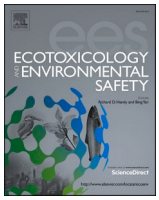
Ecotoxicology and Environmental Safety 232 (2022) 113287

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Role of endocrine disruption in toxicity of 6-benzylaminopurine (6-BA) to early-life stages of Zebrafish

Guiyi Gong [a,b,*], Hiotong Kam [b], Hanbin Chen [b], Yan Chen [b], Wai san Cheang [b], John P. Giesy [c,d,e], Qiaohong Zhou [f], Simon Ming-yuen Lee [b,**]

[a] Zhanjiang Institute of Clinical Medicine, Central People's Hospital of Zhanjiang, Zhanjiang 524045, China
[b] State Key Laboratory of Quality Research in Chinese Medicine and Institute of Chinese Medical Sciences, University of Macau, Macao, China
[c] Toxicology Centre, University of Saskatchewan, Saskatoon Saskatchewan S7N 5B3, Canada
[d] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon Saskatchewan S7N 5B4, Canada
[e] Department of Environmental Sciences, Baylor University, Waco, TX 76706, United States
[f] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

ARTICLE INFO

Edited by Professor Bing Yan

Keywords:
Plant growth regulator
Endocrine disruptors
CYP19A
Neurogenesis
Estrogenic

ABSTRACT

6-benzylaminopurine (6-BA), classified as a "plant hormone", is an important ingredient in production of "toxic bean sprouts". Although there is no direct evidence of adverse effects, its hazardous effects have received some attention and aroused furious debate between proponents and environmental regulators. In this study, potential adverse effects of 6-BA were investigated by exposing zebrafish in vivo to 0.2 – 25 mg 6-BA/L. Results indicated that, when exposure was limited to early-life stage (4–36 hpf), 20 mg 6-BA/L caused early hatching, abnormal spontaneous movement, and precocious hyperactivity in zebrafish embryos/larvae. While under a continuous exposure regime, 6-BA at 0.2 mg/L was able to cause hyperactive locomotion and transcription of genes related to neurogenesis (*gnrh3* and *nestin*) and endocrine systems (*cyp19a* and *fshb*) in 5 dpf larvae. Quantification by use of LC/MS indicated bioaccumulation of 6-BA in zebrafish increased when exposed to 0.2 or 20 mg 6-BA/L. These results suggested that 6-BA could accumulate in aquatic organisms and disrupt neuro-endocrine systems. Accordingly, exposure to 0.2 mg 6-BA/L increased production of estradiol (E2) and consequently E2/T ratio in zebrafish larvae, which directly indicated 6-BA is estrogenic. *In silico* simulations demonstrated potential for binding of 6-BA to estrogen receptor alpha (ERa) and cytochrome P450 aromatase (CYP19A). Therefore, induction of estrogenic effects, via potential interactions with hormone receptors or disturbance of downstream transcription signaling, was possible mechanism underlying the toxicity of 6-BA. Taken together, these findings demonstrate endocrine disrupting properties of 6-BA, which suggest concerns about risks posed to endocrine systems.

1. Introduction

Endocrine disruptors (EDs) are exogenous substances or mixtures that alter functions of the endocrine system and consequently cause adverse effects in intact organisms (Swedenborg et al., 2009). Known as "environmental hormones", they act either directly on receptors as hormone mimics/antagonists or disturb the processes of hormone synthesis or hormone conversion (Cheek et al., 1998). Some contaminants, that are widespread and accessible, have been found to exhibit overt endocrine disrupting effects. Some of them that have caused negative effects on reproduction of wildlife have been studied extensively (Cao et al., 2019; Meyer et al., 2018; Xiao et al., 2019; Xie et al., 2019). Since endocrine systems are integrated, EDs that interact with multiple receptors simultaneously, could also cause behavioral dysfunction and disorders by altering interacting signal transduction pathways (Chen et al., 2016). Recently, science has begun to become aware that some EDs might affect specific developmental period and elicit non-linear dose responds (Cheng et al., 2016). It has been reported that, even at low doses, bisphenol A (BPA), as well as its replacement bisphenol S (BPS), could induce precocious hypothalamic neurogenesis in

* Corresponding author at: Zhanjiang Institute of Clinical Medicine, Central People's Hospital of Zhanjiang, Zhanjiang 524045, China.
** Corresponding author.
E-mail addresses: yb67530@umac.mo (G. Gong), simonlee@umac.mo (S.M.-y. Lee).

https://doi.org/10.1016/j.ecoenv.2022.113287
Received 30 November 2021; Received in revised form 20 January 2022; Accepted 2 February 2022
Available online 8 February 2022

embryonic zebrafish (Kinch et al., 2015), which linked BPA to neuro-developmental disorders, such as anxiety and hyperactivity. They also claimed the neurogenic period might be a critical window of vulnerability to endocrine disruptors (Kinch et al., 2015). Restricted to limited exposure period and unheeded behavioral phenotype, such kinds of EDs are seldom noticed and explored. Thus, discovery of emerging EDs, especially those that target specific periods of development, are a challenge and of importance.

Plant growth regulators (PGRs), which are widely adopted in agricultural practice to improve quality of products by enlarging size or inhibiting biochemical changes to retain freshness, were generally regarded as "plant hormones" (Santner et al., 2009). Misleading by the name "hormones", some believe that these exogenous substances could also affect the endocrine system of humans and bring about detrimental effects. Although no solid evidence supported such causal relationships, increasingly, results of studies have indicated PGRs are emerging as EDs in mammal. A synthetic plant cytokinin, forchlorfenuron (CPPU), could decrease steroidogenesis in GCs and H295R cells and pathologically changed structure of ovaries in rats (Bu et al., 2019). In addition, pre-pubertal exposure to CPPU promoted secretion of estradiol, which resulted in altered vaginal opening and time to onset of first estrus in female rats (Zhu et al., 2020). In adult, male rats, gibberellic acid (GA3) could reduce their fertility by influencing the number of both live and immature sperm, and DNA integrity of chromatin in spermatocytes (Hosseinchi et al., 2013). These findings suggested risks due to application of PGRs and their endocrine disrupting effects may be underestimated (Wang et al., 2011). Therefore, toxicity of PGRs, especially on the endocrine system, deserved our more efforts and more detailed investigations.

As one of the "plant hormones", 6-benzylaminopurine (6-BA) had aroused intense scholarly debate in China, due to its potentially hazardous effects on human, especially on pregnant women and children (Wang et al., 2019). While functioning as plant growth regulator, 6-BA also prevents rooting in various crops. To take advantage of this response, Chinese farmers applied 6-BA (commonly at 0.5–25 mg/L) in their practices to obtain rootless, crispier and sturdier bean sprouts (also call "toxic bean sprouts" in China) with higher yield and shorter time of production, from 7 to 10 days to 4–5 days (Wang et al., 2019; Zhang et al., 2018). Under lax supervision and detection, abuse of 6-BA could result in its release to the environment and excessive residues in agricultural products. Concentrations as great as 3.9 μg 6-BA/g have been observed in sediments (Dong et al., 2014). In other studies, 0.1802, 0.0623, 0.0873 and 0.0200 mg 6-BA / kg were detected in samples of pitaya, peach, apple and bean sprout (Kim et al., 2016; Wang et al., 2015).

Although the government of China has banned use of 6-BA as a food additive in sprout production (CFDA, 2015), there was no adequate experimental clarification of its toxic profile. Previous literature had shown that 6-BA induced developmental toxicity in zebrafish embryos and suggested further investigation into its effect on neuro-endocrine system in the near future (Wang et al., 2019). Later, 6-BA was reported to cause alterations of locomotion and sleep/wake behavior in zebrafish larvae, which gave more clues of its influence on neuro-endocrine system (Yang et al., 2021). Considering its application worldwide and accessibility in daily life, whether 6-BA poses hazards to wildlife, is of great concern to both public authorities and the general population. More solid and reliable evidence was required to seek deeper understanding of the potential toxicity of 6-BA, particularly on neuro-endocrine system.

Zebrafish have long been regarded as a dependable sensor of endocrine disruptors (EDs) (Jarque et al., 2019), because of conservation of neuro-endocrine systems and genome with humans (MacRae and Peterson, 2015). Several zebrafish genes, such as *cyp19a*, *cyp19b*, *er*, and *vtg*, which are well-known biomarkers of endocrine disruption (Chen et al., 2018; Chen and Chan, 2016; De Oliveira et al., 2020; Kazeto et al., 2004), can be correlated to at least one human orthologue (MacRae and Peterson, 2015). Numerous EDs and their endocrine disrupting effects had been successfully identified and characterized in zebrafish in vivo (Fu et al., 2020; Maharajan et al., 2020; Teng et al., 2020). Hence, potential toxicity of 6-BA on endocrine system of developing zebrafish was investigated here, and underlying mechanisms were further determined.

