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Penetration of Organophosphate Triesters and Diesters across the Blood−Cerebrospinal Fluid Barrier: Efficiencies, Impact Factors, and Mechanisms

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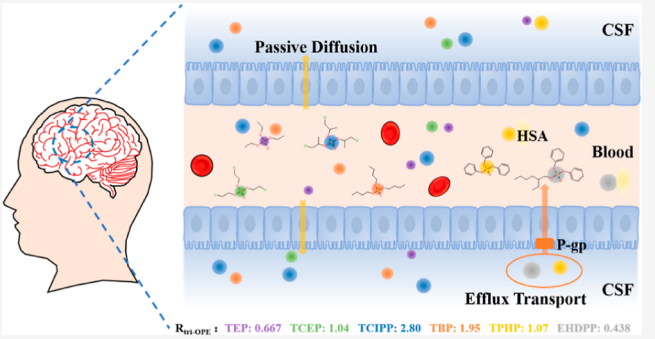
ABSTRACT: The penetration of organophosphate triesters (tri-OPEs) and diesters (di-OPEs) across the blood−brain barrier and their influencing factors remain unclear in humans. In this study, 21 tri-OPEs and 8 di-OPEs were measured in 288 paired serum and cerebrospinal fluid (CSF) samples collected in Jinan, China. Six tri-OPEs were frequently detected in both serum and CSF, with median concentrations ranging from 0.062 to 1.62 and 0.042−1.11 ng/mL, respectively. Their penetration efficiencies across the blood−CSF barrier (BCSFB) ($R_{CSF/serum}$, C_{CSF}/C_{serum}) were calculated at 0.667−2.80, and these efficiencies first increased and then decreased with their log K_{ow} values. The reduced penetration efficiencies of triphenyl phosphate (TPHP) and 2-ethylhexyl diphenyl phosphate (EHDPP) may be attributed to their strong binding affinities for human serum albumin and p-glycoprotein due to their high hydrophobicity and aryl structure, as indicated by molecular docking. This suggests that active efflux transport may be involved in the penetration of TPHP and EHDPP in addition to passive diffusion similar to the other four tri-OPEs. Di-OPEs were found in few serum samples and even fewer CSF samples, indicating their limited BCSFB permeability. This may be due to their high polarity, low hydrophobicity, and ionic state in blood. This study has important implications for understanding the neurotoxicity of tri-OPEs and di-OPEs and the underlying mechanisms.



KEYWORDS: *Tri-OPEs, diester metabolites, blood−brain barrier, cerebrospinal fluid, molecular docking, neurotoxicity*

1. INTRODUCTION

Organophosphate triesters (tri-OPEs) are widely used as flame retardants, plasticizers, and antifoaming agents in various products, such as building materials, electronics, polyurethane foam furniture, textiles, and paints.[1,2] The global consumption of tri-OPEs has increased from 186,000 tons in 2001 to 680,000 tons in 2015 and will continue to increase due to the phase-out of polybrominated diphenyl ether production.[3,4] As additives, tri-OPEs are easily released from products into the surroundings. Additionally, tri-OPEs are also oxidation products of organophosphite antioxidants, which are widely used in various manmade polymers.[5,6] Therefore, tri-OPEs have become ubiquitous in diverse environments and organisms worldwide[7−9] and typically observed at higher levels than brominated flame retardants in multiple environmental compartments.[4,10] Humans are inevitably exposed to tri-OPEs via inhalation, dust ingestion, dermal absorption, water, and food consumption.[11−13] As a result, tri-OPEs are frequently detected in human specimens (e.g., blood, urine, milk).[14−16] Organophosphate diesters (di-OPEs), in addition to being metabolites of tri-OPEs,[17,18] have been reported to have wide industrial applications[19] and could be produced by their parent tri-OPEs in the natural environments.[20,21] Di-OPEs have been widely observed in human urine[16,22,23] and blood[14,16] and have recently been found in dust and food,[13,24,25] indicating direct exposure to di-OPEs. Recent in vivo and in vitro studies implied that several di-OPEs may produce endocrine disrupting effects,[26] affect thyroid hormone transport,[27] disrupt metabolism in mice,[28,29] and inhibit the growth of male zebrafish,[30] suggesting that more attention should be given to both tri-OPEs and di-OPEs.

In addition to carcinogenicity,[1,8] developmental and reproductive toxicity,[31] and endocrine disruption,[32,33] growing evidence from laboratory animal studies and human epidemiological studies has indicated that tri-OPEs cause significant neurotoxicity.[34] For example, exposure of zebrafish/Chinese

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Rare Minnows to tri(2-chloroethyl)phosphate (TCEP), tri-(1,3-dichloro-2-propyl)phosphate (TDCIPP), tri-phenyl phosphate (TPHP), tri-*n*-butyl processes phosphate (TNBP), or tri(2-butoxyethyl)phosphate (TBOEP) induced hyperactivity,[35] impaired learning/memory performance,[36] and led to significant changes in locomotor behavior.[37] TCEP exposure resulted in a decline in the spatial learning and memory functions of rats.[38] Following the exposure of gestational mice to a mixture of TDCIPP, TPHP, and trimethylphenyl phosphate (TMPP), their offspring exhibited altered locomotor and anxiety-related behaviors.[39] Additionally, in a cross-sectional study in the US, children with higher tri-OPE concentrations in the wristbands they wore (an effective way to measure children's exposure to tri-OPEs) had less responsible behavior and more externalizing behavior problems measured using the Social Skills Improvement Rating Scale.[40] Another birth cohort study reported that urinary di-phenyl phosphate (DPHP) concentrations of pregnant women were negatively associated with the full-scale intelligence quotient and working memory of their children at age 7.[41]

