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Distinctively Different Effects of Perfluorobutanoic Acid and Perfluorononanoic Acid on Zebrafish Sex Differentiation and Androgen Receptor Activity

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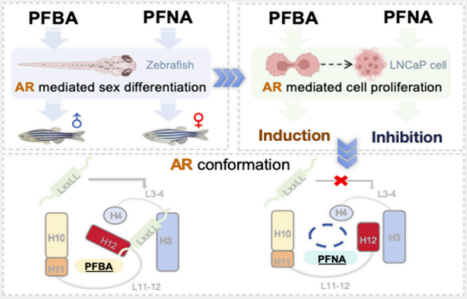
ABSTRACT: With the prohibition of perfluorooctanoic acid (PFOA), the emergence of alternative perfluoroalkyl substances such as perfluorobutanonic acid (PFBA) and perfluorononanoic acid (PFNA) in various environmental matrices has led to concerns about their adverse effects on humans and biota. This study aims to investigate the reproductive and developmental toxicity of PFBA and PFNA by combined *in vivo*, *in vitro*, and *in silico* approaches. Examination of juvenile zebrafish exposed to PFBA at environmental concentrations by histopathology, sex hormone, and gene expression revealed accelerated development of zebrafish toward males, while exposure to PFNA during sex differentiation resulted in feminization. In accordance with the *in vivo* results, PFBA activated the androgen receptor (AR) signaling pathway, but PFNA inhibited it in both prostate cancer cell proliferation and luciferase reporter gene assays. Similarly, the differential binding mode of the two chemicals to AR was shown in the molecular docking analysis, with PFBA exhibiting higher potency for the agonist conformation and PFNA favoring the antagonistic conformation. Together, these results suggest that, while PFNA exhibited similar effects on sex differentiation and AR activity as PFOA, PFBA showed distinctive effects and deserves particular attention and further investigation.



KEYWORDS: *Perfluoroalkyl substances, Zebrafish, Androgen receptor, Molecular docking*

1. INTRODUCTION

Poly- and perfluoroalkyl substances (PFASs) are widely utilized in various consumer products and industrial applications, encompassing over 200 distinct uses and subuses.[1,2] The exceptional stability of the carbon−fluorine (C−F) bonds in PFASs results in the persistence of their ultimate degradation products within the environment.[3,4] Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are the most widely used PFASs identified in diverse environmental matrices[5] and have been proven to lead to adverse effects on human health, wildlife, and ecosystems.[6−9]

Due to regulations, PFOA and PFOS concentrations in surface waters globally showed a decreasing trend in some regions,[6,10] but the use of other PFASs as alternatives increased, such as perfluorobutanonic acid (PFBA) and perfluorononanoic acid (PFNA),[11] remaining a significant environmental and health concern with detections often exceeding health advisory levels. Eighty seven water specimens from all five Great Lakes were collected in 2021−2023 and analyzed for 41 PFASs, and PFBA was the most abundant chemical among short-chain PFASs detected in lake water with median levels of 0.63, 1.2, 1.3, 1.7, and 2.5 ng/L in Lake Superior, Michigan, Huron, Erie, and Ontario, respectively.[12] The highest detected concentrations of PFBA in the surface and bottom seawater of China's Laizhou Bay were 31.54 ng/L and 57.04 ng/L, respectively.[13] Zhang et al.[14] examined surface water in Hefei City, Southeast China, and found PFBA to be one of the most prevalent PFASs, with levels spanning from 1.06 to 99.2 ng/L. The concentration range of PFNA in drinking water was 0.03−22.10 ng/L with an average of 2.61 ng/L and significant regional differences, and the concentration near the pollution source was relatively high in Pakistan.[15,16] PFBA and PFNA were detected in tap water samples across various locations in the Klang Valley (Selangor, Malaysia), with the maximum concentrations being 93.5 and 48.5 ng/L, respectively.[17]

Due to high average concentrations, the U.S. Environmental Protection Agency (EPA) has identified PFNA as one of the most concerning PFASs present in drinking water.[18] Epidemiological studies have identified associations between PFBA, PFNA, and chronic diseases such as metabolic syndrome and hyperlipidemia.[19] Sang et al.[20] also showed that PFNA exposure is negatively linked to testosterone levels

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in men across China, the US, and Denmark, suggesting reproductive health risks. Additionally, a Chinese case-control study linked higher maternal PFAS exposure, particularly to PFBA, PFNA, PFOA, and PFOS, to an increased risk of preterm birth, modified by estradiol levels.[21] These studies suggest a strong association between PFBA and PFNA and the endocrine system. Toxicological studies have found that PFBA and PFNA could cause endocrine disruption effects.[22−24] In independent research, environmentally relevant concentrations of PFBA, PFOA, and PFNA significantly impaired zebrafish antibacterial ability during their early developmental stages. This study also highlighted differences in the immunotoxicity of PFASs, particularly through the toll-like receptor signaling pathway.[18] Wasel et al.[25] found that PFBA, while less acutely toxic than PFOA, could still induce significant developmental neurotoxicity and morphological changes at sublethal concentrations in zebrafish embryos during development. The distinct effects of PFBA underscore the possible risks that it may present to the ecological system and human health, warranting further investigation into its mechanisms of action and toxicity. The androgen receptor (AR) signaling pathway is pivotal within the endocrine system, where androgens and androgenic chemicals bind to AR to initiate a cascade of biological responses.[25−27] One new research study indicated that exposure of medaka fish embryos to levonorgestrel alters the balance between androgens and estrogens, ultimately leading to masculinization or feminization. The mechanism of action is related to the transcriptional activity of medaka fish androgen receptor *in vitro*.[28]

In zebrafish exposed to 17β-trenbolone during their initial developmental phase, there is an observed rise in the male-to-female ratio within the population.[29] This effect is linked to the activation of AR as the molecular initiating event (MIE).[30] Our previous study found that PFOA and its alternative hexafluoropropylene oxides antagonized the AR signaling pathway and disrupted the sex differentiation of juvenile zebrafish.[31] Although PFBA and PFNA have been found to have endocrine disrupting effects through ER and ERRγ signaling pathways,[32−34] their effects on the AR signaling pathway have not been investigated.

