Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study

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European Centre for Ecotoxicology and Toxicology of Chemicals
4 Avenue E. Van Nieuwenhuyse (Bte 6), B-1160 Brussels, Belgium.

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EXECUTIVE SUMMARY

The current 'gold standard' for the assessment of reproductive toxicity in safety evaluation is the two-generation reproduction study, OECD Test Guideline (TG) 416[1]. This is a complex study that involves a large number of animals (ca. 2,600), takes nine months from start of treatment to necropsy, and is costly. Under the EU regulation on registration, evaluation, authorisation, and restriction of chemicals (REACH) (EU, 2006), this study may be required for substances produced or imported into the EU at more than 100 tonnes per annum if triggered by findings in other studies, and is a default requirement for substances produced or imported into the EU at more than 1000 tonnes per annum.

The standard one-generation study design (OECD TG 415) is largely disfavoured because it does not cover the full reproductive cycle, and has not been updated with the developing science. For example, many apical endpoints for the evaluation of endocrine active potential could not be evaluated in this study design. Recent developments have, however, led to a re-evaluation of the one-generation study, with the proposal that it could be extended, not only to cover more of the reproductive cycle but also to include additional evaluations of developmental toxicity (Cooper *et al*, 2006). In parallel to this, re-evaluations of the two-generation study have questioned the value of the second breeding in cases where data are available from other studies (Janer *et al*, 2007; Makris, 2004). The OECD is now considering a new test guideline describing an extended one-generation study design.

Inclusion of all endpoints suggested by Cooper *et al* (2006) for the testing of agrochemicals would result in a very complex design for routine evaluation of industrial chemicals far beyond the scope of current testing, as well as increasing the potential for additional animal usage in further studies that may be triggered by type I errors (false positive results) in the main study. Consequently, a modular study design, in which additional evaluations could be triggered or waived in the light of other available information, would appear to be optimal.

If such an extended one-generation study design is to be used within a tiered testing strategy, there must be a formal process for deciding the choice of modules. In this vein, guidance for triggers and waivers for the inclusion or exclusion of modules is required.

This document includes discussion of the following modules that might be included within an extended one-generation study design: second breeding for an F_2 generation; developmental neurotoxicity (DNT); prenatal developmental toxicity (PDT); and developmental immunotoxicity (DIT). It gives consideration to the identification and validation of the triggers for these modules, within the framework laid down by the OECD (OECD, 2005). This report also serves as the starting point for a multi-stakeholder workshop for the development of triggering and waiving criteria for the modules of the extended one-generation study.

[1] OECD test guidelines mentioned throughout this report are referenced in the Bibliography.

1. INTRODUCTION

1.1 The evaluation of reproductive toxicity: the changing shape of the 'gold standard'

Mammalian reproduction is a complex, balanced process that relies on the co-ordinated interaction of diverse organ and cell systems, and may be adversely affected by exposure to chemicals acting through a wide range of mechanisms. Given the complexity of the reproductive cycle, and the relative lack of understanding about its underlying processes, the evaluation of the impact of chemical exposure largely relies upon the conduct of apical studies *in vivo*. A variety of protocol designs have been established to assess the impact of chemical exposure on reproductive health, although many of these focus on specific phases of the reproductive cycle, rather than the cycle as a whole (ECETOC, 2002).

The OECD is currently formalising a new test guideline for an extended one-generation study design, to replace or update the current OECD TG 415 and address the guideline's shortcomings. Primary among these is that it cannot evaluate effects upon the progeny that are expressed after weaning.

The two-generation reproductive toxicity study, OECD TG 416, is considered to be the 'gold standard' for assessing the impact of substances upon reproductive health, as it covers all stages of the reproductive cycle (e.g. ECETOC, 2002). It is also the most intensive study design not only in terms of sophistication, but also animal usage, time, and cost. The main reason for this is the conduct of a second-generation breeding phase.

The Agricultural Chemical Safety Assessment (ACSA) technical committee, established by the International Life Sciences Institute's (ILSI) Health and Environmental Sciences Institute (HESI), proposed a review of the one-generation reproduction toxicity study to cover additional developmental landmarks and endpoints (Cooper *et al*, 2006). To follow up on the proposals, workshops were organised by the European Centre for the Validation of Alternative Methods (ECVAM) and the European Partnership on Alternative Approaches to Animal Testing (EPAA)[2]. Consensus was found that the described study design may also be applied to other regulatory frameworks, such as REACH. Triggering and waiving criteria should be clearly defined when assessing the need for performing additional modules (PDT, DNT, DIT), as these are not always required by legislation or for risk assessment.

It is the purpose of this ECETOC document to review the triggering and waiving criteria available for assessing the need for additional toxicological assessments.

[2] Published reports from these workshops were not available to the task force at the time of writing.

1.2 An alternative 'gold standard'

An alternative approach to the traditional assessment of effects on reproductive health has been suggested through an extension of the one-generation reproductive toxicity study design (Cooper *et al*, 2006), and although it was specifically developed for agrochemical testing, the principles are more widely applicable. This approach seeks to minimise animal usage by limiting the conduct of a second breeding, while at the same time optimising the detection of substances harmful to reproductive health. It also introduces modules to evaluate developmental neurotoxicity and developmental immunotoxicity.

Recent evaluations of two-generation studies for a wide range of chemicals have been undertaken to determine the value of the second mating phase and the F_2 generation. It has been suggested that data from the second mating of a two-generation study do not add value to hazard identification or risk assessment when an extensive assessment is made of the first generation during adulthood (Janer *et al*, 2007), or when the first-generation data are evaluated along with other toxicological data (Makris, 2004).

The reduction in animal usage that is achieved by substituting the guideline two-generation study with an extended one-generation study design is substantial (Cooper *et al*, 2006). A two-generation study conducted to OECD TG 416 will entail the use of approximately 2600 animals. If standalone PDT and DNT studies are also conducted, these will add at least 80 and 1200 animals respectively (Cooper *et al*, 2006), bringing the total to around 4000. An appropriately designed extended one-generation study would use around 1400 animals or 2600 if a second breeding is triggered.

It is important to note that this reduction in animal usage is not attained at the expense of data generation for risk assessment purposes. PDT and DNT can be evaluated in animals that will be born during the normal conduct of the study. This means that additional animals are not needed for the conduct of standalone studies. All of the data that would have been generated under the traditional testing paradigm could still be obtained, with a significant reduction in the number of animals used (up to 65%).

1.3 Guidance for triggering or waiving an extended one-generation reproduction toxicity study under REACH

Within the European Union, the development of an extended one-generation study guideline will have significant impact upon the design of testing strategies and use of animals in compliance with REACH (EU, 2006).

REACH stipulates a specific battery of reproductive studies for substances placed on the market at levels of 10 tonnes or more per annum. Both the regulation and accompanying technical guidance document on information requirements (EU, in preparation) lay out a tiered testing strategy, triggered by volume or alerts from existing studies, which starts with screening studies (OECD TG 421 and 422) at lower production volumes, and includes more substantial studies (e.g. OECD TG 414 and 416) at higher volumes. In addition, the strategy lays down criteria for waiving tests that would otherwise be required. The technical guidance document on information requirements recognises that information for evaluation of reproductive toxicity potential may be derived from other guideline or non-standard studies.

For the purposes of this evaluation, it is assumed that the need for an extended one-generation study has been established within an appropriate testing strategy. Furthermore, it is assumed that data are available from other studies confirming that repeated dosing is tolerated and that these data allow suitable dose/exposure levels to be identified.

1.3.1 Triggers for conducting an extended one-generation reproduction toxicity study

The triggers for conducting an extended one-generation reproduction toxicity study should be evaluated within the legislative framework. Indeed, the technical guidance document on information requirements (EU, in preparation) foresees that such a study design, properly validated and accepted, could be used in place of the OECD TG 416. Thus, the considerations for conducting an extended one-generation study will be the same as those for conducting an OECD TG 416, and are laid down in both the regulation and the technical guidance document on information requirements. There are additional considerations as to how such a study should be designed, and this latter concept will be examined herein.

With respect to the triggers for both conducting a study and its design, the use of available data should be considered in order to minimise animal use and stress, and studies should be evaluated to assess their suitability for use, robustness of design and quality.

