2.9**	**92,1 ± 0.88**

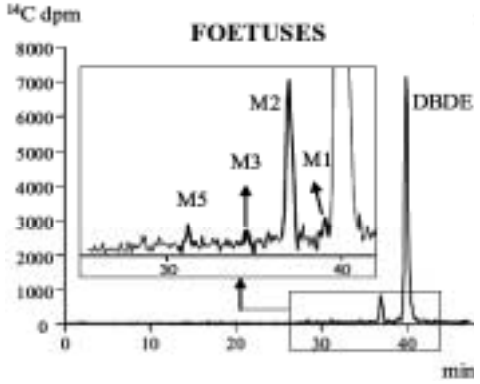
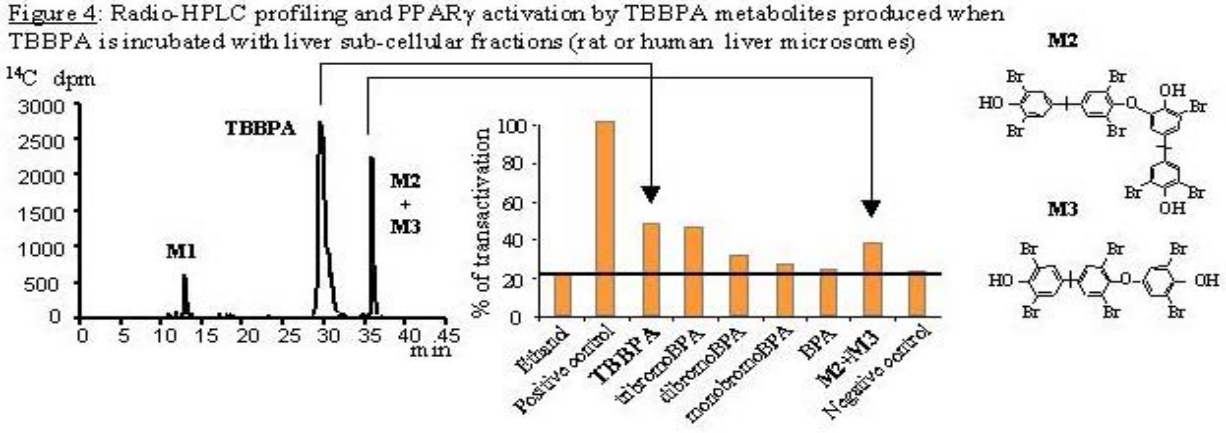


Table 1 : metabolic balance of ^{14}C-DBDE (1.95 mg/kg) and ^{14}C-TBBPA (191 µg/kg) administered orally to rats from gestation days 16 to 19.
Figure 3 : radio-HPLC profiling of deca-BDE and its metabolites in foetuses from rats dosed with ^{14}C-DBDE on gestation days 16 to 19.

In contrast, the results obtained for TBBPA demonstrate a very limited distribution of the administered radioactivity into tissues. The highest values were recorded for the liver and the intestine wall, and did not exceed 0.1% of the administered dosage. The trans-placental passage of TBBPA residues was measurable (0.3 ng/g (ppb) of TBBPA equivalents), but very limited: for comparison purposes, calculated residual concentrations were 1.3, 5.0 and 29.3 ppb in plasma, liver and the small intestine wall, respectively. It should be stressed that TBBPA's half-life in rat[6] and in human is relatively short, presumably due to the extensive conjugation and subsequent elimination of the molecule[6].

For deca-BDE, data from *in vivo* experiments in pregnant rats strongly support the data obtained in human. On the one hand, our results demonstrate an efficient absorption of deca-BDE and the fact that deca-BDE and its metabolites can cross the placental barrier in rat. On the other hand, the qualitative distribution of deca-BDE and its metabolites, *e.g.* the predominance of high molecular weight PBDE, is in very good agreement with the results obtained for umbilical cord sera. A very limited number of studies including the dosage of deca-BDE have been carried out in human. Schecter et al.[3] have been able to show the presence of deca-BDE in human foetuses liver. All together, there is a growing amount of evidence that strongly suggests the exposure of human foetuses to PBDEs, which are probably, in the majority, high molecular weight PBDE, namely octa to deca congeners.

Regarding TBBPA, *in vivo* experiments in rat show a very limited distribution and a marginal trans-placental passage (Table 1). These results may appear as somewhat contradictory with the data obtained in human, which demonstrates that quantitatively, TBBPA, is the main BFR found in the umbilical cord serum and even in the maternal serum. The residual levels of TBBPA in rat samples were measured 24 hr after the last TBBPA dosage, which may explain the small amounts detected, in good agreement with the short half-life of TBBPA.



Our results in rat confirm previous conclusions by hakk et al.[6], with an extensive conjugation of this BFR *in vivo*. However, comparative human/rat *in vitro* metabolism studies carried out on TBBPA gave additional information about the possible metabolic pathways this BFR undergoes. When incubated with liver sub-cellular fractions (microsomes or S9), TBBPA is extensively oxidised into hydroxylated dibromo-isopropyl-phenol (Fig 4, M1), resulting from the oxidative cleavage of the molecule, and into compounds exhibiting a lower hydro-solubility than TBBPA itself, identified as "dimeric" structures (Fig.4, M2 and M3). The formation of all these metabolites was shown to be P450-dependent. It occurs both in human and in rat[9]. Moreover, similar metabolites are extensively formed when TBBPA is incubated with human neutrophil granulocytes[11]. In this system, TBBPA was also shown to enhance ROS production in a concentration-depended manner[12]. These compounds, as well as TBBPA itself, are able to activate PPAR gamma receptors (Fig. 4). All together, these data confirm that TBBPA is certainly not a bio-accumulating substance in fat or any other tissue. However, human exposure to this BFR occurs, at least in France. This is also the case for the highest brominated PBDE, which are equally difficult to measure in human tissues. More research should be focused on these specific BFR, but also on their biotransformation products, in order to better understand the risks they represent for human.

Acknowledgements

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