A major target of chemical-mediated neurotoxicity is the brain. In humans, the penetration of exogenous toxic substances from the circulating blood into the central nervous system (CNS) is restricted by a complex barrier system, which typically includes the blood−brain barrier (BBB) and blood−cerebrospinal fluid barrier (BCSFB).[42] The former separates brain parenchyma from blood and is composed of brain capillary endothelial cells (ECs), pericytes, basement membrane, and astrocyte endfeet. The latter restricts circulation between cerebrospinal fluid (CSF) and blood and is formed by choroid plexus epithelial cells (CPEpiCs).[43] Although differences in composition exist between these two barriers, ECs and CPEpiCs possess similar properties, such as continuous tight junctions between adjacent cells, low levels of transcytosis, and expression of efflux and selective transporters.[42,44] These features ensure the import of nutrients and the export of metabolic wastes and limit the permeation of xenobiotics; thus, they are critical for brain homeostasis. Tri-OPEs have been documented to cross the BBB in organisms. For example, high levels of d_{15}-TPHP were detected in the brains of adult zebrafish after 11−14 days of exposure, while the metabolites were not.[17] Six tri-OPEs [TCEP, TCIPP, TDCIPP, TNBP, tri-iso-butyl phosphate (TIBP), and TPHP] were found in the brains of mice after 72 days of inhalation exposure.[45] Additionally, several tri-OPEs [TCEP, TCIPP, TNBP, TBOEP, TMPP, 2-ethylhexyl di-phenyl phosphate (EHDPP), isodecyl diphenyl phosphate (IDPP), 4-*tert*-butylphenyl diphenyl phosphate (BPDP), and 2-isopropylphenyl diphenyl phosphate (IPDP)] were detected in the brain tissue of silver carp (*Hypophthalmichthys molitrix*) from Taihu Lake, China[46] and dolphins (*Delphinus delphis*) from Southern European waters.[47] However, it remains unclear whether tri-OPEs and di-OPEs can pass through the BBB in humans. Moreover, the factors influencing the penetration efficiencies of tri-OPEs and their relevant mechanisms are poorly known, yet critical to understand potential neurotoxicity.

Considering the difficulty and ethical limitations of access to the human brain tissue, CSF is often used as a surrogate to investigate the penetration ability of chemicals into the CNS. CSF is mainly produced by the choroid plexus and fills the ventricular and subarachnoid space in the brain. The concentrations of exogenous compounds in CSF have been reported to be close to those in brain interstitial fluid.[48] Therefore, in this study, paired serum and CSF samples were collected to (1) determine the internal exposure levels and penetration efficiencies across the BCSFB of tri-OPEs and di-OPEs in humans; (2) clarify the factors influencing their penetration behaviors, such as BCSFB permeability, physical and chemical properties of compounds, binding affinities of tri-OPEs with human serum protein and transporters in the barrier, and so forth; and (3) explore the transport mechanisms of tri-OPEs and di-OPEs from serum into CSF and the potential hazards of tri-OPEs to the CNS.

2. MATERIALS AND METHODS

2.1. Sample Collection. From February to August 2021, we collected 288 paired serum and CSF samples from patients in Qilu Hospital, Shandong Province, China (n = 576). These patients were hospitalized for brain disease, headache, or fever and had a lumbar puncture. After the serum and CSF samples were collected for necessary medical examination, approximately 1 mL of each sample was transferred into a 1.5 mL PP centrifuge tube and stored at −20 °C until samples were transported to the laboratory in Beijing by a cold-chain shipment, where they were stored at −20 °C prior to extraction. The study protocol was approved for ethical review and all participants signed informed consent forms before sample collection. Demographics (e.g., sex and age) and clinical parameters of participants [e.g., red blood cell (RBC) count, white blood cell (WBC) count, and globulin in CSF; glucose (Glu), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and homocysteine (Hcy) in serum] were obtained from the associated medical records.

2.2. Sample Extraction and Instrumental Analysis. Tri-OPEs and di-OPEs in serum and CSF were extracted according to previously described methods with minor modifications.[16,49] Briefly, 0.5 mL of serum or CSF samples were spiked with deuterated internal standards and extracted with acetonitrile by ultrasonication. The extract of serum was purified with an Oasis HLB cartridge (6 mL, 200 mg; Waters) for tri-OPEs and di-OPEs. The CSF extract was cleaned on an ENVI-18 cartridge (6 mL, 500 mg; Supelco) for tri-OPEs and di-OPEs. The samples were analyzed for 21 tri-OPEs and 8 di-OPEs (listed in Table S1). The analysis was performed using an ultraperformance liquid chromatograph (HPLC, Ultimate 3000, Thermo Fisher Co., Sunnyvale, CA, USA) coupled with electrospray triple quadrupole mass spectrometry (API 4500, ESI MS/MS; Applied Biosystems, Foster City, CA, USA) with separation on an Acquity BEH C18 Column (1.7 μm, 2.1 mm × 100 mm, Waters, USA). More detailed information on the sample preparation and analytical method is provided in Supporting Information, Table S2.

2.3. Quality Assurance and Quality Control. Six-point calibration curves ranging in concentrations from 0.1 to 50 ng/mL were used in the quantitation of tri-OPEs and di-OPEs, and the calibration curves exhibited excellent correlation coefficients (r^2 > 0.995) for all chemicals. To reduce background contamination, all glass tubes used were rinsed with ultrapure water and methanol prior to use. A procedural blank was analyzed in each batch of 11 samples to check for the background contamination. Field blanks (ultrapure water, n = 10) were pretreated following the same procedure as that for the samples. The levels of the target compounds in the field blanks were comparable to those in the procedural blanks. The concentrations of target tri-OPEs and di-OPEs in all blanks

