ECETOC Guidance on Dose Selection

Technical Report No. 138

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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Technical Report No. 138

Brussels, March 2021

ISSN-2079-1526-138 (online)

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1. SUMMARY

Prior to registering and marketing any new pharmaceutical, chemical, agricultural chemical or food ingredient products manufacturers must, by law, generate data to ensure human (and environmental) safety.

The safety testing requirements to ensure human safety vary depending on sector, product type and geographical region/country and for many will require repeat-dose testing in animals. Dose level selection is the most important aspect of the specification of repeat dose toxicity studies. It is the means by which we identify if a dose response is present, those exposures leading to relevant hazard and those exposure levels that should be used as the basis for human health risk assessment, normally based on the absence of adverse effects. Repeat dose studies span a wide range of toxicology study types with duration from 28 day systemic toxicity to 2-year carcinogenicity studies in a number of species. Assessment of systemic toxicity is done in the rat and mouse and for some sectors in dogs, while the assessments of reproductive and developmental toxicity is done in rats and rabbits. The advice on dose level selection provided in test guidelines, allied guidance documents and by individual regulatory authorities globally for most sectors is illustrated in this report but is not well harmonised; the pharmaceutical sector being the closest to having a globally aligned approach through the International Council for Harmonisation (ICH). The various criteria used to set the highest dose level are even less well harmonised across the range of studies and geographies. These range from approaches that can be interpreted to allow a proportion of deaths at the highest dose tested to those that encourage high dose level selection based on signs of evident toxicity.

The data provided by toxicity studies are used in all sectors to provide a point of departure from normality which can be used as the endpoint underling a human health risk assessment, and to provide information leading to hazard based classification (although the consequence of classification will vary across sectors, from very little practical impact to a denial of a registration). The recommendations of this report represent pragmatic approaches to selecting dose levels that allow accurate risk assessment and also enable hazard-based classification based on identification of relevant hazards. Analysis of classification outcomes supports the conclusion that there is no automatic relationship between positive classification outcomes and increasing dose, especially approaching the limit dose (currently 1000 mg/kg/d) for repeat dose toxicity studies across all major endpoints measured. This

provides a reassurance that a workable and acceptable approach can be found that provides accurate information for risk assessment and assigning a hazard-based classification, and that these needs can co-exist and be served by the same study.

It is recognised that different sectors have differing degrees of freedom to operate in dose level selection and this is reflected in the overall recommended approaches. These can be applied within the current regulatory frameworks, and also provide future options and approaches to science-based dose level selection.

- As recommended in test guidelines and guidance documents, wherever practically possible an understanding of internal exposure should be developed, through the deployment of toxicokinetic (TK) approaches, and used to guide dose level selection. In the great majority of cases and situations internal exposure (blood and tissue) will be approximately linear with applied external dose. Knowing this will provide reassurance that the biological effects, including toxicities that are observed, represent true responses to increasing exposure. In a minority of cases a disproportional increase in internal exposure may be demonstrated. In such a situation this knowledge is vital in shaping approaches to dose level selection where plateaus of exposure or less than proportional exposure with increasing applied dose can be taken into account. This information must come from appropriate and rigorous TK approaches, and the optimum ways of generating and interpreting these data are illustrated and presented in the report.

- Where there are no or little data to make a dose selection decision based on internal dose, or where internal dose is linear with applied dose, then signs of toxicity (which may range from mild to severe) remain the main source of knowledge for selecting appropriate dose levels. With the possible exception of early dose range-finding studies and in the absence of any clear prior information on mode of action or structural class to guide dose level selection, then in repeat dose studies the highest dose level should be limited to a reasonable level such as that which causes evident but minimal toxicity (as an example existing guidance often points to effects such as a 10% reduction in body weight gain). There should be no situation in 21st century toxicology practice where there is any scientific justification for selecting the high dose in repeat dose studies with the intention of causing pain, distress, suffering, significant toxicity or lethality in any of the experimental subjects. In practice, laboratories and investigators

conducting studies have very clear local guidance and legislation aimed to limit or prevent the intentional use of doses that cause such effects. As an example, if any repeat dose study causes lethality, peri-lethal effects or a sustained period of reduced weight gain or weight loss at the high dose, this dose would very likely be terminated without further evaluation, often requiring further studies at more appropriate dose levels. Where such historical studies exist, they should be interpreted with great caution.

- As the science of predictive human exposure further develops and matures, this will provide exciting and novel opportunities for more relevant approaches to dose level selection. In some sectors this approach is well understood and is currently used in dose level selection (pharmaceuticals); in other sectors opportunities for the use of margin of exposure (based on predicted human exposures) to set dose levels are being considered, particularly agrochemicals.

- In circumstances where the mode of action (MoA) causing toxicity is well understood and described, and where a material can be clearly assigned to such a class, then different opportunities exist and should be considered in approaches to dose level selection. However, it is recognised that in order to base doses on MoA, one would need a quite extensive and existing knowledge-base. Similarly, the paradigm for dose level selection in studies designed to elucidate a MoA (usually based on the findings from more traditional regulatory toxicity studies), can be very different and are illustrated in the report.

2. Introduction, Background and Principles

2.1. Background and Principles

Toxicology is essentially an observational science, and toxicologists are well able to describe or even predict the effects a chemical may cause. The study designs we use and our approach to dose level selection have evolved to reinforce this focus on describing effects (hazards), be these at the cellular, organ or whole organism level. However, it is not just about identifying and characterising a hazard. As the goal is to ensure human safety, toxicologists need to understand relevance to humans and this requires an understanding of human exposure, something that can be much more difficult to accurately assess. Moreover, since toxicity studies generally rely on only a small number of dose levels (generally no more than four), it can be difficult to accurately describe the exposure levels (doses) that cause (or can be predicted to cause) adverse effects. It is therefore critically important that toxicologists pay the greatest attention to the way in which these (limited number of) dose levels are selected to provide the optimum information to serve the goal of protecting human health.

An additional challenge toxicologists face globally is the different ways in which information from toxicity studies is used in risk management. When used for risk assessment the critical data from repeat dose toxicities studies is the No Adverse Effect Level (NOAEL) which is used as the point of departure from normality. This is compared to human exposure to provide a risk assessment and a risk management judgment is made on human safety for that chemical application or exposure. When data are used for hazard-based classification the focus is purely on the effect (hazard) largely independent of any thoughts around toxicological potency and relevance to human exposure. This approach means that hazard-based classification is a rather blunt instrument for protecting human health as there is little consideration of degree of hazard or the relevance of the dose levels used in toxicity studies to human exposures.

The purpose of this guidance is to provide sector-specific recommended approaches to protect human health, respect animal welfare and provide relevant endpoints for risk assessment and information needed for hazard-based classification. Workable approaches that allow risk assessment and hazard-based classification to co-exist are a priority.

2.2. Current Regulatory Framework and Guidance

2.2.1. Historical perspectives and the evolution of test guidelines

The OECD (Organisation for Economic Co-operation and Development) Guidelines for the Testing of Chemicals is a library of the internationally agreed testing methods used by government, industry and independent laboratories to identify and characterise potential hazards of chemicals. Originally drafted in 1979, the aim was to produce a well-defined framework for each toxicity test to standardise test conduct across multiple countries to produce results that are fully acceptable to numerous regulatory agencies. This harmonised approach would (i) promote scientific aspects of toxicity testing, (ii) ensure international acceptance of test data (iii) avoid duplication/repetition, (iv) promote efficient use of laboratory animals and (v) improve the efficiency of test conduct. The guidelines were intended to align general principles of toxicity testing in order to detect and characterise hazard in a reproducible manner. They were not specifically designed for some of the uses to which the data are put today such as hazard classification and labelling purposes, and their use in this context should be treated with pragmatism and caution. In addition, the guidelines were designed to propose methodologies that could be used across chemical sectors (pharmaceuticals (although now supplanted by ICH approaches), agrochemical, biocides and industrial chemicals). The guidelines were not intended to be a test protocol, but to lay out a set of generally accepted principles that could be modified for each industry sector or purpose and to provide comparable endpoints.