2. Materials and methods

2.1. Materials

6-benzylaminopurine (6-BA, CAS Number: 1214–39–7, 99%, Fig. 1A) was obtained from Lin Guo fertilizer co. LTD (Guangzhou, China). Dimethyl sulfoxide (DMSO), Oil Red O solution (0.5% in isopropanol) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). TB green ® Premix Ex Taq™ II kit was purchased from TaKaRa (Dalian, China). All other chemicals of analytical grade were purchased from local sources.

2.2. Zebrafish maintenance and ethical approvals

The wild type (Tu or AB) zebrafish (*Danio rerio*) was raised as described previously (Gong et al., 2021). Under 14:10 h light/dark photoperiod, adult zebrafish were maintained in saltwater (electrical conductivity: 500–1500 us) with pH of 7 ± 0.2 at 28.5 ℃. They were fed with dry food once daily and live brine shrimp twice a day. By natural pairwise mating, embryos were generated and raised at 28.5 ℃ in embryo medium (E3 medium, 0.2 g/L of Instant Oceans Salt in distilled water). Ethical approvals (UMARE-030–2017) for the animal experiments were granted by the Animal Research Ethics Committee, University of Macau.

2.3. Drug treatment paradigms

Fertilized and normally developing embryos were selected under a stereomicroscope. For phenotype observation and locomotion test: embryos were arrayed in 24-well plate, with 15 embryos per well containing 1 mL solutions. Apart from the specific treatment paradigm, other experiments followed this procedure: briefly, at 4 hpf, embryos were continually exposed to 0, 0.2, 2, 5, 10, 20 or 25 mg/L of 6-BA solution till the endpoints. For experiments with restricted temporal exposure to 6-BA, embryonic zebrafish were exposed to vehicle (DMSO concentrations below 0.1% (v/v)) only or were exposed to vehicle until 16 hpf, then were exposed to 6-BA from 16 to 36 hpf, and then were returned to vehicle till endpoint (5 dpf) (Kinch et al., 2015). For quantitative real-time PCR, determination of hormone level and uptake of 6-BA assays: embryos were arrayed in 6-well plate, with 20/60/20 embryos per well containing 3 mL solutions. At 4 hpf, embryos were continually exposed to 0, 0.2, 20 or 25 mg/L of 6-BA solution till the endpoints. In all exposure experiments, embryos were randomly selected, and evenly represented in each well/sample. They were maintained in an incubator with light-dark cycle at 28.5 ℃. DMSO (concentrations less than 0.1% (v/v)) was used as solvent control. Solutions were changed daily with removal of chorion remnants and dead embryos for the duration of the experiment. Each treatment was replicated three times on different days/spawns.

2.4. Phenotype observation

After continuous exposure to 0, 0.2, 2, 5, 10, 20 or 25 mg/L of 6-BA solution till 96 hpf, the presence and morphology of swimming bladders were observed under stereomicroscope. In this study, absence, smaller sizes, or under-inflation of swimming bladders were regarded as abnormal, proportion of which were counted and presented in the bar chart.

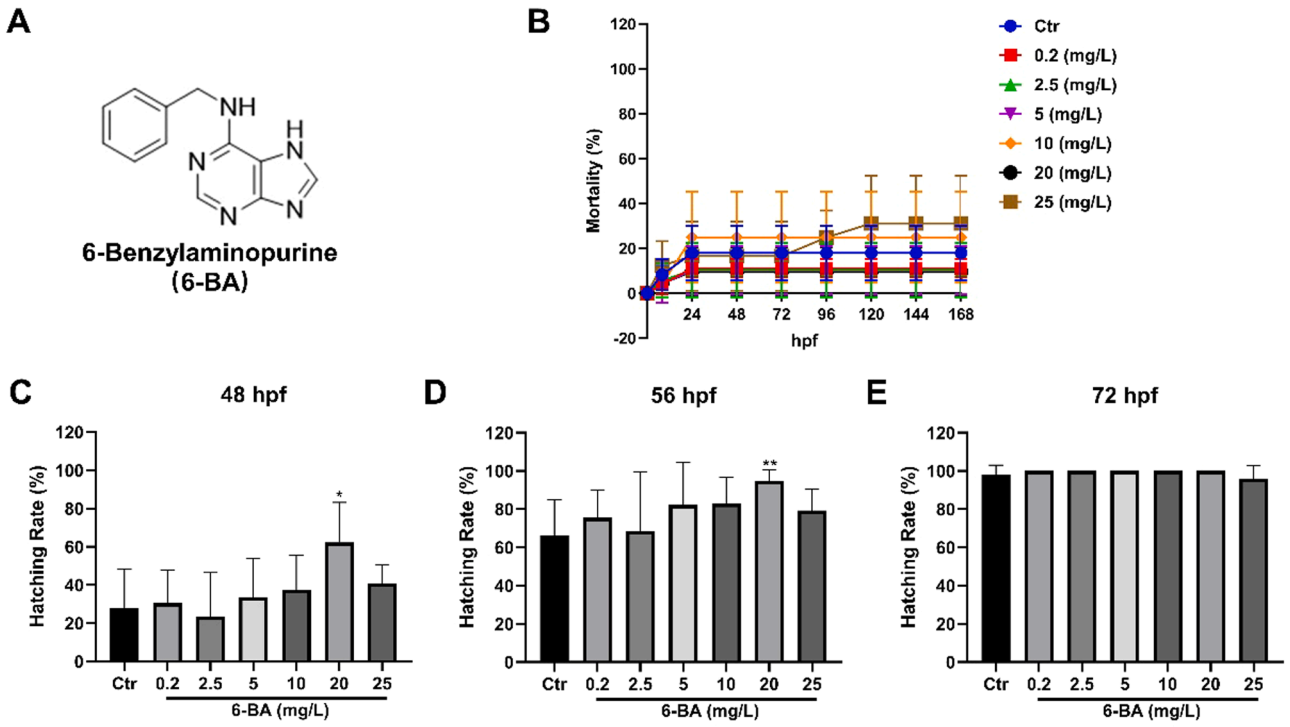


Fig. 1. 6-BA caused accelerated hatching in zebrafish at 48 and 56 hpf. (A) Chemical structure of 6-BA. (B) Mortality calculated from 8 to 168 hpf, (C, D, E) Hatching rate at 48, 56 and 72 hpf were monitored after continued exposure of 6-BA at 0.2 – 25 mg/L. Each treatment was performed in triplicate. Data are plotted as means ± SD (n = 15–20). * , $P < 0.05$ and * *, $P < 0.01$ versus the unexposed, control group.

2.5. Locomotor behavior assay. Embryonic behavior

After different doses treatment of 6-BA, embryos (24 hpf) were randomly selected (n = at least 10 per group) to determination of spontaneous movement. Briefly, after 2 min acclimatization, the total numbers of body axis rotations were counted during 1 min inside chorion. Larval behavior : After exposure to various treatments, 5 dpf zebrafish larvae were placed into 96-well plate wells (1 larva per well) and their swimming activity were monitored in the dark for 30 min with Zebralab Video-Track system (ViewPoint Life Sciences) according to previously published methods (Gong et al., 2020). Larvae were allowed to acclimate for 10 min before the test started. In total 12 larvae per group had their locomotor behavior monitored. The total distance traveled were recorded and compared between different treatments.

2.6. Quantitative real-time PCR

Embryonic zebrafish at 4 hpf were exposed to 0.2 mg 6-BA/L. At 5 dpf, total RNA was extracted from 20 zebrafish per group using Trizols Reagent (Invitrogen, USA) according to the protocol from manufacturer. Then each cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) with random primers according to reported protocol (Gong et al., 2019). Real-time PCR was performed using TB green® Premix Ex Taq™ II kit and Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA). Abundance of mRNA was normalized to elongation factor 1 α (Ef1-α) levels and expressed as percentage of the control (100%) for statistical analysis. Primer sequences of each gene were described in Table S1 (Supporting information).

2.7. Molecular docking

Molecular docking was employed to fast predict the binding potential of 6-BA, as well as BPA (for comparison) to cytochrome P450 aromatase (CYP19A) or estrogen receptor alpha (ERa). Briefly, prior to calculation, prepared three-dimensional coordinates of CYP19A (PDB ID-3S79) and ERa (PDB ID-1ERE) were retrieved from the RCSB Protein Data Bank. Respectively, their structures were imported to Auto-Dock Tools suite for docking setup (Gong et al., 2020). First, degrees of freedom of each molecule were lowered, by assigning Gasteiger partial charges to all atoms, and merging the nonpolar hydrogen with carbon atoms following the united atom representation. Then a box was created to enclose the receptors, allowing the ligand to access all the areas on their surface. By using the Auto-Dock Vina software, molecular dockings with the Lamarckian genetic algorithm and physics-based scoring function were performed. Ten best conformations were produced for each protein-ligand complex and were ranked by the estimated binding free energy. These conformations were visualized by PyMOL 1.8 and grouped by their binding poses to receptors. For each group, the conformation with the best binding energy score will be selected to represent the binding mode of ligand to receptors.

