

Effect of a two-week treatment with low dose of *ortho*-substituted polychlorinated biphenyls (PCB104 and PCB153) on VEGF-receptor system expression in the choroid plexus in adult ewes

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Abstract

Ortho-substituted polychlorinated biphenyl (PCB) congeners, which constitute a large part of PCB residues found in the environment and in animal tissues, are known to exert potent vascular effects and can activate endothelial cells in the periphery and in the brain. The choroid plexus (CP) is responsible for cerebrospinal fluid (CSF) production and its epithelial cell layer is responsible for structure and functions of the blood-CSF barrier. The aims of this study were: 1) to investigate if environmentally relevant doses of PCB153 and similar doses of PCB104 caused changes in the expression of vascular endothelial growth factor (VEGF) – receptor system, which maintains CP function, and 2) to determine the level of both congeners in blood plasma after their oral administration. Studies of both congeners were performed on ovariectomized ewes treated *per os* with low doses (0.1 mg/kg, three times a week for two weeks) of PCB153 (n=4) or PCB104 (n=4) and vehicle (control, n=4). The effects of PCB153 and PCB104 treatment on mRNA expression of two isoforms of VEGF (VEGF₁₂₀ and VEGF₁₆₄) and their receptors Flt-1 and KDR were determined using real-time PCR. Plasma concentration of PCBs was measured using high resolution chromatography/tandem mass spectrometry (HRGC/MS-MS). We observed that neither PCB153 nor PCB104 significantly altered the mRNA of the VEGF-receptor system in the CP. In PCB treated animals plasma concentration of PCB153 (1.425 ± 0.16 ng/g of dry mass, DM) was about 150 times higher than PCB104 (0.009 ± 0.007ng/g DM). In control animals the PCB153 level was 0.14 ± 0.031 ng/g DM, while the PCB104 level was below detection level. This indicates that increase in plasma PCB153 concentration to levels similar to those reported in humans and of PCB104 concentration to levels 100 times higher than those found in human plasma did not affect the VEGF-receptor system in the CP in adult ewes. The significantly lower increase of PCB104 than PCB153 concentration in blood after oral administration suggests different absorption of both congeners from the digestive tract.

Key words: polychlorinated biphenyls: PCB104, PCB153, vascular endothelial growth factor, choroid plexus, ewes

Introduction

Polychlorinated biphenyls (PCBs) are chemicals that comprise a group of 209 congeners with varying degrees of chlorination, which determines their physical, chemical, and biological properties (Safe 1994). From a structural point of view, there are at least two distinct classes of PCBs: the non-*ortho* (coplanar) and *ortho*-substituted (planar) congeners. *Ortho*-substituted PCBs constitute a large part of the PCB residue found in the environment and animal tissues (Kodavanti et al. 1998, Faroon et al. 2001, Costera et al. 2006). The most environmentally relevant *ortho*-substituted PCB – 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) has been demonstrated to affect the reproductive function and puberty onset in young goats which were natively exposed to a low dose of PCB153 (Lyche et al. 2004, Oskam et al. 2005). We recently demonstrated that a low dose of PCB153 affects gonadotropin secretion in adult female ewes through an action on both pituitary Luteinizing Hormone (LH) release and the hypothalamic Gonadotropin Releasing Hormone (GnRH) pulse generator (Skipor et al. 2012). Although the brain appears to be better protected against PCBs than other tissues because of the blood-brain and blood-cerebrospinal fluid (CSF) barriers (Skipor and Thiery 2008), *ortho*-substituted PCBs have been reported to accumulate preferentially in the brain and CSF compared to other PCBs (Kodavanti et al. 1998, Takasuga et al. 2004, Montie et al. 2009). Recently, measurement of PCB concentration in the ovine CSF showed that PCB153 was the most abundant congener in the CSF (Skipor et al. 2012).