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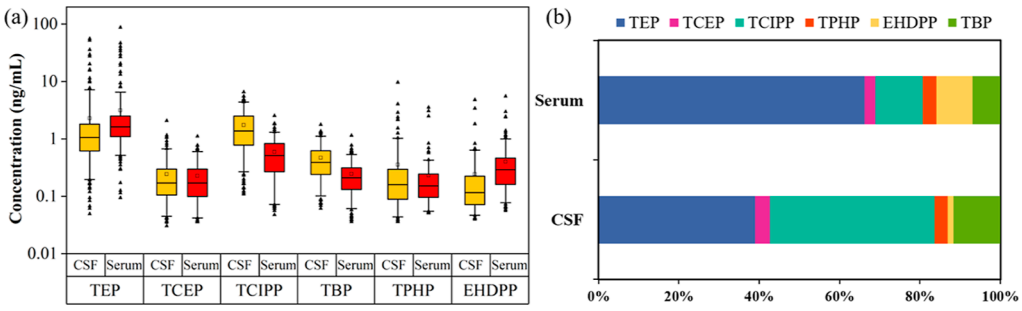

Figure 1. Concentrations (a) and compositional profiles (b) of tri-OPEs in CSF and serum samples. In panel (a), the upper and lower bounds of the boxes indicate the 75th and 25th percentiles, respectively; the horizontal lines within the boxes indicate median values; the hollow squares within the boxes indicate mean values; the upper and lower limits of the whiskers indicate the 95th and 5th percentiles, respectively; and the solid triangles above or below the whiskers indicate outlier values.

were <MDLs −0.106 and <MDLs −0.128 ng/mL, respectively (Table S3). For the target compounds not detected in the blanks, the method detection limits (MDLs) were defined as the concentrations with a signal-to-noise ratio of three. For the analytes detected in the blanks, the MDLs were defined as the 3 times the standard deviation of the blanks normalized to the sample volume. The MDLs for tri-OPEs in serum and CSF were in the range of 0.006−2.40 ng/mL. The MDLs for di-OPEs in serum and CSF were in the range of 0.017−2.00 ng/mL (Table S3). The matrix spike recoveries were determined by spiking target compounds (1, 5, and 10 ng/mL) into three replicates of a pooled serum or CSF sample. Except for B3tBPPP, the recoveries of tri-OPEs in serum and CSF were 50−127 and 68−125%, respectively, while those of di-OPEs were 57−130 and 72−123%, respectively. The recoveries of internal standards ranged from 71 to 93% for tri-OPEs and 60 to 118% for di-OPEs (recoveries of BEHP-d_{34} were 150% in CSF and 203% in serum) (Table S3). Matrix effect was observed and the specific process and results are shown in Supporting Information, Table S4. The final concentrations of target analytes were blank-corrected and not corrected according to their recoveries.

2.4. Statistical Analysis. Descriptive statistics were adopted to illustrate the concentrations of tri-OPEs and di-OPEs in serum and CSF samples. Only compounds with detection frequencies (DFs) >50% were included in subsequent statistical analyses and calculations. Concentrations of target compounds below the MDLs were assigned as 1/2 MDLs. The concentration ratios of tri-OPEs between CSF and serum (termed $R_{CSF/serum} = C_{CSF}/C_{serum}$) were calculated to estimate the penetration efficiencies of tri-OPEs across the BCSFB. Only paired CSF and serum samples with compound concentrations above the MDLs were included in the calculations. The data were not distributed normally according to a Kolmogorov−Smirnov test. Spearman's rank correlations (r_s) were used to assess correlations among the analytes, between analyte concentrations in CSF and serum, and between tri-OPE concentrations in CSF or $R_{CSF/Serum}$ of tri-OPEs and the age of participants or parameters of the serum. A Mann−Whitney U test was used to examine the differences in concentrations and $R_{CSF/Serum}$ among different analytes and among populations with different sex and parameters in CSF. The significance level was set at 0.05. All statistical analyses were performed using IBM SPSS Statistics 19.0 (SPSS Inc., USA).

2.5. Molecular Docking. To investigate the effects of the efflux transporters and human serum albumin (HSA) on the penetration efficiencies of tri-OPEs across the BCSFB in humans, the interactions of tri-OPEs with P-glycoprotein (P-gp) and HSA were investigated using molecular docking. The three-dimensional (3D) structures of P-gp (PDB ID: 6QEX) and HSA (PDB ID: 4E99) were downloaded from the RCSB Protein Data Bank (www.rcsb.org). The 2D structure of the compound was drawn by ChemBioDraw Ultra 14.0 and converted into a 3D structure by Chem3D Ultra 14.0 software. The binding mode between each tri-OPE congener and the specific proteins was simulated using AutoDock Vina 1.1.2.[50] The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (www.pymol.org). More details on the molecular docking are provided in the Supporting Information.

3. RESULTS AND DISCUSSION

3.1. Population Characteristics. A summary of the demographic characteristics and clinical parameters of the 288 participants is provided in Table S5. Participants (140 females and 148 males) were 1−82 years old, with a median age of 51 years. Globulin, RBC count, and WBC count are routine tests performed on CSF to estimate the condition of the CNS. Generally, a qualitative test of globulin is negative, and an RBC count of 0 unit/μL and a WBC count of less than 15 unit/μL are considered normal reference values, which were met by 28.3% (n = 81), 36.8% (n = 106), and 47.2% (n = 136) of participants, respectively. In addition, several parameters that may be related to BBB permeability were measured in serum, including Glu, TC, HDL-C, LDL-C, TG, and Hcy, which had median concentrations of 5.40, 4.20, 1.06, 2.55, 1.19 mmol/L, and 10.8 μmol/L, respectively.