2.8. Determination of internal levels of VTG and hormones in zebrafish larvae

After treatment of 6-BA for 5 dpf, 60 larvae were collected and homogenized in 3 mL ice-cold phosphate buffered solution (PBS; pH=7.2–7.4) by an electric grinder (TIANGEN BIOTECH (BEIJING) CO. LTD. China). Each treatment was replicated three times. Then, the homogenate was centrifuged for 10 min at 11,000 rpm, 4 °C, whose supernatant was collected and kept at − 80 °C before determination of internal levels of VTG (Vitellogenin), T (Testosterone), and E2 (Estradiol) of zebrafish larvae. The internal levels of VTG, T, and E2 were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (with Cat. No. KS13404, KS17621 and KS17632, KeShun CO. LTD. China).

2.9. Determination of uptake and bioaccumulation of 6-benzylaminopurine in zebrafish

Zebrafish embryos (in two replicates) were arrayed in 6-well plate

with 20 embryos/well and 3 mL embryo medium/well, then treated with 0.1% DMSO and 0.2 and 20 mg/L of 6-BA at 4 hpf for indicated time. The embryos from each group were washed three times with embryo medium and the corresponding embryo medium (200 μl) were individually collected at different time points into 1.5 mL micro-centrifuge tubes. Embryos then were homogenized in 0.2 mL embryo medium (with 0.2% formic acid). Meanwhile, 0.2 mL methanol (with 0.2% formic acid) was added to each tube. Then embryo homogenates and their corresponding medium were centrifuged and stored at − 80 °C until sample analysis. The chromatographic separation was achieved on an Synergi™ 4 μm Hydro-RP 80 A LC column (150 *2 mm), with a gradient elution using mobile phase consisted of water with formic acid and 15 mM ammonium acetate (pH=2.4), and acetonitrile. The gradient elution was as follows: 0–10 min, 30% acetonitrile; 10–14 min, 30%–95% acetonitrile; 14–15 min, 95% acetonitrile; 15–15.1 min, 95%–30% acetonitrile; 15.1–20 min, 30% acetonitrile at a flow rate of 0.3 mL min-1. The injection volume was 3 μl. A series of standard concentrations of 6-BA were prepared to make the standard curve for quantification. LC/MS analysis was performed using AB Sciex 4000 Q-trap with an electrospray ionization source operating in negative ion mode. The quantification mode was multiple reaction monitoring (MRM). The column temperature was maintained at 35 ℃ with a binary pump, degasser, column oven and autosampler. Ion spray voltage was set at - 4500 V and source temperature at 400 ℃. The MS parameters for ionization in MRM model were curtain gas of 30 psi, nebulizer gas of 40 psi, heater gas of 20 psi, decluttering potential of − 84 V, entrance potential of − 10 V, collision energy of − 33 V and cell exit potential of − 18 V. The values of precursor and fragment ions were set as 225.1 and 132.9 for targeted analytes, respectively.

2.10. Statistical analysis

All data are presented as means ± SD and analyzed using GraphPad Prism 8.3 software (GraphPad Software Inc., La Jolla, CA). Also, they were investigated to determine if they met the assumptions of normality by using the Shapiroe Wilks test and for homogeneity of variance by use of Levene's test. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison

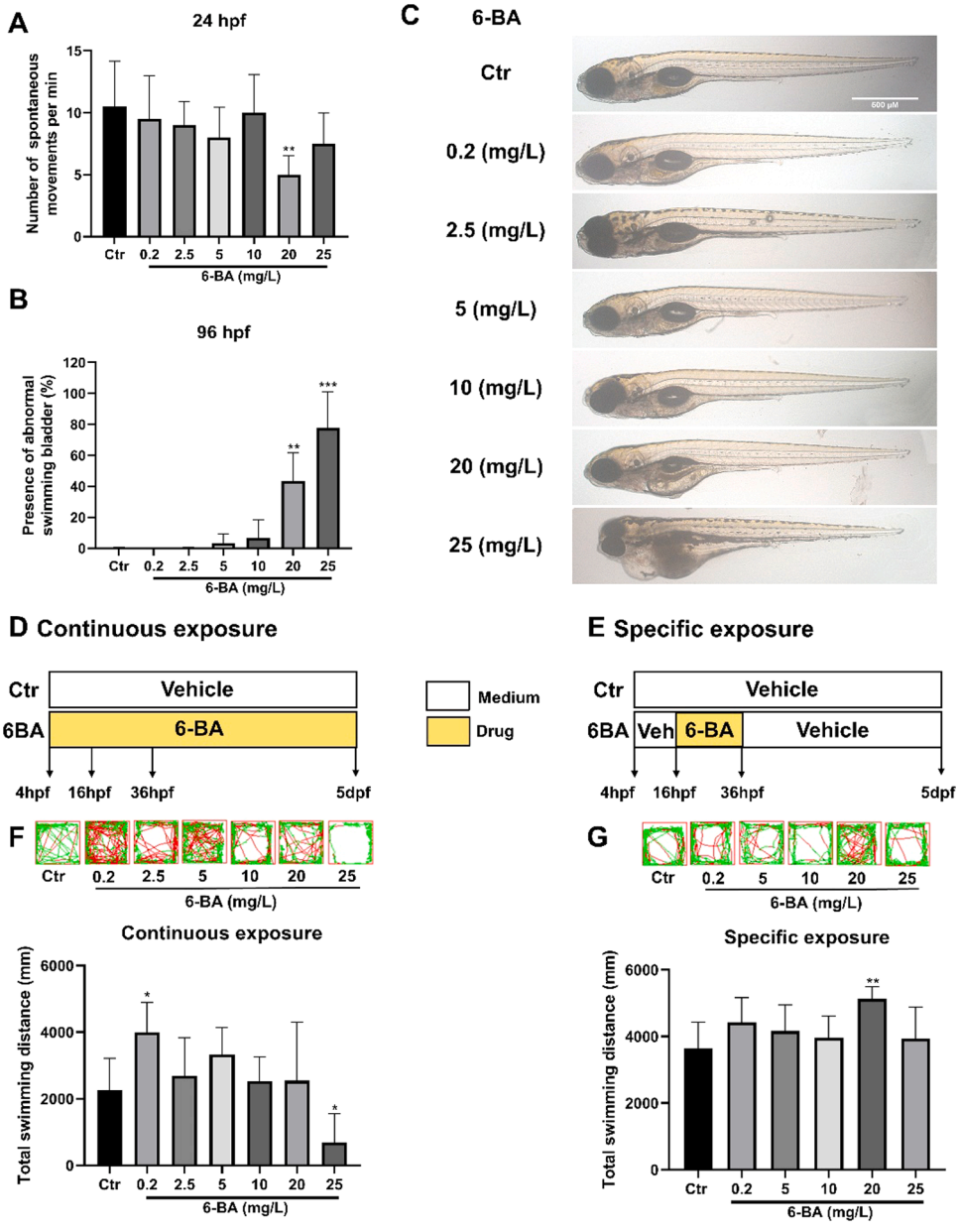


Fig. 2. 6-BA caused alterations to spontaneous movements, swimming bladder and behavior in larval zebrafish. (A) Number of spontaneous movements per min at 24 hpf, (B) Percentage of abnormal swimming bladder at 96 hpf, and (C) Representative images were recorded after continued exposure of 6-BA at 0.2 – 25 mg/L. (D and E) Paradigms of continuous and specific exposure. (F and G) Total swimming distance were calculated, and representative images were shown after different exposure paradigms of 6-BA at 0.2 – 25 mg/L. Each treatment was performed in triplicate. Data are plotted as means ± SD (n = 10 –24). *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ versus the unexposed control group.

test. Difference between two groups were determined by Student's t-test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. 6-BA caused accelerated hatching in zebrafish embryos

Mortality and hatching were observed at various durations of exposure based on results of previous studies (Wang et al., 2019; Yang et al., 2021), concentrations as great as 25 mg 6-BA/L, did not significantly alter mortality of embryonic zebrafish from 8 to 168 hpf (Fig. 1B). Although previous results found no alteration of hatching rate at 72 or 96 hpf after exposed to 0–25 mg 6-BA/L (Wang et al., 2019; Yang et al., 2021), here, we did observe at least 20 mg 6-BA/L could cause accelerated hatching in earlier period at 48 or 56 hpf (Fig. 1C and D, from 28% to 62%, 66–95%) without affecting the overall hatching rate at 72 hpf (Fig. 1E).

