1 Regulatory framework for the characterization and identification of endocrine disrupters

For decades, studies of endocrine disrupters (EDs) have challenged traditional concepts in toxicology, in particular the dogma "the dose makes the poison," as they can have effects at low doses that are not predicted by effects at higher doses. At the beginning of the 90s the term endocrine disruption first appeared in scientific literature. and a workshop sponsored by US environmental protection agency (US EPA) that gathered experts on the topic described an endocrine disrupter (ED) as "An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and or behavior" (Kavlock et al., 1996). Around the same time a workshop held in Weybridge UK emphasized emphasize that an endocrine disrupter could be adequately defined only in terms of their effects on intact living organisms: "An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine function" (World Health Organisation/ European Centre for Environment and Health, 1996). A definition that was later adopted by the European chemicals agency (ECHA) the more generalized formulation: "Endocrine disruptors are chemicals that may interfere with the hormonal system and thereby produce harmful effects in both humans and wildlife (ECHA, 2024).

Consequently, to identify an ED based on these definitions, it has to be shown that an adverse effect would occur in a test animal system, epidemiologically or clinically and it has to be demonstrated that the mechanism by which the substance is causing the adverse effect is endocrine and a plausible link between the adverse effect and the endocrine mechanism has to be established. This definition and its elements have been widely accepted and later made the basis for the ED criteria set by the European Union (Andersson et al., 2018). Since there are several elements required to fulfill the definition and derived criteria no single test provides definitive evidence of endocrine disruption. Moreover, it is important to note that the term ''intact organism'' does not necessarily mean that adverse effect and endocrine mechanism both have to be demonstrated in an intact test animal. Adequately validated alternative test systems that predictive for humans are considered acceptable as well. However, as there is a lack of alternative test systems that fulfill these criteria, one or more appropriate in vivo studies are usually required to appropriately characterize an adverse effect. In contrast, the endocrine mechanism leading to the adverse effect may be detected in an *in vitro* assay such as a receptor binding or transactivation assay.

Due to a lack of regulatory guidance for industry and assessors of competent regulatory authorities the European Commission has requested ECHA and the European Food Safety Authority (EFSA) to develop, with the support of the Joint Research Centre (JRC), a common guidance document for implementing the hazard-based criteria to identify endocrine disruptors (Andersson et al., 2018). The resulting guidance document describes how to gather, evaluate and consider all relevant information for the assessment, conduct a mode of action (MoA) analysis, and apply a weight of evidence (WoE) approach, in order to establish whether ED criteria outlined in Commission Delegated Regulation (EU) 2017/2100 and Commission Regulation (EU) 2018/605 for biocidal products and plant protection products (EU 2017/2100, 2017; EU 528/2021, 2012), respectively, are fulfilled.

For example, if an absolute and relative increase in thyroid weight is observed in a repeated dose 28-day oral toxicity study in rodents (OECD TG 407, 2008), the adversity of this finding should be investigated further according to the EU guidance for the identification of endocrine disruptors (Andersson et al., 2018). In 2008 OECD guidance for the 28-day oral toxicity study in rodents has been revised to include parameters suitable to detect endocrine activity of test substances. As thyroid hormones are involved in multiple physiological processes such as regulating the basal metabolic rate, promote the adrenergic nervous system to generate heat in response to cold exposure, stimulate gluconeogenesis as well as lipolysis and lipogenesis, the weight change of the thyroid in the 28-day oral toxicity study indicates a potential endogen disruptive effect of the investigated substance (Liu & Brent, 2010). However, it has to be borne in mind that owing to the low statistical power of the study (5 animals/group), the window of exposure and the parameters tested, the weight increase of the thyroid can only be interpreted as being indicative, whereas a negative outcome would not be conclusive for the absence of endocrine disruptive effects.

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (OECD GD 150, 2018) gives guidance on how to identify and characterize the mode of action of a potential endogen disruptive substance further. The effect on the thyroid weight in the repeated-dose 28-day rodent study indicates a potential thyroid mediate endocrine disruption. However, the potential for multiple modes of endocrine action should be considered in the planning of further experimental studies. Besides a thyroid mediated endocrine disruption, estrogen/androgen-mediated endocrine disruption and an interference with steroidogenesis should be considered. In general, no single assay provides definitive evidence of endocrine disruption, but rather a weight of evidence approach is needed in the further characterization. Before further testing all existing data such as structural information of the investigated substance should be considered. In QSAR analyses structural entities might be identified that give further indication on the mode of endogen disruption or a potential for read-across with other similar substances may be indicated. In a first step along the in vitro assays listed in the OECD guidance in section B.1.2. should be conducted to characterize the potential endocrine mechanism(s)/pathway(s) further: The hER binding assay (OECD TG 493) and the estrogen receptor transactivation assay (OECD TG 455). These assays can provide identification of possible mechanisms and MOA, prediction of adverse outcome pathways (AOPs), priority-setting and WOE-based judgements for the more in-depth test strategy.

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