In this research, the effects of PFBA and PFNA at concentrations relevant to the environment on reproductive and developmental health were assessed using zebrafish to investigate the influence of PFBA and PFNA on the sex differentiation of zebrafish. To establish a connection between the observed toxicological pathways and human health, the study also explored the impacts of tested chemicals on human prostate cancer cell proliferation and their influence on AR transcriptional activity. Ultimately, molecular docking simulations were performed to analyze the molecular-level interactions among PFBA, PFNA, and the human androgen receptor.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

PFBA (>99%, CAS#375−22−4) was purchased from Titan (Shanghai, China). PFNA (99%, CAS#375−95−1) and dihydrotestosterone (DHT, 99%, CAS#521−18−6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metribolone (R1881, ≥98%, CAS 965−93−5, Shanghai, China) and Flutamide (≥98%, CAS#13311−84−7, MedChemExpress, New York, USA) were used in the experiments.[31] The chemicals were prepared for testing by mixing the stock solution with the medium, ensuring a 0.1% (v/v) DMSO concentration.

2.2. Zebrafish Maintenance and Experimental Exposure

Zebrafish were raised in a system with a continuous water flow, utilizing water that had been filtered through activated charcoal. Zebrafish embryos were collected from three hatched males and two females. After a 2 h interval, the embryos were meticulously picked by using an optical microscope to ensure proper development. From 5 to 11 days postfertilization (dpf), the larvae were nourished with *paramecium* twice each day. Between 12 and 15 dpf, their diet was supplemented with a mixture of *paramecium* and brine shrimp. From 16 to 40 dpf, zebrafish were nourished with brine shrimp exclusively. The exposure solution was maintained at a controlled temperature of 27 ± 1 °C, and the lighting time was set to 14 h of light and 10 h of darkness.[35]

At 21 dpf, 11 randomly chosen larvae were placed into a 5 L tank containing 3 L of the solution of PFBA and PFNA. Environmental (PFBA: 0.05 μg/L, PFNA: 0.02 μg/L), medium (20 μg/L), and high (200 μg/L) concentrations were chosen for zebrafish exposure. Notably, the environmental concentrations are within the reported ranges typically found in surface water.[36−41] For every concentration of the test chemical, two zebrafish cohorts were established. The exposure mixtures were prepared by blending the stock solution with DMSO in tap water that had been filtered and oxygenated. A new exposure solution was added to half of the tank to make a semistatic exposure system everyday.[42,43] Zebrafish were anesthetized on ice and collected at 40 dpf. Out of the 11 zebrafish in one group, three fish were frozen using liquid nitrogen for subsequent RNA extraction, four fish were mixed together to measure sex hormones, and four were taken as samples for gonadal histology.[44]

2.3. Histological Examination of Gonads

The larvae were put in a 4% (w/v) paraformaldehyde solution and maintained under this condition for a day at 4 °C. Following this, they were dehydrated utilizing a series of ethanol solutions with varying levels. Afterward, the larvae were embedded in paraffin wax, and 3 μm-thick longitudinal sections were prepared from the gonadal area, yielding two slices per fish. These slices stained with Hematoxylin-Eosin were observed with Olympus light microscopes (Tokyo, Japan).[45,46]

2.4. Sex Hormone Measurement

Zebrafish were subjected to a rapid chilling process by adding ice-cold PBS at a ratio of 0.1 g of tissue to 900 μL of PBS. Subsequently, the samples of zebrafish underwent homogenization for 3 min at 4 °C utilizing a tissue analyzer (Servicebio, Wuhan, China). Posthomogenization, the samples were subjected to centrifugation at 13 000*g* for 15 min at 4 °C to separate the supernatant. The protein concentrations of the supernatant were employed to normalize the measurements of the 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) for precise comparability of the results.[31]

2.5. Expression of Sex Differentiation Related Genes Measurement

The process of extracting total RNA from all of the samples was conducted utilizing the RNAiso Plus reagent. The experimental details of the first-strand cDNA synthesis are described in the Supporting Information.[31] The expression of sex differentiation genes and the housekeeping gene β-actin were quantified.

2.6. Cell Culture

LNCaP human prostate cancer cells were procured from the China Center for Type Culture Collection (CCTCC, Wuhan, China). They were maintained in RPMI 1640 medium without phenol red from Gibco, Grand Island, New York, augmented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and antibiotics at concentrations of 100 units/mL penicillin and 100 μg/mL streptomycin. The culture conditions were maintained at 37 °C in an incubator with 5% CO_2 atmosphere. ThMDA-kb2 human breast cancer cells were grown in L-15 medium from Gibco, Grand Island, New York, enhanced with a 10% FBS solution, under humidified conditions at 37 °C without