3.2. 6-BA caused alteration in spontaneous movement and abnormality of swimming bladder in zebrafish

Exposure to 20 mg 6-BA/L significantly reduced spontaneous movements from 10 to 5 times per min with on alteration in other concentrations (Fig. 2A). In addition, 6-BA caused increasing abnormality of swimming bladder at a dose dependent manner, and about 50, 80% of larvae in 20, 25 mg 6-BA/L treatment group presented abnormal/reduced swimming bladders, respectively (Fig. 2B and C).

3.3. 6-BA caused alteration in locomotor behavior in treatments

After continuous exposure to 6-BA (Fig. 2D), 0.2 mg 6-BA/L caused hyperactivity (from 2200 to 4000 mm in total swimming distance) in zebrafish larvae, while 25 mg/L significantly impaired the locomotion (Fig. 2E). When exposure to 6-BA was restricted to 16–36 hpf (Fig. 2F), only those exposed to 20 mg/L exhibited hyperactive phenotype (from 3700 to 5000 mm in total swimming distance) (Fig. 2G).

3.4. Bioaccumulation of 6-BA in zebrafish embryos

The accumulation of 6-BA in zebrafish were determined by LC/MS method (representative peak of 6-BA was shown in Fig. S1). When exposed to 0.2 mg 6-BA/L, the accumulation of 6-BA in per embryo were 0.15 or 1.03 ng at 48 or 120 hpf, respectively (Fig. 3A). While exposure to 20 mg 6-BA/L resulted in bioaccumulation of 1.56, 4.13 and 8.86 ng 6-BA per embryo at 24, 48 and 120 hpf, respectively (Fig. 4A). The concentrations in the exposure medium (both in 0.2 and 20 mg/L exposures) remained consistent during 120 h exposure (Fig. 3B), whereas

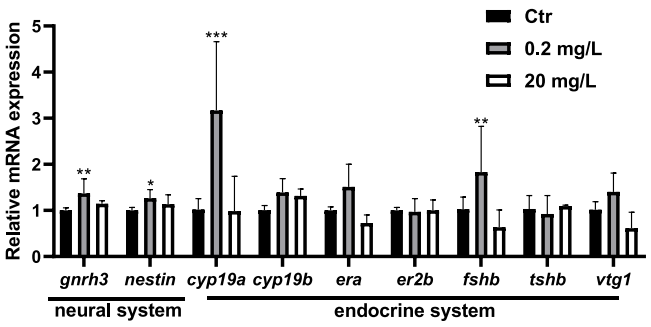


Fig. 4. Exposure of 6-BA altered expression of genes related to neurogenesis, neural and endocrine systems in zebrafish. (A and B) mRNA levels of *gnrh3*, *nestin*, *cyp19a*, *cyp19b*, *era*, *er2b*, *fshb*, *tshb*, and *vtg1* in zebrafish larvae exposed to vehicle control, 0.2 and 20 mg 6-BA/L at 5 dpf. Each treatment was performed in triplicate. Data are presented as mean ± SD. n = 3. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus the unexposed, control group.

the bioaccumulation of 6-BA in zebrafish embryos were definitely increased.

3.5. 6-BA changed expression of neurogenesis genes in zebrafish

There was a significant increase in expression of mRNA of *gndh3* and *nestin* in zebrafish exposed to 0.2 mg 6-BA/L (Fig. 4). No alterations were observed between other groups.

3.6. 6-BA altered expression of genes related to endocrine disruption in zebrafish

Expressions of biomarkers of endocrine disruption, including *cyp19a* and *fshb* were significantly up-regulated by about 3.2 and 1.8 -fold after exposure to 0.2 mg 6-BA/L (Fig. 4). Other genes were not differentially expressed between control and exposure groups.

3.7. 6-BA virtually targets to CYP19A and Era

Potential binding sites of 6-BA, as well as BPA to ERa as well as CYP19A were examined by simulations *in silico* with molecular docking models. Results indicated both of them could bind to both proteins, CYP19A and ERa, through hydrogen bonds with binding sites that shown in Fig. 5, respectively. The binding energy of 6-BA and BPA to CYP19A and ERa complexes ((A) 6-BA&CYP19A, (B) BPA&CYP19A, (C) 6-BA&ERa, (D) BPA&ERa) were respectively predicted as − 8.3, − 8.8, − 7.8 and − 8.5 kcal/mol. These results revealed potential binding ability of 6-BA toward CYP19A and ERa, with similar modes of BPA

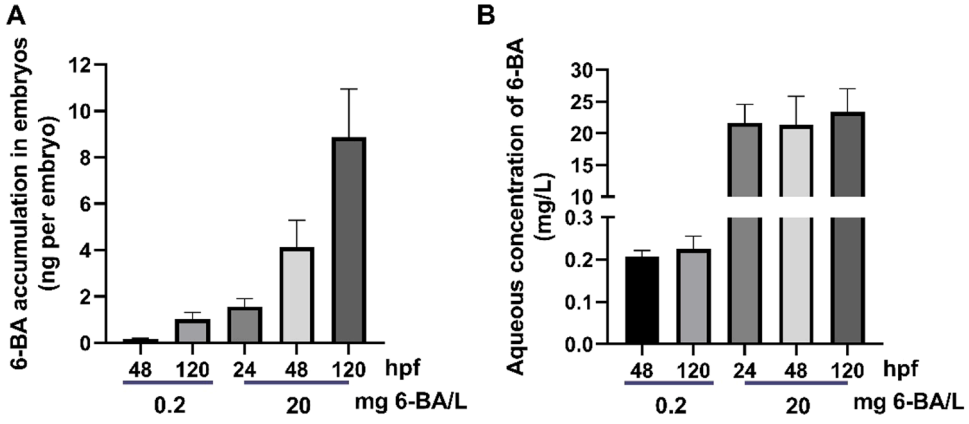


Fig. 3. Exposure concentrations of 6-BA in zebrafish. Time-course study of 6-BA accumulation in zebrafish (A) and in exposure medium (B) when dosed at 0.2 or 20 mg 6-BA/L.