1.3.2 Waivers for not conducting an extended one-generation reproduction toxicity study

Criteria for reduced testing requirements have been laid down in the REACH technical guidance document on information requirements mentioned above. Specifically, these relate to substances that show low toxicological activity, no systemic exposure through relevant routes of exposure and no significant human exposure. Furthermore, where sufficient information is available to assess the reproductive safety of a chemical, the generation of further animal data is not

warranted. This information may be in the form of a previously conducted OECD TG 416 study or the weight of evidence from existing information.

1.3.3 General considerations for the selection of modules

The inclusion (or exclusion) of modules within the overall study design should be made on a weight of evidence approach, based on external and internal triggers. This refers to available data from previously conducted studies (external triggers) and, where appropriate, data developed during the conduct of the extended one-generation study itself (internal triggers). It is important to note that the selection of a module should rarely be based on the identification of a single trigger endpoint, but rather on the basis of biological plausibility from a consistent pattern of findings. In this context, other factors such as severity of findings, magnitude of effect, dose-response relationship, and statistical significance should also be taken into consideration.

External triggers can be taken into account during the design stages of the extended one-generation study. Clearly, the weight of evidence would be greater from data generated in good quality and biologically relevant, validated and accepted studies, e.g. those designed in line with OECD test guidelines and conducted according to the principles of good laboratory practice. Although data from all available studies should be used in the weight of evidence evaluation used to trigger or waive a second generation, where there is a conflict between study results, data from high quality studies are likely to overrule data from studies of questionable quality or significance (OECD, 2005). Consequently, if no data are available to decide on the inclusion or exclusion of modules, these data must be generated either prior to or during the conduct of the extended one-generation study.

Inclusion of a module should be waived when data exist from an appropriately powered, designed, well conducted definitive study that gives a conclusive result. Further testing may however be justified to determine the mechanism of action and potential relevance for man. Unless scientifically justified, no further testing should be performed to confirm a definitive study outcome.

The extended one-generation is described further in section 2.1.3, and an overall scheme showing the modules within the extended one-generation study is given in Appendix 1.

2. THE EXTENDED ONE-GENERATION REPRODUCTION TOXICITY STUDY AND SUGGESTED MODULES

2.1 Overview of the extended one-generation study

2.1.1 Background

The current one-generation study guideline, OECD TG 415, evaluates the effect of chemical treatment upon the mating success of the parental (P) generation and prenatal through to early postnatal development of the offspring (F_1 generation). Groups of male and female animals, usually rats, are administered the test material for up to 10 weeks prior to mating, throughout gestation and lactation, to the time that the offspring are weaned on postnatal day (PND) 21. At this time, all animals are killed (males are usually killed earlier) and undergo routine examination.

The two-generation study, OECD TG 416, was conceived as one in which the mating success of the F_1 generation could be evaluated. In this context, F_1 animals are selected at weaning and remain on the same treatment schedule as the respective P generation. After at least 10 weeks of treatment, and when sexually mature, the F_1 animals are mated. The same evaluations that were made on the P generation parents and F_1 offspring are made on the F_1 parents and their offspring (F_2 generation) up to study termination around the time of weaning.

Developments in scientific understanding, particularly with respect to the perturbation of development mediated through disruption of the endocrine system, were incorporated into an update of the OECD TG 416. Many of the developmental landmarks under endocrine control occur after weaning, and in some cases only upon maturation. Consequently, the OECD TG 415 was not considered suitable for the purpose of detecting endocrine disrupting chemicals.

Another aspect in which both the one- and two-generation studies were considered deficient was in the evaluation of DNT. Standard two-generation and DNT test protocols are designed to be performed as independent studies, and even though DNT endpoints could be included within the conduct of an OECD TG 416 study, thus far this has usually been limited to the observation of pup behaviour inside and outside the home cage, not functional testing and neuropathology.

2.1.2 The proposal for an extended one-generation study

The Agricultural Chemical Safety Assessment (ACSA) technical committee, established by the International Life Sciences Institute's (ILSI) Health and Environmental Sciences Institute (HESI), developed a tiered approach to life stages testing for agricultural chemical safety assessment

(Carmichael *et al*, 2006). As part of this programme, ACSA considered how the one-generation study design might be adapted to address its shortcomings with respect to detection of endocrine disruption and developmental neurotoxicity. The result was an extended one-generation study design, in which selected F_1 offspring would be assigned to 'sets' and maintained on the same treatment regimen as the P generation until at least 10 weeks of age. Each set would be used to evaluate different aspects of development (e.g. a battery of behavioural and neuropathological investigations for DNT and DIT) and also evaluated for endocrine-mediated developmental landmarks (Cooper *et al*, 2006).

Routine application of the extended one-generation study design proposed by ACSA would present significant resource constraints in the safety evaluation of chemicals outside the specific requirements of agricultural safety assessment. However, the concept underlying the extended one-generation study has engendered substantial interest for its potential to balance both resource constraints (e.g. animal use, laboratory availability) with thorough safety assessment. In this context, a development of the ACSA extended one-generation study can be envisaged, in which some components are conducted by default (core) and others (i.e. modules) are included or waived on the basis of external or internal triggers (sections 2.2 to 2.5).

2.1.3 Core study design

The extended one-generation study (core) design should cover the reproductive cycle from a period prior to mating, through gestation and lactation, and follow the offspring to maturation (Appendix 1).

Groups of sexually mature male and female animals, usually rats, will be assigned randomly to different treatment groups, including one group that will serve as untreated control. Treatment will commence and continue before mating for a period sufficient to allow steady-state exposure conditions to be achieved, and to detect functional and behavioural changes that might affect mating and fertilisation. The duration of the pre-mating exposure for females is typically two weeks, or two oestrus cycles (Cooper *et al*, 2006; OECD 1983, 2001b). For males, a pre-mating exposure period of four weeks (Cooper *et al*, 2006; Takayama *et al*, 1995) or 10 weeks (OECD 1983, 2001b) has been used, although another study showed that two weeks may suffice (Sakai *et al*, 2000).

Males and females will then be paired for mating, with care taken to ensure non-sibling pairings.

After mating, males will continue to be treated for at least four weeks in total, or until it is known that a second mating will not be required. Females will continue to be treated during gestation and lactation, until the offspring (F_1 generation) are weaned. If there are equivocal results from

the first mating (F_{1A}), the males and females from each group may be mated with different partners from the same treatment group to produce a second litter (F_{1B}).

Prior to weaning, F_1 animals will be selected for post-weaning studies and remain on the same treatment regimen as the P generation. Selected offspring will include at least one group that will be evaluated for developmental success to maturation, at least 10 weeks. The remaining animals will be euthanized and subjected to necropsy.

2.1.4 Endocrine disruption

During the conduct of the extended one-generation study, there are several landmarks of endocrine-mediated structural and behavioural development that may be assessed to determine whether a chemical acts as an endocrine disrupter (Cooper *et al*, 2006). These may be included within the core design, or their inclusion may be prompted by internal or external triggers.

Core endpoints that may be indicative of endocrine disruption may include some or all of the following:

- Ano-genital distance at birth (F_1);
- testis descent (F_1);
- oestrus cycle length and normality (P, selected F_1);
- onset of puberty, determined by age at vaginal opening or preputial gland separation (selected F_1);
- mating behaviour (P);
- reproductive organ weights (P, selected F_1);
- morphological assessment of reproductive tract organs (P, selected F_1);
- analysis of sperm parameters: number, motility and morphology (P, selected F_1).

Triggered endpoints, or endpoints that can be determined in triggered modules, that may be indicative of endocrine disruption include some or all of the following:

- Ano-genital distance at birth (F_2);
- presence of retained areolae and nipples on the abdomen of males at PND 12 or 13 (F_1, F_2);
- testis descent (F_2);
- mating behaviour (selected F_1).

2.1.5 General advantages of evaluating developmental toxicity within a modular approach

There are significant advantages to evaluating developmental toxicity within the framework of an extended one-generation study, in contrast to standalone studies (e.g. OECD TG 414, OECD TG 426).

Reducing animal numbers in the evaluation of reproductive toxicity

The normal conduct of a guideline PDT study (OECD TG 414) requires group sizes of approximately 20 females with implantation sites at necropsy. In practice, as many as 25 presumed pregnant females may be assigned to each group to ensure adequate group sizes after failed mating pairs are accounted for. The standard study design, outside of the limit test, is to include at least three dose levels and one concurrent control, and consequently may use between 80 and 100 female animals.

