Regulatory Toxicology and Pharmacology 68 (2014) 175–192

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Anti-androgenicity can only be evaluated using a weight of evidence approach

Stephanie Melching-Kollmuß [a], Karma C. Fussell [b], Roland Buesen [b], Martina Dammann [b], Steffen Schneider [b], Henk Tennekes [c], Bennard van Ravenzwaay [b,*]

[a] BASF SE, Product Safety, 67056 Ludwigshafen, Germany
[b] BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany
[c] Experimental Toxicology Services (ETS) Nederland BV, Frankensteeg 4, 7201KN Zutphen, The Netherlands

ARTICLE INFO

Article history:
Received 22 July 2013
Available online 31 October 2013

Keywords:
Preputial separation
Anti-androgen
Two-generation toxicity study
Rat
OECD 416
Endocrine disruption
Body weight
Weight-of-evidence

ABSTRACT

Preputial separation (PPS) is a commonly used external marker for the onset of male puberty in experimental animal studies. While treatment-related delays in PPS may be indicative of specific anti-androgenic activity, impaired general growth also alters the onset of puberty. To differentiate between specific and non-specific effects on the age at PPS – and thereby evaluate the validity of the endpoint PPS-two-generation toxicity studies of 23 substances were evaluated. The 23 substances were assessed regarding anti-androgenicity using all available data and external assessments in a weight-of-evidence evaluation (WoE).

Correlation of individual pup body weight with age at PPS revealed that delays in pubertal development coincided with reduced pup body weight. After comparison with the WoE assessment, we concluded that inclusion of body weight analysis into the PPS evaluation of each study was able to correctly identify three compounds which specifically induced delayed PPS and 16 which only showed unspecific changes. A further two compounds which might be categorized as anti-androgens based on delayed PPS, were correctly regrouped using our refined methodology. Based on this analysis and in comparison to the WoE evaluation, it was found, that caution should be exercised when using the endpoint PPS in hazard assessment.

1. Introduction

The investigation and assessment of endocrine disrupting potential has become more and more important in Europe, especially after the introduction of cut-off criteria for pesticide registration. Directive 1107/2009, now requires that "an active substance . . . shall only be approved if . . . it is not considered to have endocrine disrupting properties, that may cause adverse health effects in humans. . ." (European Parliament and the Council of the European Union, 2009). Under the Biocides Directive 528/2012 endocrine disrupting compounds cannot be approved, while under the REACH regulation for chemicals (1907/2006) endocrine disrupting compounds may be included in Annex XIV and subjected to authorization. An overall accepted definition of an endocrine disrupting compound is the WHO/IPCS definition: An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (IPCS, 2002). It is expected, that a final definition on endocrine disruption criteria will be available by end of 2013, which shall be adopted by the Commission, according to the Biocides Directive. The European Food and Safety Authority as well as the Joint Research Centre (JRC) have recently published scientific opinions on criteria to conduct hazard assessments for endocrine disrupting compounds (EFSA, 2013; JRC, 2013). Criteria are not yet set, but there is ongoing discussion amongst stakeholders on how cautiously ED-related criteria shall be implemented for findings with an endocrine mode of action. However, in all current regulations, the two-generation toxicity study conducted under the most recent OECD or US Guidance is regarded as a higher tier study suitable for the evaluation of the endocrine disrupting potential of a test substance (OECD Technical Guideline for the Testing of Chemicals, 2001; US Environmental Protection Agency, 1998).

In recent years, regulatory guidelines for reproductive toxicity testing of agricultural and industrial chemicals have been extensively revised with the addition of many new end points of reproductive function and offspring development (Korenbrot et al., 1977; OECD Technical Guideline for the Testing of Chemicals, 2001; US Environmental Protection Agency, 1998; US Environmental Protection Agency, 2001; Yamasaki et al., 2005). The newly integrated parameters were:

Abbreviations: PPS, preputial separation; PND, postnatal day; p.p., post-partum.

* Corresponding author. Fax: +49 621 605 81 34.
E-mail address: bennard.ravenzwaay@basf.com (B. van Ravenzwaay).

0273-2300/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yrtph.2013.10.004

- sperm analysis,
- organ weights in offspring,
- weights, as well as more extensive and detailed histopathology of reproduction organs in parents and offspring (uterus, ovaries, testes, epididymides, prostate, and seminal vesicles with coagulating glands),
- age at sexual maturation, as defined by vaginal opening or preputial separation (PPS),
- anogenital distance (optional), and
- estrous cycle.

One important reason for these additional end points was to address concerns about the potential toxicity of hormonally active chemicals (Carney et al., 2004). Chemicals inhibiting androgen activities (by blocking androgen receptors, suppressing androgen hormone synthesis, or both) may cause irregular reproductive development (Fisher, 2004; Gray et al., 2001; Ostby et al., 1999; Wong et al., 1995). Specific anti-androgenic responses include decreased reproductive organ weights and/or histopathological changes, affected sperm characteristics, undescended testes, decreased male fertility, delayed puberty onsets, retention of nipples or areolas, penile malformations (e.g. hypospadias) and decreased anogenital distances in male offspring. *In utero* exposure of laboratory rats to androgen receptor antagonists such as vinclozolin may feminize male offspring (Gray et al., 1994; Schneider et al., 2008; Hellwig et al., 2000; Matsuura et al., 2005; van Ravenzwaay, 1992; Wolf et al., 2000); direct exposure of the young male animals before puberty may lead to delayed pubertal development (Monosson et al., 1999), as visualized by a delayed onset of androgen-dependent PPS (Blystone et al., 2009). Delayed preputial separations are also observed in studies with other compounds known to have an anti-androgenic mode of action (Blystone et al., 2009; Wolfe and Patel, 2004).