Although test guidelines are subject to periodic revision and additions, the fundamental core generally remains unchanged, and forty years later the results and endpoints derived from these studies are being used within the regulatory arena to identify and characterise hazards and in risk assessment. There are, however, a number of acknowledged limitations and important considerations, including:

- The animal models used in repeat dose studies may not always be relevant when extrapolating to humans.
- It is not possible to devise “standard” test methods appropriate to all chemicals, expert toxicological judgement should be considered when assessing the suitability of each method.

- The final purpose of the study should be considered prior to conducting each test i.e., is it for (i) risk assessment or (ii) hazard characterisation? How will the resulting data inform decision-making?

- Dose relevance for the use pattern and exposure scenario. For example, what is the probability (the risk) of a human ever encountering acute exposures up to 2000 mg/kg bw/day or chronic exposures up to 1000 mg/kgbw/d?

- Is the top down approach of starting with an excessively high dose fit for purpose or would a bottom up approach of using a knowledge or prediction of human exposure to set dose ranges be more relevant?

The OECD test guidelines do not contain the detail of a Standard Operating Procedure or Test Protocol, although they are often viewed this way; this was acknowledged by OECD and considered intentional because "toxicology was a developing science and excessive rigidity or over-detailed specification of methods could inhibit scientific initiative and be counter-productive." It is unfortunate that forty years later this important insight is not considered by many stakeholders (regulators and industry alike) who use the procedures or data in decision making. It is important that toxicological skill and pragmatic judgment are utilised. It is noteworthy that in the current version of OECD test guidelines the stated purpose is primarily to provide information on hazard and to characterise that hazard in order to provide a point of departure for a risk assessment, although there is no tangible guidance on how the results should be used.

2.2.2. The evolution of guidance on dose level selection

Some advice on dose level selection is given in individual test guidelines (See Table 5). The following excerpt taken from OECD 408, 2018 (90 day Repeated Oral Toxicity study in Rodents), summarises the test purpose and is typical of the guidance given for repeat dose studies:

> *"…. 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).”

Taking this same guideline as an example the advice on dose level selection has a focus on hazard characterisation, that is identifying a point of departure for use in risk assessment:

“At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see paragraph 18). 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 chemical, the highest dose level should be chosen* ***with the aim to induce toxicity but not death or severe suffering*** *(see OECD, 2000. Series on Testing and Assessment No. 19). A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and a NOAEL at the lowest dose level. Two- to four-fold 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.”*

It is worth specifically recognising that for many years the need to select dose levels in order to ensure a humane approach to the use of animals has been enshrined in the OECD guidance. Outcomes such as a test article-related reduction in body weight gain has been specifically mentioned in the context of animal suffering (OECD, 2000. Series on Testing and Assessment No. 19). This is covered in more detail below.

Further advice on dose level selection is given in the Guidance Document 116 (OECD 2014), and is most relevant to long term and carcinogenicity studies. The challenges in setting a high dose that satisfies all needs (especially in carcinogenicity assessments) are highlighted:

“Dose selection should be based on the findings of subchronic or range-finding studies. The highest dose level to be used in a chronic toxicity or carcinogenicity study needs to be carefully considered and the reasons for the final choice clearly defined. Ideally, the dose levels selected will maximise the detection of dose–response relationships and facilitate the extrapolation of

these to potential hazards for other species, including humans. The selection of the highest dose level to be used in a chronic toxicity or carcinogenicity study has long been a matter of controversy. At the time when long-term animal bioassays began to be routinely used to assess the qualitative potential of a test substance to cause chronic toxicity and cancer, the emphasis was on testing at high levels in order to maximise the potential of such studies to detect effects. The concept of the Maximum Tolerated Dose (MTD), conventionally defined as the highest dose to produce toxic effects without causing death and to decrease body weight gain by no more than 10% relative to controls (OECD, 2002 - GD No. 35) became well established. The MTD is often used in the assessment of a chronic toxicity or a carcinogenicity study to decide whether the top dose tested was adequate to give confidence in a negative result. This Guidance Document focuses on the selection of the top dose, rather than attempting to define an MTD. While some regulatory bodies or organisations interpret an adequate high dose to be a minimally toxic dose, others emphasise the need to select a dose level that is a maximally tolerated dose (i.e., more severe toxicity should be demonstrated). Thus, because of differences in views regarding the severity of toxic effects that are interpreted as providing evidence that an adequate high dose has been attained or exceeded, a completed carcinogenicity bioassay may be considered to be acceptable by one organisation but not by another. Many carcinogenicity studies can be challenged on the basis of selection of a top dose that is too high, particularly if there is a large interval to the next highest dose. This results in data that are difficult to interpret and may be of limited use for regulatory purposes. If the main objective of the study is to identify a cancer hazard, there is broad acceptance that the top dose should ideally provide some signs of toxicity such as slight depression of body weight gain (not more than 10%), without causing e.g., tissue necrosis or metabolic saturation and without substantially altering normal life span due to effects other than tumours. Excessive toxicity at the top dose level (or any other dose level) may compromise the usefulness of the study and/or quality of data generated. Criteria that have evolved for the selection of an adequate top dose level include: (in particular) toxicokinetics; saturation of absorption; results of previous repeated dose toxicity studies; the MOA and the MTD. Toxicokinetic non-linearity should also be considered in the selection of the top dose to be used. Although top dose selection based on identification of inflection points in toxicokinetic non-linearity may result in study designs that fail to identify traditional target organ or body weight effects, ***it must be appreciated that metabolic saturation in fact represents an equivalent indicator of biological stress. In this***

case, the stress is evidenced by appearance of non-linear toxicokinetics rather than appearance of histological damage, adverse changes in clinical chemistry, haematology parameters or decrease in body weight gain."

2.2.3. The Maximum Tolerated Dose concept

- The concept of the MTD was initially established in the 1960s as part of the dose selection process for carcinogenesis studies (McConnell, 1995). The National Cancer Institutes (NCI) defined the MTD as "the highest dose of the test agent during the chronic study that can be predicted not to alter the animals' longevity from effects other than carcinogenicity" (Sontag et al., 1976), and further suggested that the choice of the MTD should be based on the results of a 90-day study where the highest dose caused "no more than a 10% weight decrement, as compared to the appropriate control group; and does not produce mortality, clinical signs of toxicity, or pathologic lesions (other than those that may be related to a neoplastic response) that would shorten the animal's life span.

- Amongst the drawbacks inherent in the MTD approach is use of the term "maximum" which automatically connotes that an "excess" amount of the chemical is used to produce a given effect; this can be misleading and cause confusion. Proposals to refine the term as a "minimally toxic dose," but use the same definition (Huff et al., 1994) have done little to inform or to clarify.

- A further important disadvantage is that it is difficult to determine the relevance to humans of effects that are found at doses that exceed the MTD. For example, if carcinogenic activity is only observed at doses that clearly exceed the MTD, should that particular chemical be considered an animal carcinogen? This is a particular problem for regulators and agencies that have to classify chemicals on the basis of hazard. When the compounds are not genotoxic there is no logic in classifying chemicals simply on a carcinogenic response at very high doses.

- Where it is practically impossible to attain an MTD (e.g., for chemicals with low toxicity), practical limits (referred to as the "upper limit") have been accepted for studies using some routes of exposure, for example: - 1% of the diet, extent of solubility in water, or limit of acceptance (palatability). Basically, dose influences mechanism and, over a wide range of doses, mechanism can change with changing dose (Counts and Goodman, 1995). Thus, a

carcinogenic effect observed at a high dose is not necessarily expected to occur at lower doses, especially when dealing with nongenotoxic chemicals (McClain, 1994).