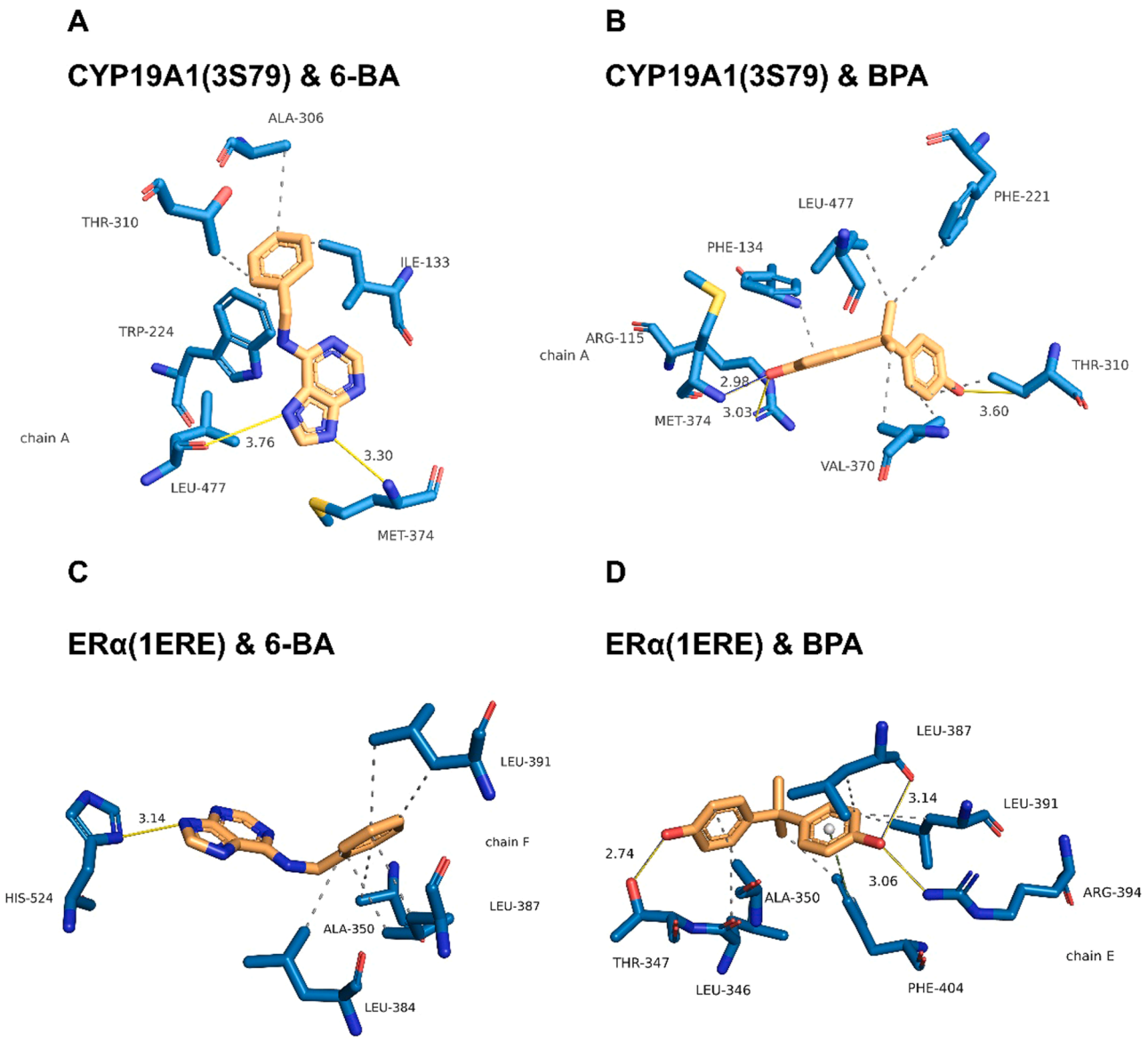


Fig. 5. 6-BA virtually binds to estrogen receptor alpha (ERa) and cytochrome P450 aromatase (CYP19A), in comparison with bisphenol A (BPA). Docking complexes of 6-BA and BPA with ERa and CYP19A are shown in (A), (B), (C) and (D), respectively, with affinities of − 8.3, − 8.8, − 7.8 and − 8.5 kcal/mol, whose interactive strengths are through hydrogen bonds with the bind sites. Yellow lines indicate the hydrogen bonds.

(Fig. 5).

3.8. Exposure of 6-BA disturbed the hormone homeostasis in zebrafish larvae

As shown in Figs. 6, 6-BA at 0.2 mg/L significantly enhanced production of E2, which might catalyze by activation of CYP19A. Although the T and VTG level did not significantly change in the treatments with 6-BA, the E2/T ratio increased significantly upon exposure to all tested doses of 6-BA.

4. Discussion

Apart from impairment of the endocrine system, endocrine disruption can also interfere with developmental processes, especially when exposure occurs during early life stages (Ramakrishnan and Wayne, 2008). In turn, developmental toxicity could be possibly attributed to endocrine disruption (Perugini et al., 2020). Developmental toxicity of 6-BA has been demonstrated, although at relatively great concentrations ($\geq$ 25 mg/L), which elicited obvious cardiac malformation and behavior alterations (Wang et al., 2019; Yang et al., 2021). In this study, profound change of swimming bladders was observed, failure of its inflation could be consequences of cardiac malformation and cardiac edema, meanwhile, since swim bladder functions as a hydrostatic organ and helps the fish to maintain its depth, impairment definitely causes behavioral alteration. After meticulous examination, we found that 6-BA affected early development and accelerated hatching after exposure to approximately 20 mg/L. Generally, hatching correlates closely to properties of the chorion and its interface tension, and accelerated hatching might be a consequence of impairment of chorionic structure or interface tension. In zebrafish, the herbicide, glyphosate (Zhang et al., 2017) and insecticides, acephate and deltamethrin (Liu et al., 2018), all reduced the interface tension of the chorion, which increase the rate of hatching at 72 hpf. Previously, 6-BA was found to reduce interface tension of the chorion (Yang et al., 2021), which might account for the accelerated hatching observed during this study. Based on previous knowledge, several EDs, such as BPA (Qiu et al., 2016), estradiol-17 (Kishida et al., 2001) and chlorpyrifos (Yu et al., 2015), could accelerate time to

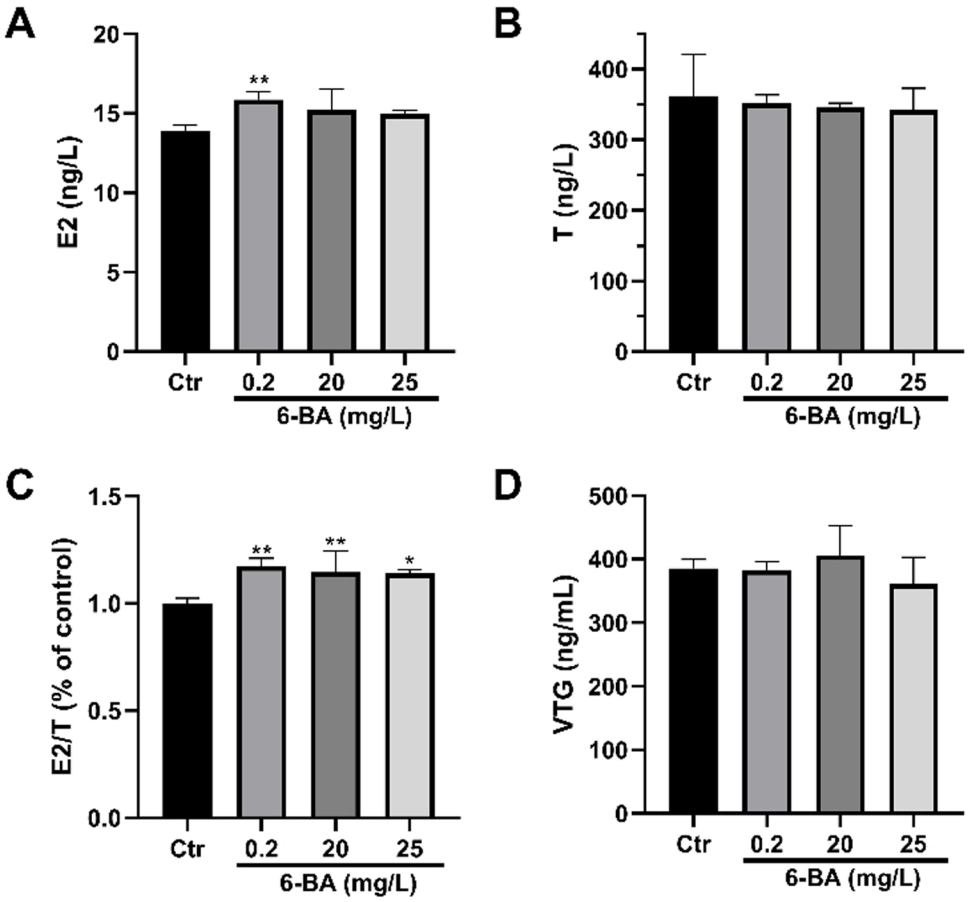


Fig. 6. Exposure of 6-BA disturbed the hormonal homeostasis in zebrafish larvae. (A) E2, (B) T, (C) E2/T, and (D) VTG levels in homogenate of whole body of zebrafish larvae after exposure to 6-BA (0.2, 20 and 25 mg/L) for 5 days. Each treatment was performed in triplicate. Data are plotted as means ± SD (n = 60). * , $P < 0.05$ and * *, $P < 0.01$ versus the unexposed, control group.

hatching after either continuous exposure or exposure during a critical period of development. These EDs might affect the development of neuroendocrine system of zebrafish, which might be another reason that explains a decrease time to hatch. Although the exact reason accounting for the accelerating hatching behavior of 6-BA is awaited proven, given the similar hatching behavior of 6-BA, its potential function on endocrine system deserves more investigations.

The same as BPA (a well-known endocrine disruptor), 0.2 mg 6-BA/L also significantly up-regulated expression of *fshβ* (encoding for follicle-stimulating hormone beta subunit) (Chen and Chan, 2016), which could drive embryonic neurogenesis via androgen receptor-mediated up-regulation of aromatase (Qiu et al., 2016). Since the enzyme aromatase catalyzes conversion of androstenedione to estradiol intermediate estrone (Hinfray et al., 2006; Kazeto et al., 2004), findings of significant increase of aromatase genes *cyp19a*, suggests increased production of estradiol after exposure to 6-BA. Indeed, 6-BA treatment increased E2 content, as well as E2/T ratio in zebrafish. Although 6-BA did not decrease content of T significantly, there is a trend of decreased concentration of T after exposure to 6-BA. Since T is an important precursor for synthesis of E2, the ratio of E2/T concentrations is one of the sensitive indicators of estrogenic effects in zebrafish, thus it is direct evidence that 6-BA is an endocrine disruptor. Although docking modeling could not predict the agoniztic or antagonistic effect of a chemical, its results do reveal the computational binding capacity toward a receptor. Thus, docking results shown the binding potential of 6-BA toward ERa and CYP19A and further confirm its endocrine disrupting property.