3.2. Occurrence of Tri-OPEs in Serum and CSF. The concentrations and profiles of tri-OPEs in the serum and CSF samples are summarized in Figure 1 and Table S6. Among the 21 target tri-OPEs, TEP, TBP (TNBP + TIBP), TCEP, TCIPP, TPHP, and EHDPP were detected in 56.6−99.7% of the serum samples, while the remaining tri-OPEs showed low DFs (<37%) or were not detectable. TEP (median: 1.63 ng/mL) was the most abundant tri-OPE in serum, with a contribution of 66.3% to the ∑tri-OPEs, followed by TCIPP (0.291 ng/mL, 11.8%), EHDPP (0.220 ng/mL, 9.0%), TBP (0.170 ng/mL, 6.9%), TPHP (0.085 ng/mL, 3.5%), and TCEP (0.062 ng/mL, 2.5%) (Figure 1). The median TEP concentration in serum in this study was higher than that reported in serum from four cities in Jiangsu Province (0.15 ng/mL)[51] or whole blood from Beijing (0.432 ng/mL)[16] and Shenzhen (0.49 ng/mL).[52] The TCIPP concentrations in this

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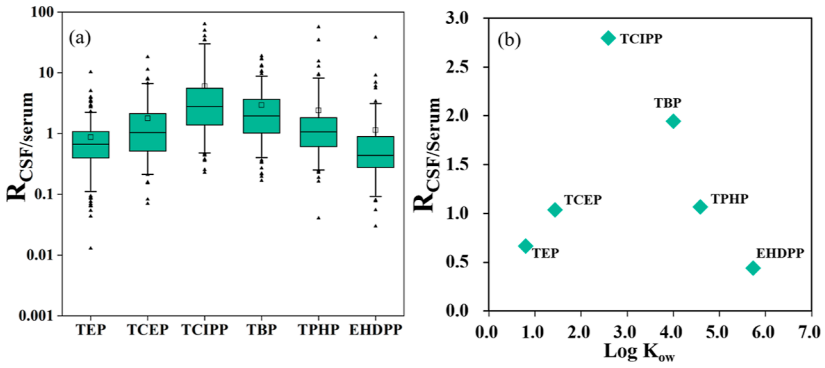

Figure 2. $R_{CSF/serum}$ values of tri-OPEs based on paired serum and CSF samples (a) and the relationships between median $R_{CSF/serum}$ values of tri-OPEs and their log K_{ow} values (b). In panel (a), the upper and lower bounds of the boxes indicate the 75th and 25th percentiles, respectively; the horizontal lines within the boxes indicate median values; the hollow boxes within the boxes indicate mean values; the upper and lower limits of the whiskers indicate the 95th and 5th percentiles, respectively; and the solid circles above or below the whiskers indicate outlier values.

study were lower than those in Hengshui (0.36 ng/mL)[14] and Shenzhen (0.71 ng/mL)[52] but higher than those in Beijing [not detected (ND)][16] and Jiangsu Province (0.05 ng/mL),[51] China. Different from TEP being the main tri-OPE in this study, TNBP, TPHP, TCIPP, EHDPP, and TCEP were reported to be the dominant tri-OPEs in blood samples from Shenzhen,[52] Hengshui,[14] Jinan,[13] four cities in Jiangsu Province,[51] and Bohai Bay,[53] China, respectively. Although the most abundant tri-OPEs in blood have differed among studies due to variation in the blood matrices investigated, pollution characteristics among areas, and metabolic characteristics of various populations, the above 6 tri-OPEs have been typically detected at higher levels in the blood than other congeners. This finding indicates that the widespread use of these tri-OPEs in China has resulted in high human exposure. Therefore, we recommended prioritizing attention to these traditional tri-OPEs in future studies. Several emerging tri-OPEs measured in our study had low DFs (0−11.5%) in serum samples, although they were highly detected in environmental samples.[24,54,55] This may be attributed to their relatively lower concentrations compared to traditional tri-OPEs in an indoor environment in China.[24,55] Among the 288 serum samples analyzed, significant positive correlations were found among TBP, TCEP, TCIPP, TPHP, and EHDPP (r_s = 0.159−0.504, p < 0.01) (Table S7), suggesting similar sources and pharmacokinetics in humans. For example, chlorinated tri-OPEs are usually used as flame retardants in polyurethane foam in furniture,[56] while TCEP, TPHP, and EHDPP were added in PVC as plasticizers.[8] Additionally, TBP, TPHP, and EHDPP are also used in hydraulic fluids and lubricants.[1]

Similar to the serum samples, TEP, TBP, TCEP, TCIPP, TPHP, and EHDPP were frequently found in CSF samples, with DFs ranging from 51.7 to 91.2%, while the other tri-OPEs had low DFs (<38.9%). TCIPP (median: 1.11 ng/mL) and TEP (1.06 ng/mL) were the dominant tri-OPEs in CSF samples, accounting for 41.0 and 39.0% of the ∑tri-OPEs, respectively. Additionally, TBP (0.315 mg/mL), TCEP (0.099 ng/mL), TPHP (0.086 ng/mL), and EHDPP (0.042 ng/mL) contributed 11.6, 3.6, 3.2, and 1.6% to the ∑tri-OPE concentrations, respectively (Figure 1). TCIPP and TBP showed significantly higher contributions in CSF than in serum (p < 0.001), while the opposite was true for TEP and EHDPP (p < 0.001). No significant differences were observed for TCEP and TPHP. The above results indicate that these tri-OPEs can penetrate through the BCSFB in humans and that the penetration efficiencies from serum to CSF may vary among compounds. To our knowledge, no study has investigated tri-OPE levels in CSF in humans. One previous study reported the existence of several tri-OPEs in the brain tissue of dolphins from the Alboran Sea, Spain[47] and showed that TCIPP [median: 41.2 ng/g dry weight (dw)] and TNBP (18.3 ng/g dw) exhibited higher concentrations than TCEP, EHDPP, TMPP, and TBOEP (0.71−4.69 ng/g dw),[47] results that were consistent with higher proportions of TCIPP and TBP in CSF in this study. However, different phenomena were observed in another two research studies, one of which reported higher concentrations of TBOEP [10 ng/g wet weight (ww)] than TNBP (2.20 ng/g ww), TCIPP (0.776 ng/g ww), TCEP (0.246 ng/g ww), and EHDPP (0.178 ng/g ww) in the brain tissue of silver carp from Taihu Lake[46] and the other showed more accumulation of chlorinated tri-OPEs (TCIPP, TCEP, and TDCIPP) in mouse brain tissue than TNBP, TIBP, and TPHP after 72 days of inhalation exposure.[45] Exposure differences may help to explain the discrepancies among these studies. For example, the low DFs of TDCIPP and TBOEP and lower levels of TCEP than TBP in CSF in this study may be related to low exposure to these tri-OPEs among the participants. In addition, differences among species in the structure and function of the BBB may also account for the different tri-OPE compositions in CSF or brain tissue between studies. The six frequently detected tri-OPEs were significantly correlated with each other in CSF (r_s = 0.117−0.566, p < 0.05) (Table S7). For emerging tri-OPEs, higher DFs were found for bisphenol-A bis(diphenyl phosphate) (BPA-BDPP), bis(3-isopropylphenyl)phenyl phosphate (B3IPPPP), and 3-*tert*-butylphenyl diphenyl phosphate (3tBPDPP) in CSF (8.3, 9.0, and 13%) than in serum (1.7, 0, and 6.3%) in our study. Previous studies have also reported the presence of BPDP and IPDP in the brain tissue of silver carp[46] and IDPP and tris(isopropyl-phenyl) phosphate (IPPP) in the brain tissue of dolphins.[47] These findings suggest that these novel aryl-tri-OPEs may penetrate the BBB and cause neurotoxicity, which needs to be verified. No significant associations were observed between tri-OPE levels in serum or CSF and the sex and age of the participants.