- Looking to the future, the concept of using the MTD as the "highest" dose may still be valid, but the definition needs to reflect state-of-the-art science. Effects on body weight, morbidity, mortality, or pathology, when present, will continue to be valid endpoints when evaluating the results of short-term studies for selecting the MTD for longer term studies. However, a more science-based rationale is required. Concerns with the MTD approach include how the resulting data are used in the risk assessment process (and in hazard-based classification), especially in the area of carcinogenicity and are well described (McConnell, 1989; Counts and Goodman 1995; Gaylor, 2005).

- The definition of the MTD should also incorporate the findings of properly conducted absorption, distribution, metabolism, and excretion (ADME) studies. For example, if a chemical is poorly absorbed, then an upper limit approach to selecting the MTD would be used. Or if, at a given dose, metabolic saturation is achieved, then that dose would be defined as the MTD. These concepts are not new and were initially described in guidance in the U.S. EPA (Environmental Protection Agency) Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005): "Other signs of treatment-related toxicity associated with an excessive high dose may include (a) significant reduction in body weight gain (e.g., greater than 10%), (b) significant increases in abnormal behavioural and clinical signs, (c) significant changes in hematology or clinical chemistry, (d) saturation of absorption and detoxification mechanisms, or (e) marked changes in organ weight, morphology, and histopathology."

- A limit dose of 1000 mg/kg/day is used for most repeat dose studies. The origin of this value is not apparent, but it was probably chosen as an arbitrary value lower than single-dose limits of 2000mg/kg or 5000mg/kg. However, the reduction of the recommended limit dose as study duration increases stops at 28 days. In practice, the limit dose of 1000 mg/kg/d results in a very high metabolic load on laboratory animals, and it can lead to liver enlargement and kidney toxicity with otherwise relatively nontoxic chemicals. Note, for a 70kg human this is equivalent to an intake of 70g compound per day, every day for a lifetime, a situation which is extremely unlikely. In turn, this can lead to problems in assessing the results of specific toxicity studies. Doe *et al*. (2006) suggested that it may be possible to set limit doses based on an assessment

of the maximum theoretical human exposure level, followed by application of a margin of exposure of 1000 from this level.

2.2.4. The use of toxicity studies for Classification and labelling

Classifications based entirely on hazard are made according to the Globally Harmonised System (GHS) and regional adaptations of this guidance. GHS is designed to ensure the consistent and standardised classification of chemical hazard. In reviewing the GHS document on classification and labelling (8th revision ed, 2019), it is clear that when a specific classifiable adverse event or specific target organ toxicity is identified from standard well conducted studies then it should be evaluated and classified and when necessary placed in a specific category. Some categories are associated with a specific need to provide a label on the safety data sheet, container, or notify relevant personnel when transporting. These are well established and internationally agreed.

Classification is based on having a relevant finding in the appropriate toxicology studies. There is no requirement to classify each molecule. Some approaches to classification can be taken to imply that each molecule evaluated must be classified and therefore, it must be tested in such a way that one creates a classifiable effect. This can be contrasted with the intent of the GHS which is to evaluate each molecule in order to assess if a classification is warranted. In many cases (and especially for the key classifications related to cancer and reproduction), classification is not required. To consider effects that are directly attributable to dose and time as an intrinsic feature of the chemical conveying the concept of an intrinsic hazard may be considered a flawed premise. While it is true that some acute toxicities such as corrosivity may be intrinsic to the molecular structure it is equally true that carcinogenicity and reproductive toxicity are not intrinsic features of the molecule. This is because the development of cancer and adverse effects on reproduction for example are complex multistage processes where each stage (or key event) has its own dose response and temporal relationship.

Recently concerns have been expressed that insufficient dosing in some toxicity studies (particularly those relating to assessments of reproductive toxicity and cancer) may provide inadequate data for classification and labelling purposes (Heringa *et al.,* 2020; Woutersen *el al.,* 2020). The over-riding concern being, that by missing elements of hazard it may not be possible to fulfil the precautionary protection goal served by classification and labelling. Specific examples include:

- Registrants providing studies that fall short of demonstrating an 'MTD' with the consequence that adverse effects that may be observed only at higher dose levels, and which would attract a classification, are not being identified.

- A lack of familiarity leading to low confidence in the use of kinetics to guide dose level selection in industry sectors where this approach has not previously been used

- A failure to conclude the classification process with the implied need for registrants to repeat studies at higher dose levels (and the associated increase in animal numbers).

Whilst these concerns are driven by a wish to ensure adequate human health protection (albeit from a hazard-based perspective), this approach may be overly conservative and there are concerns that it could lead to the use of unnecessarily high doses in animal studies that do not add scientific value (Sewell *et al.*, 2020; Smith and Perfetti, 2020; Terry *et al.*, 2020).

Key questions and assumptions to consider in dose level selection include:

- Do classification outcomes always follow a linear relationship to applied external dose in experimental animals? Are more positive classification and labelling outcomes seen the closer one gets to the limit dose?

- Are the hazards associated with high dose testing relevant to protect human health? Will testing at lower doses miss important hazards?

- Which other considerations for dose level selection could better identify relevant hazards? e.g., use of kinetically-driven approaches.

2.2.5. Classification outcomes and their relationship to applied dose

In order to investigate whether classification outcomes follow a linear relationship to applied external dose in experimental animals and as the limit dose is approached more positive classification and labelling outcomes are detected, one would need an evaluation of the doses that lead to positive classification outcomes for a wide range and number of chemicals.

Muller *et al. (*2012) evaluated the potency of substances classified (comparing NOAELs, LOAELs (Low Observed Adverse Effect Levels) and ED10 (Effective Dose 10 (Dose causing a 10% effect)) values for the effect leading to a classification) in the EU (European Union) for effects on development and reproduction in order to better understand how the degree of hazard (potency) could be used in setting specific concentration limits (SCLs) for chemicals. They assessed the experimental data for 93 substances classified based on adverse effects on foetal development. The analysis showed a wide range of potency covering seven orders of magnitude. When the LOAELs for the effect driving the classification were considered, >70% were in the range 0.01 – 316 mg/kg/d. When the NOAELs for the effect driving the classification were considered, >95% were in the range 0.01 – 316 mg/kg/d. Effects detected at doses around or above the limit dose (1000 mg/kgbw/d) contributed only a very minor number of classification outcomes (1% for the NOAELs and 3% at the LOAEL).

A similar picture is provided by Muller's analysis of chemicals classified for effects on fertility.
The analysis again showed a wide range of potency covering five orders of magnitude. When the LOAELs for the effect driving the classification were considered, >75% were in the range 0.01 – 316 mg/kg/d. When the NOAELs for the effect driving the classification were considered, 90% were in the range 0.01 – 316 mg/kg/d. Effects detected at doses around or above the limit dose (1000 mg/kgbw/d) contributed only a very minor number of classification outcomes (0% for the NOAELs and 4% at the LOAEL), further challenging the assumption that the higher the dose the greater the number of classifiable outcomes.

2.3. Maternal toxicity in developmental toxicity studies

Developmental toxicity studies represent a specific case in study design and dose selection, in that the developing embryo is dependent on the maternal system for its metabolic needs. From the perspective of dose-response, these studies are intended to establish a dose-response for both mother and offspring. However, the top dose is generally selected based on maternal toxicity. A frequent observation in developmental toxicity studies is toxicity to the fetus only in the presence of maternal toxicity. Interpretation of this result is contentious, as it is not possible in guideline studies to distinguish between the possibility that the developmental effects are a response to the maternal toxicity or are a direct effect of the test agent on the embryo. The answer to this question matters a great deal, as developmental effects, even in the presence of maternal toxicity, are being used as the

basis for classifying agents as reproductive hazards, although the C&L (Classification and Labelling) guidelines indicate that the relevance of maternal toxicity on developmental effects should be evaluated.

