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Advice on dose-level selection for the conduct of sub-acute and sub-chronic assays under REACH

Background

The REACH legal text now includes provisions about dose selection in the introductory part of each annex from VII to X[1]:

'Where a test method offers flexibility in the study design, for example in relation to the choice of dose levels, the chosen study design shall ensure that the data generated are adequate for hazard identification and risk assessment. ***To this end, testing shall be performed at appropriately high dose levels.*** *If dose (concentration) selection is limited by the physicochemical properties or biological effects of the test substance, justification shall be provided.'*

Examples of such physicochemical properties are explosivity and flammability, but also viscosity, solubility, vapour pressure and particle size may be limiting factors for certain routes of administration. Examples for limiting biological effects are lethality, (respiratory tract) irritation/corrosivity or any other manifestation of toxicity which leads to death or severe suffering. The lack of biological effects of a substance would normally lead to a limit test.

The sub-chronic toxicity study is a standard information requirement (SIR) at Annex IX, 8.6.2 (Sub-chronic toxicity study (90-day)) and the sub-acute toxicity study is a SIR at Annex VIII, 8.6.1 (Short-term repeated dose toxicity study (28 days)); the OECD 422 study may also be used to fulfil the SIR of Annex VIII, 8.6.1.

The aim of such studies[2,3] is to collect suitable information for hazard identification (i.e. classification and labelling [specifically for specific target organ toxicity – repeated exposure (STOT RE) and for reproductive toxicity], for characterising the major toxic effects and target organs of the substance and, hence, for detecting triggers for further studies), for risk assessment (i.e. for assessing a no-observed-adverse-effect level (NOAEL) and the dose-response relationship) and whether the substance meets the criteria for a substance of very high concern regarding endocrine disruption according to Article 57(f) of REACH[4].

[1] COMMISSION REGULATION (EU) 2021/979 of 17 June 2021 amending Annexes VII to XI to Regulation (EC) No 1907/2006.

[2] Annex I Section 1.0.1. to REACH: "the objectives of the human health hazard assessment shall be to determine the classification of a substance in accordance with Regulation (EC) No 1272/2008; and to derive levels of exposure to the substance above which humans should not be exposed".

[3] From, for example, OECD TG 408: paragraph 3. "The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth into adulthood of the test animals. The study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation of test chemical, and can provide an estimate of a no-observed-adverse-effect level (NOAEL) of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. Alternatively, this study yields dose related response data that may be used to estimate point of departure for hazard assessment using appropriate modelling methods (e.g., benchmark dose analysis)." Paragraph 4. "This study should allow for the identification of chemicals with the potential to cause neurotoxic, endocrine, immunological or reproductive organ effects, which may warrant further in-depth investigation."

[4] The OECD TG 422 study also provides information on reproductive toxicity; please see ECHA's "Advice on dose-level selection for reproductive toxicity studies (OECD TGs 414, 421/422 and 443) under REACH"

The wording of OECD TG 408 (oral 90-day) on dose-setting is similar to other OECD TGs involving repeated dosing:

"At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see paragraph 16). Dose levels may be based on the results of repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test compound or related materials. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. Two to fourfold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of about 6 - 10) between dosages."

There are similar considerations regarding dose level setting for the 28-, 90-day and reproductive screening studies, and for oral, dermal and inhalation routes of exposure in these studies. Consequently, the advice in this note applies to repeated-dose studies with 28–90-day durations and all three routes of exposure. Severe suffering in animal studies should be avoided; identification of severe suffering is described in *OECD guidance document 19*[5], and reference should be made to national legislation on animal experiments and associated guidance on severe suffering.

How to comply with the provisions about dose selection in the annex preamble

Justification

The test guidelines indicate that the dose-setting should consider any existing toxicological and toxicokinetic data available for the test compound, and the preamble to the annexes states that justification has to be provided if the dose (concentration) selection is limited by the physicochemical properties or biological effects of the test substance.