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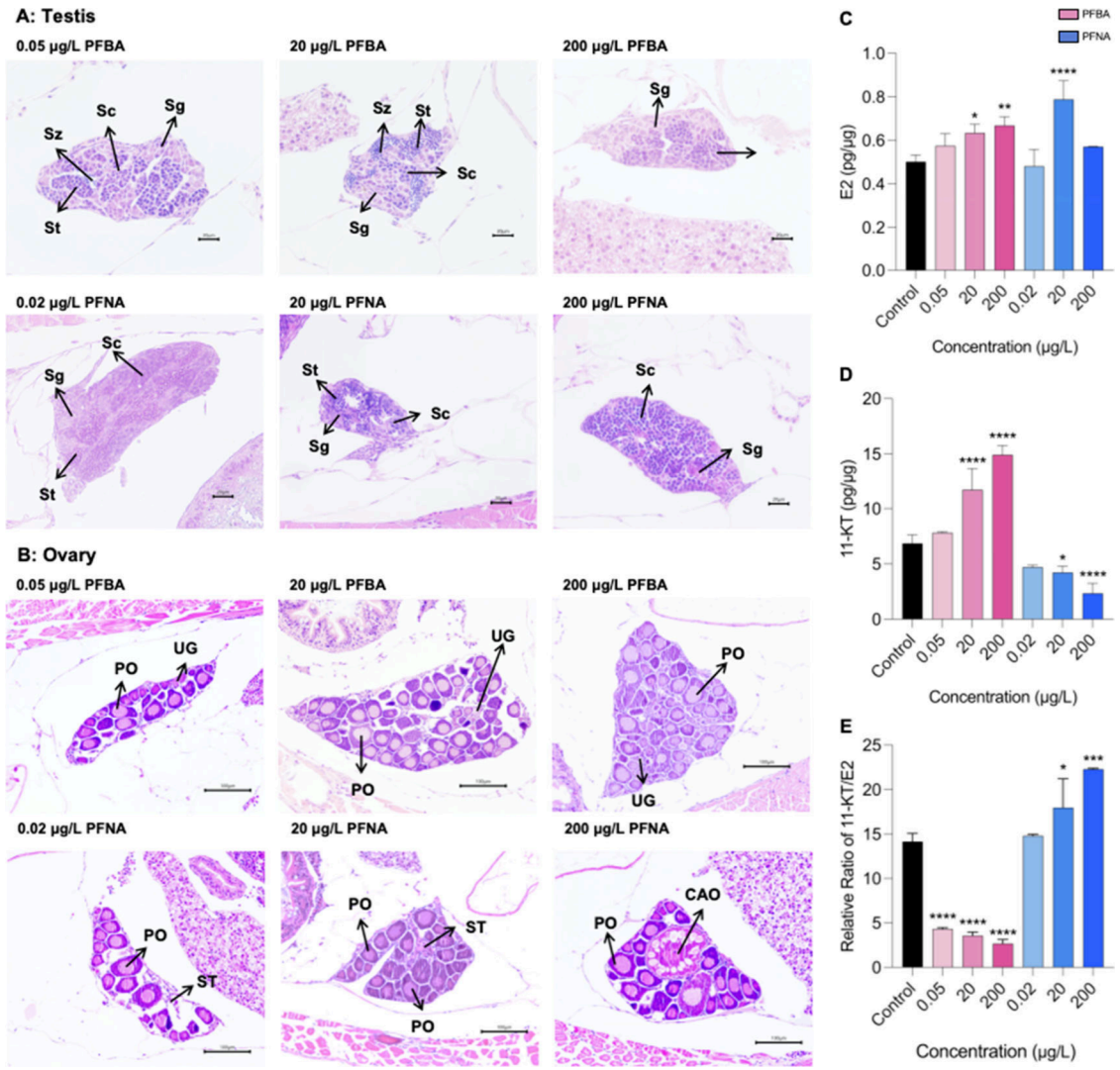

Figure 1. Gonadal histopathology and alterations of 11-KT and E_2 concentrations in zebrafish following exposure to PFBA and PFNA between 20 and 40 dpf. (A, B) The histological changes of gonads followed exposure to PFBA and PFNA. (C–E) Effects of PFBA at concentrations of 0.05, 20, and 200 μg/L and PFNA at concentrations of 0.02, 20, and 200 μg/L on the levels of 11-KT, E_2, and the 11-KT/E_2 ratios in zebrafish. Sg: Spermatogonia; Sc: Spermatocytes; St: Spermatids; Sz: Spermatozoa. PO: Perinuclear-stage oocyte; CAO: Cortical alveolar oocyte; UG: Undifferentiated gonocyte. The scale bars for testis = 20 μm. The scale bars for ovaries = 100 μm. The data are depicted as the mean value derived from three separate experiments, each including a group of 4 fish. The error bars indicate the standard deviation derived from three independent experimental measurements. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, compared with the control group.

CO_2 supplementation. Human embryonic kidney cells (HEK293) were sustained in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% FBS in an environment with 5% CO_2 and controlled humidity.

2.7. Cell Proliferation

LNCaP cells were put into 96-well plates overnight so that there were 1×10^4 cells in each well. The medium was then changed to 1640 medium without phenol red, and 2 mmol/L L-glutamine was added. These cancer cells were subjected to a 24 h deprivation period to enhance their responsiveness to androgens. After being introduced to chemicals, the cells were kept warm for 48 h. Five times of each exposure quantity were tested. After this, medium with 1 mg/mL MTT was added to these cells. Following the addition of 100 μL of DMSO to each well and allowing it to incubate for 4 h, the optical density was quantified at 490 nm by using a multipurpose microplate reader.

2.8. AR-Mediated Transcriptional Activity Assay

The transcriptional activity mediated by the human androgen receptor was quantified through luciferase-based reporter gene assays conducted in MDA-kb2 cells. The cells were put into 24-well plates with 2×10^5 cells per well and allowed to settle for 24 h.

The effects of PFBA and PFNA on the transcriptional activity of the zfAR was evaluated using a dual luciferase reporter system in transiently transfected HEK293 cells. pBIND-zfAR plasmid and the pGL4.35 plasmid were transfected into the HEK293 cells using Lipofectamine 3000. The subsequent steps for measuring luciferase activity were described in the Supporting Information.[31,47,48]

2.9. Molecular Docking Simulation

To forecast the possible interactions between the tested chemicals and AR, the CDOCKER module in Discovery Studio (Accelrys Software Inc., San Diego, CA, USA) was utilized for molecular docking simulation. The structures of human AR agonistic (PDB ID: 3L3X) and antagonistic (PDB ID: 2AX6) conformations have been obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). The 3D structures of small molecules were downloaded from PubChem. The docking process consists of several stages. First, the small molecules and AR were prepared by eliminating water molecules from the structure and adding hydrogen atoms to the AR. Both the small

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molecules and AR are optimized using the CHARMm force field. Then, the active site of the AR is identified based on the binding site of an internal ligand. Subsequently, the small molecules are positioned into the active site of AR using appropriate parameter settings and a series of algorithms.

2.10. Statistical Analysis

The data of all of the results are presented as the mean value ± standard deviation. Statistical analysis was conducted using GraphPad Prism version 6.01, which included a one-way ANOVA and the Dunnett's multiple comparison test to assess significant differences in mean values posthoc.