However, delay of sexual maturation can also occur as a secondary, non-specific, effect of general systemic toxicity caused by a compound administered in a toxicological study (Delemarre-Van de Waal et al., 2002). There is broad evidence in the literature, that lower body weights of the offspring, as well as the decreased food consumption or lower body weights of dams during pregnancy, correlate with the age at preputial separation of its male offspring and may cause delayed onset of puberty in male and female rats (Carney et al., 2004; Chernoff et al., 2009; Laws et al., 2007; Leonhardt et al., 2003; Marty et al., 2003). In addition, it is known from human medical observation and epidemiological studies that the status of nutrition plays an important role on reproduction functions and onset of puberty (Dunger et al., 2006; Baird et al., 2006; Frisch, 1987; Van Weissenbruch et al., 2005; Warren, 1983; Kaplowitz, 2008). Furthermore, as test guidelines require that systemic toxicity is evident, at least at the highest dose level, and that effects on body weight development (reductions in the range of 10–20%) are the most common effects noted at maximum tolerated dose levels, there is a significant chance that delayed preputial separation is observed as a result of general systemic toxicity rather than a consequence of an endocrine disruption mode of action. It is probable then that delays in male sexual maturation can occur which are distinct from those due to hormonal disruption. Thus, the dosing requirements of the test guidelines assure that putative endocrine disruptive effects will often be identified; without a critical analysis of the specific or unspecific nature of their mode(s) of action, many of these will be false-positive artifacts of systemic toxicity.

Meanwhile guidelines have become available to specifically study androgenic/anti-androgenic modes of action *in vivo*. In the Hershberger assay (OECD Technical Guideline for the Testing of Chemicals, 2009) castrated testosterone-supplemented rats are dosed for 10 days and the weights of the accessory male reproductive organs are determined, while in the male pubertal assay (US Environmental Protection Agency, 2009) young male rats are peripubertally administered the test compound under investigation for 15 days. These two study types are included in the Tier 1 battery of the US EPA Endocrine Disruptor Screening Program (http://www.epa.gov/endo/pubs/assayvalidation/tier1battery.htm#assays) and also mentioned in the OECD conceptual framework as lower tier studies, giving certain evidence for a specific mode of action, which would have to be further investigated in higher tier studies, like the two-generation toxicity study (OECD Technical Guideline for the Testing of Chemicals, 2001; US Environmental Protection Agency, 1998) or the extended one-generation study (Fegert et al., 2012; OECD Technical Guideline for the Testing of Chemicals, 2012). Studies conducted according to these guidelines provide results from a variety of endocrine-related endpoints. Similarly, it has been acknowledged that the evaluation of the endocrine disrupting potential of a test compound shall only be done by applying a WoE approach using all available data (Bars et al., 2011; Boobis et al., 2008; European Centre for Ecotoxicology and Toxicology of Chemicals, 2009).

An important feature of these studies is testing for anti-androgenicity. Several definitions for anti-androgenicity have been described, but a broader understanding of what constitutes an anti-androgen is required here. Therefore in the context of this paper the following working definition, based on the observed effects rather than defining a specific mode of action, is used: Anti-androgens are substances which are capable of inhibiting the biological effects of androgen hormones. Relevant targets are summarized in Fig. 1 below.

Among other measures, the entry into puberty (age at onset of preputial separation in males) is one of the mandatory investigative endpoints in these higher tier studies which is widely-regarded as a sign of anti-androgenicity in reproductive toxicity

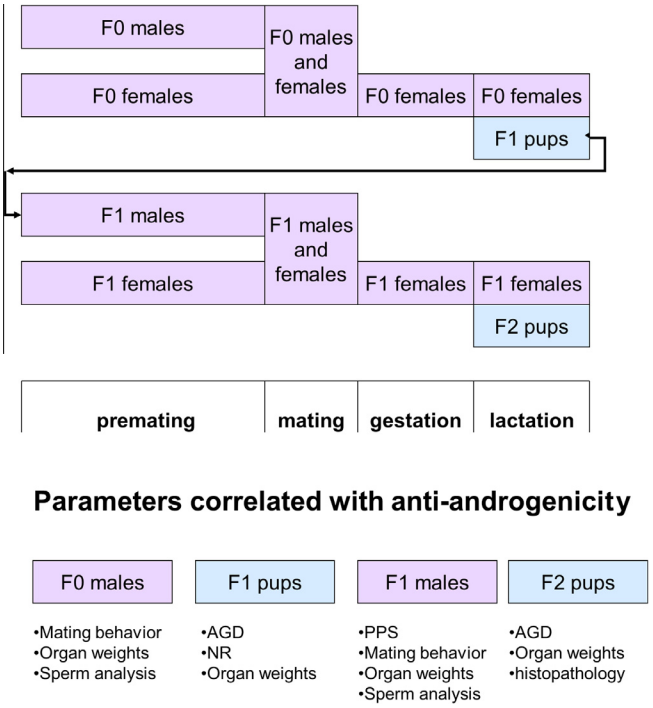

Fig. 1. Sequence of a two-generation toxicity study. This study design provides general information about the effects of a substance on male and female fertility, mating behavior, conception, gestation, parturition, lactation, weaning, as well as the development of the offspring. The parameter specifically correlated with anti-androgenicity and the respective animal group, where they are investigated, are mentioned in the bottom part of this figure.