The normal conduct of a guideline DNT study (OECD TG 426) requires group sizes of at least 20 litters for neurotoxicity evaluation. In practice, as many as 30 presumed pregnant females may be assigned to each group, to ensure adequate offspring after failed mating pairs, litter standardisation, and the proper assignment of animals for behavioural tests and neuropathology are accounted for. The standard study design includes at least three dose levels and one concurrent control, and consequently may use between 100 and 120 female animals.

The inclusion of PDT and DNT assessment as modules in the extended one-generation study would eliminate the need for additional animals in the evaluation of these endpoints. The selected females will be bred during the normal course of the study.

Identifying effects related to sustained blood concentrations

The standard approach to the PDT study is to use oral gavage as the mode of test substance administration to the dam. In the case of DNT studies, although not explicitly stipulated in test guidelines, direct gavage dosing of pups is frequently used to ensure exposure to offspring during all early periods of brain development, if exposure through milk is considered to be inadequate or is unknown.

Bolus administration of test material results in a relatively high systemic concentration as the test material is absorbed from the gastro-intestinal tract, followed by a reduction as it is eliminated. The variance between these peaks and troughs over time will depend upon the pharmacokinetic characteristics of the test substance and the dosing regimen. Oral administration of test material

in feed or drinking water results in absorption over a longer period of time. Although temporal variations in blood levels will follow from dietary administration, these will generally be less extreme in nature. The difference in patterns of systemic exposure between bolus and sustained dosing regimens has implications for the identification of developmental toxicity in both PDT and DNT studies.

Organogenesis is a staged process, and different stages may be more or less sensitive to toxic insult, depending on the mechanism by which a substance interacts with its target. If the concentration of a substance at the target peaks during a critical window of organogenesis, then this may result in the development of abnormalities, but if it is below a critical level, then the potential to detect these effects is diminished. Consequently, bolus administration favours the identification of toxic effects only if the timing of dosing and the pharmacokinetic characteristics of the test substance favour sufficient dose delivery at the target during the critical window. In some cases developmental toxicity is actually more closely related to total integral exposure to a toxicant rather than to peak levels (O'Flaherty and Scott, 1997). In the case of DNT studies, peak blood concentrations may lead to acute toxic effects in the offspring that interfere with the conduct or interpretation of the study itself.

Gavage dosing, therefore, may favour the identification of effects that occur at high, often non-physiological blood concentrations as long as peak systemic levels coincide with critical windows of development, and do not induce confounding systemic toxicity in the dams or in the pups themselves. In contrast, sustained dosing through the diet or drinking water may favour the identification of effects that occur over a longer period of exposure, or those for which the window of susceptibility might otherwise coincide with lower systemic exposure levels following administration by oral gavage.

Simplification of risk assessment: integrated evaluation of prenatal, postnatal, and sub-chronic toxicity

Although the guidelines do not stipulate a route or mode of application of the test material, the oral route is favoured (OECD, 2001a,b) and application is largely through gavage bolus for sub-acute studies (such as OECD TG 414 and OECD TG 426) and via diet or drinking water for sub-chronic studies (such as OECD TG 416). Therefore, it is often the case that results from these studies are obtained under different conditions of systemic dose delivery. As noted above, bolus dosing results in greater extremes of systemic exposure compared to continuous dosing, and this complicates the overall assessment of toxicity findings and identification of critical endpoints for risk assessment.

The combination of prenatal, postnatal and sub-chronic evaluations within a single study design obviously counters this disparity. When all observations are made under the same conditions of

dose delivery, animal husbandry and laboratory environmental conditions, inter-study as well as protocol variability is eliminated. Therefore, it becomes more realistic to interpret PDT and DNT findings within a wider frame of reference.

Test material application more relevant to human environmental exposures

During the conduct of standard PDT and DNT studies the test material is usually administered by oral bolus (gavage) dosing, because this presents a reliable and convenient way of achieving systemic exposure. As a consequence, the plasma concentration of the test substance follows a pattern of peaks and troughs, depending on the substance's pharmacokinetic characteristics. This pattern of systemic exposure may favour the identification of effects related to peak blood concentrations (C_{max}), including dose-limiting maternal toxicity, as noted above.

Human exposure to chemicals rarely follows this pattern, but instead is characterised by lower levels of exposure experienced either intermittently or over an extended period. Peaks of plasma concentration, following bolus oral administration, are therefore largely unrepresentative of environmentally relevant levels of exposure. Studies that are conducted to emphasise the 'worst-case' exposure scenario may identify effects that are of questionable physiological relevance, may be influenced through saturation of rate-limiting transformation pathways, or may be restricted by dose-limiting systemic toxicity (Conolly *et al*, 1999).

In contrast, longer-term breeding studies are more often conducted with the test material administered via the diet or drinking water, which will result in greater consistency of the plasma concentration profile and be more representative of patterns resulting from environmental exposure.

2.2 The second-generation module

It has long been assumed that, in order to enable a thorough investigation of reproductive toxicity, F_1 generation animals—which have been exposed to the test substance *in utero*—should be mated to ensure that their reproductive function is unimpaired. This is because the F_1 generation is potentially exposed during the critical periods of development of the endocrine system, as well as other systems known to be sensitive to perturbation by certain xenobiotics.

This assumption is being increasingly challenged. Janer *et al* (2007) investigated 176 data sets for 148 substances (46 of which had been classified by the EU or California EPA as toxic to reproduction or fertility). From this database it was found that a previous generation was just as likely to provide a lower no observed adverse effect level (NOAEL) than was a subsequent

generation; of the 176 studies, the NOAEL was derived from the F_1-F_2 generation mating data on only two occasions. This low incidence was explained by the authors as the result of chance, since for any given generation there is a probability that treatment-related effects may not attain statistical significance. In a separate review of 44 chemicals classified as toxic to reproduction (fertility) in the EU, it was found that in a weight of evidence approach to hazard identification, a mating phase was rarely needed to provide alerts for effects on fertility (Dent, 2007). Preliminary findings from a review conducted by the US EPA indicated that the F_2 generation does not impact the risk assessment of substances compared to data from the F_1 generation and data from other studies (Makris, 2004).

The results of the F_1 mating rarely affect the overall NOAEL in a two-generation study and differences in NOAEL values between generations are a matter of chance. Therefore, a study in which F_1 generation animals are exposed to the test item into adulthood without mating should be sufficient for classification and risk assessment. Consequently, the purpose of the F_1 mating phase would be to confirm or refute equivocal results from a previous mating, rather than to show any differences in sensitivity between generations (Janer *et al*, 2007). This assumption underlies the recommended design elements outlined in this section.

2.2.1 Description of the module

The design of a triggered second-generation module should be based on the OECD TG 416, which describes a two-generation reproduction test design in rodents, and the proposal of Cooper *et al* (2006). The second generation module requires that selected F_1 adults should continue to be treated with the test material beyond PND 70, paired for mating and their progeny, the second (F_2) generation, examined at study termination, typically at weaning (PND 21). The external and internal examinations performed on the P generation animals and the F_1 postnatal animals should also be applied to the F_1 parental animals and the F_2 postnatal animals until and including study termination.

2.2.2 Triggers for adding a second-generation module

Further assessment of reproductive toxicity beyond the scope of the extended one-generation reproduction toxicity study can be triggered following review of existing data using a weight of evidence approach. Where data conclusively support the classification criteria of a chemical or NOAEL for risk assessment, then the need for a second-generation module is waived. Typically, the second-generation module is triggered by equivocal effects in the first generation for reproduction endpoints. These could be further investigated with the second-generation module added to the extended one-generation reproduction toxicity study. It should be noted that

equivocal findings in the P generation may be confirmed with a second breeding of those animals to produce a F_{1B} generation, as described previously.

2.2.3 Examples of triggers and waivers for a second-generation module

Compound 1[3]

A four-week range finding study resulted in reduced weights of epididymis and cauda epididymidis, prostate and seminal vesicles with no corroborating histopathological changes. In a prenatal developmental toxicity study no relevant developmental effects were observed. A subsequent one-generation range finding study showed reduced weights of epididymis and cauda epididymis, prostate, seminal vesicle with no corroborating histopathological changes, reduced sperm parameters as well as decreased litter size were noted in the P generation at the top dose. Delayed sexual maturation and decreased size of prostate and seminal vesicles were evident in top dose F_1 males.

