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# Dose- and time-dependent changes in tissue levels of tetrabromobisphenol A (TBBPA) and its sulfate and glucuronide conjugates following repeated administration to female Wistar Han Rats

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ABSTRACT

Tetrabromobisphenol A (TBBPA), a nongenotoxic flame retardant, causes uterine tumors in female rats. A proposed mode of action (MoA) for these tumors involves an increase in the bioavailability of estradiol as a result of TBBPA inhibiting estrogen sulfotransferases (ES), the enzymes responsible for inactivating and enhancing the elimination of estradiol. The objective of this study was to evaluate the effect of dose and repeated administration of TBBPA on the level of TBBPA, TBBPA-glucuronide (GA) and TBBPA-sulfate (S) conjugates in plasma, liver and uterus of female Wistar Han rats administered TBBPA (50, 100, 250, 500 or 1000 mg/kg) for 28 consecutive days. In accordance with this objective, TBBPA sulfation was used as a surrogate for evaluating the potential for estradiol sulfation to be limited at high dose levels of TBBPA. Blood samples were collected at 4 and 8 h post-dosing on study day 7, 14, and 28, while liver and uterus were collected at the same time points following 28 days of dosing. Tissue samples were analyzed for TBBPA, TBBPA-GA and TBBPA-S by LC–MS/MS. A dose-related increase in the concentration of all three analytes occurred in plasma (day 7, 14, and 28) as well as liver and uterus tissue (day 28) at both 4 and 8 h post dose. The plasma concentration of TBBPA-GA and TBBPA-S was higher in animals dosed for 28 days compared to those dosed for 7 or 14 days showing an increase in systemic circulation of these conjugates with repeated administration. The balance of these conjugates was also different in tissues with TBBPA-S > TBBPA-GA at high doses in the liver and TBBPA-GA > TBBPA-S in both plasma and uterus. In all three tissues the ratio of TBBPA-S/TBBPA-GA showed a decreasing trend with dose, suggesting that at high TBBPA dose levels sulfation of TBBPA becomes limited. This effect was most apparent in the liver and plasma at 28 days of administration. Together these data show that administration of high doses of TBBPA associated with the induction of uterine tumors, results in a disruption in the balance of conjugates reflected by a decrease in the TBBPA-S/TBBPA-GA ratio. A limitation in the sulfation of TBBPA *in vivo* supports *in vitro* data defining TBBPA as an inhibitor of ES activity, thus providing further support that the proposed MoA occurs under conditions of high dose, chronic TBBPA administration to Wistar Han rats. Given that the uterine tumors observed in rats (250–1000 mg/kg-day) only occur at very high doses that perturb homeostatic control, it is unlikely such effects would occur in humans given that current TBBPA exposure levels are approximately eight orders of magnitude lower than these doses that are associated with exceeding the capacity of conjugation pathways in animal studies.



## 1. Introduction

Tetrabromobisphenol A (TBBPA) is the most widely produced brominated flame retardant, primarily because of its effectiveness and low hazard profile (http://www.bsef.com). It is used in epoxy, polycarbonate and phenolic resins in circuit boards, as well as in acrylonitrile–butadiene–styrene (ABS). Examples of products con-

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taining TBBPA include printed circuit boards, communications and electronics equipment, appliances, transportation devices, sports and recreation equipment, automotive parts, pipes and fittings [2]. Humans can be exposed to TBBPA during manufacturing and production, *via* use of TBBPA-containing products, and *via* recycling of TBBPA-containing products. TBBPA has been detected in the ppb range in human serum in cases of exposure in occupational and non-occupational settings, as well as in breast milk, demonstrating that TBBPA is in fact absorbed in exposed human populations [19,34,32,33].

The acute oral toxicity of TBBPA in rats is low ($LD_{50}$ > 5 g/kg) [18], with little toxicity identified overall in a number of robust standard 28- and 90-day toxicology studies [8,28,38,29]. In a recent cancer bioassay conducted by the National Toxicology Program (NTP), TBBPA was administered to Wistar Han rats at dose levels of 250, 500, and 1000 mg/kg for 2 years. The NTP reported an increased incidence of uterine epithelial tumors in female rats (combined adenoma, adenocarcinoma, or malignant mixed Mullerian tumor) at the mid- and high dose levels of 500 mg/kg-day (32%) and 1000 mg/kg-day (38%), when compared to the vehicle control group (12%) [28]. Given that TBBPA has been shown not to be mutagenic or genotoxic [15,8], several investigators have proposed a mode of action (MoA) for uterine tumors that involves endocrine mediated events that would result in a disruption of estrogenic activity [6,26]. A review of both the published literature and high throughput screening (HTS) data (http://actor.epa.gov/edsp21/) has shown that TBBPA does not directly interact with the estrogen receptor (ER) [42]. Based on the lack of ability of TBBPA to be an ER agonist, the focus of this current investigation has been on the ability of TBBPA to directly disrupt the metabolism of estrogen.

To date, toxicokinetic data on TBBPA are limited to relatively traditional kinetic studies. Existing data reflect administration of varying dose levels of TBBPA for different durations in order to characterize TBBPA's major metabolites in multiple strains of rats and humans [12,25,31,20,24]. Overall, TBBPA is rapidly absorbed and efficiently metabolized by glucuronyltransferase and sulfotranferase enzymes in the liver. The major route of elimination of administered TBBPA is in feces, with minor elimination in urine resulting in low bioavailability [12,25,24]. Together the data from these studies support the conclusion that TBBPA is cleared *via* bile for fecal elimination with the two major metabolites identified as a TBBPA-sulfate and TBBPA-glucuronide conjugate.

