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Commentary

Fish biomarkers for regulatory identification of endocrine disrupting chemicals

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ABSTRACT

Demonstrating chemical-induced adverse effects, endocrine mechanisms/modes of action (MOAs) and their causal link is needed for regulatory identification of endocrine disrupting chemicals (EDCs). This paper addresses critical issues over MOAs and their causal link to changes in endpoints. Vitellogenin (VTG), secondary sex characteristics (SSC), and sex ratio (also an apical endpoint) are indicative of chemicals interfering with EAS (estrogen, androgen and steroidogenesis) pathways. These biomarkers, however, can be changed by non-EAS chemicals, systemic toxicity and the stress response. Examples are shown that proving causal link between MOAs and changes in endpoints may be difficult for regulatory identification of EDCs. The paper concludes that both in vitro and in vivo data are needed to define MOAs for regulatory identification of EDCs. Further development of guidance documents for data interpretation and for defining the level of evidence is needed for regulatory identification of EDCs.

1. Introduction

Identification of endocrine disrupting chemicals (EDCs) is needed under the EU chemical regulation REACH (Registration, Evaluation, Authorization and restriction of Chemicals, EC No 1907/2006), the revised regulation for plant protection products (EU 1107/2009) and the biocide regulation (EU 528/2012). The starting point for regulatory identification of EDCs is the WHO definition for endocrine disruptors (WHO, 2002), which consists of three key elements, chemical-induced adverse effects (adversity), chemical-specific endocrine modes/mechanisms of action (MOAs) and the causal relationship (causality) between adverse effects and endocrine MOAs (Munn and Goumenou, 2013). These three key elements of the WHO definition form the basis for developing criteria for the regulatory identification of EDCs. The element of adversity is not unique to EDCs. Data interpretation for adversity has been developed for the routine hazard and risk assessment practice, including classification and labeling. The other two elements, i.e. endocrine MOAs and causality, are unique to the regulatory identification of EDCs. It is acknowledged that regulatory identification of EDCs should be based on evidence of the biologically plausible causal relationship between endocrine MOAs and adverse effects (EFSA, 2013).

The recently revised OECD conceptual framework (CF) is a toolbox, in which test guidelines (TGs) for regulatory screening and testing of EDCs are listed at five levels (OECD, 2012). At level 2, in vitro transcriptional activation assays and steroidogenesis assay are used to test whether or not a chemical of interest could interfere with estrogen, androgen, and steroidogenesis activity (EAS pathways). Fish short term reproduction assays (TG229, 230) at level 3 include biomarkers vitellogenin (VTG) and secondary sex characteristics (SSC) to indicate EDCs interfering with EAS pathways. In addition to VTG, the fish sexual development test (TG234) at level 4 uses apical endpoint sex ratio that can also have some diagnostic value for EAS pathways. These fish biomarkers vitellogenin (VTG), secondary sex characteristics (SSC) and sex ratio are also included in fish partial/full-life cycle toxicity tests at level 4 and 5 (OECD, 2012). In contrast to the indication of EAS pathways, these fish biomarkers also respond to non-endocrine mediated variables and stressors (Hutchinson et al., 2009; Wheeler et al., 2013). For example, changes in fish biomarkers could be influenced by systemic toxicity (Hutchinson et al., 2009; Wheeler et al., 2013) and pathogenic infection (Burki et al., 2012). Recently, several studies show that chemicals that do not act directly via EAS pathways are also capable of inducing changes in fish biomarkers. For example, melatonin, progesterone, and dexamethasone may not directly interfere with estrogen receptors (ERs). However, these chemicals induce changes in VTG in zebrafish and in fathead minnow (Carnevali et al., 2011; DeQuattro et al., 2012; LaLone et al., 2012). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) may inhibit VTG via an

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aryl hydrocarbon receptor (AhR) other than directly via the ER-mediated pathway in zebrafish (Bugel et al., 2013). Apparently, changes in fish biomarkers are not always linked to a direct interference with EAS pathways. MOAs of a chemical could be mistakenly concluded if changes in fish biomarkers are the exclusive basis and if these confounding factors are not considered. So far, data requirements for the regulatory identification of EDCs have not yet been specified. The questions are raised how much evidence is needed to define MOAs of a chemical or whether both in vitro and in vivo tests are needed for define MOAs; whether an indirect effect induced by a chemical of interest should be considered for regulatory identification of EDCs; how much evidence is enough to show causal relationship between MOAs and adverse effects?

To this end, publicly available data on positive responses of fish biomarkers and underlying endocrine MOAs have been collected and analyzed. The analysis was based on our database (Dang et al., 2011a,b) and additionally collected papers. The strategy for collecting additional papers and data quality consideration can be found in our previous papers (Dang et al., 2011a,b). Furthermore, the stress response in fish will be highlighted because it appears to be a particular contributor to a change in fish biomarkers. Finally, the results from these analyses will be discussed in the context of regulatory identification of EDCs. The focus of this paper is on the small model fish recommended by the OECD for regulatory laboratory tests, i.e. fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and three-spined stickleback (*Gasterosteus aculeatus*). Only when necessary, information on other species of fish will be included.

2. Direct and indirect effects of chemicals on fish biomarkers

Fish biomarkers may directly respond to a chemical with specific MOAs or be indirectly influenced by systemic toxicity (Hutchinson et al., 2009; Wheeler et al., 2013) or by the stress response. There is increasing recognition of the need to identify specific sublethal effects and specific MOAs of chemicals (Munn and Goumenou, 2013). It is therefore important to set test concentrations not exceeding maximum tolerated concentration (MTC) for testing EDCs. Details over setting MTCs as well as the systemic toxicity and endocrine fish biomarkers can be found in two excellent review papers (Hutchinson et al., 2009; Wheeler et al., 2013). This paper focuses on fish biomarkers influenced by chemicals at concentrations below the MTC.

