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Endpoint sensitivity in fish endocrine disruption assays: Regulatory implications

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ABSTRACT

Identifying potential endocrine disrupting chemicals (EDCs) needs screening and testing for mode of action (MOA) and intrinsic toxicological properties. MOA is often indicated by biomarker endpoints, whereas toxicity by apical endpoints. Risk assessment is mainly based on apical but not on biomarker endpoints. The 21-day fish assay (OECD TG229) is considered a screening test. But it includes both biomarker and apical endpoints. This study explores the utility of results of the 21-day fish assay for risk assessment purposes. Endpoint sensitivity was analysed by compiling 142 data sets for 21-day fish assays and 38 data sets for the fish sexual development test (FSDT), encompassing 62 chemicals with different MOAs. Conclusions from this analysis include: (1) vitellogenin (VTG), fecundity and gonad histology are the most sensitive endpoints for fathead minnow, medaka and zebrafish in 21-day fish assays; secondary sex characteristics (SSC) are a less sensitive endpoint and is likely inadequate to detect all known MOAs. (2) Biomarker endpoints like VTG and apical endpoints like fecundity from the 21-day fish assay can be used for risk assessment. (3) Lowest observed effect concentrations (LOECs) of the most chemicals are comparable for the 21-day fish assay and for the FSDT, further supporting that results of 21-day fish assays can be used for risk assessment. However, a significant difference in LOECs was observed for some chemicals, suggesting that chemical specific effects should be taken into account. This paper emphasizes that a weight of evidence approach is important for interpretation of results of the 21-day fish assay.

1. Introduction

Endocrine disrupting chemicals (EDCs) may act like hormones in the endocrine system, disrupt the normal physiological function and eventually lead to adverse effects (Hutchinson et al., 2006; Dang and Lowik, 2005; Dang, 2009). Endocrine disruption as mode or mechanism of action (MOA) is often indicated by biomarker endpoints in a battery of *in vitro* and *in vivo* screens; whereas adverse effects are evidenced by apical endpoints in a suite of apical tests (ECETOC, 2009; OECD, 2010). Screening assays for the estrogen receptor (ER) mediated pathway, for example, include the *in vitro* ER binding assay, the *in vitro* ER transcriptional assay (e.g. test guideline 455, TG455), the *in vivo* Uterotrophic assay (TG440) for human health and the *in vivo* 21-day fish assay (TG230) for the environment (OECD, 2010). Biomarkers of these assays could indicate possible MOAs but cannot supply direct information on toxicity of chemicals. Adverse effects of concern should be further shown in apical tests like the two generation test (TG416) and the fish full life cycle test (FFLC), which indicate toxicity of the chemical and form a basis for determining the no observed adverse effect level (NOAEL) for human health and the no observed effect concentration (NOEC) for the environment. Both screening and testing are needed for elucidating MOAs and intrinsic toxicological properties of chemicals and are also essential for identification of EDCs for regulatory purposes.

Evaluation of endocrine disrupting properties of chemicals becomes a regulatory need under the current EU revised regulation for plant protection products (Regulation (EC) No. 1107/2009). According to the OECD (Organisation for Economic Co-operation and Development) conceptual framework for the testing and assessment of EDCs, diagnostic *in vitro* and *in vivo* screening assays like TG 455 and TG230 are used as a trigger for further testing if the results of these assays are positive. Definitive testing assays like the TG416 generation test and the FFLC are needed for deriving NOAELs or NOECs for risk assessment purposes (OECD, 2010). As current risk assessment of chemicals is mainly based on apical endpoints but not on MOA endpoints, MOA data may not be directly

Abbreviations: 11-KT, 11-ketotestosterone; AR, androgen receptor; E2, 17β-estradiol; EDC, endocrine disrupting chemical; EPA, environmental protection agency; ER, estrogen receptor; FFLC, fish full-life cycle test; FSDT, fish sexual development test; HSI, hepatosomatic index; GSI, gonado-somatic index; LOEC, lowest observed effect concentration; MOA, mode or mechanism of action; NOAEL, no observed adverse effect level; NOEC, no observed effect concentration; OECD, Organisation for Economic Co-operation and Development; SSC, secondary sex characteristics; T, testosterone; TG, test guideline; VTG, vitellogenin.