Assessment: If such clear findings had been observed in an extended one-generation study, they would not trigger further breeding.

Compound 2

In a three-month study, slight changes in the testes and epididymides weights were recorded at the high dose-levels. In a subsequent one-generation study, mating and fertility indices were slightly decreased in the P generation starting at the mid-dose. These changes were associated with a slight decrease in the number of pregnant females.

Assessment: Trigger for a two-generation evaluation based on equivocal findings.

Compound 3

No signs of maternal toxicity were observed in an OECD TG 422 study except for a significant dose-related delay in parturition of P generation females. This was associated with a lower number of live born pups at the low and mid-dose groups. No live pups were recorded at the high dose. A dose-related increase in post-implantation loss was recorded in all groups. The lowest observed adverse effect level (LOAEL) was identified.

Assessment: If these findings were made in an extended one-generation study, they would not trigger further breeding.

[3] Note: The examples given herein relate to test data on commercial substances. It is the finding and the decision process which are important and not the identity of the substance itself. Therefore, the chemical formulae of the compounds are not revealed.

2.3 The developmental neurotoxicity (DNT) module

The triggers in the following discussion are also summarised in Appendix 2.

Specific DNT testing is designed to provide data, including dose-response characterisations, about the potential functional and morphological effects on the developing nervous system of the offspring that may arise from exposure of the mother during pregnancy and lactation. The evaluation of DNT within an extended one-generation reproductive toxicity study design has specific advantages and disadvantages in comparison to the conduct of a traditional 'standalone' DNT study, in addition to the general advantages already noted above (section 2.1.5).

2.3.1 Description of the module

In the DNT module, selected F_1 males and females will be subject to a functional observational battery (home cage observation, open field observation, sensory motor and reflex tests) and assessed for motor activity a few weeks after weaning. Gross necropsy of these animals at the age of 10 weeks includes detailed neuropathology after perfusion fixation. Detailed neuropathology after perfusion fixation is also done in selected pups at the age of three weeks.

2.3.2 Specific advantages of evaluating DNT within a modular approach

Both DNT test guidelines, the OPPTS 870.6300 (US EPA, 1998) and the recently adopted OECD 426 (OECD, 2007), specifically indicate a preference for combined testing: "A developmental neurotoxicity study can be conducted as a separate study, incorporated into a reproductive toxicity and/or adult neurotoxicity study (e.g. Test Guidelines 415, 416, 424), or added onto a prenatal developmental toxicity study (e.g. Test Guideline 414)". The REACH technical guidance document on information requirements makes similar recommendations (EU, in preparation).

In fact, the general design of DNT testing is somewhat redundant in comparison to a standard two-generation protocol, because, apart from recording general clinical parameters for both studies, there is a significant overlap in the developmental life stages assessed across the various study designs; in particular, each study includes exposures during gestation, parturition and through early postnatal life.

Another example is the globally recognised ICH S5(R2) guidance for pharmaceuticals (ICH, 1993), which provides for a limited assessment of DNT such as functional development parameters (motor activity, memory, auditory startle) in a pre- or postnatal study. The guidance

was acknowledged to likely provide an adequate screen for the potential of a test substance to cause DNT effects.

2.3.3 Specific disadvantages of evaluating DNT within a modular approach

Difficulties in dose selection arising from potential interference from secondary effects of toxicity

Testing for DNT is intended to investigate changes in behaviour due to effects on the central and peripheral nervous systems. However, behaviour also may be affected by the function of other organs—e.g. liver, kidneys and the endocrine system—such that systemic toxicity in offspring may result in secondary changes in behaviour. Thus, higher doses that cause target organ toxicity may impair the assessment of DNT by introducing non-specific behavioural changes. On the other hand, doses which do not induce excessive toxicity in the offspring may be adequate for DNT testing but disfavour the identification of effects on target organs or on fertility and reproduction that occur only at higher doses.

Developmental toxicity may also be manifested as apparent changes in behaviour which are unrelated to, but may be erroneously attributed as DNT. For example, changes in the musculo-skeletal system may be misinterpreted as neurotoxicity. This potential confounder of DNT testing may be difficult to detect if no *in utero* and neo-natal deaths or macroscopically obvious malformations were observed.

Therefore, the selection of doses that are optimal for the identification of primary functional and behavioural effects on the nervous system may not be the same as that for other toxicological evaluations, including PDT. Conversely, dose selection that is optimised for general toxicity or PDT may result in secondary effects that are not truly indicative of DNT.

Exposure to offspring less well-defined

As discussed previously, DNT studies are often conducted with the test material administered to dams and pre-weaning pups by bolus gavage. This approach is to ensure a well-defined exposure to offspring during all early periods of brain development. In contrast, in longer-term reproductive toxicity studies, test substances are usually administered via the diet or drinking water. This mode of administration results in an exposure profile for pre-weaning offspring that varies throughout lactation. During early lactation, the pups are solely exposed via the milk. Later, a gradually increased exposure is added via the medicated feed. It needs to be noted that the actual dosage received by the offspring can usually not be quantified, but can only be inferred. Thus, the evidence of DNT in offspring depends on the kinetic profile of each

individual compound. For example, if the compound excretion via milk is high, pups might receive higher dosages (per kg body weight) during adolescence than adult animals which makes it difficult to compare sensitivity for neurotoxic effects between adults and pups.

Different duration of exposure compared to a standard DNT study

The exposure of the progeny in the standard DNT study (OECD 426) usually ends at weaning. This is in contrast to the extended one-generation study, where exposure continues after weaning into adulthood. The extended duration of exposure in the latter study may add uncertainty to the interpretation of neurotoxicity detected in adulthood, as it will be more difficult to differentiate between developmental and acute effects. However, this may be overcome by cessation of exposure at weaning for the animals selected for later DNT evaluation.

2.3.4 Triggers for adding a DNT module

The evaluation of DNT needs to be undertaken within the context of available data, and the overall testing strategy. The technical guidance document on the information requirements for REACH (EU, in preparation) states "In exceptional cases when relevant triggers are met testing for developmental neurotoxicity effects should be considered. Relevant triggers could be if the substance has been shown to (1) cause structural abnormalities of the central nervous system, (2) cause clear signs of behavioural or functional adverse effects of nervous system involvement in adult studies e.g. repeated-dose toxicity studies or (3) have a mode of action that has been closely linked to neurotoxic or developmental neurotoxicity effects, e.g. cholinesterase inhibition or thyroid effects. However, in the case of (3) targeted testing on the specific mode of action in developing animals may provide sufficient information for regulatory purposes."

Thus, possible triggers comprise chemical structure or mode of action (if known) of the test compound, data from *in vitro* and non-mammalian tests and predominantly toxicity data from adults indicating potential neurotoxicity.

Structure-activity relationships

Known DNT effects of a compound similar in structure to the test compound may be a trigger for DNT testing.

Mode of action (if known)

Interferences of the test compound with synaptic signal transmission which may contribute to triggering the DNT module include the following:

- Interaction with transmitter synthesis, release and re-uptake;
- interaction with catabolising enzymes (e.g. cholinesterases);
- interaction with neurotransmitter receptors (e.g. cholinergic, adrenergic, dopaminergic).

Data from in vitro and non-mammalian tests

A number of *in vitro* and non-mammalian models exist for DNT-relevant endpoints. However, none of them have been formally validated or used specifically for DNT testing up to this date. There are caveats with regard to the predictive capacity and inherent limitations of established *in vitro* and non-mammalian models. For example, there may be false positives or false negatives because of interspecies differences and because *in vitro* systems do not reflect *in vivo* absorption, distribution, metabolism, and excretion of test compounds. Further, they usually cannot reflect interaction/interplay between target organ toxicity, endocrine and immune function, as well as the importance of blood-brain barrier and choroid plexus in a developing individual (Coecke *et al*, 2007). Therefore, positive data from these models should not be used as triggers on their own. Nevertheless, these models merit consideration in a weight of evidence approach to drafting DNT testing strategies, as long as one is aware of their limitations and caveats.

Neurotoxicity in adults

Findings in adults which may act as triggers for DNT testing are treatment-related direct effects on the peripheral and central nervous systems, based on evaluation of functional (behaviour, electrophysiology, neurochemistry) and morphological findings. It is not only persistent effects that are of concern for possible DNT but also transient findings, because transitory adverse effects on the nervous system from exposure during early childhood may be expressed at any time during the lifespan of the exposed individual. For example, early attachment failure may result in problems in later physical and psychological development (Kaufmann, 2000; Zimbardo, 1995). Lesions or changes should not be considered in isolation but always in the context of all other findings and data.