3. RESULTS AND DISCUSSION

3.1. PFBA and PFNA Significantly Altered the Gonadal Development in Juvenile Zebrafish

Due to the high degree of genetic similarity of 85% with humans and the close resemblance of their signaling pathways, zebrafish were chosen as model organisms. The sex differentiation in zebrafish is a critical biological process that holds significant importance for understanding the reproductive development, typically taking place between 20 and 40 dpf during developmental stages.[49,50] This process in zebrafish is influenced by exposure to endocrine-disrupting chemicals (EDCs), such as Bisphenol A[51] and 17α-ethinylestradiol,[52] which can disrupt the process and cause an imbalance of sex ratio. Consequently, we set up an exposure period ranging from 20 to 40 days to investigate the effects of PFBA and PFNA on sexual differentiation of zebrafish.

The zebrafish testis develops from spermatogonia (Sg) through spermatocytes (Sc) to spermatids (St), culminating in the formation of mature spermatozoa (Sz) for fertilization. In the control group, juvenile zebrafish formed Sg, Sc, and St at 40 days postfertilization (dpf) (Figure S1). At this point, Sz had not yet formed, indicating that testis had not reached the mature stage. Figure 1A shows the histological sections of juvenile zebrafish testis after exposure to PFBA and PFNA. In the 0.05 μg/L PFBA group, in addition to Sg, Sc, and St, the formation of Sz was also observed, suggesting a more mature stage than that observed in the control group. The 20 μg/L PFBA group also formed Sg, Sc, St, and Sz and produced more mature sperm than that observed in the 0.05 μg/L group. However, in the 200 μg/L PFBA group, testes development was hindered. Only Sg and Sc were formed in the testes, and there were also a significant number of undifferentiated gonadal cells present, which is presumed to be a result of the high concentration of the chemical. For PFNA, the results of the 0.02 μg/L group closely aligned with those of control groups, with the formation of only Sg, Sc, and St. The 20 μg/L group also formed Sg, Sc, and St; however, large interstitial voids were observed in this group, indicating damage in the testis. The 200 μg/L group only had Sg and Sc and undifferentiated gonad cells in the testis. These observations suggest that testis development was impeded after exposure to 20 and 200 μg/L PFNA, which is similar to the results of 200 μg/L PFOA exposure obtained in our previous study.[31]

In zebrafish ovarian development, perinuclear oocytes (POs) initiate the process, laying the groundwork for further development. They are succeeded by cortical alveolar oocytes (CAOs), an intermediate stage of maturation with alveoli formation. Subsequently, the oocytes advance to early and late vitellogenic stages, signifying the progression to more mature states. From the developmental perspective of the control group, only POs were formed in the ovary, indicating that they are still at the initial stage of ovarian development (Figure S1). Figure 1B illustrates the histological sections of ovaries from juvenile zebrafish after exposure to PFBA and PFNA. For PFBA, the development in the 0.05 and 20 μg/L groups was similar to that of the control group with only PO being formed. In the 200 μg/L PFBA group, the inhibition of ovarian development was more pronounced, and more undifferentiated gonadal cells were observed surrounding the perinuclear oocytes with the morphology of ovarian-like tissues being retained, suggesting that PFBA inhibits ovarian development at the early stage of sex differentiation. For PFNA, ovarian development in the 0.02 and 20 μg/L PFNA groups was similar to that in the control group, with only PO being formed. However, in the 200 μg/L PFNA group, CAOs were observed, indicating that they are in an intermediate stage of development and suggesting that ovarian development is faster than in control groups. This was also observed in the 200 μg/L PFOA group. Based on the experimental results of testis and ovaries, it was found that the effects observed at high concentrations of PFOA and PFNA were similar.[31]

3.2. PFBA and PFNA Disrupted the Balance of Sex Hormone Expression of Juvenile Zebrafish

Sex hormones are vital for a range of physiological functions in zebrafish, and disruptions in the balance between androgens and estrogens can serve as indicators of the internal status. The predominant hormone in these fish, 11-KT, is crucial for reproduction, including sexual behavior, sexual differentiation, and the maturation of testis.[53,54] E_2 is the key estrogen involved in the development of the ovaries. Consequently, the expressions of 11-KT, E_2, and their ratio were analyzed to determine whether PFBA and PFNA could perturb the balance between 11-KT and E_2.

As shown in Figure 1C, medium and high concentrations of PFBA significantly upregulated the E_2 level with a 25% and 31% increase compared to the E_2 level of the control group (0.50 pg/μg), respectively. In the case of PFNA, the 0.02 μg/L group did not affect the expression level of E_2 compared to the control group, but 20 μg/L PFNA significantly increased E_2 expression (0.78 pg/μg). The average concentration of E_2 in the 200 μg/L PFNA group was 0.57 pg/μg. Zhang et al.[23] reported that long-term exposure of 100 μg/L PFNA also increased E_2 levels in adult zebrafish, suggesting that zebrafish in their juvenile stage are more sensitive to the external environment and EDCs compared to their adult stage.

For 11-KT (Figure 1D), there was no significant change in the environmental concentration of PFBA after exposure compared to the level of the control. Medium and high concentrations of PFBA significantly increased 11-KT expression, with the average concentration rising to 11.70 and 14.921 pg/μg, respectively. Similarly, 0.02 μg/L PFNA had no significant effect on 11-KT expression, while at 20 and 200 μg/L, it caused a significant decrease in 11-KT expression. As for the 11-KT/E_2 ratio (Figure 1E), PFBA significantly reduced the 11-KT/E_2 ratio at the environmental concentration (0.05 μg/L), while the results showed that PFNA increased the ratio in a dose-dependent manner. This suggested that exposure to the two chemicals disrupted the balance of the sex hormones in zebrafish.