Identification of EDs represents a challenge, since their effects can be subtle or inconspicuous and depend on both the level and timing of exposure (Kinch et al., 2015). Therefore, differential effects of 6-BA on the neuro-endocrine were investigated after various magnitudes and timing. After continued exposure, 0.2 mg 6-BA/L caused hyperactivity in 5 dpf zebrafish, though greater concentrations did not significantly affect locomotion (2–20 mg/L) or causes hypoactivity (25 mg/L). This might be attributed to the morbidity caused by greater concentrations of 6-BA. However, when exposure restricted to critical neurodevelopmental period of 16–36 hpf, 20 mg 6-BA/L elicit hyperactive locomotion rather than morbidity in larval zebrafish (5 dpf). We surmised such coincidence was derived from the same level accumulation of 6-BA between two exposure groups. Then we gained evidence through LC/MS analysis, the concentration of 6-BA accumulated in zebrafish after 0.2 mg 6-BA/L exposure for 5 days (1.03 ng/embryo) was indeed similar to that in 20 mg 6-BA/L treatment group at 24 hpf (1.56 ng/embryo). Exposure of such level of 6-BA might stimulate neurogenesis, accordingly, transcription of genes *gnrh3*, encoding GnRH3 neurons, was up-regulated after exposure to 6-BA, at 5 dpf, demonstrated neurogenesis during this developmental stage (usually at early-life stage, from 16 to 36 h) (Qiu et al., 2016). This effect could also be attributed to the estrogenic effect of 6-BA. 6-BA provoked production of E2, which is a main feedback signal to the hypothalamus, and stimulated genesis of extrahypothalamic GnRH3 neurons. Such effect caused latent effects as well as abnormalities in zebrafish even it was washed out, which suggested that improper fine-tuning of the brain consequently caused potential behavior deficits (Kinch et al., 2015; Yun et al., 2016). Although contribution of thyroid disruption to functional abnormalities remains to be proven, several EDs, such as polychlorinated

biphenyls (PCBs) or organophosphate pesticides, caused over-represented incidence of attention deficit / hyperactivity disorder (ADHA) in exposed populations (Eubig et al., 2010; van den Dries et al., 2019). Thus, risks of 6-BA on neurodevelopment require our greater concern.

Potency of EDs on endocrine systems are dependent upon various factors (Solecki et al., 2017). Generally, EDs produce non-linear dose-response curves both in vitro and in vivo, though the underlying mechanism is still unknown. Recent results have indicated such non-linear, dose-response relationship for some contaminants is hormesis, and expressed concerns that it might shift the No-Observed-Adverse-Effect Level (NOAEL) (Agathokleous et al., 2021), which discredited the guideline and covered the reality of ecological risk assessment (Zhang and Lin, 2020). More distinct hormesis was observed for PGRs, including 2,4-D and CPPU, which promote growth of crops at lesser concentrations, while functioning as herbicides at concentrations as great as 500 mg/L (Li et al., 2017). The results we reported here, estrogenic effects of 6-BA depended on both magnitude and timing of exposure. Thus, hormetic effects of PGRs, such as 6-BA, require greater cautiousness when evaluating risks posed to environments, especially to algae or other aquatic organisms (Sun et al., 2019). Moreover, since EDs might work together to disrupt endocrine system even at lesser doses that individually do not elicit observable effects, elucidation of synergetic effects of EDs deserved additional efforts (Guo et al., 2021). Nanoparticles could augment the toxicity of various EDs, including pentachlorophenol (Lei et al., 2020) and tetrabromobisphenol A (TBBPA) (Zhu et al., 2021), on zebrafish larvae. Investigations on whether 6-BA acts synergistically with other PGRs (or EDs) to produce additive toxic effects or not, will be investigated in future studies by our group. Some EDs have been found to interact with multiple receptors simultaneously, indeed, 6-BA virtually binds to ERa and CYP19A, but further experimental evidence will be needed for additional receptors. Accessibility of 6-BA in the diet needs to be considered when assessing risks to endocrine systems, additive toxicity during embryonic exposure, and its potential adverse health effects on different organisms are worth heeding and careful scrutiny.

5. Conclusions

In conclusion, through different magnitudes and timing of exposure, 6-BA caused accelerated hatching, abnormal spontaneous movement, and hyperactivity in zebrafish, which are subtle and seldom investigated. LC/MS analysis verified the bioaccumulation of 6-BA in zebrafish were time-dependent. Transcription data suggested neurogenesis and endocrine disruption in zebrafish after embryonic exposure of 6-BA. Production of E2 and consequently E2/T ratio were increased after 6-BA treatment, directly indicating the estrogenic effects of 6-BA in zebrafish larvae. The binding potential and detailed pattern between 6-BA and ERa/CYP19A were further revealed by *in silico* analysis. Therefore, induction of estrogenic effects, via potential interactions with hormone receptors or disturbance of downstream transcription signaling, was possible mechanism of the toxicity of 6-BA. Our study had proposed and investigated the estrogenic effects of 6-BA for the first time, though more solid evidence is still required to determine whether there are in fact changes in neurogenesis. In addition, our findings addressed concerns about hazardous effects of 6-BA, and its risks posed on endocrine systems, at low or even environment relevant doses, after embryonic exposure.

CRediT authorship contribution statement

Guiyi Gong: Conceptualization; Formal analysis; Investigation; Methodology; Validation; Roles/Writing – original draft;, **Hiotong Kam**: Data curation; Software;, Validation; Investigation; Formal analysis;, **Hanbin Chen**: Visualization; In silico study; Methodology;, **Yan Chen**: LC/MS analysis;, **Wai san Cheang**: Writing – review & editing., **John P. Giesy**: Writing – review & editing., **Simon Mingyuen Lee**: Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Research at University of Macau was funded by The Science and Technology Development Fund, Macau SAR (File no. 0058/2019/A1 and 0016/2019/AKP), and University of Macau (MYRG2019-00105-ICMS). Prof Giesy was supported by a distinguished visiting professorship from the Department of Environmental Sciences, Baylor University, Waco, Texas, USA.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113287.

References

Agathokleous, E., Saitanis, C., Markouizou, A., 2021. Hormesis shifts the no-observed-adverse-effect level (NOAEL). Dose Response 19, 15593258211001667.
Bu, Q., Wang, X., Xie, H., Zhong, K., Wu, Y., Zhang, J., Wang, Z., Gao, H., Huang, Y., 2019. 180 day repeated-dose toxicity study on forchlorfenuron in sprague-dawley rats and its effects on the production of steroid hormones. J. Agric. Food Chem. 67, 10207–10213.
Cao, J., Wang, G., Wang, T., Chen, J., Wenjing, G., Wu, P., He, X., Xie, L., 2019. Copper caused reproductive endocrine disruption in zebrafish (Danio rerio). Aquat. Toxicol. 211, 124–136.
Cheek, A.O., Vonier, P.M., Oberdörster, E., Burow, B.C., McLachlan, J.A.J.E.H.P., 1998. Environmental signaling: a biological context for endocrine disruption. Environ. Health Perspect. 106, 5–10.
Chen, J., Wang, X., Ge, X., Wang, D., Wang, T., Zhang, L., Tanguay, R.L., Simonich, M., Huang, C., Dong, Q., 2016. Chronic perfluorooctanesulphonic acid (PFOS) exposure produces estrogenic effects in zebrafish. Environ. Pollut. 218, 702–708.
Chen, M., Zhang, J., Pang, S., Wang, C., Wang, L., Sun, Y., Song, M., Liang, Y., 2018. Evaluating estrogenic and anti-estrogenic effect of endocrine disrupting chemicals (EDCs) by zebrafish (Danio rerio) embryo-based vitellogenin 1 (vtg1) mRNA expression. Comp. Biochem. Physiol. C Toxicol. Pharm. 204, 45–50.
Chen, Y.Y., Chan, K.M., 2016. Regulation of vitellogenin (vtg1) and estrogen receptor (er) gene expression in zebrafish (Danio rerio) following the administration of Cd(2)(+) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Chemosphere 147, 467–476.
Cheng, J., Lv, S., Nie, S., Liu, J., Tong, S., Kang, N., Xiao, Y., Dong, Q., Huang, C., Yang, D., 2016. Chronic perfluorooctane sulfonate (PFOS) exposure induces hepatic steatosis in zebrafish. Aquat. Toxicol. 176, 45–52.
De Oliveira, J., Chadili, E., Piccini, B., Turies, C., Maillot-Marechal, E., Palluel, O., Pardon, P., Budzinski, H., Cousin, X., Brion, F., Hinfray, N., 2020. Refinement of an OECD test guideline for evaluating the effects of endocrine disrupting chemicals on aromatase gene expression and reproduction using novel transgenic cyp19a1a-eGFP zebrafish. Aquat. Toxicol. 220, 105403.
Dong, S., Huang, G., Hu, Q., Shi, L., Yao, Y., Huang, T., 2014. Evaluation of IL-ATPS and IL-MAE for simultaneous determination of herbicides and plant growth regulators in sediment. Chromatographia 77, 923–931.
van den Dries, M.A., Guxens, M., Pronk, A., Spaan, S., El Marroun, H., Jusko, T.A., Longnecker, M.P., Ferguson, K.K., Tiemeier, H., 2019. Organophosphate pesticide metabolite concentrations in urine during pregnancy and offspring attention-deficit hyperactivity disorder and autistic traits. Environ. Int. 131, 105002.
Eubig, P.A., Aguiar, A., Schantz, S.L., 2010. Lead and PCBs as risk factors for attention deficit/hyperactivity disorder. Environ. Health Perspect. 118, 1654–1667.
Fu, J., Guo, Y., Wang, M., Yang, L., Han, J., Lee, J.S., Zhou, B., 2020. Bioconcentration of 2,4,6-tribromophenol (TBP) and thyroid endocrine disruption in zebrafish larvae. Ecotoxicol. Environ. Saf. 206, 111207.
Gong, G., Kam, H., Tse, Y., Lee, S.M., 2019. Cardiotoxicity of forchlorfenuron (CPPU) in zebrafish (Danio rerio) and H9c2 cardiomyocytes. Chemosphere 235, 153–162.
Gong, G., Chen, H., Kam, H., Chan, G., Tang, Y.X., Wu, M., Tan, H., Tse, Y.C., Xu, H.X., Lee, S.M., 2020. In vivo screening of xanthones from garcinia oligantha identified oliganthin H as a novel natural inhibitor of convulsions. J. Nat. Prod. 83, 3706–3716.
Gong, G., Kam, H., Tse, Y.C., Giesy, J.P., Seto, S.W., Lee, S.M., 2021. Forchlorfenuron (CPPU) causes disorganization of the cytoskeleton and dysfunction of human umbilical vein endothelial cells, and abnormal vascular development in zebrafish embryos. Environ. Pollut. 271, 115791.