Similar to TBBPA, estrogen and its metabolites are conjugated to both sulfate and glucuronic acid, with sulfation serving as the main pathway for the inactivation of estrogen [43]. Kester et al. [21] reported that TBBPA inhibits estrogen sulfotransferase (ES) *in vitro* with $IC_{50}$ values ranging from 12 to 33 nM. Another study conducted by Hamers et al. [13] confirmed that TBBPA acts as a potent inhibitor of ES *in vitro* ($IC_{50}$ = 16 nM), with it being approximately 13 times more potent than pentachlorophenol (PCP), a known inhibitor of sulfotransferase. A QSAR study evaluated binding to ES and found that TBBPA fulfilled the chemical structure criteria that would predict potent inhibition of ES [14]. A more recent evaluation of the crystal structure of ES involving computation modeling found that TBBPA binds to the same site as estradiol [10]. Taken together, these studies provide evidence to support the hypothesis that TBBPA inhibits ES, and more specifically the isoforms SULT1E1 and SULT1A1. When these data are considered, and metabolic pathways for TBBPA and estrogen compared (Fig. 1), it appears that TBBPA could compete *in vivo* with the same enzyme systems as estrogens. However, currently there are no data available to characterize such a relationship *in vivo*—and, importantly, to characterize the doses at which ES inhibition may occur and affect the sulfation of estrogens. These data are critical for determining the feasibility for TBBPA to influence estrogenic potential through a disruption of its metabolism. Several studies [6,26] proposed that the TBBPA-inhibition of ES, a key enzyme in the inactivation and elimination of estrogens, would potentially result in an increased bioavailability of estrogens, which, in both humans and rats, is associated with endometrial tumors [16].

Kinetic data are not yet available to determine if the administration of TBBPA at dose levels used in the NTP study that were associated with uterine tumors in rats, inhibits ES *in vivo*, and subsequently, if such an inhibition could result in an increased bioavailability of "unconjugated" estradiol in the uterus (an estrogen responsive tissue). Based on the challenges associated with directly measuring estrogens and its metabolites in tissues (*e.g.*, assay sensitivity, specificity, and variability), the objective of this study was to determine if conjugation of TBBPA to sulfate would be limited at dose levels associated with the development of preneoplastic and neoplastic changes in the uterus of rats following chronic administration of TBBPA. TBBPA sulfation can be used as a surrogate for indirectly measuring the potential disruption in estradiol conjugation since both are metabolized through the same enzyme systems. Investigating the dose level at which the sulfation of TBBPA exceeds its capacity would provide further support that the sulfation of estradiol *in vivo* would be impacted following repeated high dose levels of TBBPA.

## 2. Materials and methods

### 2.1. Chemicals

Tetrabromobisphenol A (TBBPA) [CAS No. 79-94-7] was obtained from Albemarle Corporation (Baton Rouge, LA) with a purity of 98.83%. Corn oil [CAS no. 8001-30-7], used as the vehicle, was obtained from MP Biomedical, LLC (Solon, OH). All reagents used for LC–MS/MS analysis were HPLC grade. $^{13}C_{12}$-TBBPA was purchased from Cambridge Isotope Laboratories and control rat plasma was purchased from Innovation Research Inc.

### 2.2. TBBPA dose formulation

TBBPA dose formulations were prepared in corn oil at concentrations of 0, 10, 20, 50, 100, and 200 mg/mL to administer dose levels of 0, 50, 250, 500, and 1000 mg TBBPA/kg body weight, respectively, at a volume of 5 mL/kg. TBBPA dose formulations were reported to be stable in corn oil at room temperature for up to 40 days [28]. As such, dose formulations were prepared once every 2 weeks (2×) during this 28-day study. Dose concentration and homogeneity were confirmed by sampling the top, middle and bottom of each formulation and analyzing by HPLC-UV (285 nm) using a Shiseido Capcell Pak C18 column, 3 × 100 mm, 3 μm with a flow of 0.6 mL/min. Briefly, the HPLC method consisted of a mobile phase of 0.1 mL of trifluoroacetic acid (TFA) in deinonized water (A) and (B) 0.1 mL of TFA in acetonitrile. The gradient was 0 to 40% B over 4 min with a linear increase to 100% B up to 6 min. Approximate retention time for TBBPA was 5.6 min. Concentration and homogeneity results were acceptable if the mean concentration was within 15% of the target and the coefficient of variation in the homogeneity was less than 15%.

### 2.3. Animal model

Female Wistar (CRL:W(Han)) rats were obtained from Charles River Laboratory International, Inc (Kingston, NY) at approximately 9 weeks of age and supplied with NTP-2000 wafer diet (Zeigler Bros., Gardners, PA) and reverse-osmosis-treated tap water (City of Durham, NC) *ad libitum*. Animals were acclimated for 8 days prior to the initiation of the study and housed 3 per cage in polycarbonate cages with absorbent heat-treated hardwood bedding. The temperature and humidity of the animal rooms were held between