3.3. PFBA and PFNA Influenced the Expression of Genes Related to Sex Differentiation Mediated by zfAR

The process of sexual differentiation in zebrafish is intricately regulated at the molecular level, involving a complex

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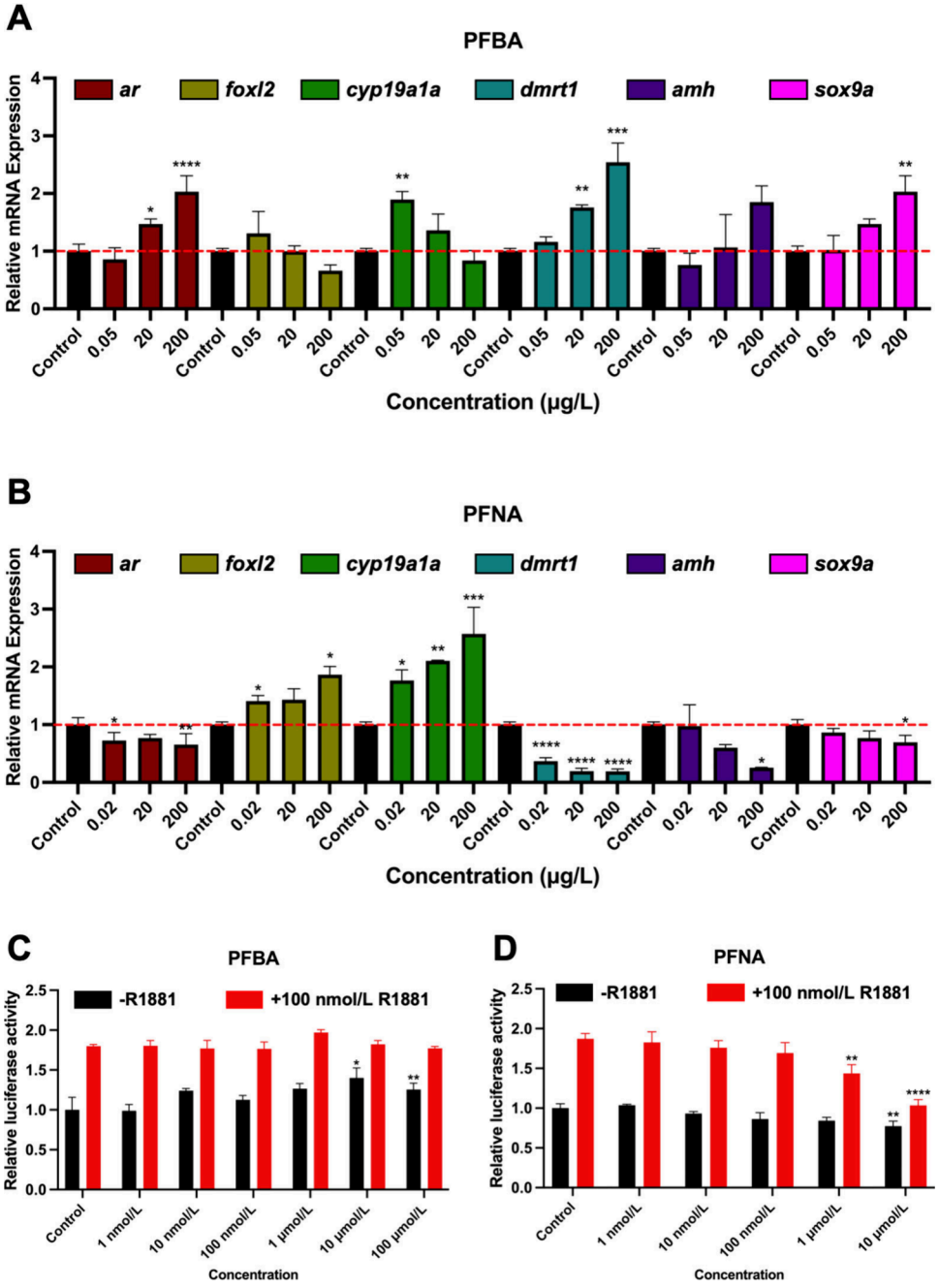

Figure 2. Effects of PFBA and PFNA on the zebrafish AR signaling pathway and transcriptional activity. (A, B) Alterations in the expression of genes specific to ovaries (*foxl2*, *cyp19a1a*) and testes (*dmrt1*, *amh*, *sox9a*) following exposure to PFBA and PFNA during 20−40 dpf. (C, D) HEK293 cells were exposed to a range of concentrations of PFBA and PFNA for 24 h in the presence or absence of 100 nmol/L R1881. The error bars indicate the standard deviation derived from three independent experimental measurements. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, compared with the control group.

interaction of various key genes such as androgen receptor (*ar*), genes specific to the testis (doublesex and mab-3 related transcription factor 1 (*dmrt1*), anti-Müllerian hormone (*amh*), sex determining region Y-box 9a (*sox9a*)),[55,56] and genes specific to the ovary (cytochrome P450 family 19 subfamily A polypeptide 1a (*cyp19a1a*), forkhead box L2 (*foxl2*)).[56−58] *dmrt1* acts as a pivotal transcription factor for sex determination and differentiation, playing an essential role in the formation of testes. *sox9a* activates the expression of *amh*, which induces the regression of the Müllerian ducts and inhibits the expression of *cyp19a1a*, an aromatase enzyme crucial for ovarian development. *foxl2* is vital for the development and maintenance of ovaries and may have compensatory interactions with *dmrt1*.