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used for the determination of NOAELs or NOECs for risk assessment purposes. Screening for EDCs may lead to additional testing with animals resulting in ethical problems as well as economic burdens.

In terms of *in vivo* OECD 21-day fish assays, there are two TGs specifically designed and validated for EDCs. These assays, i.e. TG229 and TG230, use changes of two core biomarker endpoints vitellogenin (VTG) and secondary sex characteristics (SSC) to indicate EDCs with estrogenic and androgenic activity, and aromatase inhibition potential. In contrast to TG230, TG229 includes more endpoints. Plasma steroid concentrations (E2, 11-KT, T) have been used in the literature as biomarker endpoints for EDCs. Apical endpoints like somatic growth (weight, length and condition factor), hepatosomatic index (HSI), gonado-somatic index (GSI), gonad histology, fecundity, fertilization success, and hatching success (hatchability) are also used for the 21-day fish assay. Some apical endpoints like gonad histology may indicate the specific effects of EDCs; other apical endpoints like fecundity indicate population effects which may not be specific for EDCs. Although TG229 includes apical endpoints, both TGs are considered as screening assays. Results of these assays function as a trigger for further testing but cannot be directly used in risk assessment. In comparison with the 21-day fish assay, the fish sexual development test (FSDT) or FFLC may have similar sensitivity in detecting EDCs (OECD, 2008; Knacker et al., 2010). Interestingly, the FSDT using VTG and sex ratio as core endpoints is considered as a definitive test directly used in risk assessment (Knacker et al., 2010). As the 21-day fish assay is comparable to the mammalian one-generation reproduction test which is considered as a definitive test for risk assessment, the question is whether the results of TG229 including biomark endpoints can be directly used for risk assessment.

Till now, there is little guidance regarding what constitutes the MOA of concern and how to incorporate MOA information into risk assessment of EDCs for regulatory purposes. Currently, both the European Commission and the OECD are developing guidance documents for identifying EDCs. This paper intends to compile data available on the 21-day fish assay in order to effectively use both diagnostic biomarker endpoints and apical endpoints for risk assessment of chemicals. Systematic evaluation of the data was performed so as to compare endpoint sensitivity for chemicals of different MOAs. In addition, endpoint sensitivity was compared for 21-day fish assay and for the FSDT. We believe that this study reveals important information on how to use the results of the 21-day fish assay for regulatory purposes. These results are of importance for the testing strategy and evaluation of EDCs. From both an ethical and an economic perspective, our results are likely to contribute to the reduction and refinement of animal testing.

2. Materials and methods

This paper focuses on three species of fish: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*), which are recommended for use in screening assays by OECD TG229 and TG230. Publications containing VTG data for these three species were first selected from PubMed. There is a fair body of available information with aforementioned criteria. Publications clearly indicating that changes in VTG are due to general toxicity or hepatotoxicity were not included. For reason of comparison, publications were further selected for studies carried out with sexually mature male and spawning female fish exposed to chemicals of at least two concentrations for 21 days. Papers identified were used as an additional searching source. As TG229 and TG230 were just approved by OECD in 2009, most studies in the selected papers were conducted neither according to these guideline methods nor in compliance with GLP guidelines. Data quality of these publications was therefore not scored according to the regulatory criteria but the publications were selected based on the availability of the most important parameters including chemical identity, description of fish and the test methods, testing with serial doses, and the quality of data reporting. The selection criteria conform to the basic regulatory data quality requirement. The second source of data was the OECD validation of the 21-day fish screening assay for the detection of endocrine substances, in which the TG229/TG230 like protocol was used. The third source of data was a medaka database from the Japanese Ministry of Environment that is available at http://www.env.go.jp/en/chemi/ed/rt_medaka.pdf. The database has been recommended by OECD for peer-review (OECD, 2006c). This database includes 38 chemicals tested by using the TG 230 like protocol and partial life cycle test protocol. The latter is comparable to the OECD draft protocol for the fish sexual development test (FSDT).