Several modes of action have been identified by which maternal perturbations lead to secondary effects on the embryo and fetus. These include effects on maternal oxygen carrying capacity and cardiovascular function, perturbations in maternal nutrient homeostasis and changes in maternal osmoregulation (reviewed by Daston, 1994; Carney, 1997). In each case, the toxicant has little or no effect on the embryo; the adverse developmental effects are attributable to the changes in maternal physiology. For example, the non-steroidal anti-inflammatory diflunisal causes hemolytic anemia in the pregnant rabbit. This can be sufficiently severe that it leads to hypoxia in the embryo, which is developmentally adverse. However, the adverse developmental effects can be prevented by reversing the hypoxia (through the use of hyperbaric oxygen), proving that they are secondary to maternal toxicity (Clark et al., 1984). In another example, the toxicity of many chemicals elicits an acute phase response, a generic physiological response to systemic inflammation and tissue injury. Induction of metallothionein, a metal-binding protein, is often part of the acute phase response. Metallothionein binds many divalent metal ions but has a strong affinity for zinc, such that the acute phase response is often accompanied by significant but transitory decreases in circulating zinc concentration (Taubeneck et al., 1994). These brief excursions into zinc deficiency have no long-term detrimental effects to the adult animal but are devastating to the embryo as it relies on numerous zinc-dependent transcription factors and other proteins for cell differentiation and other developmental processes. As with the previous example, the effect of the chemical is on the maternal system not the embryo. Correction of the zinc deficiency with supplemental zinc is sufficient to prevent the developmental toxicity.

One useful technique for determining whether a chemical has the potential to affect development directly is rodent whole embryo culture. Embryos are grown for up to two days during the most active phase of embryonic development. Because the embryo is isolated from the dam, they are isolated from maternal influences. The technique has been employed to show, for example, that urethane and alpha-hederin, two agents that induce maternal metallothionein and developmental toxicity *in vivo* have no effect on embryos *in vitro* (Daston et al., 1991; 1994). To demonstrate that this was not attributable to a lack of metabolising enzymes in the embryo, embryos were cultured in serum from rats treated with alpha-hederin. As long as the serum was supplemented with zinc to reverse the

acute phase response-induced deficiency, there were no effects of the serum on development (Daston et al., 1994).

These examples illustrate that excessive maternal toxicity leads to responses in the embryo that are not an intrinsic property of the chemical being tested and do nothing but confound attempts to characterise the chemical's hazard. The subject of appropriate dose-setting for developmental toxicity studies has been the subject of consensus workshops. One of the more useful workshops (Beyer et al. 2011) concluded that in addition to maternal lethality and significant clinical signs, decreased maternal weight gain during pregnancy of more than 20% would make a study uninterpretable and should be avoided.

2.4. Animal Welfare considerations

The principles of the 3Rs (**R**eplacement, **R**eduction and **R**efinement) were developed over 60 years ago providing a framework for performing more humane animal research (Russell and Burch, 1959). These have recently been updated by the UK (United Kingdom) National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (Table 1).

Table 1: Definitions of the 3Rs (https://www.nc3rs.org.uk/the-3rs)

	Standard	**Contemporary**
Replacement	Methods which avoid or replace the use of animals	Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals
Reduction	Methods which minimise the number of animals used per experiment	Appropriately designed and analysed animal experiments that are robust and reproducible, and truly add to the knowledge base
Refinement	Methods which minimise animal suffering and improve welfare	Advancing animal welfare by exploiting the latest *in vivo* technologies and by improving understanding of the impact of welfare on scientific outcomes

The OECD has set a framework for recognition of signs as humane endpoints for experimental animal use (OECD, 2000) and within this, the harmonised work from FELASA (Federation for Laboratory Animals Science associations) is key (Guillen, 2012). Guidance on euthanasia and recognition of pain

are also useful to ensure the appropriate implementation of humane endpoints (Hawkins et al., 2016; Leary et al., 2020), as well as regional legislation and guidance (e.g., European Directive 2010/63/EU). A pragmatic endpoint guidance for mild, moderate and substantial severity signs is available (FELASA, 1994; reviewed and updated in LASA/NC3Rs, 2009 Table 2). In the more recent version, the authors point out that "Defining the MTD in the studies of shortest duration informs dose setting in subsequent studies and is crucially important in application of the 3Rs since this reduces the chances of larger numbers of animals that are used in regulatory studies being exposed to unanticipated suffering". Essentially, when a single substantial effect or a combination of moderate effects (e.g., effects prolonged in nature such as a body weight loss up to 20%, or a reduction of feed consumed higher or equal than 60% for more than 72 hours), is observed, these should result in immediate actions including, where appropriate, euthanasia. Mild effects, such as reduced weight gain, transient signs (postural, neurological, respiratory, cardiac) appearing as effect of dosing, and mild transient reduction (25-60%) of feed consumption could be considered acceptable for short term studies. However, this guidance is now over 10 years old, and current thinking is that toxicity should be limited to mild clinical signs and that there is no value in exceeding this or in demonstrating moderate toxicity. More recent studies have shown that, in terms of body weight loss, this guidance is conservative. The UK NC3Rs has conducted a survey including 151 studies from 15 companies or contract research organisations and has proposed to reduce the body weight loss limit for short term dosing (up to 7 days) to 10% for rat and dog and 6% for non-human primates (Chapman, 2013). This guidance clearly indicates that for even for initial repeat dose studies there is no justification for exceeding dose levels that cause only mild effects.

Body weight loss as an objective indicator of MTD is supported by a similar cross-company initiative within the chemicals industry (mainly agrochemicals), where data on clinical signs observed during acute inhalation toxicity studies (up to 14 days duration) in rats was shared. Statistical analyses showed that body weight loss (BWL) in excess of 10% is highly predictive (positive predictive value of 94%) of death or severe toxicity at higher doses, showing that the MTD had already been reached or exceeded (Sewell *et al.,* 2015). In an ILSI-HESI (International Life Sciences Institute – Health and Environment Science Institute) workshop dealing with maternal toxicity (Beyer et al., 2011) there was no consensus on the amount of body weight gain. However, regarding developmental and reproductive toxicity studies a 20% decrease in body weight gain was considered excessive.

Table 2: Guidance on severity limits for repeat dose studies (adapted from 'FELASA, 1994' and '**LASA/NC3Rs, 2009). Toxicity should be limited to mild signs - there is no value in demonstrating moderate or substantial toxicity in repeat dose toxicity studies.***

MILD	MODERATE	SUBSTANTIAL
Reduced weight gain	Weight loss up to 20%	Weight loss greater than 50%
40-75% of normal food consumption for up to 72 hours	Less than 40% of normal food consumption for up to 72 hours	Food consumption less than 40% for 7 days or anorexia/ total inappetence for 72 hours
Partial piloerection	Staring coat	Staring coat with signs of dehydration
Subdued but responsive; shows normal provoked pattern of behaviour	Subdued' shows subdued behaviour patterns even when provoked	Unresponsive to entraneous activity and provocation
Interacts with peers	Little peer interaction	
Hunched transiently especially after dosing	Hunched intermittently	Hunched persistently
Transient vocalisation	Intermittent vocalisation when provoked	Distressed – vocalisation unprovoked
Transient oculo- nasal discharge	Oculo- nasal discharge persistent	Oculo- nasal discharge persistent and copious
Normal respiration	Intermitted abnormal breathing pattern	Laboured respiration
Transient tremors	Intermittent tremors	Persistent tremors
No convulsions	Intermittent convulsions	Persistent convulsions
No prostration	Transient prostration (<1 hour)	Prostration (> 1 hour)
No self-mutilation	No self-mutilation	Self-mutilation

2.5. Scientific Considerations

2.5.1. Relevance of high dose testing to humans

It has long been known that certain toxicities observed in experimental animals and often at high dose level are species specific (e.g., alpha-2u globulin accumulation causing kidney toxicity in male rats, and

rodent liver growth leading to liver tumours and compensatory thyroid hyperplasia secondary to liver toxicity). Moreover, in some industry sectors (e.g., Pharmaceuticals and Food), high dose testing in animals is often considered irrelevant to informing hazard and risk decisions for *human relevant* doses. Rather than focus testing and attention on high dose phenomenon, more value in protecting human health would be served by paying greater attention to the precision of dose level selection and the relevance of effects in the sub MTD range as this can provide more relevant information on target organ toxicity.