3.3. Penetration Efficiencies of Tri-OPEs and Impact Factors. To investigate the penetration of tri-OPEs from serum to CSF, we conducted correlation analyses between tri-OPE concentrations in CSF and serum and calculated the concentration ratios of each tri-OPE in paired CSF and serum

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Table 1. $R_{CSF/serum}$ Values and Related Impact Factors for Tri-OPEs

compound	$R_{CSF/serum}$[a]	MW[b]	log K_{ow}[c]	S_v[d]	$BE_{P\text{-}gp}$[e]	$BE_{HSA(SI\ binding\ site)}$[e]	$BE_{HSA\ (SII\ binding\ site)}$[e]	$t_{1/2}$ (day)[f]
TEP	0.667	182.15	0.80	167.32	−3.8	−4.5	−4.3	
TCEP	1.04	285.49	1.44	212.95	−4.0	−4.6	−4.6	17.4
TCIPP	2.80	327.57	2.59	264.84	−4.9	−5.4	−5.1	15.2
TBP	1.95	266.31	4.00	271.10	−4.6	−5.4	−5.4	4.76
TPHP	1.07	326.28	4.59	281.37	−7.7	−8.1	−7.1	9.58
EHDPP	0.438	362.41	5.73	347.13	−7.0	−7.7	−6.4	

[a]The median $R_{CSF/serum}$ values of tri-OPEs. [b]MW: molecular weight. [c]log K_{ow}: the log-transformed octanol−water partition coefficient of tri-OPEs. [d]S_v (Å^3/molecule): van der Waals volume calculated using a model provided in the study of Zhao et al.[74] [e]$BE_{P\text{-}gp}$, $BE_{HSA\ (SI\ binding\ site)}$, and $BE_{HSA\ (SII\ binding\ site)}$ (kcal/mol): the binding energies between tri-OPEs and P-glycoprotein, HSA (SI binding site), and HSA (SII binding site), respectively. [f]The estimated half-life ($t_{1/2}$, day) of tri-OPEs in humans in the study of Wang et al.[14]

samples ($R_{CSF/serum}$). Significant correlations were observed for the six frequently detected tri-OPEs (r_s = 0.186−0.454, p < 0.002) (Table S6). The $R_{CSF/serum}$ values of TEP, TBP, TCEP, TCIPP, TPHP, and EHDPP were in the ranges of 0.013−10.3, 0.167−18.8, 0.070−18.2, 0.226−63.2, 0.040−56.5, and 0.030−38.1, respectively, with median values of 0.667, 1.95, 1.04, 2.80, 1.07, and 0.438, respectively (Figure 2a). Among the participants, large variation in permeability for each tri-OPE (2−3 orders of magnitude) may be related to the differences in individual barrier function.[57] Due to the lack of an albumin ratio between CSF and serum (R_{Alb}) in this study, an indicator of barrier integrity (BBB or BCSFB permeability index), we were unable to accurately describe the relationship between brain barrier function and tri-OPE penetration efficiency in the manner reported previously for polyfluoroalkyl substances (PFASs).[57] However, in addition to R_{Alb}, the amount of globulin or the WBC count in CSF, markers of nervous system disease and infection, were also thought to indicate BCSFB function to a certain extent as these large molecules cannot easily cross the BBB in health.[42] In this study, significantly higher R_{TPHP} values and TPHP levels in CSF were found for participants with a WBC count greater than 15 unit/μL in CSF (p = 0.039 and p < 0.001) (Tables S8 and S9). In addition, participants with positive globulin in their CSF had higher TPHP levels in CSF than those with weakly positive or negative globulin in their CSF (p < 0.001) (Table S9), implying that nervous system disease and infection can impair brain barrier function and thus affect penetration of TPHP from serum to CSF. It is also possible that TPHP exposure increased the BCSFB permeability, as has been demonstrated in fish,[58] leading to an increase in TPHP in CSF. Additionally, positive associations were found between R_{TEP} (r_s = 0.319, p < 0.01, n = 72) or TCEP levels in CSF (r_s = 0.322, p < 0.01, n = 74) and triglycerides in serum. TPHP levels in CSF were also significantly correlated with total cholesterol (r_s = 0.256, p = 0.028, n = 74) and low-density lipoprotein cholesterol (r_s = 0.295, p = 0.011, n = 74) in serum (Tables S8 and S9), indicating that hyperlipidemia may compromise the integrity of brain barriers.[59,60] Additionally, significantly higher levels of TPHP in CSF and R_{TEP} values were observed for participants with impaired brain barrier function compared with those without impairment of the BCSFB (n = 42; participants met the following three criteria: globulin was negative, the WBC count was below 15 unit/μL and the RBC count was 0 unit/μL in CSF). No other significant relationships were found between the penetration efficiencies of tri-OPEs and medical parameters in serum or CSF, which may be because the brain barrier permeability was affected by many concurrent factors (e.g., sex, age, various diseases, drugs, and other unforeseen factors).[42,61−63]

