Reproductive Toxicology 34 (2012) 331–339

Contents lists available at SciVerse ScienceDirect

Reproductive Toxicology

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

# Feasibility of the extended one-generation reproductive toxicity study (OECD 443)

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ARTICLE INFO

*Article history:*
Received 17 November 2011
Received in revised form 16 May 2012
Accepted 25 May 2012
Available online 5 June 2012

*Keywords:*
OECD443
Feasibility
Vinclozolin
Methimazole
Lead
2,4-D

ABSTRACT

The extended one-generation reproduction toxicity study (OECD 443, adopted 28-July-2011) produces more information with fewer animals than the two-generation study (OECD 416), by including F1 neurotoxicity and immunotoxicity assessments, and omitting an F2 generation if there are no relevant F1 findings. This saves >1000 animals per compound. Feasibility studies based on draft OECD443 were conducted in industrial GLP laboratories in Europe and USA, using vinclozolin, methimazole and lead acetate. A fourth study was conducted with 2,4-dichlorophenoxyacetic acid (2,4-D) in response to a regulatory request for reproduction and developmental neurotoxicity data.

The studies effectively profiled vinclozolin as an anti-androgenic developmental toxicant, methimazole as a developmental anti-thyroid agent, and lead acetate as a systemic and developmental toxicant. The 2,4-D study demonstrated the value of toxicokinetic data in dose setting and data interpretation. These results illustrate the variety of reproductive and developmental endpoints which can be captured in this complex but manageable study design. Time constraints for triggering further (F2) testing are summarized.



## 1. Introduction

The extended one-generation reproductive toxicity study design was first described by Cooper et al. [1], based on a proposal by the International Life Science Institute-Health and Environmental Sciences Institute (ILSI/HESI) Agricultural Chemical Safety Assessment (ACSA) Technical Committee (Cooper 2009) [2]. Its main objectives are to evaluate specific life stages not covered by other types of toxicity studies to provide hazard characterization data for risk assessment, and to avoid the excessive use of experimental animals. In a single study, it tests parental (P) fertility and reproductive function, and offspring (F1) development through sexual maturity, including assessment of sexual landmarks (Cohort 1), nervous system (Cohort 2), and immune system (Cohort 3). If there are indications of potential adverse F1 offspring effects that require clarification, Cohort 1 may be mated to produce an F2 generation. This study design has now been adopted as OECD Health Effects test guideline no. 443 [3].

### 1.1. Advantages

Compared to the existing two-generation study (OECD 2001 and USEPA 1998) [4,5], the extended one-generation design increases the number, extent and duration of F1 offspring assessments. Significantly more F1 offspring are retained for assessments at juvenile and/or young-adult life stages as compared to the two-generation study. Furthermore, the additional offspring retained in the extended one-generation study are evaluated for additional

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http://dx.doi.org/10.1016/j.reprotox.2012.05.096

specific developmental end points (nervous and immune system function), which are typically evaluated only in separate studies. The data obtained are thus greatly enhanced in the extended one-generation study. The breadth of complementary information provided in this single study will assist with the interpretation of findings and to guide decisions regarding the most sensitive system and additional investigation.

The inclusion of additional developmental endpoints and the omission, in most cases, of an F2 generation potentially reduces the number of animals required by several thousand per compound tested, relative to running separate two-generation and developmental neurotoxicity studies, as well as immunotoxicity and adult neurotoxicity when otherwise required. This would potentially be of particular importance in the context of the new EU REACH legislation (registration, evaluation and authorization of chemicals), which requires safety testing of all industrial chemicals on a production tonnage basis. It has been estimated that reproductive toxicity testing will be required for 7.5% of all substances under REACH. Testing using the present two-generation guideline (OECD 2001) [4] alone would require approximately 1 million laboratory animals [6].

The justification for reduced use of animals by elimination of a second generation has been presented in several extensive data reviews. Janer et al. [6] retrospectively analyzed available data from 176 two-generation studies with 148 chemicals, and concluded that the F2 generation data in these studies had no impact on either classification and labeling or on risk assessment. A re-analysis of the Janer data by Rudén and Hansson [7] highlighted three studies (1-bromopropane, 2-ethylhexyl phthalate, and piperazine) in which effects (additional, more pronounced, or at lower doses) were identified in the F2 relative to the F1 or P generation. However, Janer et al. [8] responded that the NOAELs used for risk assessment, and classification and labeling of these three substances would not have been altered by omission of the F2 generation.

In a review of 329 multigeneration studies in the U.S. EPA ToxRefDB dataset, Martin et al. [9] concluded that the F2 generation in these 329 studies would rarely impact either qualitative or quantitative evaluations. Of the sixteen chemicals demonstrating higher sensitivity of the F2 generation, only three (carbaryl, fenarimol, and 2-(thiocyanomethylthio)-benzothiazole) produced F2 effects not seen in the F1 generation. Of these three chemicals only fenarimol effects (F2 litter size) were used to set the chronic reference dose.