There is evidence that PCB153 disrupts expression of tight junction proteins in the blood-brain barrier which restricts most paracellular movement of ions and solutes across the brain barriers (Eum et al. 2008, Seelbach et al. 2010). Among *ortho*-substituted PCBs, 2,2',4,5,6'-pentachlorobiphenyl (PCB104) also affects tight junction proteins expression in brain endothelial cells, stimulates inflammatory mediators in vascular endothelial cells and induces prometastatic responses (Choi et al. 2003, Sipka et al. 2008). It has been demonstrated that vascular endothelial growth factor (VEGF) is involved in PCB104 induced endothelial hyperpermeability (Eum et al. 2004). VEGF is continuously and highly expressed in the choroid plexus (CP), in which the blood-CSF barrier is located (Skipor and Thiery 2008). In the ovine CP two isoforms, VEGF₁₂₀ and VEGF₁₆₄, are expressed (Szczepkowska et al. 2012). VEGF plays an important role in regulation of the stability of the endothelial cells in the CP (Maharaj et al. 2008) and is

involved in maintaining endothelial cells' fenestrated phenotype in the CP capillaries (Roberts and Palade 1995, Esser et al. 1998). The effects of VEGF are transduced mainly by two high-affinity receptors belonging to the tyrosine kinase-family: the *fms*-like tyrosine kinase (Flt-1) and the fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR). The CP has been shown to contain mRNA (Nico et al. 2004) and protein (Witmer et al. 2002, Maharaj et al. 2008, Yang et al. 2010) of VEGF receptors.

Studies on PCB toxicity are mainly based on *in vitro* models or *in vitro* experiments in which mice or rats are intraperitoneally injected with PCBs (Sipka et al. 2008). The *in vivo* effects of PCB153 and PCB104 on expression of the VEGF system in the CP in domestic animals exposed to PCBs through the food chain, the most common route of PCB exposure, has not yet been reported. Therefore, the aim of this study was to evaluate the effect of chronic oral administration of low doses (0.1 mg/kg of body weight, bw) of PCB104 and PCB153 on expression of the VEGF system in the CP in ewes. Additionally, the concentration of both PCB congeners was measured in blood plasma.

Materials and Methods

Animal management and experimental design

The experiment was conducted on adult Polish Lowland ewes (n=16, 60-70 kg bw). To prevent variability linked with the estrous cycle or interaction with steroids the animals were ovariectomized (under xylazine anaesthesia (0.25 ml/kg, im; Rometar, Spofa, Prague, Czech Republic) about 20 days before PCB administration. All animals were maintained indoors under natural lighting conditions (October-November), each group being in separated pens in order to avoid contamination through the ingestion of PCB-contaminated faeces. The ewes were fed a diet of hay, straw, and commercial concentrates with water and mineral licks available *ad libitum*. Experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals (1997) and approved by the Local Ethics Committee (agreement no 4/2008).

After recovery, the animals were randomly allocated to three groups: group 1 – control (control), group 2 – PCB104 treated (PCB104) and group 3 – PCB153 treated (PCB153). Animals in the PCB104 and PCB153 groups were treated with appropriate PCB congeners by oral gavage three times weekly for 14 days, at a dose of 0.1 mg/kg of bw.

Table 1. Sequences of oligonucleotide primers used for Real Time – PCR analyses.

Gene	Primers (5'→3')	Product size	Literature source
VEGF-A ₁₂₀	Forward: AAGGCCAGCACATAGGAGAG Reverse: CCTCGGCTTGTCACATTTTT	101bp	Kaczmarek et al. (2008)
VEGF-A ₁₆₄	Forward: GAGGCAAGAAAATCCCTGTG Reverse: TCACATCTGCAAGTACGTTCG	150bp	Kaczmarek et al. (2008)
Flt-1 VEGF receptor 1	Forward: TGGATTTTCAGGTGAGCTTGGGA Reverse: TCACCGTGCAAGACAGCTTC	68bp	Redmer et al. (2005)
KDR VEGF receptor 2	Forward: CTTCCAGTGGGCTGATGACC Reverse: GCAACAAACGGCTTTTCATGT	67bp	Redmer et al. (2005)
NRP-1 neuropilin	Forward: GATTGCGGTGGACGATATTAGC Reverse: GGTTTTGCGCAGTCCTCTTG	60bp	Vonnahme et al. (2006)
RPL19 ribosomal protein L19	Forward: AATCGCCAATGCCAACTC Reverse: CCCTTTCGTTACCTATAACC	156bp	Drouilhet et al. (2010)
PPIC peptidyl-prolyl cis- trans isomerase C	Forward: TGGCACTGGTGGTATAAGCA Reverse: GGGCTTGGTCAAGGTGATAA	145bp	Herman et al. (2010)

Animals from the control group were treated with sunflower oil used as a vehicle for PCBs. Blood samples (20 ml) were collected from the jugular vein 15 days after beginning the PCB treatment (1 day after the end of PCB treatment) and then centrifuged and stored at -20°C for further analysis of PCBs. The animals were then sacrificed and, immediately after decapitation, the brains were dissected out and the CP from the lateral ventricles were collected and stored at -80°C until further analysis.