As illustrated in Figure 2A, PFBA at 0.05 μg/L had no significant effect on either ovary-specific or testis-specific genes. Twenty μg/L PFBA significantly promoted *ar* expression. 200 μg/L PFBA significantly upregulated the expressions of *ar*, *dmrt1*, *amh*, and *sox9a* in a concentration-dependent manner with 2.0-, 2.5-, 1.9-, and 2.0-fold change, compared with the control group. However, there is no significant difference in the expression of ovarian-specific genes

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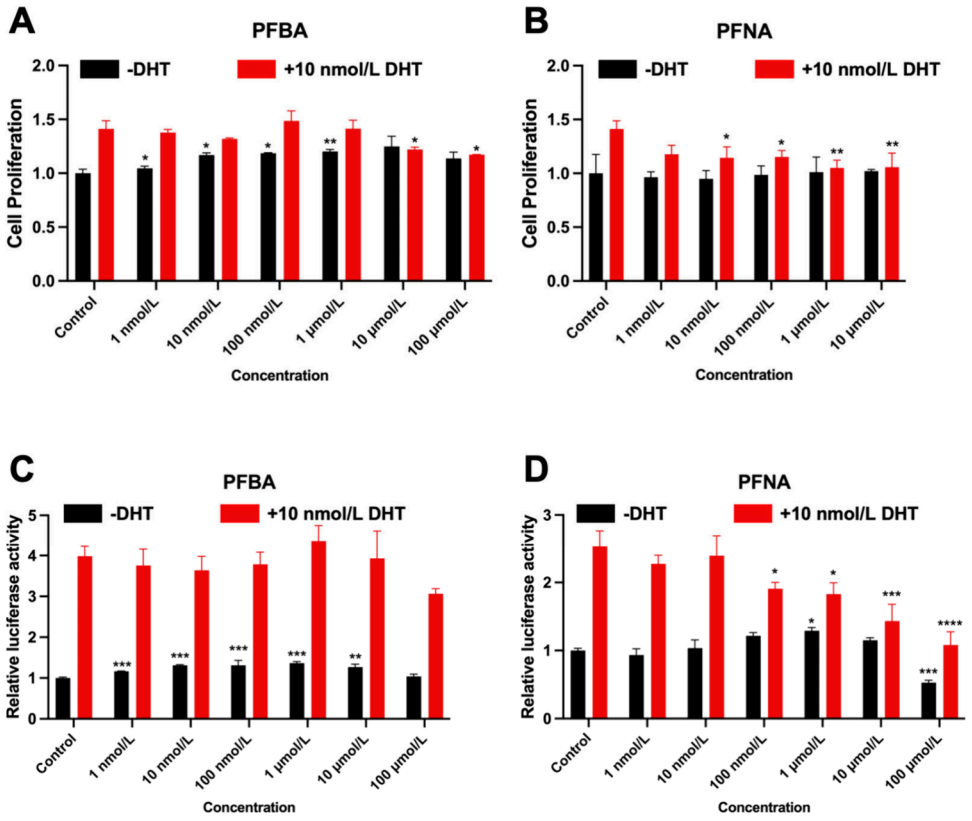

Figure 3. Effects of PFBA and PFNA on LNCaP cell proliferation and human AR transcriptional activity. LNCaP cells were treated with different concentrations of (A) PFBA and (B) PFNA in the presence or absence of 10 nmol/L DHT for 48 h. MDA-kb2 cells were treated with different levels of (C) PFBA and (D) PFNA in the presence or absence of 10 nmol/L DHT for 24 h. The error bars indicate the standard deviation derived from three independent experimental measurements. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, compared with the control group.

(*foxl2*, *cyp19a1a*). This result is consistent with the known AR agonists R1881 and 17β-Trenbolone.[29,31]

For PFNA (Figure 2B), 0.02 μg/L significantly decreased the expression of sperm-specific genes *ar*, *foxl2a*, *cyp19a1a*, and *dmrt1*. At 20 μg/L, there was a notable upregulation in the expression of *cyp19a1a* and a downregulation in *dmrt1*. When exposed to 200 μg/L of PFNA, the expression of *ar*, *dmrt1*, and *amh* was significantly suppressed, whereas the expression of *foxl2* and *cyp19a1a* was upregulated.

Combining all findings from *in vivo* assays indicated that the tested chemicals have the potential to impact sexual differentiation in zebrafish, with the effects potentially mediated through pathways involving AR.

3.4. PFBA Induced Zebrafish AR Transcriptional Activity while PFNA Inhibited

In vivo experiments have shown that PFBA and PFNA have different effects on the sex differentiation of zebrafish, and it is speculated that this is caused by the AR signaling pathway. To delve deeper into the mechanisms of action of these two chemicals, a zebrafish AR mediated-luciferase reporter gene assay was employed to investigate the effects of PFBA and PFNA on AR transcriptional activity. The nontoxic levels of PFBA and PFNA under examination were tested using MTT assays (Figure S2).

Figure 2C–D shows the results of the zebrafish AR luciferase reporter gene assays. To distinguish (anti-)androgenic effects of tested chemicals in zebrafish, the artificially synthesized androgen R1881 was selected as the positive control. When exposed alone, 10 and 100 μmol/L PFBA could significantly activate zfAR transcriptional activity, while PFNA inhibited zfAR transcriptional activity at 10 μmol/L. When coexposed with 100 nmol/L R1881, PFBA did not inhibit AR transcriptional activity induced by 100 nmol/L R1881. PFNA inhibited zfAR transcriptional activity at 100 nmol/L. Compared with the experimental results of hAR, we found that PFBA is more sensitive to the activation effect of hAR, which can be significantly promoted at 1 nmol/L.

3.5. PFBA and PFNA Exhibited Different Effects in Human AR Specific Cells

The results of the zebrafish experiments demonstrated that PFNA and PFBA influence the sex differentiation of zebrafish through the mediation of the AR signaling pathway. To further evaluate the human health risks associated with these two chemicals, we additionally carried out human AR-mediated cell proliferation and luciferase reporter gene assays to investigate their toxic effects and underlying mechanisms.

In toxicological studies, there have been several works using LNCaP cells as model cells to evaluate the (anti-)androgen effects of environmental chemicals.[59−61] Androgen can activate the AR signaling pathway by binding to AR and lead to the proliferation of LNCaP cells. Therefore, we used the LNCaP cell proliferation assay to assess androgen effects of PFBA and PFNA. Prior to this, MTT assays were conducted to determine the cytotoxicity of these chemicals. None of PFBA and PFNA showed any cytotoxic effects on the viability of LNCaP cells under 100 μmol/L. Consequently, the concentrations of PFBA and PFNA were maintained under 100 μmol/L throughout the cell proliferation experiments (Figure S3).