2.5.2. Kinetically informed dose level selection

This approach to aiding selection of dose levels and interpreting study outcomes can be useful in situations where it can be empirically shown that increasing the applied (external) dose does not lead to a proportional increase in internal exposure (where a plateau has been reached for example). This subject is covered in full in section 3 of this guidance.

In summary, this analysis shows that in the overwhelming majority of cases, the lowest adverse effect level on which classification judgments are made is well below the current limit dose of 1000 mg/kgbw/d. This relationship applies to classifications for cancer, reproductive and developmental toxicity. It logically follows therefore that rather than increase the limit dose above the current level as has been suggested (Woutersen et al., 2020), a more scientific approach and one that uses experimental animals most wisely would be to focus testing more towards the range of human exposure. This approach would provide benefits:

- In more accurately identifying a point of departure for risk assessment,
- Allowing relevant classification decisions to be made using the data,
- Potentially providing information that could be used in a more accurate classification paradigm where the degree of hazard (potency) is a key determinant. (Muller *et al.,* 2012; Hennes *et al*.,2014).

2.6. Summary

As indicated earlier the purpose of this guidance is to provide sector specific recommended approaches to protect human health, respect animal welfare and provide relevant endpoints for risk assessment and the information needed to assign hazard-based classification. However, it is recognised that different sectors have differing degrees of freedom to operate in dose level selection as shown below in Table 3 below. The degree of freedom to set dose levels can vary significantly between industry sectors. In cases where chemicals are designed to be biologically active (Pharma and Agrochemicals), there is usually a large amount known about the chemical and/or mode of action class that can be used to guide dose level selection. In other situations (Industrial Chemicals and Food ingredients) there may be much less or even and absence of any information to guide dose level selection. In addition, the different sectors are regulated in different ways and the type of information accepted for use in dose level selection across sector varies accordingly. These differences on freedom to operate is reflected in the individual sector recommended options and approaches. Recommendations take into account the industry sector, the customs, practice and expectations of regulators in that sector, the likely route of any human exposure as well as other factors such as route specific ADME.

Table 3. Cross-Sector Freedom to Operate in Dose Level Selection

Sector	Dose selection guidance provided	Ability to use TK to understand internal dose	Knowledge of MoA	Ability to use QSAR	Dialogue with regulators	Consequence of classification (and of dose level selection)
Pharma	Generally harmonised	Always	Yes	More developed	Encouraged	Little consequence
Agrochem	Some but not harmonised	Understanding and data are increasingly being provided	Pesticidal MoA generally known	Limited	Not usual and lack of consistency in positions	Can be banned on threshold toxicities

Industrial chemicals	Some but not harmonised	Data generally not generated	Rarely	Limited	Not usual	Can be banned on threshold toxicities
Food ingredients	Some but not harmonised	Data generally not generated	Rarely	Limited	Not usual and lack of consistency in positions	Can be banned on threshold toxicities

3. Use of toxicokinetics to inform dose selection

Toxicokinetic (TK) data can be obtained during toxicology studies to provide information on the internal exposure of a chemical and/or its metabolites. In the pharmaceutical sector this has involved the use of satellite animals, but in other sectors modern microsampling and analytical techniques mean that these data can be generated in the main study animals. This allows applied doses to be correlated to levels of circulating moieties (parent substance/metabolites) and linked to apical endpoints/effects observed in the same animal. Thus, TK data are useful as a tool to inform dose level selection and improve interpretation of toxicity studies, human exposure assessment and risk characterisation. Toxicokinetics displaying non-dose proportionality should also be considered in the selection of the top dose to be used, including the formation of metabolites at high doses which are not relevant to human exposures.

To obtain the key determinants of the pharmacokinetics of a xenobiotics (absorption, distribution, metabolism and excretion), several *in vivo* experiments are required, including blood/plasma-kinetics, mass balance, excretion and tissue distribution experiments. However, smaller scale experiments (e.g., plasma-kinetics) may already provide plasma concentration-time profiles to assess the extent of drug exposure in vivo. Thus, TK can be integrated into already planned *in vivo* toxicological studies, to enrich the dataset and provide information to inform subsequent studies. *In vitro* studies can also be used to provide further data on specific aspects of TK such as metabolism. These studies can be carried out using samples from both the test species and samples from humans, to facilitate interspecies comparisons (e.g., metabolite profile, metabolic rate constants).

3.1. TK requirements in each sector

Acceptance and practical use of systemic exposure determinations across sectors is variable. There are varying regulatory requirements and acceptance dependent on sector, region, purpose and intended use (e.g., hazard/classification purposes, risk assessment). Consequently, the perceived degree of value in pursuing these TK determinations for informing dose level selection, study interpretation and risk assessment is variable from both industry and regulatory perspectives. In some sectors, the use of kinetic information to inform study designs is common practice (e.g., pharmaceuticals) and in others uptake of these techniques is beginning to be recognised as bringing value to safety/risk assessment

(see examples for Agrochemicals, i.e., Dorne, 2005; Terry et al., 2015). For others, such as industrial chemicals and food industries, TK data is not generally generated (see Table 3). Various OECD test guidelines and guidance documents relevant to *all* sectors reference the potential use of TK data for interpretation of findings and dose level setting (see Table 4).

Table 4: Examples of Regulatory Guidance indicating Use of TK in Dose Selection

Region/ Authority	**Document**	**Summary of Requirements/Recommendations**	**Reference**
EU	**EC 1107/2009**	TK required in short-term & long-term studies: "For dose selection, toxicokinetic data such as saturation of absorption measured by systemic availability of substance and/or metabolites shall be taken into consideration." https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0283&from=LT	REGULATION (EU) No 283/2013
	REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals)	*Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.7c: Endpoint specific guidance* Use TK to support Dose Setting Decisions for Repeated Dose Studies. TK data, especially information on absorption, metabolism and elimination, are highly useful in the process of the design of repeated dose toxicity (RDT) studies. The highest dose-level should not exceed into the range of non-linear kinetics. https://echa.europa.eu/documents/10162/13632/information_requirements_r7c_en.pdf	ECHA, 2017.
OECD	**GD 116**	*Guidance document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting test guidelines 451, 452 and 453* TK studies may provide useful information for determining dose levels for toxicity studies (linear vs. non-linear kinetics). https://www.oecd-ilibrary.org/docserver/9789264221475-en.pdf?expires=1594381783&id=id&accname=guest&checksum=FE8C13FEB8772081DCBA1B6735D19975	OECD, 2012
	GD 151	*Guidance document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test* Aid selection of the route of administration, choice of vehicle, selection of animal species, selection of dosages, information on probable	OECD, 2013