Guo, D., Liu, W., Yao, T., Ma, M., Wang, Q., Qiu, J., Qian, Y., 2021. Combined endocrine disruptive toxicity of malathion and cypermethrin to gene transcription and hormones of the HPG axis of male zebrafish (Danio rerio). Chemosphere 267, 128864.
Hinfray, N., Palluel, O., Turies, C., Cousin, C., Porcher, J.M., Brion, F., 2006. Brain and gonadal aromatase as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (Danio rerio). Environ. Toxicol. 21, 332–337.
Jarque, S., Ibarra, J., Rubio-Brotons, M., Garcia-Fernandez, J., Terriente, J., 2019. Multiplex analysis platform for endocrine disruption prediction using zebrafish. Int. J. Mol. Sci. 20.
Kazeto, Y., Place, A.R., Trant, J.M., 2004. Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juveniles. Aquat. Toxicol. 69, 25–34.
Kim, K.G., Park, D.W., Kang, G.R., Kim, T.S., Yang, Y., Moon, S.J., Choi, E.A., Ha, D.R., Kim, E.S., Cho, B.S., 2016. Simultaneous determination of plant growth regulator and pesticides in bean sprouts by liquid chromatography-tandem mass spectrometry. Food Chem. 208, 239–244.
Kinch, C.D., Ibhazehiebo, K., Jeong, J.H., Habibi, H.R., Kurrasch, D.M., 2015. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. Proc. Natl. Acad. Sci. USA 112, 1475–1480.
Kishida, M., McLellan, M., Miranda, J.A., Callard, G.V.J.C.B., Biochemistry, P.P.B., Biology, M., 2001. Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (Danio rerio). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 261–268.
Lei, L., Qiao, K., Guo, Y., Han, J., Zhou, B., 2020. Titanium dioxide nanoparticles enhanced thyroid endocrine disruption of pentachlorophenol rather than neurobehavioral defects in zebrafish larvae. Chemosphere 249, 126536.
Li, K., Wu, J.Q., Jiang, L.L., Shen, L.Z., Li, J.Y., He, Z.H., Wei, P., Lv, Z., He, M.F., 2017. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. Chemosphere 171, 40–48.
Liu, X., Zhang, Q., Li, S., Mi, P., Chen, D., Zhao, X., Feng, X., 2018. Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (Danio rerio): a comparative study of deltamethrin, acephate, and thiamethoxam. Chemosphere 199, 16–25.
MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14, 721–731.
Maharajan, K., Muthulakshmi, S., Karthik, C., Nataraj, B., Nambirajan, K., Hemalatha, D., Jiji, S., Kadirvelu, K., Liu, K.C., Ramesh, M., 2020. Pyriproxyfen induced impairment of reproductive endocrine homeostasis and gonadal histopathology in zebrafish (Danio rerio) by altered expression of hypothalamus-pituitary-gonadal (HPG) axis genes. Sci. Total Environ. 735, 139496.
Meyer, D.N., Baker, B.B., Baker, T.R., 2018. Ancestral TCDD exposure induces multigenerational histologic and transcriptomic alterations in gonads of male zebrafish. Toxicol. Sci. 164, 603–612.
Perugini, M., Merola, C., Amorena, M., D'Angelo, M., Cimini, A., Benedetti, E., 2020. Sublethal exposure to propylparaben leads to lipid metabolism impairment in zebrafish early-life stages. J. Appl. Toxicol. 40, 493–503.
Qiu, W., Zhao, Y., Yang, M., Farajzadeh, M., Pan, C., Wayne, N.L., 2016. Actions of bisphenol A and bisphenol S on the reproductive neuroendocrine system during early development in zebrafish. Endocrinology 157, 636–647.
Ramakrishnan, S., Wayne, N.L., 2008. Impact of bisphenol-A on early embryonic development and reproductive maturation. Reprod. Toxicol. 25, 177–183.
Santner, A., Calderon-Villalobos, L.I., Estelle, M., 2009. Plant hormones are versatile chemical regulators of plant growth. Nat. Chem. Biol. 5, 301–307.
Solecki, R., Kortenkamp, A., Bergman, A., Chahoud, I., Degen, G.H., Dietrich, D., Greim, H., Hakansson, H., Hass, U., Husoy, T., Jacobs, M., Jobling, S., Mantovani, A., Marx-Stoelting, P., Piersma, A., Ritz, V., Slama, R., Stahlmann, R., van den Berg, M., Zoeller, R.T., Boobis, A.R., 2017. Scientific principles for the identification of endocrine-disrupting chemicals: a consensus statement. Arch. Toxicol. 91, 1001–1006.
Sun, H., Zheng, M., Song, J., Huang, S., Pan, Y., Gong, R., Lin, Z., 2019. Multiple-species hormetic phenomena induced by indole: a case study on the toxicity of indole to bacteria, algae and human cells. Sci. Total Environ. 657, 46–55.
Swedenborg, E., Ruegg, J., Makela, S., Pongratz, I., 2009. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. J. Mol. Endocrinol. 43, 1–10.
Teng, M., Wang, C., Song, M., Chen, X., Zhang, J., Wang, C., 2020. Chronic exposure of zebrafish (Danio rerio) to flutolanil leads to endocrine disruption and reproductive disorders. Environ. Res. 184, 109310.
Wang, F., Zhang, W., Chen, Y., Xu, G., Deng, Z., Du, H., Wang, Y., Zhang, S., Zhao, W., 2015. Simultaneous determination of six plant growth regulators in fruits using high performance liquid chromatography based on solid-phase extraction and cleanup with a novel mixed-mode functionalized calixarene sorbent. Anal. Methods 7, 6365–6371.
Wang, K.S., Lu, C.Y., Chang, S.H., 2011. Evaluation of acute toxicity and teratogenic effects of plant growth regulators by Daphnia magna embryo assay. J. Hazard Mater. 190, 520–528.
Wang, W., Wang, B., Liu, Z., Xia, X., 2019. Developmental toxicity and alteration of gene expression in zebrafish embryo exposed to 6-benzylaminopurine. Chemosphere 233, 336–346.
Xiao, X., Zhang, X., Zhang, C., Li, J., Zhao, Y., Zhu, Y., Zhang, J., Zhou, X., 2019. Toxicity and multigenerational effects of bisphenol S exposure to Caenorhabditis elegans on developmental, biochemical, reproductive and oxidative stress. Toxicol. Res. 8, 630–640.
Xie, X., Jin, Y., Ma, Z., Tang, S., Peng, H., Giesy, J.P., Liu, H., 2019. Underlying mechanisms of reproductive toxicity caused by multigenerational exposure of 2, bromo-4, 6-dinitroaniline (BDNA) to Zebrafish (Danio rerio) at environmental relevant levels. Aquat. Toxicol. 216, 105285.
Yang, M., Qiu, J., Zhao, X., Feng, X., 2021. 6-benzylaminopurine exposure induced development toxicity and behaviour alteration in zebrafish (Danio rerio). Environ. Pollut. 278, 116887.
Yu, K., Li, G., Feng, W., Liu, L., Zhang, J., Wu, W., Xu, L., Yan, Y., 2015. Chlorpyrifos is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in zebrafish. Chem. Biol. Interfaces 239, 26–33.
Yun, S., Reynolds, R.P., Masiulis, I., Eisch, A.J.J.N.M., 2016. Re-evaluating the link between neuropsychiatric disorders and dysregulated adult neurogenesis. Nat. Med. 22, 1239–1247.
Zhang, P., Wang, L.M., Zheng, D.W., Lin, T.F., Wei, X.D., Liu, X.Y., Wang, H.Q., 2018. Surface-enhanced Raman spectroscopic analysis of N(6)-benzylaminopurine residue quantity in sprouts with gold nanoparticles. J. Environ. Sci. Health B 53, 561–566.
Zhang, S., Xu, J., Kuang, X., Li, S., Li, X., Chen, D., Zhao, X., Feng, X., 2017. Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (Danio rerio). Chemosphere 181, 270–280.
Zhang, X., Lin, Z., 2020. Hormesis-induced gap between the guidelines and reality in ecological risk assessment. Chemosphere 243, 125348.
Zhu, B., Han, J., Lei, L., Hua, J., Zuo, Y., Zhou, B., 2021. Effects of SiO2 nanoparticles on the uptake of tetrabromobisphenol A and its impact on the thyroid endocrine system in zebrafish larvae. Ecotoxicol. Environ. Saf. 209, 111845.
Zhu, D., Ping, L., Shen, X., Hong, Y., Weng, Q., He, Q., Wang, J., Wang, J., 2020. Effects of prepubertal exposure to forchlorfenuron through prenatal and postnatal gavage administration in developing Sprague-Dawley rats. Reprod. Toxicol. 98, 157–164.