Calculation of doses and PCB administration

PCB104 (2,2',4,5,6'-pentachlorobiphenyl) and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) from AccuStandard (New Haven, CT, USA) were purchased locally at Tusnovics Instruments Polska and were 99.9% pure. Each PCB was dissolved in sunflower oil to a concentration of 1 mg/ml. Literature data (Lyche et al. 2004b) indicated that similar doses of PCB153 given orally in sheep for 30 days resulted in a plasma concentration of PCB153 of 30 ng/g (wet-weight). Costera et al. (2006) demonstrated that, in goats exposed to contaminated hay (concentration of PCB153 of about 460 ng/kg of dry mass (DM)), the time to reach a steady state concentration in milk was 15 days for most PCB congeners.

Relative gene expression assays

CP tissue, frozen and cut into small pieces (20 mg), was homogenized in Fenozol in Lysing Matrix D (MP Biomedicals, Illkrich, France) with a FastPrep-24 instrument (MP Biomedicals). RNA was

extracted using a Total RNA Kit (A&A Biotechnology, Poland), and Amplification Grade DNase 1 (Sigma Aldrich, Germany) was used to eliminate possible genomic DNA contamination, according to the manufacturer's instructions. The concentration and quality of RNA isolated from the CP tissue were determined using a NanoDrop (Thermo Scientific, USA) and 2% agarose gel electrophoresis. One microgram of total RNA was retained for further use in RT (reverse transcription) reaction. The RT reaction was performed with Quanti Tect Reverse Transcription Kit according to the protocol supplied by the manufacturer (Qiagen, USA). The resulting cDNA was stored at -20°C until further analysis.

To evaluate the effects of PCB treatment on mRNA expression of components of the VEGF-receptor system, real-time PCR was performed with the use of an ABI Prism 7900 sequence detection system and a Power SYBR green PCR master mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Specific primer pairs for the different genes were used according to the literature (Table 1). All primers were synthesized by IBB PAN (Poland). PCR-derived DNA fragments (VEGF-A₁₂₀, VEGF-A₁₆₄, Flt-1, KDR, NRP-1, reference genes- RPL19 and PPIC) were separated by electrophoresis on 2% agarose gels supplemented with 0.01% ethidium bromide and examined under UV light (Gel Logic100, KODAK). Each real-time PCR reaction well (20 µl) contained 2 µl of diluted RT product, 0.2 µM forward and reverse primers each and 10 µl of Power SYBR green PCR master mix. The following protocol was used: 95°C for 15 min for Hot Start AmpliTaq Gold DNA polymerase and 40 cycles of 95°C for 10 s (denaturation), 55°C for 20 s (annealing), and 72°C for 20 s (extension).