Accordingly, a justification must be provided for the setting of dose levels, and the information on any preliminary (existing) studies (relied upon for dose-setting) must provide a detailed summary providing sufficient information to make an independent assessment of the study and its contribution to the setting of dose levels. The justification for dose level setting should include specific rationale for setting high dose, middle dose and low dose levels in view of the requirements of the test guideline, together with justification for dose-spacing. Deviations from this approach, for reasons such as a steep dose-response to mortality, have to be justified.

Range-finding studies provide the basis for choice of dose levels

A specific dose of a substance is chosen *with the aim* to induce a given level of toxicity[6], with the level of toxicity being different for high, medium and low dose groups. There must be an adequate basis for establishing the aim to induce a given level of toxicity in a repeated dose toxicity study. Acute toxicity studies are not an adequate basis to justify dose-setting for a 28-day (or longer) study. Studies with seven days exposure to a test substance and basic investigations are a minimum basis for establishing the aim to induce

[5] GUIDANCE DOCUMENT ON THE RECOGNITION, ASSESSMENT, AND USE OF CLINICAL SIGNS AS HUMANE ENDPOINTS FOR EXPERIMENTAL ANIMALS USED IN SAFETY EVALUATION. ENV/JM/MONO(2000)7

[6] Toxicity is defined as an adverse effect, i.e. a change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.

a given level of toxicity in a 28-day study, and 14-day studies are the minimum basis for establishing the aim to induce a given level of toxicity in a 90-day study. However, longer periods of dosing give a better basis for setting an informative dose (e.g. 14 days for a 28-day study; a 28-day study for a 90-day study). A study is also compliant if the given level of toxicity is achieved in the high, medium and low dose groups (or the limit dose is reached/there is a limit in the dose for physicochemical reasons) of the main study, even if there is not an adequate basis for establishing the aim to induce a given level of toxicity from dose-range finding studies.

High dose group

The highest dose level is chosen with the aim to induce toxicity but not death or severe suffering[7], unless limited by the physical-chemical nature or biological effects of the test substance. Accordingly, the high dose level for a study should correspond to a dose level used for the dose range-finding study where the toxicity induced by the substance is known.

Where it is not possible to set appropriate dose levels based on the dose range-finding study due to e.g. unacceptably severe suffering/lethality below the limit dose (1 000 mg/kg bw/day) and/or unacceptably large dose-spacing in the range-finding study, consideration should be given to conducting a new dose range-finding study.

Where there are deviations from this approach, then these should be justified. For example, where a dose range-finding study shows borderline moderate to severe toxicity at high doses, and toxicokinetic information demonstrates continuing accumulation of the substance over the course of the range-finding study, it may be appropriate to moderately reduce the high dose from the dose used in the range-finding study.

For the high dose level, it must be demonstrated that the highest possible dose level without severe suffering or death was either:

1. achieved based on the actual results of the main study; or

2. justified based on the results from the range-finding study.

Alternatively, the limit dose concept has to be used, or it has to be demonstrated that the physicochemical properties of the test substance limit the dose achieved. If the high dose selection is not limited by severe suffering or death[8], or by the physicochemical properties of the test substance, the highest dose level has to be the limit dose/concentration (e.g. 1 000 mg/kg bw/day for oral studies, 5 mg/L for aerosols [for particles aerosol, testing >2 mg/L should only be attempted if a respirable particle size can be maintained/achieved], 20 mg/L for vapours, and 20 000 ppm for gases; see OECD TGs 408 and 413). These limit doses/concentrations can be exceeded if there is direct relevance for protecting human health, e.g. due to higher human exposure/sensitivity, and provided it does not result in severe suffering or deaths of the test animals.

Deviations from this approach (the highest dose level being the highest possible dose level without severe suffering or deaths) require justification; for example, an extremely steep dose-response curve with death as the response and no obvious responses at doses twofold below. Where there are multiple dose-levels in the dose-range finding study which do not have lethality or severe suffering, the use of the lowest possible dose level in the

[7] See the considerations when the substance may fulfil the criteria for classification as 'Specific target organ toxicity – repeated exposure' (STOT RE)
[8] See the considerations when the substance may fulfil the criteria for classification as 'Specific target organ toxicity – repeated exposure' (STOT RE)

range-finding study with minimal adversity as the high dose in the main study is not acceptable (unless these doses exceed the limit dose).