Most recently, Piersma et al. [10] conducted a detailed assessment of study reports from 498 rat multi-generation studies, representing 438 different test substances. Consistent with previous analyses, the data indicate that the second generation mating and F2 offspring data rarely, if ever, provided critical data that would alter risk assessment or classification and labeling.

### 1.2. Purpose of the present studies

Various components of the extended one-generation study are routinely performed in industrial GLP laboratories as stand-alone studies; however, the four studies reported here represent the first time all components have been integrated into a single study. These studies were commissioned to evaluate the experimental and logistical feasibility of the study design, in terms of conduct and the suitability of the assays to detect critical effects, based on the study design originally described by Cooper et al. [1] or slight modifications thereof. They were not intended to be formal validation studies as defined in OECD [11].

The following reference chemicals were used:

- Vinclozolin, an anti-androgenic substance. In utero/lactational exposure in rats results in demasculinized male offspring, with reduced anogenital distance, retained areola, hypospadias, hypoplastic penis, reduced testicular size, and aplasia/agenesis or reduced size of male accessory sex glands [12,13].
- Methimazole, a selective inhibitor of thyroid hormone production. The induction of hypothyroidism by methimazole produces a delay in CNS development as well as behavioral deficits in rat pups [e.g. 14].
- Lead(II) acetate; lead is a developmental neurotoxicant in young children, a cardiovascular and nephrotoxicant in adults [15], and a developmental immunotoxicant [28].
- 2,4-Dichlorophenoxy-acetic acid (2,4-D) served as an example of using a protocol based on the ACSA study design [1] for regulatory purposes, including the use of toxicokinetics and triggers based on study data to determine whether to breed the F1 animals.

## 2. Method

According to OECD 443 [3], administration of test substance via the diet is the preferred route, although other routes may be utilized if appropriate. In the studies reported here, all substances were admixed to diet except lead acetate, which was added to the drinking water. As summarized in Table 1, parental animals were exposed to test substance during a premating period and through cohabitation, gestation, parturition and lactation, totaling 12 weeks in males and 10 weeks in females. Selected F1 pups were exposed through lactation until necropsy at 8.5–20 weeks of age. After a pre-mating period of 2–4 weeks, parental animals were cohabitated overnight for mating until positive mating up to a maximum of 14 days. Females were allowed to rear their litters until weaning on postnatal day (PND) 21, when randomly selected F1 pups were assigned to one of three cohorts. The cohorts according to OECD 443 are as follows:

*Cohort 1*: Reproductive/developmental toxicity. Cohort 1A is for primary assessment of effects upon reproductive organs and function (estrous cyclicity, ovarian follicle counts, sperm analyses; reproductive organ histopathology) and general toxicity, including thyroid hormone assays for triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH). Cohort 1B is for conditional follow-up assessment of reproductive performance by mating F1 animals to produce an F2 generation and/or for additional histopathology data, if triggered by findings during the study.
*Cohort 2*: Developmental neurotoxicity (functional observational battery, motor activity, auditory startle, neurohistopathology).
*Cohort 3*: Developmental immunotoxicity.

*Note*: The design of these studies was based on Cooper et al. [1]; they were initiated before finalization of the OECD extended one-generation study guideline. Their cohort assignments were not in accordance with OECD 443; see Table 1. For developmental immunotoxicity, OECD 443 specifies analysis of splenic lymphocyte subpopulation (CD4+ and CD8+ T lymphocytes, B lymphocytes, and natural killer cells) which was not done in the studies reported here. When these studies were initiated, the draft design specified phenotypic analysis of splenic subpopulations only if there was an altered primary antibody response [2] (see Tables 2 and 3).

## 3. Results

The results are summarized in Tables 4 and 5.

### 3.1. Methimazole

In the P generation, thyroid-stimulating hormone (TSH) and thyroid weight were increased in a dose-related fashion beginning at the low dose with modest effects on triiodothyronine (T3) and thyroxine (T4), consistent with compensation to maintain thyroid hormone levels. In nearly all treated animals at all dose levels, minimal to marked diffuse follicular cell hyperplasia/hypertrophy was noted in both sexes; mean severity was dose-related. Minimal to slight focal follicular cell hyperplasia and diffuse follicular cell vacuolation were also noted in both sexes at all dose-levels. There were no histopathological effects in liver. Mating, fertility, gestation and lactation were unaffected by treatment.

In the F1 generation, all doses increased TSH and thyroid weight in a dose-related fashion on PNDs 4 and 21 and at term, with a moderate decrease in T4 and variable effects on T3. F1 body weight