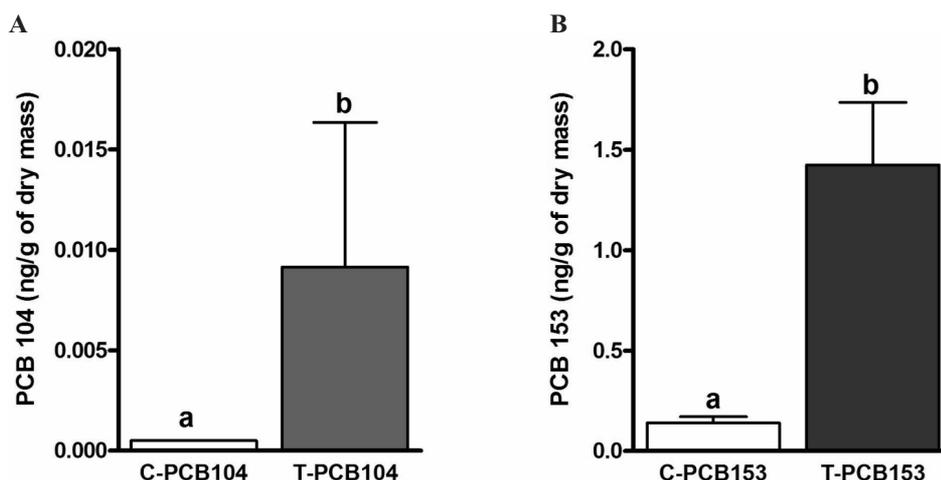


Fig. 1. Mean levels (\pm SD) of PCB104 (A) and PCB153 (B) in blood plasma of ovariectomized ewes receiving vehicle (C-PCB104 and C-PCB153), PCB104 (T-PCB104 – A) or PCB153 (T-PCB153 – B) *per os* (0.1 mg/kg of body weight; 6 doses over 14 days). Note that PCB104 in control group was below detection limit.

After the cycles, a final melting curve analysis under continuous fluorescence measurement was performed to evaluate the specific amplification. The results were analyzed using Real-time PCR Miner (available on-line: <http://www.miner.ewindup.info/version2>), based on the algorithm developed by Zhao and Fernald (2005).

PCB determination in blood plasma

Blood plasma samples (20 ml) were freeze dried and spiked with clean-up standards using 1 ml of ^{13}C -PCBs/g of the sample with standard mixture containing 10 ng/ml of ^{13}C -PCB104 and 20 ng/ml of ^{13}C -PCB153, and extracted with toluene for 16 h in 20 ml Soxhlet apparatus. The extract was cleaned up according to the previously published procedure (Surma-Zadora and Grochowalski 2008). The cleaned-up extract was evaporated to approximately 1 ml, transferred to 5 ml of dichloromethane and placed in a polyethylene semipermeable membrane. Dialysis was performed within 24 h with hexane as a recovery solvent. The hexane dialysate was then cleaned-up on a column which contained 10 g of 50% H_2SO_4 on silicagel. The column was eluted with 100 ml of hexane, evaporated to approximately 1 ml and transferred to another column containing 5 g of acidic Alumina (Merck 101078, 0.063 – 0.200 mm of activity I) and eluted firstly with 15 ml of hexane (fraction was discarded). The PCBs were then eluted in the second fraction of 50 ml of 2% dichloromethane in hexane. The eluate was evaporated just to dryness and reconstituted into 50 μl of nonane containing 100 pg/ml of ^{13}C -1,2,3,4-tetrachlorodiben-

zodioxin (^{13}C -1,2,3,4-TCDD) as a precision and recovery standard.

The determination of PCBs was performed in an accredited laboratory (Laboratory for Trace Organic Analyses, Krakow University of Technology, Poland) using the isotope dilution method, high resolution chromatography/tandem mass spectrometry (HRGC/MS-MS) on a Thermo Scientific GCQ-1100/Trace2000 system adjusted to double fragmentation mode equipped with Xcalibur data acquisition and analysis software. Separation of PCB congeners was performed on a 30 m \times 0.25 mm i.d. DB5MS capillary column of 25 μm film and DB17 30 m \times 0.25 mm i.d. A sample of 2 μl volume was injected into a SSL injector at 260°C. The GC oven temperature was held at 100°C for 1 minute, and then raised to 180°C at 20°C/min, then 2°C/min to 260°C, and then 20°C/min to a final temperature of 300°C. The temperature was held at 300°C for 5 minutes.

The method is of high specificity and gives non-interfered signals in the determination of PCB104 and 1CB153 congeners. Recoveries of PCB104 and PCB153 were in the range of 65 – 90% and 85-120%, respectively.

Data analysis

PCB data are expressed as the mean \pm SD. The real-time PCR results are presented as the relative gene expression (mean \pm SEM) of the target gene *vs.* the reference gene (RPL19). The significance of differences were statistically analyzed using one-way ANOVA (PRISM 4, Graph Pad, USA).