The median $R_{CSF/serum}$ values of different tri-OPEs, from highest to lowest, were TCIPP (2.80) > TBP (1.95) > TPHP (1.07) > TCEP (1.04) > TEP (0.667) > EHDPP (0.438) (Figure 2a). Significant differences were observed between the penetration efficiencies of these tri-OPEs (p < 0.001), except for TPHP and TCEP. In addition to brain barrier integrity, the penetration behaviors of organic compounds across the BCSFB are influenced by many other factors, including the physicochemical properties of compounds, binding to plasma proteins and efflux transporters.[57,64,65] To understand the influence of chemical properties on the penetration behavior of tri-OPEs across the BCSFB, correlation analyses were conducted between the $R_{CSF/serum}$ values of individual tri-OPEs and the related physicochemical properties, including molecular weight (MW), molecular volume (S_v), and hydrophobicity (log K_{ow}) (listed in Table 1). The results showed that no linear relationships were observed between the $R_{CSF/serum}$ values and these parameters (p > 0.05). The penetration efficiencies of the six tri-OPEs tended to first increase and then decrease with the increase in log K_{ow}, MW, and S_v (Figures 2b and S1). Additionally, considering that a positive correlation was reported between the permeability of 27 drugs across the BBB and their lipophilicity ($K_{ow}/\sqrt{MW}$) in a previous study,[65,66] we also analyzed the relationship between the $R_{CSF/serum}$ values of the tri-OPEs and their $K_{ow}/\sqrt{MW}$ values. A similar trend was observed as mentioned above; the $R_{CSF/serum}$ values of TEP, TCEP, and TCIPP increased as lipophilicity increased, while those of TBP, TPHP, and EHDPP did not follow this trend but instead decreased. Similarly, many drugs have shown low BBB penetration despite their low MW and high lipophilicity.[64,67] This may be associated with the abundant efflux transporters that exist at the BBB and BCSFB, which can transport certain compounds that have entered the brain parenchyma or CSF back into the blood.[42] Therefore, the binding affinity and mode of each tri-OPE to P-glycoprotein, a main and important transporter at the brain barrier,[65] were investigated using a molecular docking method in this study. The results showed that the binding affinities of TPHP (−7.7 kcal/mol) and EHDPP (−7.0 kcal/mol) to P-gp were higher than those of the other four tri-OPEs (from −4.9 to −3.8 kcal/mol) (Table 1), suggesting the possibility of efflux of TPHP and EHDPP from CSF via the transporter. As a result, the net penetration of TPHP and EHDPP from serum to CSF is reduced. Additionally, the binding energies of tri-OPEs to HSA were calculated, as the compound bound to protein cannot pass through the BCSFB. Stronger binding affinities were still found for TPHP (binding

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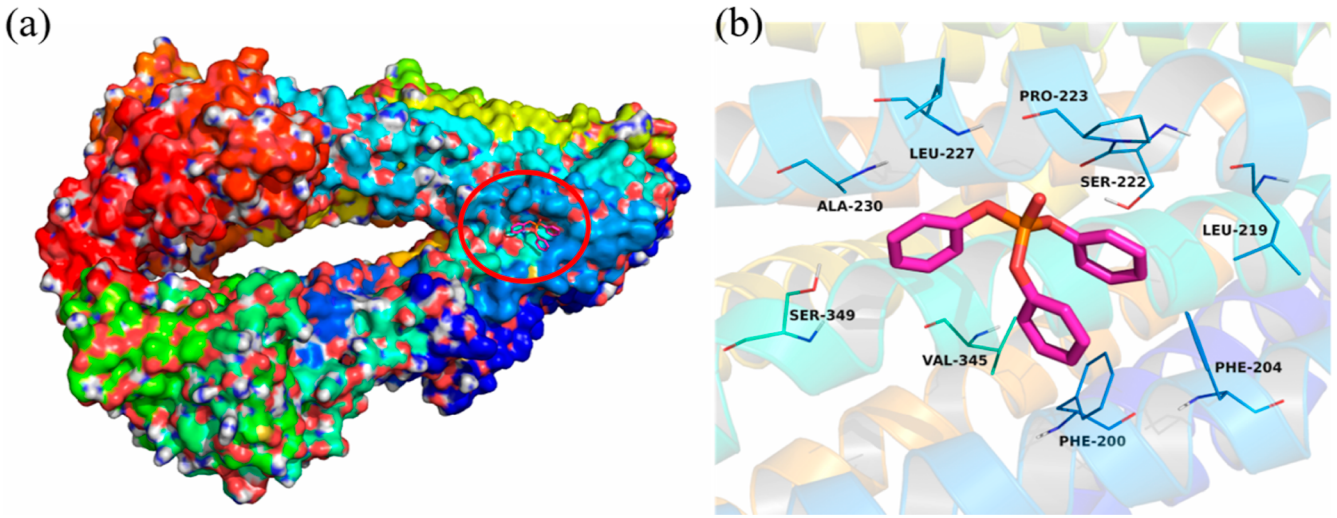

Figure 3. Ttotal view (a) and detailed view (b) of the molecular docking results for the binding between TPHP and P-gp. Figures for EHDPP docking into the binding pocket of P-gp are shown in Figure S2.