Functional effects

Clinical observations:
DNT testing may be triggered by selective treatment related clinical findings in adults that are evident from cage-side observations or during handling, are observed in the functional observational battery, are evident in motor activity testing or in further behavioural tests. These include the following:

- Changes in motor function (e.g. disturbances of gait, abnormal posture, muscle tone, or stereotypic movements);
- effects on level of arousal (e.g. hyperactivity, lethargy);
- effects of autonomic functions (e.g. salivation, lacrimation, urination, defecation);
- emotional effects (e.g. stereotypy, aggression, biting, licking, self-mutilation);
- impairment of reflexes;
- impairment of learning and memory;
- impairment of habituation, conditioning.

Electrophysiology and neurochemistry:
Treatment related findings during electrophysiology and neurochemistry studies, which may support triggering DNT testing, include changes in the following parameters:

- Electroencephalographic activity;
- evoked potentials (visual, auditory, somatosensory);
- conduction velocity of peripheral nerves;
- electromyographic activity;
- neuropathy target esterase;
- biochemical markers for neurons or glial cells;
- neurotransmitters or catabolising enzymes.

It should be noted, however, that data for these endpoints are rarely available for industrial chemicals.

Effects on morphology

Any kind of morphological treatment related changes in the peripheral or central nervous system as well as treatment related changes in brain weight will trigger DNT testing.

Effects on the thyroid

Chemicals that perturb thyroid homeostasis and result in hypothyroidism are known to be associated with neurological disorders and alterations in neurological development, both in animals and humans (Zoeller and Rovett, 2004). Treatment related effects on the rat thyroid, if they were caused by a mechanism relevant to humans (e.g. not secondary to liver toxicity), may include the following:

- Effects on thyrotropin and thyroxine;
- effects on thyroid weight, supported by histopathological findings;
- histopathological findings in the thyroid.

Such findings in adult animals may trigger DNT testing. However, disruption of thyroid homeostasis of mothers and offspring *in utero* is the initial, critical effect that may lead to adverse effects on the developing nervous system. Thus, it may be more appropriate to include a thyroid assay in the extended one-generation study, as is requested by the US EPA OPP for compounds which produces evidence of effects on thyroid function or structure (US EPA, 2005).

2.3.5 Waivers for a DNT module

For industrial chemicals, DNT testing is only justified in exceptional cases, i.e. for compounds that have been found to trigger criteria predictive of possible neurotoxic activity. The assessment whether a certain chemical meets these criteria should be made with a weight of evidence approach, taking into account the available toxicity information for each chemical or chemical class on a case by case basis.

An additional consideration is the likelihood that target organ/systemic toxicity, fertility or developmental effects may confound the interpretation of data produced in concurrent developmental neurotoxicity testing. In this case, the evaluation of DNT in a standard study according to OPPTS 870.6300 (US EPA, 1998) or OECD TG 426 would be indicated.

2.3.6 Examples of triggers for a DNT module

Isolated effects in adults or results from *in vitro* testing should generally not be considered as triggers for the DNT module but should be seen in combination with other findings. The following examples are given on how to assess the need for DNT testing.

Compound 4

In a repeated dose study, animals showed a decreased number of rearings (FOB) starting at the mid dose and a marginally reduced motor and locomotor activity at the high dose. There were no pathological changes in the nervous system. As the test compound had vasodilatory properties, clinically evident by reddening of ears starting at the mid dose, and as the high dose animals showed severely decreased body weight gain and emaciation, activity findings are considered to be related to the substance's hypotensive properties and/or systemic toxicity.

Assessment: There is no trigger for DNT testing.

Compound 5

In a repeated dose study, decreased locomotor activity occurred in females with high variability starting at the mid dose. Borderline (sciatic) nerve fibre degeneration was evident at the low dose and more clearly evident starting at the mid dose. The high dose animals revealed very mild axonal swelling in the nerve roots of the spinal cord. Although the mid dose already showed severe systemic toxicity including piloerection and emaciation, the findings in the nervous system cannot be conclusively linked to systemic toxicity as the low dose did not show clear systemic toxicity.

Assessment: Trigger for DNT testing, which could be accomplished in a subsequent extended one-generation study.

Compound 6

Animals showed reduced rearing activity starting at the mid dose in a repeated dose study. High dose animals showed salivation and a reduced number of rearings (FOB). Pathological examination revealed a slightly reduced brain weight, neuronal degeneration with reactive gliosis in several parts of the brain, slight sciatic nerve fiber degeneration with moderate thigh muscle atrophy at the high dose. Body weight was slightly reduced in high dose animals after the first treatment (by 2g) but overall weight gain was only slightly reduced (8.5 % in males, unaffected in females) and findings on other targets were only moderately expressed. Therefore, findings in the nervous system cannot be explained as secondary due to systemic toxicity.

Assessment: Trigger for DNT testing.

Compound 7

In a multi-generation reproductive toxicity study in rats with an insecticide, clinical signs of toxicity (abnormal gait) were observed in P females. In addition, clinical signs of toxicity were observed in pups (abnormal gait and tremors) during lactation as well as decreased body weights on the day of weaning (PND 21).

Assessment: If these clinical signs, indicative of potential neurotoxicity, had been observed in both the P females and the F_1 offspring of a one-generation reproduction study, these clinical signs would trigger the addition of a DNT module.

Compound 8
In a one-generation reproductive toxicity study via inhalation, convulsions were observed in the exposure chamber that resulted in death for several animals. In addition, liver and pituitary gland weights were increased compared to a control group in P females.

Assessment: Recommend a DNT module based on neurological signs observed for P animals during exposure (especially if the substance is known or suspected to cross the placental barrier).

2.4 The prenatal developmental toxicity (PDT) module

The triggers in the following discussion are also summarised in Appendix 2.

The PDT study (e.g. OECD TG 414) and two-generation breeding study (OECD TG 416) may be conducted as part of an overall triggered testing strategy. If both studies are triggered at the same level, e.g. production volume, and if the two-generation study is replaced by an extended one-generation design, there is potential to include the evaluation of PDT as an additional module within the breeding study rather than as a 'standalone' test. The evaluation of PDT within an extended one-generation reproductive toxicity study design has disadvantages in comparison to the conduct of a traditional 'standalone' PDT study, in addition to the abovementioned advantages (section 2.1.5).

It must be recognised that, in terms of risk assessment, PDT data are a core requirement for substances that warrant reproductive toxicity evaluation–whether this is due to production volume, application, exposure pattern or other considerations. This requirement applies broadly across many regulatory instruments and chemical sectors (e.g. Cooper *et al*, 2006; EU, 2006). Consequently, triggers and waivers for a PDT should be evaluated within the scope that such a module may be a default requirement.

2.4.1 Description of the module

In this module, F_1 males and females would be selected for future breeding, in a similar manner to how breeding pairs are selected for the second mating phase to raise an F_2 generation in the current OECD TG 416. One male and one female animal from each litter will be selected for future mating, and the test material will continue to be administered to the animals during the

pre-mating and mating phases. Mating will be conducted when the animals reach approximately 10 weeks of age, and planned such as to avoid sibling pairings. The dams will be sacrificed on gestation day (GD) 20, will undergo caesarean section, and the offspring will be evaluated for developmental findings as per normal procedure (OECD TG 414).

An alternative approach for this module would be to mate the P generation animals after successful weaning and selection of the F_1 generation.

2.4.2 Specific disadvantages of evaluating PDT within a modular approach

May disfavour the identification of effects related to high blood concentrations

As discussed previously, PDT studies are commonly conducted with the test material administered by bolus gavage. This approach is favoured for convenience and to establish a 'worst-case' exposure scenario for purposes of hazard identification. In contrast, although the oral route of exposure is commonly used in longer-term reproductive toxicity studies, and gavage dosing is not precluded (OECD, 2001b), test substances are usually administered via the diet or drinking water. This mode of administration results in plasma concentrations that follow a less extreme pattern of peaks and troughs than occurring with bolus dosing, which may disfavour effects that occur only at high peak plasma concentrations (section 2.1.5).