Results

PCB determination in blood plasma

The mean level of PCB congeners in blood plasma of sheep after oral administration of a low dose (0.1 mg/kg/per day) of PCB153 and PCB104 is presented on Fig. 1. In control sheep concentration of PCB153 was 0.14 ± 0.031 ng/g of DM, while the PCB104 level was below the detection limit (0.0001 ng/g DM). After oral administration of PCB153 and PCB104, their concentrations in blood plasma were 1.425 ± 0.16 ng/g DM and 0.009 ± 0.007 ng/g DM, respectively.

Real Time PCR analysis

PCB104 and PCB153 treatment had no significant effect on the expression of all tested genes: VEGF-A₁₂₀, VEGF-A₁₆₄, Flt-1 and KDR (Fig. 2) in comparison with the control group.

PCB153 action was compared with that of PCB104, formerly documented as affecting the VEGF system *in vitro* (Eum et al. 2004). Neither PCB153 nor PCB104 exposure significantly altered the mRNA of the VEGF-receptor system expression in the CP in ovariectomized adult sheep. The high variability of PCB concentration (78.5% for PCB104 and 21.8% for PCB153) observed in our study may account for large individual differences in PCB action on VEGF-receptor system mRNA expression. An earlier study demonstrated that maternal exposure to low doses (98 µg/kg bw) of PCB153 during gestation and lactation suppressed prepubertal plasma LH concentrations, delayed the onset of puberty of the female offspring, altered bone composition, and suppressed neonatal immunity in goats (Lyche et al. 2004a, b, Lyche et al. 2006, Lundberg et al. 2006). Recently we observed symptoms of estrogenization (enlarged vulva and edematous uterus) in PCB153 treated ewes, in which the plasma concentration of PCB153 was 196 pg/ml, equivalent to 2.4 ng/g DM

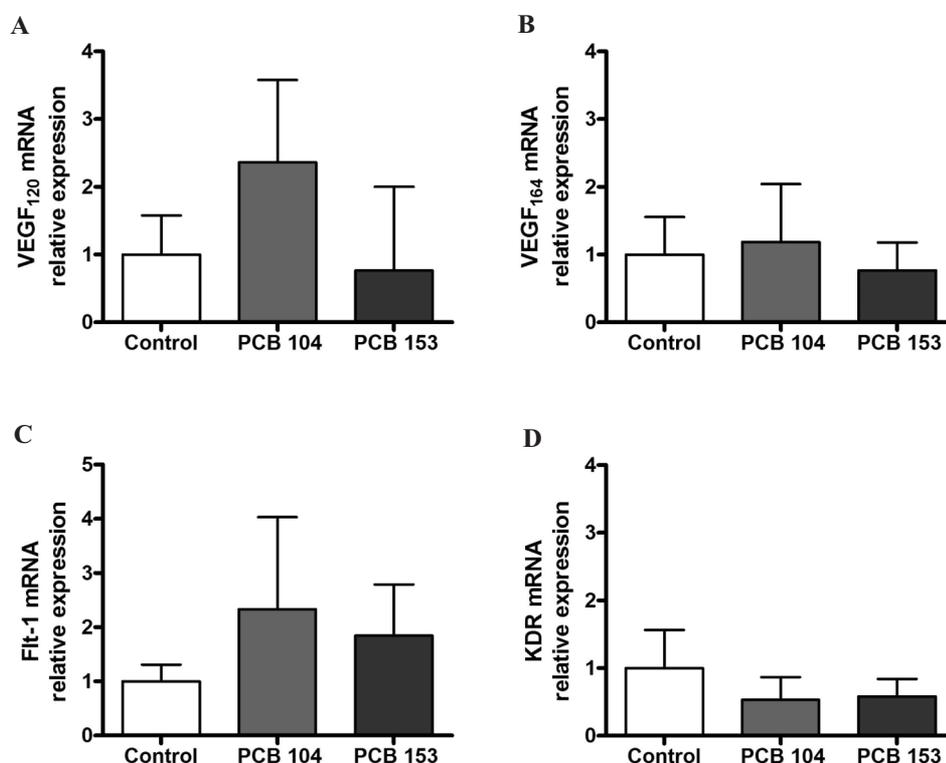


Fig. 2. Effect of PCB104 and PCB153 on mRNA expression of VEGF120 (A), VEGF164 (B), and its receptors Flt-1 (C) and KDR (D) in the choroid plexuses of adult ovariectomized sheep. Results were normalized to control group receiving vehicle (control) which was considered as 100%.