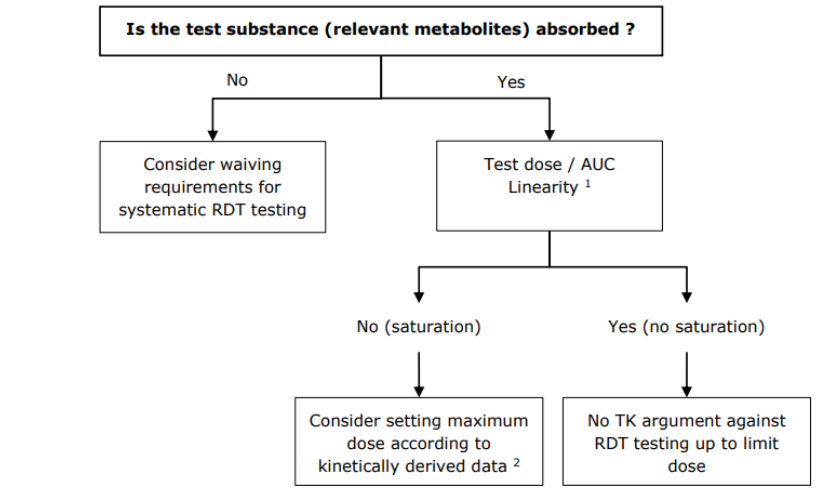
		offspring exposure (in utero or via breast milk) and for interpretation of data obtained from the conduct of TG (test guidelines) 443. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2013)10&doclanguage=en	
USEPA	**Guidance**	*Rodent carcinogenicity studies: Dose selection and evaluation* Recommends 'use of innovative approaches' The highest dose tested should not be above a dose that results in saturation of absorption.	US EPA, 2003
	Guideline	*Guidelines for Carcinogen Risk Assessment* The high dose in long-term studies is generally selected to provide the maximum ability to detect treatment-related carcinogenic effects while not compromising the outcome of the study through excessive toxicity or inducing inappropriate toxicokinetics (e.g., overwhelming absorption or detoxification mechanisms). https://www.epa.gov/sites/production/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf	US EPA, 2005
ICH	**S3A**	*Toxicokinetics: A guidance for assessing systemic exposure in toxicology studies.* The high dose levels in toxicity studies will normally be determined by toxicological considerations. However, the exposure achieved at the dose levels used should be assessed. Where toxicokinetic data indicate that absorption of a compound limits exposure to parent compound and/or metabolite(s), the lowest dose level of the substance producing the maximum exposure should be accepted as the top dose level to be used (when no other dose-limiting constraint applies. Very careful attention should be paid to the interpretation of toxicological findings in toxicity studies (of all kinds) when the dose levels chosen result in non-linear kinetics. However, non-linear kinetics should not necessarily result in dose limitations in toxicity studies or invalidate the findings; toxicokinetics can be very helpful in assessing the relationship between dose and exposure in this situation. https://database.ich.org/sites/default/files/S3A_Guideline.pdf //www.ema.europa.eu/en/documents/scientific-guideline/ich-s-3-toxicokinetics-guidance-assessing-systemic-exposure-toxicology-studies-step-5_en.pdf	ICH, 1994

3.2. How TK can be used for dose selection?

TK data are useful to inform the design of toxicity studies. The first question to be addressed is whether the substance is absorbed. If it can be demonstrated that absorption in animal species does *not* occur, no human exposure is anticipated and so the chemical cannot induce direct systemic effects. Therefore, there is no need for further *in vivo* repeated dose testing (RDT: see Figure 1). However, if absorption *does* occur it will be important to determine the relationship between the administered dose and the systemic exposure. If that relationship is proportional, for example, doubling the administered dose doubles the systemic exposure. However, a non-dose proportional relationship may result from saturable processes in the absorption, distribution, metabolism or excretion of the substance. This applies to both the parent compound as well as its metabolites. Therefore, it is important to assess, not just parent, but *all* toxicologically meaningful circulating metabolites to understand their dose proportionality and how they contribute to the toxicity profile (Rhomberg, 2007). When extensive metabolism of parent compound is expected, early identification and quantification of major metabolites in plasma/blood and urine can become crucial.

When it comes to dose selection for mammalian toxicity studies, human relevance of exposure previously evaluated in animals should be assessed and understood where possible. The systemic exposure to parent compound and major, measurable and/or relevant/toxic metabolites may change with increasing dose, sex, duration, route of exposure, *etc.*, in a manner that may or may not be of human relevance. Typically, the risk of non-relevance may be higher at higher dose levels, where A) high systemic exposures may disrupt physiological detoxification processes or other homeostatic processes leading to overt toxicity, jeopardising appropriate evaluation of the toxicological results, B) high systemic exposures may be quantitatively and qualitatively different from potential human systemic exposure. Both aspects may impact the relevance of the observed high dose effects, for human safety hazard identification and risk assessment. It is recommended that evaluation of target organ toxicity is performed in a dose range covering dose proportional TK, thus avoiding use of high doses in the non-dose proportional range. This method of dose selection can be described as the kinetically-driven maximum dose (KMD), as opposed to dose selection based on the MTD, which is selected on the basis of demonstrable toxicity (i.e., clinical signs and body weight loss / reduction in body weight gain (see section 2.2.3 and 2.3). Therefore, the highest dose level should be set in the dose-proportional range without reaching the toxicity MTD based on apical endpoints.

1A



[1] In the dose-range under consideration for RDT testing

[2] Meaning that the highest dose-level should not exceed into the range of non-linear kinetics.

1B

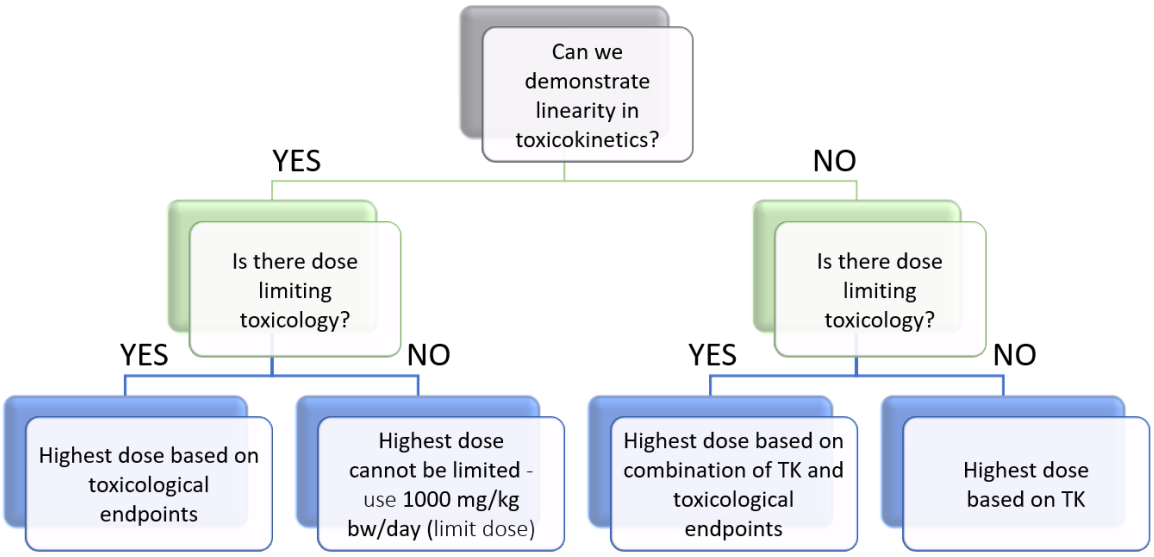


Figure 1: Use of TK data in the design of toxicity studies (Panel 1A, adapted from Figure R.7.12-1 in ECHA, 2017; Panel 1B: high dose selection decision flowchart)