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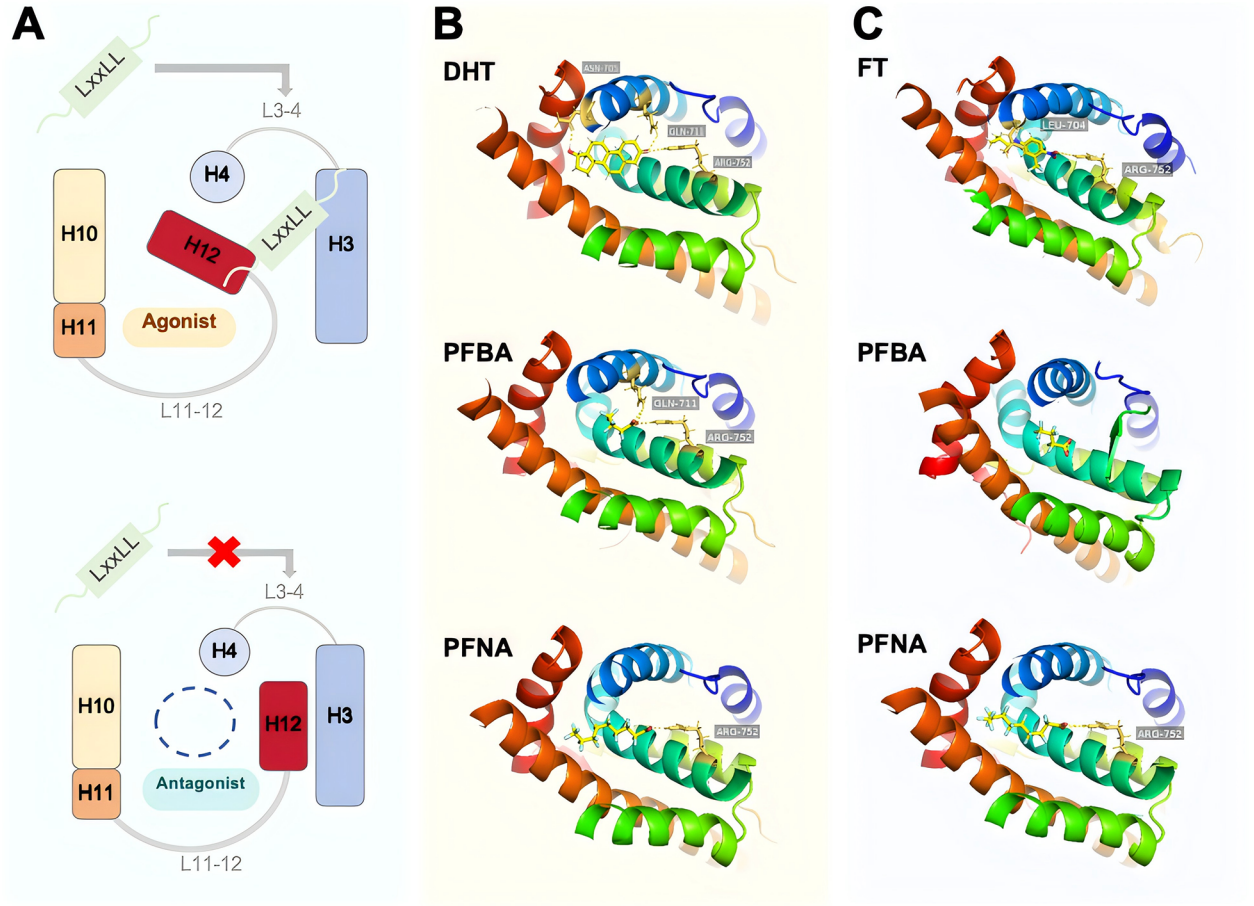

Figure 4. Interactions of the structures between human AR and PFBA and PFNA using molecular docking simulations. (A) Schematic representation of the crystal structures of different NR conformations induced by agonist and antagonist ligands. (B) Simulated structure of DHT, PFBA, and PFNA binding with AR agonist conformation. (C) Simulated structure of FT, PFBA, and PFNA binding with AR antagonist conformation. The multicolored ribbons symbolize the α-helices within the androgen receptor. The dashed yellow lines denote the hydrogen bonds established between PFBA and PFNA and the amino acids. The gray labels represent abbreviations for amino acids.

When exposed alone, PFBA could promote LNCaP cell proliferation in a concentration-dependent manner, and the LOEC value was 1 nmol/L. The proliferation effect was the most obvious at 1 μmol/L (1.3-fold) compared with that of the control. In addition, the inhibition of cell proliferation at high concentrations of PFBA is similar to that of DHT, suggesting that PFBA has an androgenic effect (Figure 3A). PFNA did not significantly promote the proliferation of LNCaP cells (Figure 3B). When coexposed with 10 nmol/L DHT, PFBA, and PFNA, all could inhibit LNCaP cell proliferation triggered by different concentrations of DHT. The LOEC values were determined to be 10 μmol/L for PFBA and 10 nmol/L for PFNA. It is worth mentioning that 100 μmol/L PFBA inhibited the proliferation of LNCaP cells when coexposed with 10 nmol/L DHT, and this phenomenon was also observed under 10 μmol/L DHT exposure, which was consistent with the androgen-dependent characteristics of LNCaP cells.[62]

The MDA-kb2 cells were utilized to assess transcriptional activity of the human androgen receptor. Neither PFBA nor PFNA showed cytotoxicity on MDA-kb2 cells viability under 100 μmol/L (Figure S4). When exposed alone, PFBA could significantly induce AR transcriptional activity with an LOEC value of 1 nmol/L and the strongest potency at 100 nmol/L (Figure 3C). PFNA had a minimal effect on activating human AR-mediated transcription at 1 μmol/L, but it significantly suppressed AR transcription at higher concentrations of 100 μmol/L (Figure 3D). When coexposed with 10 nmol/L DHT, there was no significant change after PFBA exposure, indicating that PFBA does not have the ability to inhibit AR transcriptional activity. PFNA demonstrated a dose-dependent inhibition of AR transcriptional activity, with the LOEC value of 100 nmol/L. This aligns with prior research identifying PFNA as an AR antagonist,[63] indicating that our results were credible.