Middle dose group

The setting of a middle dose should aim to induce clear but mild toxicity as a means of demonstrating any dosage-related response (unless limited by the physical-chemical nature or biological effects of the test substance). The middle dose level may be chosen by reference to a dose level in a range-finding study which induces clear but mild toxicity.

An alternative is that the top dose of a study is chosen by reference to a dose level in a range-finding study which induces the highest possible toxicity but not death or severe suffering, and the middle dose level is set two- or threefold below this level, depending on the expected dose level for clear but mild toxicity.

Low dose group

The low dose level is chosen to demonstrate a no-observed-adverse-effect level (NOAEL); the NOAEL is used as the point of departure for risk assessment. The importance of the NOAEL is reflected in the column 2 provisions for repeated dose toxicity, e.g. Annex VIII 8.6.1 provides that further studies may be required if there is a 'failure to identify a NOAEL in the 28- or the 90-day study, unless the reason for the failure to identify a NOAEL is absence of adverse toxic effects'. It is important, therefore, to justify the choice of dose for the low dose group in relation to results from range-finding studies, while taking cognisance of the decreased sensitivity and/or exposure time of the range-finding studies.

The identification of mild toxic effects in a low dose group does not by itself mean that the information requirement cannot be fulfilled by the data from the study, since it might be possible to compensate for the lack of NOAEL by including additional assessment factors in the risk assessment. However, it is necessary to consider the severity of the toxic effects seen in the low dose group, the shape of the dose response curve and the concerns in risk assessment to conclude whether the data are adequate for fulfilling the information requirement.

Specific considerations for 'Specific target organ toxicity – repeated exposure' (STOT RE)

Where preliminary data indicate that criteria for classification as 'Specific target organ toxicity – repeated exposure' (STOT RE)[9] may be met, the study design must ensure that the potential of the substance to cause significant and/or severe toxic effects below the dose/concentration guidance values[10] for STOT-RE is robustly assessed. The regulatory purpose of achieving classification mandates that, under the dose/concentration guidance values, it is necessary to induce significant and/or severe toxicity[11]. The guidance values for STOT RE should be considered and the selection of doses relative to the guidance values should facilitate the comparison of the results to the STOT RE criteria and should be specifically justified.

Where preliminary data indicate that criteria for classification as STOT RE may be met, dose-spacing should only exceptionally, and with specific justification, be more than threefold intervals. Where appropriate, the characterisation of a NOAEL may require the use of additional dose groups.

[9] See Regulation (EC) No. 1272/2008, Annex I, section 3.9 Specific target organ toxicity — repeated exposure
[10] See Regulation (EC) No. 1272/2008, Annex I, section 3.9 Table 3.9.2 and 3.9.3
[11] Including but not limited to morbidity/death and other effects specified in CLP 3.9.2.7.3. Examples of severity of effects that do not justify classification are given in CLP 3.9.2.8.1.

Dose-spacing

Dose-spacing must be justified in light of the aim to characterise the dose-related response. Two- to fourfold intervals are generally optimal. Dose-spacing of more than fivefold in a sub-acute study or sub-chronic study will require exceptional substance-specific justification, such as a shallow dose-response curve, and the use of additional dose groups should be preferred.

Toxicokinetics as a basis for dose setting

Setting the dose level by toxicokinetic considerations only is not allowed under REACH because toxicokinetic considerations do not ensure that the data generated are adequate for hazard identification, i.e. toxicokinetic data per se cannot provide a basis for an aim to induce a given level of toxicity.

Example cases for dose selection

The following examples are illustrative to reflect dose level selection rationales under REACH for different scenarios.

Example 1: Dose range-finding study shows minimal toxicity

- A substance shows no mortality in acute oral toxicity at 2 g/kg. A dose range-finding study is conducted in rats up to a top dose of 300 mg/kg/day for 7 days. The study provides relevant information on repeated dose toxicity, including selected organ weights and histopathology.