However, as discussed earlier (section 2.1.5), PDT findings observed under unrealistic physiological conditions are largely of questionable relevance for human exposures to industrial chemicals (Conolly *et al*, 1999). Furthermore, depending on the coincidence of troughs of systemic exposure with critical windows of development, bolus dosing may actually disfavour the identification of PDT effects.

Limitation of dose levels by sub-chronic toxicity

Dose levels set for sub-chronic studies may be lower than those for sub-acute studies (such as OECD TG 414) on the same substance, if exposure tolerance is reduced by the induction of effects that occur over a longer period of exposure. This may disfavour the identification of effects that occur only at higher doses. Again, this may be of limited relevance in terms of human exposure to industrial chemicals and risk assessment.

Impact of fertility and early post-implantation loss

Agents affecting fertility (e.g. mating behaviour, oocyte release) or inducing pre-implantation loss may not be as rigorously evaluated in the post-implantation phase when exposure starts before implantation, compared to a study in which exposure is initiated after implantation has taken place. This potential confounder may be signalled by poor reproductive performance in the P generation, such as low litter size or *post-mortem* observation, and would either serve as a waiver for a PDT module in favour of a separate study to OECD TG 414, or indicate a modification of the study design so that exposure of selected F_1 animals for PDT evaluation ceased from the time of selection at weaning through to implantation (GD6), at which time exposure would recommence.

Further complication of the assessment of prenatal toxicity data from two species

Where guidelines or legislation call for PDT to be evaluated in two species, the two species of choice are the rat and rabbit. The choice is usually made on the basis of available historical control data, ease of handling, duration of gestation and regulatory acceptance. Studies are usually conducted by oral gavage dosing in both species.

It is often the case that findings in one species are not replicated in the other. In such cases, it is evident that the discrepancy lies in a fundamental interspecies difference, and further investigation may be made to understand which species is most relevant as a model for human risk assessment, or simply the data from the most sensitive species may be used. However, should the PDT evaluation in the rat be conducted as a module in the extended one-generation study design, differences in findings from the second species may no longer be ascribed to species sensitivity.

To counter this, an alternative mode of administration to bolus dosing might be considered for the second species. In this case, special consideration may need to be given to the formulation of feed if the test material is given in the diet.

2.4.3 Triggers for adding a PDT module

The evaluation of PDT needs to be undertaken within the context of available data and the overall testing strategy. In cases where PDT data are not required prior to conducting a breeding study, or where the available data are of limited value, such as derived from a study assigned a reliability score of 3 or 4 (Klimisch *et al*, 1997), the lack of robust PDT data in the rat will not preclude the acquisition of that data through an appropriate modification of the breeding study.

This is not to say that the core design of the breeding study should be modified, but that careful consideration should be given to include a PDT module.

Structure-activity relationships

Known PDT of a compound similar in structure to the test compound may be a trigger for PDT testing.

Postnatal evaluation of the F_1 generation

The inclusion of a PDT module may be triggered by observations made during the conduct of the one-generation reproduction toxicity study itself. These internal triggers include:

- Low pup birth weight;
- reduced litter size;
- malformed pups;
- early postnatal pup death.

It should be noted that postnatal examination will be limited to the identification of external malformations, unless pups found dead or sacrificed moribund are subjected to internal examination. Thus, routine assessment of offspring post-partum is not a replacement for a comprehensive evaluation of structural development. In addition, rare malformations are likely to be overlooked, as the affected offspring may be cannibalised by the dam before observation. The absence of these animals may not be noticed as a reduction in litter size, due to relatively large intra- and inter-group variations of this endpoint that can mask small but biologically significant losses.

Data from other studies

Other data that might act as external triggers for such a module are:

- The substance being classified, or meeting the criteria for classification, as a mutagen category 2 (according to GHS[4]) or 3 (in EU)[5];
- positive mutagenicity data in mammalian cells *in vitro*, unless contradicted by negative data *in vivo*;
- positive results from developmental toxicity tests conducted *in vitro*.

[4] Globally Harmonised System of Classification and Labelling of Chemicals (UN, 2007).
[5] Classification and Labelling Requirements for Dangerous Substances and Preparations (EU Commission, 1967).

2.4.4 Waivers for a PDT module

The presence of an adequate study conducted in the rat according to OECD TG 414 would obviously negate additional testing in the breeding study, as would other conditions that eliminate the need for such a study in the rat. Furthermore, if positive findings in a study according to OECD TG 414 in the rabbit are enough for classification for developmental toxicity in category 1B (according to GHS)[4] or 2 (in the EU)[5] and if the data are sufficient for risk assessment, a rat PDT module may be waived. Otherwise, confirmation that PDT in rat does not influence the risk assessment or classification (e.g. lower NOAEL, higher degree of classification) might be needed, but evaluation within the breeding study may suffice.

An additional consideration is the likelihood that fertility effects may confound the interpretation of a PDT module, for example, if treatment is expected to increase the incidence of pre-implantation loss (section 2.4.2; OECD, 2001a). In this case, the evaluation of developmental toxicity in a standard study according to OECD TG 414 may be indicated, although the inclusion of a module may suffice if exposure of selected F_1 animals was ceased from weaning until after implantation (GD6).

2.4.5 Examples of triggers for a PDT module

Compound 9

A 90-day study was conducted in rats, with the addition of one-generation reproductive evaluations. There was an increase in testicular spermatid numbers in P generation males. In addition, there were effects on implantation efficiency, number of pups born, and number of pups born alive. Pre-implantation losses were not accounted for in this study, but the implantation efficiency was significantly reduced at the two highest doses. As a consequence, there was a significant reduction in the number of pups born, born alive and alive at 4 days post partum in the highest dose group. Reductions of the same magnitude were observed at the mid dose for the number of pups born and born alive (not significant). The number of pups alive at day 4 in the mid dose however was significantly reduced. Pup bodyweight was also significantly reduced at day 4, 7, 14, 21 of lactation.

Assessment: PDT module should be conducted as a result of effects observed in offspring survival in the aforementioned reproduction study.

Compound 10
In a one-generation reproduction toxicity study, there were substance-related increases in gestation length indicative of dystocia, lower fertility, and lower maternal and offspring viability during the lactation period in all treatment groups. At the highest dose administered, additional evidence of test substance-related toxicity included effects on teeth (P males), reductions in body weight and food consumption parameters (P males and females), and reductions in offspring body weights (F_1 males and females).

Assessment: PDT would be triggered as a result of lower offspring viability and body weight reduction in offspring.

Compound 11
In a one-generation reproductive toxicity study in rats, there were effects on number of pups born and number of pups alive on day 4 of lactation. In addition, pup weights were reduced during lactation. Increased foetal skeletal alterations were observed in a PDT study in rats.

Assessment: A one-generation study and PDT study were conducted using the same compound. However, if the PDT study did not exist and if one only saw the effects on pups at birth and during lactation, findings such as these would be a reasonable trigger to add a PDT module to a one-generation study.

2.5 The developmental immunotoxicity (DIT) module

The triggers in the following discussion are also summarised in Appendix 2.

DIT is currently not a standard requirement for the safety evaluation of chemicals, although possible impact upon the developing immune system may be considered in the light of available data. While attempts have been made to define a schedule for assessing DIT (e.g. Cooper *et al*, 2006; Holsapple *et al*, 2005; Luster *et al*, 2003), there are as yet no agreed international guidelines for the design and conduct of a DIT study. This is in contrast to PDT and DNT (OECD 2001a, 2007).

Given the lack of an OECD test guideline for DIT at this time, no attempt will be made herein to describe the design of a DIT module in terms of endpoints to be evaluated and testing schedule, as this is outside the remit of this task force. However, the conduct of a DIT module should only be considered in broad terms until such time as consensus on a validated study design and conduct is reached.

2.5.1 Advantages of evaluating DIT within a modular approach

There is no standard requirement for the conduct of a DIT evaluation under REACH, and consequently the reduction in animal usage will not be as evident in the testing strategies performed in compliance with this regulation. However, in cases where a DIT study is warranted, conduct within the extended one-generation study will reduce the overall number of animals used for a given substance.

The developing immune system is largely seen as more sensitive than that in adults (Luster *et al*, 2003). Therefore, negative findings in a DIT study should obviate the need for further specific testing of immune function in adults.