3.6. PFBA and PFNA Bind to AR in Different Conformations

In vivo and *in vitro* experiments revealed (anti-)androgenic effects of the three PFASs through AR mediated pathways. To further clarify the interaction between PFBA and PFNA with AR, a molecular docking simulation was performed to investigate the binding interactions.

AR is a nuclear receptor with two key functional domains: a DNA-binding domain (DBD) and a ligand-binding domain (LBD). The DBD identifies and binds to specific DNA sequences in target gene promoters, mediating transcriptional activity.[64] Crystallography research reveals that agonist binding to the LBD locks it in the active conformation with helix 12 (H12) integrating into a hydrophobic groove on the receptor surface, serving as a docking site for coactivator proteins' conserved motifs.[65] Antagonist binding repositions H12,

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covering the coactivator binding site and blocking interaction. The binding mode and ability of a chemical with NR's conformations determine if it is an agonist or antagonist (Figure 4A).

The three-dimensional models of the complexes formed between the small molecules and the LBD of the human AR in both agonistic and antagonistic conformations were simulated (Figure 4B,C). Figure 4B shows the docking results of the tested chemicals with hAR agonistic conformation, and the binding free energy and the residues involved are detailed in Table 1. Upon binding of agonist molecule (DHT) to the ligand-binding pocket (LBP), AR undergoes an important conformational change that notably affects the position of the carboxyl-terminal α-helix 3, 5, and 12 (H3, H5, and H12) located in the LBD.[66,67] In keeping with the conformation between DHT and AR, PFBA and PFNA were inserted into the ligand binding pockets of AR. The carboxylic acid moieties of PFBA and PFNA were located within the interior of the binding pockets, whereas their hydrophobic tails were oriented toward the openings of these pockets. PFBA was able to interact with H3 and H5, establishing hydrogen bonds with two specific amino acid residues, resulting in a calculated binding free energy of −11.7175 kcal/mol. Only one hydrogen bond was formed between PFNA and the ARG752 residue of helix 5.

Table 1. CDOCKER Interaction Energy between Human AR Agonistic Conformation and Tested Chemicals and Amino Acids Participating in Hydrogen Bonds

	hAR (3L3X)	
chemicals	CDOCKER interaction energy (kcal/mol)	amino acid
PFBA	−11.7175	GLN711, ARG752
PFNA	−7.4832	ARG752
DHT	−16.9755	ASN705, GLN711, ARG752

Figure 4C shows the docking results of the tested chemicals with hAR antagonistic conformation and the binding free energy and amino acids involved in hydrogen bonding detailed in Table 2. Previous molecular dynamic simulation studies have shown that, after the antagonist binds to AR, it could move H12 away from the LBP of AR, resulting in an increasing binding pocket space.[68] Figure 4C illustrates the simulation of FT with the LBD of the human androgen receptor in its antagonistic conformation (2AX6). The simulated structures revealed two hydrogen bonds with LEU704 and ARG752 residues, which are part of H3 and H5, respectively. These interactions are similar to the results of other known antagonists.[69] No hydrogen bonds were formed when PFBA binds to antagonistic conformation, and this may be related to the increase in the binding pocket. A single hydrogen bond was established between PFNA and the ARG752 residue within H5, with a calculated binding free energy of −13.1402 kcal/mol. The simulation results suggest that PFBA tends to bind to AR in a conformation that is conducive to an agonist, while PFNA binds to AR in an antagonistic conformation. Overall, the observed distinct effects of PFBA and PFNA on AR suggest that the structure−activity relationship of PFASs is complex and chain length may play a crucial role.

Table 2. CDOCKER Interaction Energy between Human AR Antagonistic Conformation and Tested Chemicals and Amino Acids Participating in Hydrogen Bonds

	hAR (2AX6)	
chemicals	CDOCKER interaction energy (kcal/mol)	amino acid
PFBA	−7.0661	
PFNA	−13.1402	ARG752
FT	−31.5658	LEU704, ARG752

4. CONCLUSIONS

To conclude, the use of PFBA and PFNA in production leads to an increase in their detection in environmental media, but health risks of them to humans have not been fully assessed. Our study combined *in vivo* and *in vitro* experiments to investigate their toxic effects. We found that exposure to these chemicals in zebrafish alters sex differentiation gene expression and sex hormone levels, with PFBA promoting testis development at 20 μg/L and PFNA promoting ovary development at 200 μg/L. Human LNCaP cells showed that PFBA activated the AR signaling pathway, while PFNA acts as an antagonist. Molecular docking studies confirmed these results. Chen et al.[70] investigated exposure to PFBS throughout the life cycle at concentrations that are representative of environmental levels (0, 1.0, 2.9, and 9.5 μg/L), leading to a shift in the sex ratio of fish populations, favoring males, and significantly compromising the reproductive capabilities of female fish. This result is like the *in vivo* results of PFBA in our study, which speculated that a 4 carbon chain length may be the key cause of masculinization. However, the results of PFNA are similar to those of PFOA, which has been studied in our previous work. Gustafsson et al.[71] exposed zebrafish embryos to two hydroxylated polybrominated diphenyl ethers (OH-PBDEs) (6-OH-BDE47 and 6-OH-BDE85), and the results revealed minimal commonality in the affected metabolites. This indicates that chemicals with analogous structures may trigger distinct metabolic responses that could be connected to their varied toxicological pathways. To sum up, there is an urgent need to bridge the current gap in understanding by examining a wider range of PFASs with varying chain lengths. This is essential for conducting comprehensive ecological and health risk assessments related to PFASs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00240.

Text: additional experimental details, materials, and methods; figures: gonadal histology of testis and ovary in juvenile zebrafish after R1881 and FT exposure; effects of tested chemicals on viability of HEK293, LNCaP, and MDA-kb2 cells (PDF)

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Notes

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