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Abstract of Thesis/Dissertation

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Title : Research on the endocrine disrupting potential of environmental contaminants and their developmental toxicity using zebrafish

(ゼブラフィッシュを用いた環境汚染物質の内分泌撹乱作用と発生毒性に関する研究)

Abstract

A significant and varied array of endocrine-disrupting chemicals (EDCs) has been released into the environment since World War II. EDCs have the capacity to interfere with the normal functioning of the endocrine and reproductive systems by mimicking or impeding the actions of endogenous hormones. Researchers have recognized the potential harm posed by EDCs for numerous years. Notably, exposure to anti-androgens and estrogens has been linked to the feminization of wild fish. Considering the reproductive toxicity and endocrine system disruption by anti-androgens in fish, it is crucial to identify environmental chemicals with anti-androgenic potential and to develop sensitive detection methods to assess anti-androgenic responses in fish. Several techniques have been developed to identify anti-androgenic effects using fish species, such as three-spined stickleback (*Gasterosteus aculeatus*), Japanese medak, and *spiggin-gfp* medaka. However, these testing methods require longer exposure durations and specific maintenance systems.

Zebrafish, as a cost-effective and high fertility animal model, has been widely used in various research fields, including drug screening, gene function analyzing, developmental toxicity measuring, and endocrine-disrupting study. In recent years, molecular docking

simulations have become essential in structure-based computational studies aimed at enhancing our understanding of receptor-ligand interactions at the atomic level. Therefore, it is both possible and crucial to develop rapid and quantitative assessment methods for identifying anti-androgens *in vivo* and *in silico* using zebrafish.

To address this gap, Chapter I aimed to establish zebrafish-based *in vivo* and *in silico* assay systems to evaluate the anti-androgenic potential of environmental chemicals. Zebrafish embryos were exposed to 17α-methyltestosterone (TES) alone or in combination with the anti-androgen flutamide (FLU), as well as various pesticides known for their anti-androgenic activities, such as *p,p'*-DDE (DDE), vinclozolin (VIN), linuron (LIN), and fenitrothion (FEN). In order to explore the potential correlation between anti-androgenic potency and developmental toxicity, this chapter additionally conducted morphological assessments.

The expression of sulfotransferase family 2, cytosolic sulfotransferase 3 (*sult2st3*), was measured as an indicator of anti-androgenic effects. The expression of *sult2st3* mRNA was significantly induced by TES in the later developmental stages of embryos. However, the TES-induced expression of *sult2st3* was inhibited by anti-androgen FLU in a concentration-dependent manner with IC_{50} of 5.7 μM, suggesting that the androgen receptor (AR) plays a role in *sult2st3* induction. Similarly, DDE, VIN, and LIN repressed the TES-induced expression of *sult2st3* with IC_{50} of 0.35, 3.90, and 52.00 μM, respectively. At the highest concentration tested (100 μM), FEN also suppressed sult2st3 expression almost completely, although its IC_{50} could not be calculated. Notably, DDE and LIN did not inhibit *sult2st3* induction due to higher concentrations of TES; instead, they potentiated TES-induced *sult2st3* expression. FEN and LIN, which had relatively low anti-androgenic potentials in terms of *sult2st3* inhibition, induced broader toxicities in zebrafish embryos; thus, the relationship between developmental toxicities and anti-androgenic potency was unclear. Additionally, an *in silico* docking simulation showed that all five chemicals interact with the zebrafish AR at relatively low interaction energies and with Arg702 as a key amino acid in ligand binding, whereas several chemicals classified into other groups (e.g., potassium permanganate, 4-hydroxytamoxifen, and 20-hydroxyecdysone) have higher interaction energies. This indicates that anti-androgenic compounds exhibit a higher stability in bonding with zfAR compared to other compounds.