The KMD approach uses relevant departures from linear TK in one or more biomarkers (i.e., parent compound and/or major metabolites) to limit dose level selection in toxicology studies. Thus, TK information is used to determine at what dose level systemic exposures become *non-dose*

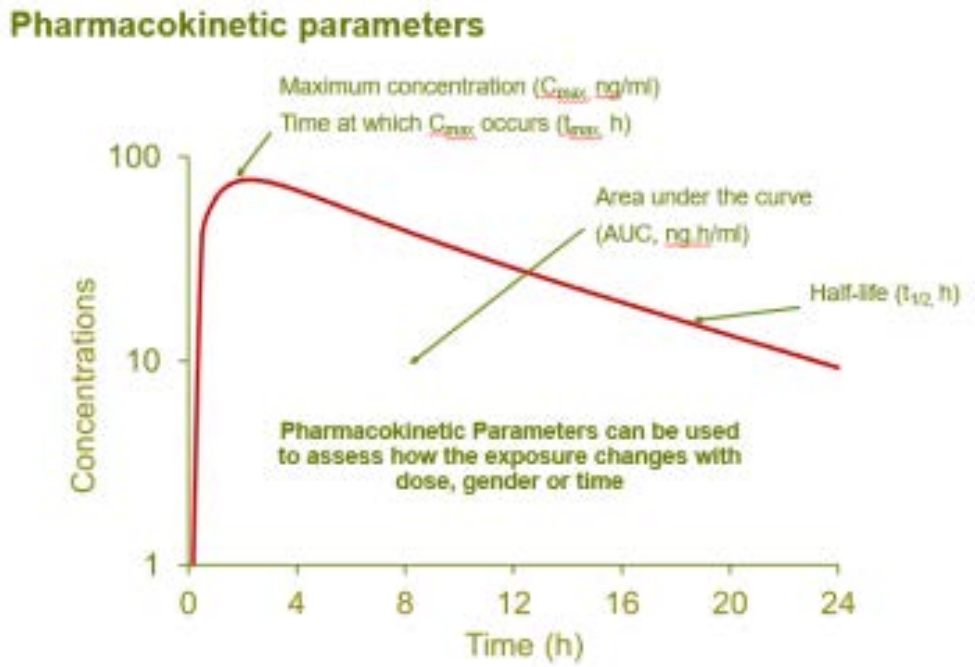
proportional (i.e., due to saturation of metabolic or excretion processes), providing a scientifically-defensible biological basis for selection of lower doses than might otherwise be used in conventional MTD-based testing. The KMD approach uses kinetic information to scientifically derive a limit dose. There is limited value in administering doses at levels where increases only correspond to minimal increases in systemic exposure or, vice versa, where the dose increase may lead to exaggerated systemic exposure not compatible with life over longer exposure durations inducing unnecessary suffering.

Graphical examples may be useful to qualitatively highlight trends and departures from proportionality, for example by plotting concentration-related PK (pharmacokinetics) parameters such as the area under the blood/plasma concentration time curve (AUC) or the maximum blood/plasma concentration (C_{max})) (Figure 2) versus dose. These TK parameters can be displayed either as AUC *vs.* dose (Figure 2B); or as dose-normalised parameters e.g., AUC / Dose *vs.* dose (Fig 2C) and initially examined visually for deviations from dose-proportionality. Note, the AUC is usually preferred to C_{max}, because it relates to both absorption and clearance.

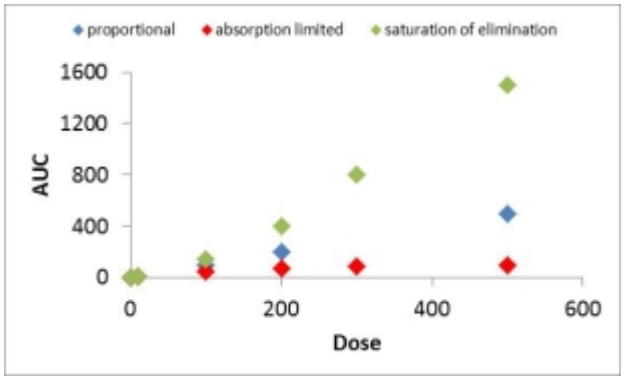
For example, in Figure 2C, the following trends can be seen:

- Dose proportional exposure - the AUC/Dose ratio is constant over the dose range (blue diamonds).
- Absorption limited exposure - Less than proportional increase in exposure (i.e., AUC/dose ratio decreases) with increasing dose (red diamonds).
- Saturation of clearance - more than proportional increase in exposure (i.e., AUC/dose ratio increases) with increasing dose (green diamonds).

2A



2B



2C

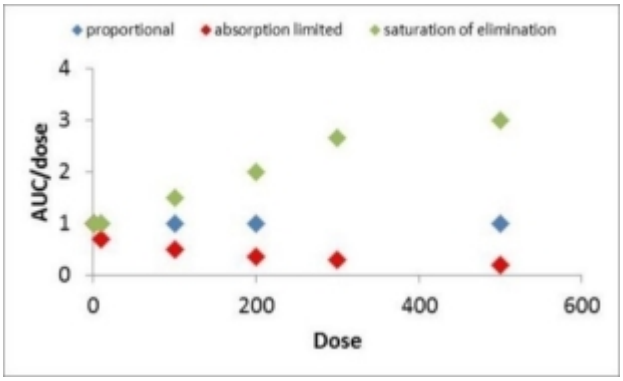


Figure 2: Toxicokinetic parameters (Panel 2A: nomenclature of most commonly used kinetic parameters; Panel 2B and 2C: visual representation of dose proportionality case examples).

While, graphically and statistically (see following sections) it is possible to identify departure from proportionality, it is useful to understand the underlying mechanisms as these may influence the choice of a top dose above or below the statistical range of non-proportionality. It is possible that one or more saturation mechanisms may occur following high dose exposure so that a chemical may appear to display dose-proportionality. For example, both a decrease in oral absorption and saturation of renal active transport processes (excretion) may offset each other.

3.3. TK testing strategy

There are a variety of effective tools to characterise the kinetic behaviour of a substance comprising *in silico, in vitro, in chemico, ex vivo* methods to whole animal *in vivo* models (see the sets of similar "modules" in Figure 3). These studies have been organised describing first the commonly used *in vivo* studies, covering early *in vivo* studies (module 1), integrated TK (module 2) and full ADME studies (module 3) (see Figure 3). The supporting modules (non-testing, *in vitro, in silico*) demonstrate how alternative non-animal tools can aid dose selection and how the incorporation of TK in animal studies improve their interpretability. While recognising that sector specific data requirement exists, in this document the focus will be mostly on the *in vivo* TK testing strategy with some details given for *in silico*, *in vitro* and *ex vivo* models.