2.5.2 Triggers for adding a DIT module

Triggers for the evaluation of DIT were identified at a workshop held under the auspices of ILSI HESI (Holsapple *et al*, 2005). For a modular one-generation reproduction study triggers will need to be validated for sensitivity and specificity in context with the design of the module itself. This implies that the elements of the module must be defined before triggers can be identified.

Structure-activity relationships

Known immunotoxicity or DIT of a compound similar in structure to the test compound may be a trigger for DIT testing.

Immunotoxicity in adults

Observations in adults that may act as triggers for DIT testing are treatment-related direct effects on immuno-competent organs, like the spleen, thymus, local lymph nodes, liver and Peyer's patches, based on the evaluation of the following criteria:

- Morphological, histopathological and immunohistological findings;
- changes in immunoglobulins (in particular IgM and IgG);
- stimulation or suppression of blood cell (e.g. polymorphonuclear granulocytes, monocytes, B-lymphocytes, T-helper cells, T-cytotoxic cells, natural killer cells) and bone marrow counts (e.g. large unstained cells);
- positive findings in an immunotoxicity assay;
- weight changes (if observed in combination with one or more of the above findings).

2.5.3 Waivers for a DIT module

DIT testing is only justified in exceptional cases, i.e. for compounds found to trigger criteria viewed as predictive of possible immunotoxic activity. The assessment as to whether a certain chemical meets these criteria should be made with a weight of evidence approach, taking into account the available toxicity information for each chemical or chemical class on a case-by-case basis.

3. VALIDATION CRITERIA TO BE APPLIED TO TRIGGERS AND WAIVERS

3.1 Introduction and rationale

The regulatory acceptance of a new or revised test method by the OECD, such as the extended one-generation reproduction toxicity study, requires an adequate validation in accordance with internationally recognised principles and criteria as laid down in the OECD Guidance document (GD) 34 (OECD, 2005; Appendix 3). The proposed study design of the extended one-generation study combines various modules which are mostly already included in other regulatory accepted standard studies. However, a retrospective assessment of the validation status (Art. 36, 37 of OECD GD 34) of the various endpoints identified as potential triggers should be performed, in order to select only the parameters that contribute to regulatory decisions.

For example, retrospective analyses have been performed to evaluate the benefit of including an assessment of the second generation (Janer *et al*, 2007; Makris, 2004). Other evaluations, such as developmental immunotoxicity, have yet to be addressed in depth. Also, the extent to which experimental data can be pooled for the purpose of a single analysis, if obtained with different variants of a test method, still needs to be determined (Art. 38).

This chapter reviews the validation requirements for the triggers for the proposed modules, not the extended one-generation study as a whole. The validation status of the triggers will be assessed as described in Art. 40 of OECD GD 34. It is expected that a complete dossier addressing the validation criteria of all components of the extended one-generation study will be submitted to the OECD. The necessary supporting documentation for a test submission to the OECD is defined in chapter VII Articles 143-166 of the OECD GD 34. The complex design of the extended one-generation study means that a pragmatic approach is necessary to apply the required validation principles (Art. 137).

3.2 Test definition

Within an extended one-generation study, additional modules are triggered or waived on the basis of external or internal trigger criteria. The mechanistic basis that relates to the triggering endpoints and the modules that they trigger should be described in the context of the broader scientific understanding of each assessed endpoint. Hereby, the triggers and waivers are linked to well defined tests or test batteries. Emphasis should be laid on the test system compared to the species of interest (Art. 146).

Currently the data interpretation procedure is based on expert judgment since the complexity of the assessed endpoints and the potentially high intra- and inter-laboratory variability makes a

quantitative assessment difficult to achieve. However, a series of key aspects have been identified for each trigger allowing a harmonisation of the data interpretation procedure (Art. 26, 148):

- Consistency of findings within the study (i.e. is a 'picture' or a pattern of effects developing, or is the alert a single finding?);
- biological plausibility (i.e. are the findings consistent with a particular mode of action?);
- statistical significance (although non-statistically-significant changes may be biologically significant and vice versa);
- magnitude of the effect;
- whether the effect occurs at doses that cause general toxicity;
- supporting data (e.g. structure activity relationships, *in vitro* or *in vivo* data).

Based on this information, the development of scoring according to the 'level of concern' is desirable for supporting an objective judgment of data (OECD, 2005). The level of concern will depend on the type and incidence of response to treatment, as well as the overall conditions under which the response is elicited. Selected supporting studies of the various modules demonstrate how to proceed with the decision making process for the various extensions (Art.150).

3.3 Intra- and inter-laboratory variability

Due to the complicated study design, the number of various toxicological mechanisms involved, the amount of laboratory animals, the costs, the variability of studies and animal strains within the laboratories and between laboratories, as well as newly introduced endpoints (e.g. DIT), a sufficient number studies is not yet available in order to perform a retrospective analysis of the individual modules. A rigorous application of the validation principles in a prospective validation cannot be required for ethical reasons, specifically considering the number of animals that would be needed, Art. 149.

3.4 Relevance of triggers and waivers

Art. 33 of OECD GD 34 highlights that if a rigorous assessment of the predictive capacity may not be possible, the relevance of the tests in question should be assessed using all available information and a periodical review of the validation status is recommended.

Currently, a sufficient number of studies to analyse the relevance of all of the proposed triggers and waivers are not available. More information may be expected from the feasibility studies assessing prototype substances with known toxicological modes of action that are relevant for the

proposed modules. However, a retrospective analysis of data from tests that are proposed as modules, as well as from other reproduction toxicity studies, could provide a better understanding of the accuracy of the internal triggers and waivers (Art. 158).

Data generated in good quality, biologically relevant, validated and accepted studies, e.g. those designed in line with OECD technical guidelines and conducted according to the principles of good laboratory practice, should be used preferentially to define a trigger. However, data from all available studies should be used in the weight of evidence evaluation used to trigger or waive a particular module. In case of a conflict between study results, data from these good quality studies are likely to overrule data from studies of questionable quality or significance (Art. 161).

3.5 Additional considerations

The validation of triggers and waivers that form the connections between the core study and the various extensions is more complex than the validation of a standalone test method and involves, besides the application of general validation principles, additional considerations such as:

- An analysis of the sensitivity of the trigger towards the envisaged module (e.g. higher sensitivity of histopathology of the testis compared to effects on male fertility [Ulbrich and Palmer, 1995]);
- a specificity analysis of the trigger (e.g. *in vitro* tests with a low specificity—a high rate of false positives—should be cautiously considered as triggers, due to the unnecessary generation of confirmatory testing);
- the assumption that all *in vitro* tests (especially non-validated ones) are mechanistically relevant needs to be scientifically assessed case by case, since they may be based on irrelevant mechanisms. The same holds true for non-validated and non-regulatory *in vivo* studies. Sufficient information on the sensitivity and specificity of the tests should be provided.

It is envisaged that these questions should be addressed by a workshop of experts. The purpose of the workshop will be to define the applicability of external and internal triggers, both in terms of sensitivity and specificity, and future research requirements to substantiate and validate them where this has not already been achieved.

4. CONCLUSIONS

The extended one-generation reproduction toxicity study can contribute significantly to animal welfare by substantially reducing the number of animals needed for chemical risk assessment without compromising information requirements or data quality. The study design may be refined by the use of modules to evaluate specific aspects of development. The complexity of the study can be minimised by adding modules to a core design through the evaluation of external or internal triggers, i.e. endpoints observed in other studies or during the conduct of the extended one-generation study itself.

Endpoints have been identified that may be used within a structured, modular design—based on an extended one-generation reproduction toxicity study—to trigger or waive the inclusion of modules for a second (F_2) generation, and the assessment of DNT, PDT and DIT. Many of these endpoints have yet to be validated in terms of their sensitivity and specificity towards the modules that they may trigger. Nevertheless, some examples are given of triggers that could have been applied within the design or conduct of an extended one-generation study.

In order to overcome these limitations, a consensus among stakeholders needs to establish the data requirements for linking modules to endpoints that trigger them. A workshop will be organised in conjunction with ECVAM to address the validation of endpoints as triggers and waivers, and potential research needs to address open questions.