The *in vivo* and *in vitro* anti-androgenic and anti-estrogenic effects of bisphenol A (BPA) have been reported. Still, there is limited research on its analogs about these aspects. Therefore, Chapter II aimed to measure the anti-androgenic potency of BPA and its various analogs applied the established method in Chapter I, and to test their anti-

estrogenic potentials both *in vivo* and *in silico*. To better understand the involvement of estrogen receptor (ER) signaling in developmental toxicity of BPA and its analogs, this chapter also performed morphological assessments of cardiovascular toxicity, such as pericardial edema and blood flow reduction.

For gene expression analysis, zebrafish embryos were exposed to TES or 17β-estradiol (E2) alone or in combination with BPA or each of its analogs, such as bisphenol AF (BPAF), bisphenol E (BPE), bisphenol F (BPF), bisphenol B (BPB), bisphenol C2 (BP C2), 2,2′-bisphenol F (2,2′-BPF), 4,4′-(1,3-dimethylbutylidene)diphenol (Bis-MP), bisphenol Z (BPZ), and bisphenol S (BPS), at 72 h postfertilization. After 24 h of exposure, all samples were collected to measure the mRNA expression levels of *sult2st3* and *CYP19A1b*, which were used to assess the anti-androgenic and estrogenic potential of the tested compounds, respectively. To better understand the ligand binding affinity, we analyzed the binding mode of bisphenols to the zebrafish AR and estrogen receptor subtypes (ERα, ERβ1, and ERβ2). For morphological assessment, embryos were exposed to BPA or its analogs in the presence or absence of ER antagonist fulvestrant (ICI) at 72 hpf and were observed at 96 hpf.

BPAF, BPE, BPA, BPF, and BPB inhibited the expression of TES-induced *sult2st3* with IC_{50} values of 0.53, 3.7, 4.7, 12, and 87 μM, respectively. BP C2, 2,2′-BPF, and Bis-MP showed an inhibitory effect on TES-induced *sult2st3* at the higher tested concentrations. However, BPZ and BPS did not exhibit inhibitory effects on TES-induced *sult2st3*. These results indicate that the anti-androgenic effect of BPA and its analogs follows this order: BPAF > BPE > BPA > BPF > BPB > BP C2 > 2,2′-BPF > Bis-MP >> BPZ ≈ BPS. In AR ligand binding domain, BPA formed a hydrogen bond with Met695, while most of analogs hydrogen-bonded to Asn655 and/or Gln661, indicating that these amino acid residues are essential for exhibiting anti-androgenic activity of bisphenols. Furthermore, BPAF, BPA, BPF, BPB, BP C2, and Bis-MP showed inhibitory effects on E2-induced *CYP19A1b* expression in a concentration-independent manner, indicating their anti-estrogenic effects. Compared to our previous study, the binding mode of BPA and its analogs differed between their antagonistic and agonistic effects on ER subtypes. Specifically, ERβ2 exhibited completely distinct binding modes for bisphenols in the agonistic and antagonistic modes, suggesting the potential role of ERβ2 in distinguishing agonistic and antagonistic activities of bisphenols. The concentration effects of BP C2, Bis-MP, and BPAF on cardiovascular toxicity are much stronger than BPA, BPE, and BPF. The incidence of pericardial edema and blood flow reduction induced by BP C2, Bis-MP, and BPAF was large/completely reduced by ICI, whereas this effect was not observed with

BPA, BPE, and BPF. This suggests that ER signaling may not mediate the cardiovascular toxicity induced by BPA, BPE, or BPF.

In summary, the majority of tested bisphenols exhibited both anti-androgenic and anti-estrogenic effects, while BPZ and BPS did not demonstrate either. Some BPA alternatives, such as BP C2, Bis-MP, and BPAF, showed stronger cardiovascular toxicity than BPA, BPE, and BPF. The cardiovascular toxicity caused by BPA, BPE, and BPF was unlikely to be mediated by ER signaling, but further investigations are needed to confirm the involvement of ER in BP C2-, Bis-MP-, and BPAF-induced cardiovascular toxicity. Based on our findings, utilizing zebrafish-based *in vivo* and *in silico* assessments appears to be a promising approach for evaluating the antiandrogenic or anti-estrogenic potentials of environmental chemicals. Careful consideration should be given to the toxicities of alternative itself when selecting BPA replacement.