Supporting information

Role of endocrine disruption in toxicity of 6-benzylaminopurine (6-BA) to early-life stages of zebrafish

Guiyi Gong [a, b,*], Hiotong Kam [b], Hanbin Chen [b], Yan Chen [b], Wai san Cheang [b], John P. Giesy [c,d,e], Qiaohong Zhou [f], Simon Ming-yuen Lee [b,*],

[a] Zhanjiang Institute of Clinical Medicine, Central People's Hospital of Zhanjiang, Zhanjiang, 524045, China

[b] State Key Laboratory of Quality Research in Chinese Medicine and Institute of Chinese Medical Sciences, University of Macau, Macau, China

[c] Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B3, Canada

[d] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

[e] Department of Environmental Sciences, Baylor University, Waco, TX 76706, United States

[f] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[*]Corresponding authors:

Simon Ming Yuen Lee, E-mail addresses: simonlee@umac.mo

Guiyi Gong, E-mail addresses: yb67530@umac.mo

Table S1

Prime	Sequence
gnrh3 forward	5’-CACTGGTCATACGGTTGG-3’
gnrh3 reverse	5’-ATCCTGAATGTTGCCTCC-3’
nestin forward	5’-ATGCTGGAGAAACATGCCATGCAG-3’
nestin reverse	5’-AGGGTGTTTACTTGGGCCTGAAGA-3’
gap43 forward	5’-TGCTGCATCAGAAGAACTAA-3’
gap43 reverse	5’-CCTCCGGTTTGATTCCATC-3’
elavl3 forward	5’-AGACAAGATCACAGGCCAGAGCTT-3’
elavl3 reverse	5’-TGGTCTGCAGTTTGAGACCGTTGA-3’
mbpa forward	5’-AATCAGCAGGTTCTTCGGAGGAGA-3’
mbpa reverse	5’-AAGAAATGCACGACAGGGTTGACG-3’
syn2a forward	5’-GTGACCATGCCAGCATTTC-3’
syn2a reverse	5’-TGGTTCTCCACTTTCACCTT-3’
ngn1 forward	5’-TGCACAACCTTAACGACGCATTGG-3’
ngn1 reverse	5’-TGCCCAGATGTAGTTGTGAGCGAA-3’
cyp19a forward	5’-GCTGACGGATGCTCAAGGA-3’
cyp19a reverse	5’-CCACGATGCACCGCAGTA-3’
cyp19b forward	5’-GTCGTTACTTCCAGCCATTCG-3’
cyp19b reverse	5’-GCAATGTGCTTCCCAACACA-3’
erα forward	5’-GGTCCAGTGTGGTGTCCTCT-3’
erα reverse	5’-AGAAAGCTTTGCATCCCTCA-3’
er2β forward	5’-TTCACCCCTGACCTCAAGCT-3’
er2β reverse	5’-TCCATGATGCCTTCAACACAA-3’
fshβ forward	5’-TGAGCGCAGAATCAGAATG-3’
fshβ reverse	5’-AGGCTGTGGTGTCGATTGT-3’

tshb forward	5’-TGCATGGGCTTCTGTTTCT-3’
tshb reverse	5’-TTCTCCTCGGGGTACAGATGA-3’
vtg1 forward	5’-TCCATTGCTGAAAACGACAA-3’
vtg1 reverse	5’-TGCATTCAGCACACCTCTCA-3’
cyp1b forward	5’-CCACCCGAACTCTGA AACTC-3’
cyp1b reverse	5’-AAACACACCATCAGCGACAG-3’

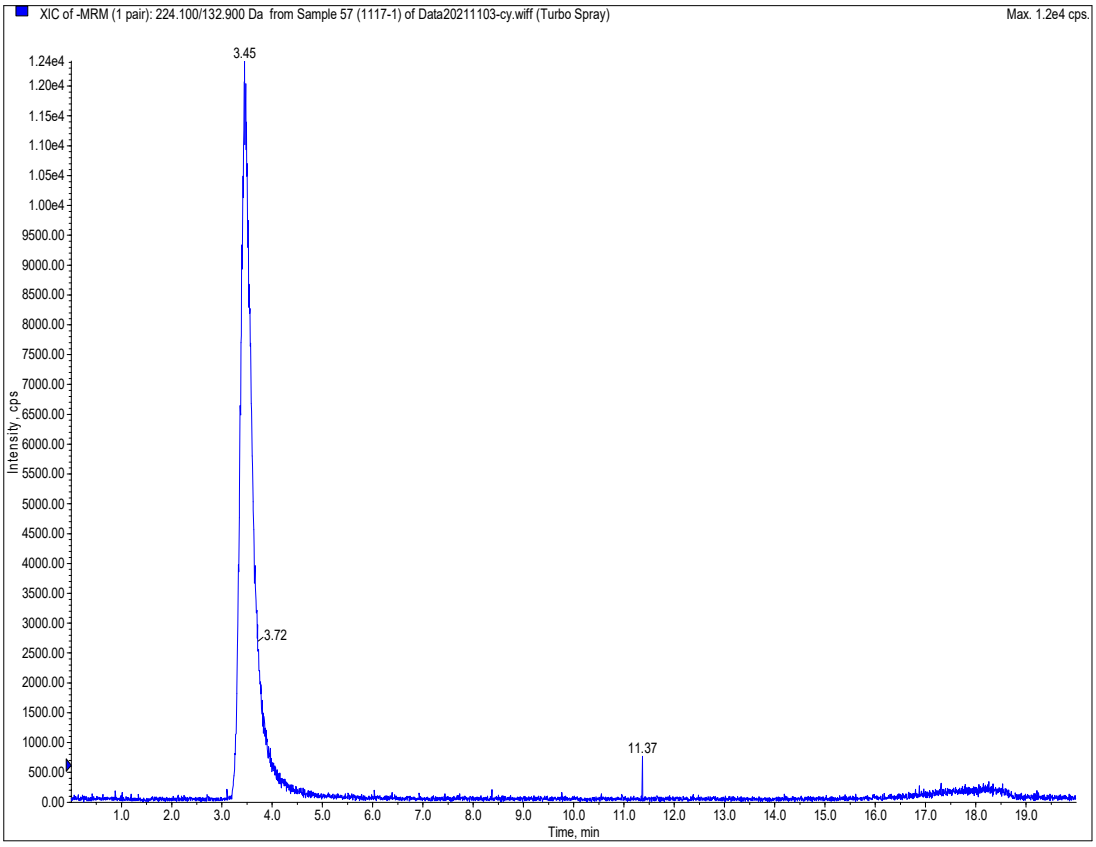


Fig. S1. Representative image of analyte peak and retention time of 6-BA.