- The results of the study show mild liver enlargement (ca. 10 %) at 300 mg/kg/day but no other effects.

- Conclusion. Testing is mandated up to the level of toxicity or the limit dose of 1 000 mg/kg bw/day; the toxicity at 300 mg/kg/day is not the highest possible toxicity without death or severe suffering, and 1 000 mg/kg/day has not been reached. Hence a new dose-range finding study with higher doses should be conducted, to establish selection of the highest possible dose level without death or severe suffering.

Example 2: Main study doses are not set appropriately based on range-finding study

- A substance is tested in an oral 28-day study at 100, 300 and 1 000 mg/kg/day. This is a valid range-finder for a 90-day study.

- The results of the 28-day study show mild renal basophilia and liver enlargement (ca. 15 %) at 1 000 mg/kg/day and statistical significance but minimal liver enlargement at 300 mg/kg/day. There are no other effects.

- A 90-day study is conducted with at top dose of 200 mg/kg/day, and the top dose is justified by reference to the toxicity seen in the 28-day study at 300 mg/kg/day. There is statistically significant but minimal liver enlargement at 200 mg/kg/day in the 90-day study, but no other effects.

- Conclusion. The toxicity seen in the 28-day study at 1 000 mg/kg/day is not severe toxicity and has not been shown to be the highest possible toxicity without death or severe suffering, but 1 000 mg/kg/day is the limit dose, and a higher dose is generally not needed to be tested. As such, a dose of 300 mg/kg/day cannot be considered as being chosen with the aim of being the highest possible dose level without severe suffering or death, and consequently the lower dose of 200

mg/kg/day has the same problem. The high dose chosen in the 90-day study is not compliant with the REACH information requirements, and an additional 90-day study must be performed to include a high dose of 1 000 mg/kg/day.

Example 3: Main study shows severe toxicity

- A substance shows no mortality at 2 000 mg/kg in an acute toxicity study. There is no repeated-dose range finding study.

- A 28-day study is performed in rats at 60, 200 and 600 mg/kg/day. The results of the study show that at 600 mg/kg/day, there is ulceration of the stomach in all animals, moderate to severe fatty change in the liver associated with evidence of cell death, accompanied by a body weight decrease of 18 % compared to controls. The 200 mg/kg/day shows milder toxicity and no toxicity is seen in the low dose group.

- Conclusion. There is not an adequate basis for choosing the doses in the main study, as no dose range finding study was performed, and the high dose is not the limit dose. However, the high dose group shows severe toxicity (e.g. ulceration in all animals per se demonstrates severe toxicity) and the study is, therefore, compliant with the test guideline.

Example 4: Relationship to STOT-RE

- A substance was tested in a 28-day study at 900, 450 and 225 mg/kg/day. The 28-day study is a valid range-finder for a 90-day study.

- The results of the 28-day study show that the 900 mg/kg/day group was terminated because of excess toxicity. At 450 mg/kg/day, there was severe fatty change and degenerative lesions in the liver. At 225 mg/kg/day, there are elevated serum transaminases, but liver damage was mild.

- According to the *Guidance on the Application of the CLP Criteria*, Table 3.16 states that 300 mg/kg/day is the guidance value for a 28-day study which corresponds to the guidance value of 100 mg/kg/day for Category 2 STOT-RE for a 90-day study. The substance causes severe target organ toxicity at 450 mg/kg/day, but not at 225 mg/kg/day. The substance does not currently meet the criteria for classification as STOT-RE 2, but it is realistic to anticipate that it might do so in a 90-day study.

- Conclusion. The substance should be robustly tested for classification as STOT-RE. The high dose level for the 90-day study should be set at 450 mg/kg/day, in accordance with the corresponding dose level from the 28-day study. Dose intervals should not be set more than threefold apart, and the guidance value for STOT-RE 2 should be evaluated. Therefore, there should be doses of 200 and 100 mg/kg/day, and an additional dose level of 50 mg/kg/day should be considered to ensure a NOAEL can be established.