VTG is a phospholipoglycoprotein normally produced by the liver of female oviparous vertebrates under stimulation of estrogens. It is almost undetectable in the plasma of immature female and male fish because they lack sufficient circulating estrogen; however, the liver in males is also capable of synthesizing and secreting vitellogenin in response to exogenous estrogen stimulation. According to OECD TGs 229, 230 and 234, the measurement of VTG serves to detect chemicals interfering directly with EAS pathways. Indeed, the majority of the studies in the OECD validation tests and in the literature have been conducted with typical EAS chemicals (Dang et al., 2011a). These EAS chemicals include estrogens (e.g. 17β-estradiol, 17α-ethinyloestradiol, bisphenol A, 4-tert-octylphenol), anti-estrogens (e.g. tamoxifen), androgens (e.g. 17β-trenbolone, methyltestosterone), anti-androgens (flutamide, vinclozolin) and chemicals interfering with steroidogenesis (e.g. fadrozole, prochloraz). Extensive in vitro and in vivo evidence shows that these chemicals directly target EAS pathways (Dang et al., 2011a). There are some chemicals influencing VTG without information on directly targeting EAS pathways. For example, there is no information on whether butachlor, a chloracetamide herbicide, can directly target ERs or not. This chemical increases plasma VTG level in male zebrafish. Based on this evidence, the authors concluded that butachlor might have estrogenic activity (Chang et al., 2013). There are also chemicals that may not have direct effects on the EAS pathways but still influence VTG in fish. For example, hormones melatonin and progesterone exert their primary action through the melatonin and progesterone receptors, respectively. The main mechanism proposed for acetaminophen or paracetamol is the inhibition of cyclooxygenase (COX, Kim et al., 2012). Ibuprofen, non-steroidal anti-inflammatory drug (NSAIDs), inhibits COX, too (Han et al., 2010). TCDD directly inhibits the vitellogenin pathway in zebrafish through activation of the AhR2 (Heiden et al., 2006; Bugel et al., 2013). This anti-estrogenic mechanism is complicated and is far from being understood. It is speculated that AhR-ER cross-talk can occur through (1) direct inhibition of the ER pathway by AhR binding to cisregulatory elements near estrogen responsive elements required for ER binding; (2) interaction with common cofactors required by both the AhR and the ER; (3) AhR-induced synthesis of an inhibitory factor; (4) proteasomic degradation of ERs; (5) induction of enzymes involved in estrogen metabolism/clearance; and (6) competition for resources necessary for synthesis mRNA and proteins (Bugel et al., 2013).

SSC in male fish of fathead minnow and medaka are externally visible and quantifiable traits, which are responsive to circulating levels of endogenous androgens. Females maintain the capacity to develop male SSC and can do so after exposure to androgenic EDCs. Similar to VTG, the majority of the studies in the OECD validation tests and in the literature have been conducted with typical EAS chemicals like 17β-estradiol, 17α-ethinyloestradiol, 4-tert-octylphenol, 17β-trenbolone, methyltestosterone, flutamide, fadrozole, and prochloraz (Dang et al., 2011a). These chemicals interfere directly with EAS pathways and influence SSC in fathead minnow and/or in medaka (Dang et al., 2011a). Changes in SSC of male fathead minnows have also been observed for an antidepressant of the selective serotonin re-uptake inhibitor fluoxetine. This effect may not act via interfering directly with EAS pathways but rather via modulating brain serotonin activity, which may in turn influence the hypothalamus–pituitary–gonadal (HPG) axis (Schultz et al., 2011).

Sex differentiation in fish is labile and can be modulated, for example, by exposure to EDCs. According to OECD TG234, this fish test is intended to use both VTG and a shift of sex ratio to detect chemicals with androgenic and estrogenic properties as well as anti-androgenic, anti-estrogenic and steroidogenesis inhibiting properties. An increase in VTG of both males and females as well as in female biased sex ratio has been reported for typical estrogenic chemicals like 17β-estradiol, 17α-ethinyloestradiol, bisphenol A, 4-nonylphenol, 4-tert-octylphenol, and 4-tert-pentylphenol (references see Table 1). A decrease in VTG of males and females and an increase in male biased sex ratio have been observed for typical androgenic chemicals like 17β-trenbolone and dihydrotestosterone as well as for steroidogenesis inhibitor prochloraz (references see Table 1). Some chemicals, e.g. malathion, tribromophenol, tributyltin chloride, may induce changes in VTG and/or sex ratio, different from those of typical estrogenic, androgenic and steroidogenesis chemicals (references see Table 1). In the literature, there are chemicals described that may have no direct effects on the EAS pathways but still influence VTG and sex ratio in fish. For example, PFOS may target the peroxisome proliferator-activated receptor α (PPARα), whereas the primary mechanism of action for perchlorate is inhibition of iodine uptake thereby interfering with thyroid hormones. PFOS changes VTG and the sex ratio in zebrafish (Du et al., 2009; Wang et al., 2011); perchlorate induces hermaphroditism in threespine stickleback (Bernhardt et al., 2006).

This study focuses on three fish biomarkers. The other endpoints like gonadal histology may provide mechanistic information. Some