site SI: −8.1 kcal/mol; SII: −7.1 kcal/mol) and EHDPP (SI: −7.7 kcal/mol; SII: −6.4 kcal/mol) with HSA than for other tri-OPEs (SI: −4.5−5.4 kcal/mol; SII: −4.3−5.4 kcal/mol) (Table 1). Consistent finding has been reported in a previous study, in which TPHP has higher plasma protein binding ratio and binding constant with HSA than TCIPP, TCEP, and TNBP.[14] These findings may further explain the lower penetration efficiencies of TPHP and EHDPP. In addition to the factors discussed above, the metabolic rates of tri-OPEs may also affect their permeability across the BCSFB because several tri-OPEs have been found to metabolize easily in the human body.[18] Chlorinated tri-OPEs were the most accumulated tri-OPEs in mice after chronic PM2.5 exposure.[45] The estimated half-lives ($t_{1/2}$) of TCIPP (15.2 days) and TCEP (17.4 days) in humans based on in vitro metabolic experiments were higher than those of TNBP (4.76 days) and TPHP (9.58 days).[14] Previous studies have reported that the amount of peptides across the BBB was highly dependent on their half-lives in plasma.[68,69] Therefore, the relatively long half-life of TCIPP may enhance its penetration into the CSF, which may be one reason for the higher permeability of TCIPP compared to TBP despite TCIPP having a lower lipophilicity than TBP. In addition, it cannot be ruled out that there are other unknown factors increasing the penetration of TCIPP, which requires further investigation. The low DFs of other tri-OPEs in serum and CSF limit our full understanding of the impacts of physicochemical properties and affinities with transporters of tri-OPEs on their penetration efficiencies. Two previous studies investigated the penetration efficiencies of PFASs across the BCSFB in neonates[70] and adults.[57] Lower $R_{CSF/serum}$ values (0.008−0.029) were reported for eight PFASs, with MWs > 400 Da, compared to the six tri-OPEs in this study, which may be attributed to their higher MWs, stronger affinities for HSA,[71,72] and ionized form in the blood.[73] Further relevant research should focus on additional organic compounds to better evaluate the influence of various factors on the BCSFB permeability of these chemicals.

3.4. Penetration Mechanisms. The BCSFB is formed by choroid plexus epithelial cells that are connected to each other by tight junctions[44] and exhibit extremely low levels of pinocytosis/transcytosis.[42] This particular structural feature prevents the paracellular diffusion of exogenous compounds from blood into CSF, resulting in the penetration of chemicals mainly via the transcellular route.[75] Therefore, molecules with large MW or high hydrophilicity usually have low permeability through the phospholipid bilayer. Numerous studies on drug transport across the BBB have indicated that molecules with MWs less than 400 Da and fewer than eight H-bonds with water can cross the BBB via passive diffusion, and their barrier permeability increases with the increase in lipophilicity.[64] The six tri-OPEs (TEP, TCEP, TCIPP, TBP, TPHP, and EHDPP) conform to the above criteria; thus, we inferred that they could enter the CSF from circulating blood by free diffusion. This was confirmed by the frequent detection of these tri-OPEs in CSF samples and the increased permeability of the BCSFB to TEP, TCEP, and TCIPP with lipophilicity increase. However, passive diffusion cannot explain the reduced penetration efficiencies of TPHP and EHDPP given their greater lipophilicity. Therefore, binding to transporters was suspected to be involved in the brain barrier penetration of these two tri-OPEs. The strong affinities of TPHP and EHDPP for P-gp suggest that binding to efflux transporters causes the active export of these two tri-OPEs back into the blood. The molecular docking results showed that both TPHP and EHDPP adopted a compact conformation to bind at the binding pocket of P-gp, forming a strong hydrophobic interaction (Figures 3a and S2a). This suggests that hydrophobicity is an important factor affecting the protein binding ability of tri-OPEs given the higher log K_{ow} of TPHP (4.59) and EHDPP (5.73) than the other four tri-OPEs (0.8−4.0). Similarly, a previous study reported that TPHP and tri-*o*-cresyl phosphate (ToCP) (log K_{ow}: 6.34) exhibited stronger affinities for transthyretin (TTR) (−7.7 and −8.4 kcal/mol) and CYP3A7 liver metabolic enzyme (−8.6 and −9.4 kcal/mol) than TCEP (log K_{ow}: 1.44; TTR: −4.1 kcal/mol; CYP3A7: −4.2 kcal/mol) and TPP (log K_{ow}: 1.87; TTR: −5.3 kcal/mol; CYP3A7: −5.4 kcal/mol).[76] Additionally, the phenyl groups of TPHP and EHDPP formed CH−π interactions with several residues of P-gp (Figures 3b and S2b), indicating that aryl-tri-OPEs could bind more strongly to P-gp than alkyl- and chlorinated tri-OPEs, thus showing lower BCSFB permeability. In addition to P-gp, there are many other transporters present at the brain barrier, but the interactions between tri-OPEs and these proteins are currently unclear. Therefore, future studies

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using in vitro models or computational toxicological methods, and focusing on more transporters, are needed to explore the role of efflux transporters in the barrier penetration behaviors of different tri-OPEs and related influencing factors. In addition, some of the participants in this study had brain diseases, implying that brain barriers were compromised, such as structural alternation or downregulation of TJs.[42] Thus, in addition to passive diffusion and active efflux transport, some tri-OPEs in the blood of these participants may have penetrated the CSF via paracellular passage, which needs to be explored in the future.