Discussion

This is the first study investigating the effect of environmentally relevant PCB153 *in vivo* on the VEGF-receptor system in the CP. In this study

(Skipor et al. 2012). Moreover, in these sheep basal plasma LH and pulsatile LH release was also affected (Skipor et al. 2012). It should be noted that the mean plasma concentration of PCB153 found in our study (1.4 ng/g DM, corresponding to 0.116 ng/ml) as well

as in our earlier study (0.196 ng/ml) is similar to the PCB153 concentration measured in the human population in the United States (<http://www.cdc.gov/exposurereport>). According to a national report on human exposure to environmental chemicals, the geometric mean of serum PCB153 concentration in humans was 0.167 ng/g (0.151-0.185 ng/g, 95% confidence interval, CI) in 2001-2002 and 0.121 ng/ml (0.114-0.128 ng/ml, 95% CI) in 2003-2004.

In contrast to PCB153, PCB104 is not environmentally and tissue relevant (<http://www.ewg.org/reports/bodyburden2>, Fielden et al. 1997), mainly due to the low distribution in commercial PCB mixtures (Frame 1997). In our studies, treatment of ewes with a low dose of PCB104 increased its concentration in blood plasma from an undetectable level to 0.009 ng/g DM (corresponding to 0.75 pg/ml). In humans, measurement of PCB104 in umbilical blood plasma gave 0.9 pg/g of lipid weight, corresponding approximately to 0.007 pg/ml (<http://www.ewg.org/reports/bodyburden2>). Therefore, we demonstrated here that a 100 times higher concentration of PCB104 than those found in humans did not affect the VEGF-receptor system in the CP. The dose of PCB104 used in *in vitro* studies promoting an increase in the microvascular permeability and the transmigration of breast cancer cells following PCB104 treatment ranged from 2-15 μ M, which corresponds to 650-4860 ng/ml. Such high concentrations of total PCBs (3-6 μ M) were detected in human blood plasma in industrialized areas of the Netherlands and in the Aland/Turku Archipelago (Koopman-Esseboom et al. 1994, Hagmar et al. 1998).

In our studies, treatment of ewes with the same low doses (0.1 mg/kg bw) of PCB104 and PCB153 resulted in about 150 times higher concentration of PCB153 in blood plasma compared to PCB104. This could result from feeding ewes with hay containing PCB153 since the concentrations of PCB153 congener in hay is among the highest compared to other congeners (Chauhan et al. 2000). Costera et al. (2006) showed that amounts of PCB153 in hay may reach 460 ng/kg/DM. It is also possible that our findings may result from the lower resistance to the biotransformation for PCB104 than for PCB153, which was demonstrated to be very resistant (Thomas et al. 1999). Indeed, about 22% of the total PCB residue in human tissue consists of PCB153 (Jensen and Sundstrom 1974). It has been suggested that binding of PCB with transthyretin in blood plasma protects against their rapid removal from circulation. PCB153 binds with transthyretin more effectively than PCB104 (IC₅₀, 90 vs. >1000) (Chauhan et al. 2000). In our study, the mean concentration of PCB153 in blood plasma was about 1.4 ng/ml. This is below the levels measured in

the plasma of pregnant goats (30 ng/g wet weight) long-term treated with a similar dose of PCB153 (Lyche et al. 2004b). Such discrepancy between two closely related animal families could be due to species difference, as differences in reactivity to PCB treatment has also been reported between rats and mice (Craft et al. 2002). Alternatively, it could stem from the use of pregnant goats by Lyche et al. (2004b) and ovariectomized sheep in our study. Finally, the endocrine status of the female rather than a difference in regimen of the treatment may be responsible for such a discrepancy, since we demonstrated that in sheep two-week oral administration of low doses of PCB153 (0.3 mg/kg bw) induced higher levels of PCB153 in short days than long days (Skipor et al. 2012).

In summary we demonstrated that increase of plasma PCB153 concentration to the level present in humans and PCB104 concentration to the level exceeding 100 fold that present in human plasma did not affect the VEGF-receptor system in the CP in adult ewes.

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