Mortality of fish in the 21-day fish assays has been reported in the literature. As tested concentrations were not sufficiently high to cause systemic toxicity, mortality was not included as an endpoint in our database of 21-day fish assays. This endpoint, however, was included for the FSDT because exposure begins at fish early life stage and lasts for 2 months. Core endpoints reported in the literature include VTG, somatic growth (wet weight, standard length and condition factor), hepatosomatic index (HSI), gonado-somatic index (GSI), secondary sex characteristics (SSC), gonad histology, fecundity, fertilization success, and hatching success (hatchability). Besides, steroid concentrations (plasma E2, 11-KT, T) were often reported in fathead minnows. For medaka tested in the FSDT, additional endpoints like time to hatching, testis-ova were included in the database. Measurement of VTG has been conducted in different tissues (e.g. blood for fathead minnows, liver for medaka) by using different methods. The differential measurement of VTG and other endpoints is not the focus of this paper and therefore is not noted in our database though it may contribute to variability of the data. Assays of TG229 and TG230 are capable of detecting multiple MOAs, i.e. estrogenic and androgenic activity, and aromatase inhibition. Some endpoints are sex-related and may be observed in either males or females or both. Sex of fish is not specified in the database of this paper. It is noted that some endpoint effects are apparently nonmonotonic. For such effects, the lowest concentration that induced endpoint effects was indicated in the database.

Table 1 summarizes the possible MOAs and the different tests available for a total of 62 chemicals. Some of these chemicals have been employed in the evaluation of the 21-day fish reproduction screen and characterized as typical agonists or antagonists of ERs or ARs or typical steroid metabolism modulators (including aromatase inhibitors). Chemicals like atrazine may act via the hypothalamus–pituitary axis. Due to the relative scarcity of MOA information in fish, these chemicals have been grouped as uncharacterized or uncertain MOAs (US EPA, 2006), which is also noted in Table 1. Many chemicals in the Japanese database belong to this group although some evidence shows that the chemicals may act via ERs, ARs or steroid metabolism modulating. The uncertain MOA was not indicated for these chemicals from the Japanese database in Table 1, but was labeled in Table 5. Potassium permanganate, sodium pentachlorophenol and n-octanol may have other toxic effects but have been considered as negative control because these chemicals are known not to be disruptive of reproductive endocrine processes (OECD, 2006a,b; US EPA, 2006). In this paper, they are also indicated as negative controls.

Tables 2–5 show dose-dependent effects of chemicals on fathead minnow, medaka and zebrafish. Endpoint effects in this paper were divided into three effective categories, effects at lowest observed effect concentration (LOEC, marked with a red colour), effects at concentrations above the LOEC within the same study (yellow colour), and absence of effects at the maximum tested concentrations (>the maximum concentration, blank). The number of observations for these three groups is plotted and shown in Figs. 1A, 2A, 3A and 4. Except the endpoint VTG, the other endpoints were not reported in all studies of the 21-day fish assay collected in the literature and OECD validations. For reason of comparison, the relative contribution of each effective category was additionally calculated and plotted as the ratio between the number of observations in each effective category and the number of total observations in three effective categories of each endpoint and was expressed as percentage (Figs. 1B, 2B and 3B).

3. Results

Data were generated for 62 chemicals tested with the protocols of 21-day fish assays for fathead minnow, medaka and zebrafish and of the FSDT for medaka. These 62 chemicals include typical agonists and antagonists of ERs and ARs, typical steroid metabolism modulators, chemicals that do not influence AR, ER and steroid metabolism, and chemicals with other MOAs (e.g. dopamine inhibitor) or uncertain MOAs. Some chemicals have even multiple MOAs. Some chemicals have been considered as EDCs but their MOAs have not yet been fully supported by the experimental data and therefore not indicated in Table 1.

As shown in Table 2, 21-day fish assays have been carried out in 46 fathead minnow studies for 25 chemicals, among which 10 chemicals were tested at least two times. Chemicals with estrogenic and androgenic activity as well as aromatase inhibition induced changes in VTG (cells with red and yellow colours). The anti-androgenic chemical flutamide did not consistently influence VTG among 5 studies, with VTG changes reported only in two studies. Chemicals with other MOA or negative controls did not have effects on VTG. Changes in VTG (yellow and red colours) are accompanied with at least one other endpoint effects for almost all