3.5. Penetration Behaviors of Di-OPEs from Serum to CSF. The DFs of eight di-OPEs in serum and CSF samples were all lower than 40% (Table S10). BEHP, DBP, and DPHP were detected in 39.6% (n = 114), 35.4% (n = 102), and 25.3% (n = 73) of serum samples, while the other di-OPEs had DFs <10%. Similar findings were reported for the serum of adults in Norway[77] and for the whole blood of puerpera in Hong'an, Hubei Province, China,[76] which may be due to the easy excretion of di-OPEs from the human body via urine.[16] In serum, the 95th percentile levels of BEHP, DPHP, DBP, BBOEP, and BMPP were 0.829, 0.410, 0.231, 0.164, and 0.050 ng/mL, respectively. Similarly, BEHP was also the dominant di-OPE in the serum of adults from Beijing[16] and four cities in Jiangsu Province, China.[51] Additionally, relatively higher DFs for DPHP and DnBP were observed in serum samples from Norway[77] and Jiangsu Province, China.[51] This may be related to the direct industrial applications,[78] larger production volumes,[19] and higher concentrations in indoor dust[13,24] and food[25] of BEHP, DPHP, and DnBP than other di-OPEs. Among CSF samples, BEHP had a DF of 35.8%, followed by DBP (13.5%), DPHP (12.8%), and DMPP (7.6%). The other di-OPEs were detected in <2.1% of the samples. Similar to the profiles of di-OPEs in serum, BEHP (0.644 ng/mL), DPHP (0.412 ng/mL), and DBP (0.132 ng/mL) showed higher 95th percentile levels than BMPP (0.047 ng/mL) in CSF samples.

The penetration efficiencies of di-OPEs across the BCSFB were not analyzed due to their low DFs in paired serum and CSF samples. However, the lower DFs and levels of di-OPEs in CSF than in serum indicate that di-OPEs cannot pass through the brain barrier easily, which was in line with a previous finding that DPHP was not detected in zebrafish brain tissue after 11−14 days of exposure to d_{15}-TPHP.[17] The low BCSFB permeability of di-OPEs may be associated with their high polarity and low hydrophobicity (log K_{ow} < 3.97), except for BEHP, which makes them easily excreted from the human body. The low hydrophobicity of di-OPEs may also directly limit their permeability through the phospholipid bilayer of epithelial cells. Additionally, di-OPEs are ionized in human serum due to their pK_a values being less than 2 (Table S1), which may be another reason for their low BCSFB permeability. Finally, binding to the organic anion transporter, an important efflux transport protein,[79] may also influence the transport of di-OPEs from serum to CSF. Additional relevant studies are warranted to verify these results. Overall, although the DFs of di-OPEs in CSF were low, the detection of some di-OPEs (e.g., BEHP) in partial samples suggests that di-OPEs can penetrate the BCSFB under certain conditions, such as an impaired brain barrier. In this study, however, we did not find any associations between the penetration of di-OPEs and parameters in serum or CSF, which may be largely due to the low DFs of di-OPEs.

3.6. Implications and Limitations. This study is the first to investigate the occurrence of tri-OPEs and di-OPEs in paired human CSF and serum samples, and we confirmed that tri-OPEs can pass through the BCSFB, while di-OPEs showed low brain barrier permeability in humans. Similar to many drugs, the BCSFB penetration efficiencies of tri-OPEs were affected by a combination of barrier integrity, physicochemical properties of compounds, binding of serum protein and transporters, and metabolic characteristics. Based on these findings, passive diffusion was proposed to be the main penetration mechanism for six tri-OPEs across the BCSFB, and active efflux transport was involved in the penetration of TPHP and EHDPP. These results are important for evaluating the neurotoxicity of tri-OPEs. Many previous animal experiments (e.g., zebrafish and mice)[38,39,58] and human epidemiological studies[41,80] have shown that exposure to tri-OPEs (e.g., TNBP, TCEP, TDCIPP, TPHP, and TBOEP) can cause neurotoxicity, such as impaired learning or memory function, altered locomotor activity, and cognitive deficits. However, the underlying mechanisms responsible for these neurotoxic effects are unclear. The frequent detection of several tri-OPEs in CSF in this study suggests that tri-OPEs may penetrate the brain barrier and then directly cause damage to the CNS, which is worthy of attention. However, it is not clear whether the current burden of tri-OPEs in CSF will have any adverse effects on the CNS. These aspects need to be explored and verified in further in vitro and in vivo studies. In addition, as clarified in fish in a previous study,[58] it is also possible that human exposure to tri-OPEs can increase brain barrier permeability by activating inflammatory factors or disrupting tight junctions. This may lead to increased penetration of various exogenous substances, including tri-OPEs, from the blood to the brain parenchyma or CSF and thus increase the potential for neurotoxicity. Relevant toxicological studies are necessary to better understand the neurotoxic mechanisms of tri-OPEs.

Several limitations of our study should be noted. First, most of the participants in this study had or were presumed to have brain diseases because CSF collection is not a routine test. Therefore, the levels and penetration behaviors of tri-OPEs and di-OPEs in our study may not be generalized to the general population. Additionally, due to a lack of accurate indicators of BCSFB permeability in the participants, such as albumin concentration ratios, we could not determine whether brain barrier integrity was the primary cause of individual differences in penetration efficiencies of tri-OPEs across the BCSFB. Studies with larger sample sizes and more detailed clinical information on participants are needed to improve our understanding of the brain barrier permeability to tri-OPEs and potential neurotoxicity. Additionally, the mechanisms underlying the neurotoxicity of tri-OPEs in humans can be further explored by using CSF samples and metabolomics approaches.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01850.

Details of chemicals and reagents, sample preparation, instrumental analyses, quality control measures, and molecular docking; demographic characteristics and medical parameters of study participants; tri-OPE and di-OPE concentrations in CSF and serum; correlations

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among tri-OPEs, between analyte concentrations in CSF and serum, and between tri-OPE concentrations in CSF or $R_{CSF/serum}$ of tri-OPEs; age and sex of participants or parameters in serum and CSF; relationships between $R_{CSF/serum}$ of tri-OPEs and their S_v and MW; and molecular docking results for the binding between EHDPP and P-gp (PDF)

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Notes

The authors declare no competing financial interest.

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