ABBREVIATIONS

ACSA	Agricultural Chemical Safety Assessment
C_{max}	Maximum plasma concentration
DIT	Developmental immunotoxicity
DNT	Developmental neurotoxicity
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EPAA	European Partnership on Alternative Approaches to Animal Testing
EU	European Union
F_1	First generation of offspring
F_2	Second generation of offspring
FOB	Functional observation battery
GD	Gestation day
GD	Guidance document
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
GLP	Good laboratory practice
HESI	Health and Environmental Sciences Institute
ICH	International Conference on Harmonisation
ILSI	International Life Sciences Institute
LOAEL	Lowest observed adverse effect level
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
OPP	(US EPA) Office of Pesticide Programs
OPPTS	(US EPA) Office of Prevention, Pesticides and Toxic Substances
P	Parental (generation)
PDT	Prenatal developmental toxicity
PND	Postnatal day
REACH	Registration, evaluation, authorisation and restriction of chemicals
TG	Test guideline
US EPA	United States Environmental Protection Agency

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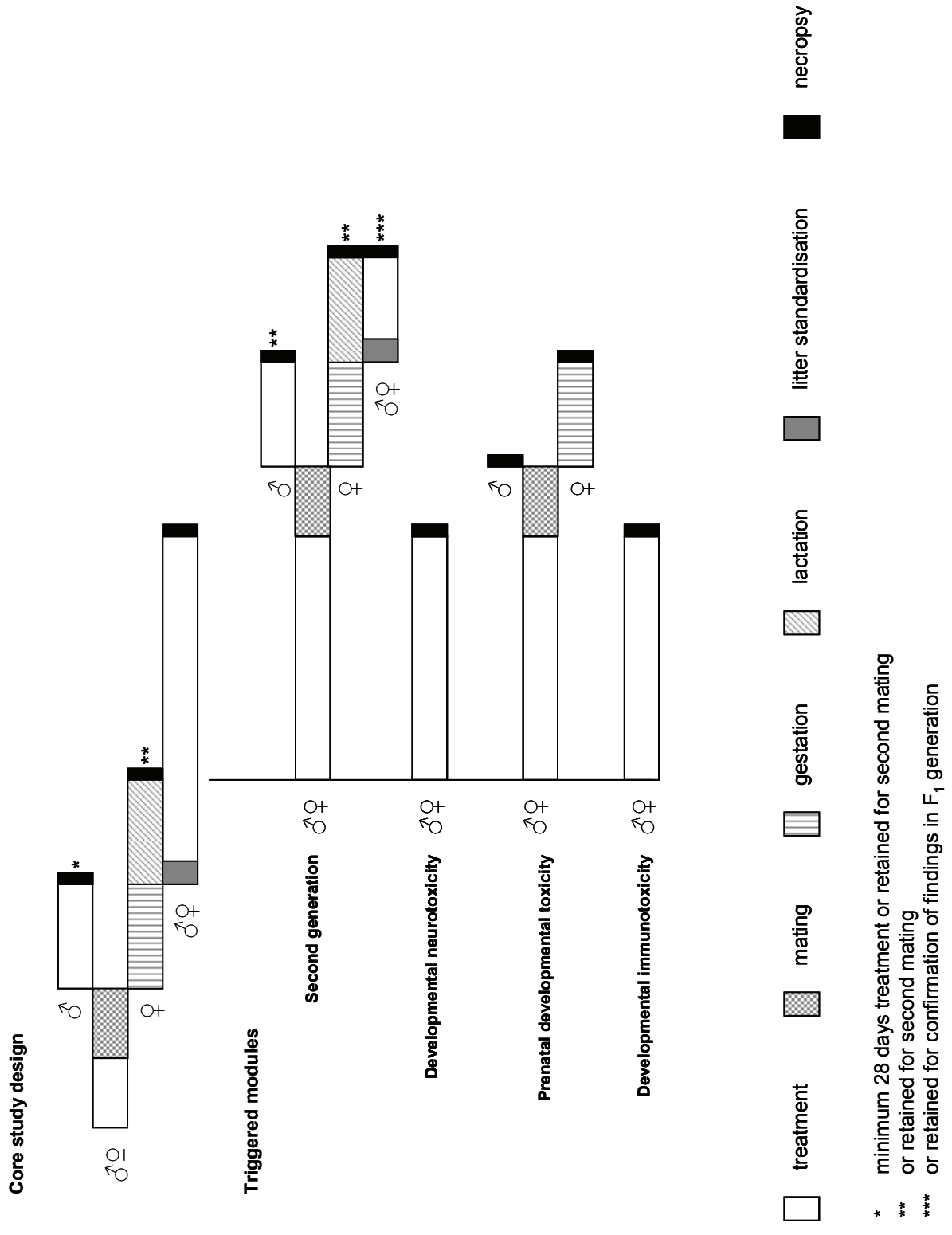
APPENDIX 1

Extended one-generation study design: core design and triggered modules (schematic, not to time scale)



APPENDIX 2

Table 1: Non-exhaustive list of triggers (as mentioned in sections 2.2-2.5)

Second-generation module	
The only trigger is equivocal findings in first generation breeding	
Developmental neurotoxicity (DNT) module	
Structure-activity relationships	
Mode of action (if known)	- Interaction with transmitter synthesis, release and re-uptake - Interaction with catabolising enzymes (e.g. cholinesterases) - Interaction with neurotransmitter receptors (e.g. cholinergic, adrenergic, dopaminergic)
Data from in vitro *and non-mammalian tests*	
Neurotoxicity in adults:	
Functional effects	
Clinical observations	- Changes in motor function (e.g. disturbances of gait, abnormal posture, muscle tone, or stereotypic movements) - Effects on level of arousal (e.g. hyperactivity, lethargy - Effects of automatic functions (e.g. salivation, lacrimation, urination, defecation - Emotional effects (e.g. stereotypy, aggression, biting, licking, self-mutilation) - Impairment of reflexes - Impairment of learning and memory - Impairment of habituation, conditioning
Electrophysiology and neurochemistry	- Electroencephalographic activity - Evoked potentials (visual, auditory, somatosensory) - Conduction velocity of peripheral nerves - Electromyographic activity - Neuropathy target esterase - Biochemical markers for neurons or glial cells - Neurotransmitters or catabolising enzymes
Effects on morphology	
Effects on the thyroid	- Effects on thyrotropin and thyroxine - Effects on thyroid weight, supported by hispathological findings - Hispatological findings in the thyroid

Table 1: Non-exhaustive list of triggers (as mentioned in sections 2.2-2.5) *(cont'd)*

Prenatal developmental toxicity (PDT) module	
Structure-activity relationships	
Postnatal evaluation of the F_1 generation	- Low pup birth weight - Reduced litter size - Malformed pups - Early postnatal pup death
Data from other studies	- Substance being classified, or meeting criteria for classification, as mutagen category 2 (GHS) or 3 (EU) - Positive mutagenicity data in mammalian cells *in vitro*, unless contradicted by negative data *in vivo* - Positive results from developmental toxicity tests conducted *in vitro*
Developmental immunotoxicity (DIT) module	
Structure-activity relationships	
Immunotoxicity in adults	*Treatment-related direct effects on immuno-competent organs, like the spleen, thymus, local lymph nodes, liver and Peyer's patches, based on the evaluation of:* - Morphological, hispathological and immunohistological findings - Changes in immunoglobulins (in particular IgM and IgG) - Stimulation or suppression of blood cell (e.g. polymorphonuclear granulocytes, monocytes, B-lymphocytes, T-helper cells, T-cytotoxic cells, natural killer cells) and bone marrow counts (e.g. large unstained cells) - Positive findings in an immunotoxicity assay - Weight changes (if observed in combination with one or more of the above findings)

APPENDIX 3

Principles and Criteria for Test Method Validation (OECD, 2005)

a) The rationale for the test method should be available.
This should include a clear statement of the scientific basis, regulatory purpose and need for the test.

b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.
This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.

c) A detailed protocol for the test method should be available.
The protocol should be sufficiently detailed and should include e.g. a description of the materials needed, such as specific cell types or construct or animal species that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analysed, decision criteria for evaluation of data and what are the criteria for acceptable test performance.

d) The intra- and inter-laboratory reproducibility of the test method should be demonstrated.
Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed.

e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.
A sufficient number of the reference chemicals should have been tested under code to exclude bias (see paragraphs on "Coding and Distribution of Test Samples").

f) The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.
In the case of a substitute test method adequate data should be available to permit a reliable analysis of the performance and compatibility of the proposed substitute test method with that of the test it is designed to replace.

g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.

Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated.

h) All data supporting the assessment of the validity of the test method should be available for expert review.

The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organised and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol.

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