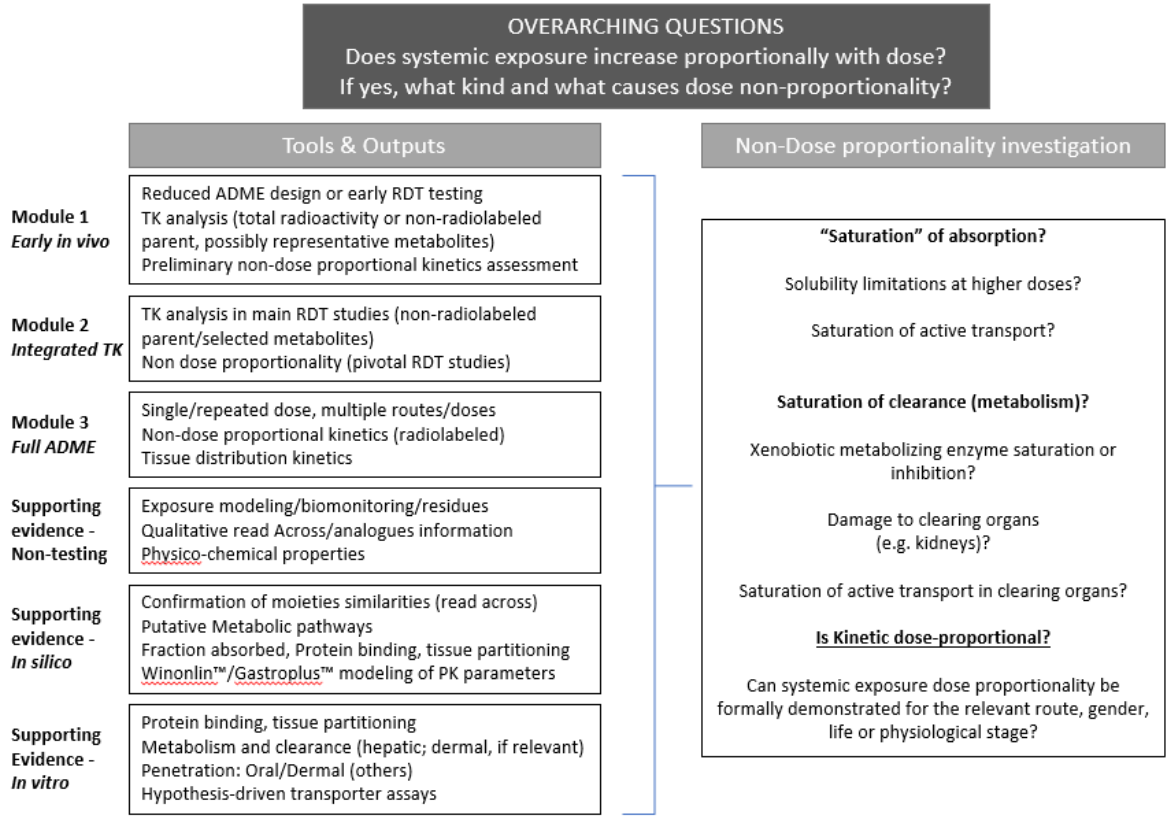


Figure 3: Framework to Integrate Systemic Exposure Evidence into Dose Level Selection

3.4. TK studies and TK models

At each stage of compound development, results influence and inform dose selection and study designs in subsequent investigations; this is also applicable to TK. Usually at early stages, systemic exposure data obtained in subacute dose range finder (DRF) studies can be compared to elucidate species-differences or physiological life-stage adaptations (such as pregnancy for example). Later in the program, TK evaluations in the subchronic studies (e.g., 90-day) can be used to inform dose level selection for longer term carcinogenicity studies (such as carcinogencity and generational studies). Evidence for (non-)dose proportionality in systemic exposure will be generated along the program and may become an important factor for the latter purpose.

3.4.1. Module 1 - Early *in vivo* studies

At early stages of a toxicological program several approaches could be used to obtain first indications whether the parent and/or metabolites show non-dose proportionality. However, at early stages of toxicological programs, the availability of radiolabeled compound or analytical standards may vary. The following examples are given as general guidance for the oral route (one of the most commonly used for testing), but flexibility in study design, route, solvent and other may be driven by sector, or molecule-specific consideration.

3.4.1.1. Single dose TK studies with non-radiolabeled compounds

A single dose plasma TK study via oral gavage with unlabelled test item can be conducted in rats (or species of interest). A group size of n=3 animals per dose (and per sex, if both are used) is recommended, with 2-3 doses. Typically, aqueous suspension with 0.5% to 1% carboxy methyl cellulose (typically solvent) may be preferable for oral bolus; however, test material physical properties must be considered. Use of cannulated animals is optional and blood is collected using a minimum of 6 to 9 time points with animal use and welfare considerations considered. Urine/faeces and target tissue collection is optional, but the resulting data may be useful later in the toxicology assessment program.

Typical study objectives include obtaining initial information on:

- TK parameters (Cmax, AUC, $t_{½}$ (Apparent terminal phase half-life), etc)
- Dose-proportionality – for parent compound and eventual metabolites;
- Metabolism and eventual quantification of major metabolites with elucidation of sex differences;
- Appropriate blood sampling times for subsequent repeat dose toxicology studies;
- Metabolite concentrations in urine or other matrices (optional);
- Metabolite concentrations in target tissues (optional).

3.4.1.2. Probe ADME studies with radiolabeled compounds

Typically, probe ADME studies are carried out in a preferred toxicological species, using a single low (and eventually a high) dose of radiolabeled compound. Similar consideration to the non-radiolabeled design above described are taken into account. In addition, to the above-described parameters, study objectives using a radiolabeled compound are to gain information on:

- Identification and quantification of circulating metabolites (especially their structures)
- Determination of the C_{max}, t_{max} and the area under the plasma/blood concentration/time curve (AUC) of ^{14}C-dose equivalent concentrations for the parent, and for any metabolite
- Evaluation of the concentration ratio of radioactivity in plasma and blood to obtain an indication of preferred partitioning into red blood cells;
- Evaluation of sex-specific differences in metabolism and/or parent/metabolite body burden;
- Determination of the major routes of excretion
- Indications of distribution/accumulation in tissues (optional; depending on the study design)

Results from radiolabeled ADME studies are instrumental to implement bioanalytical methods to support the interpretation of TK. Information from these studies are an important first step in conducting integrated TK studies and informing the design of full ADME studies.

3.4.1.3. Early repeated dose toxicity (or TK) studies

Short-term repeat dose studies (such as a dose-range finder, possibly up to a 28-day treatment duration) are conducted as part of the regulatory toxicology program and it is recommended that

PK/TK measurements are integrated to characterise dose *vs.* exposure relationship and the range of dose proportionality. Options for oral administration include gavage and/or dietary regimens (for further guidance on route differences see also section 3.3.4.4). These studies are normally conducted with non-radiolabeled compound in the rat (or relevant species of interest) of both sexes. If dose proportionality is to be investigated, then usually 3 to 5 dose levels are used (see later section), with plasma-concentration time profiles taken once or twice during the study.

Study objectives are to:

- Assess dose proportionality of parent compound under steady state conditions (see further guidance on steady state in section 3.4.4.1)
- Establish gender effects (differences in systemic exposure (AUC) between the sexes; see further guidance in section 3.4.4.2)
- Establish temporal effects (changes in systemic exposure (AUC) appearing with time that cannot be explained from single dose data: see further considerations on time effect and accumulation, in section 3.4.4.3)

It is recognised that study objectives for these early studies may differ across sectors. Generally, the dose-exposure relationship (i.e., dose-proportionality) is assessed once a steady state concentration has been reached, which for repeat-dosing generally takes approximately five half-lives (Derendorf and Schmidt, 2019). Preliminary information on the half-life would be typically available from previous studies and should be considered in study design. Similarly, dose level selection for these early repeated dose studies should incorporate prior knowledge from early TK studies so that there are at least two dose levels covering the presumed area of dose proportionality, with one dose ranging around the departure from dose proportionality and the other in the non-dose proportionality range. Interpretation of non-dose proportionality of circulating parent and metabolites should be done in conjunction with the initial toxicity endpoints observed and other available data, in order to understand the underlying processes triggering TK non-linearity. Together with the toxicodynamic responses (toxicity apical endpoints), the toxicokinetic evaluation from the dose range finder studies will form the basis of the dose setting rationale for the subsequent toxicity studies.
Finally, comparison of circulating parent/metabolites profiles across early studies will contribute to an overall understanding of the variability in systemic exposure and dose-proportionality in different test system (I.e., multiple species/physiological states (life stages; see section 3.4.4.5 for further guidance).

3.4.2. Module 2 - Integrated toxicokinetics

The integration of pharmacokinetic/toxicokinetic measurements in repeated dose toxicity studies is often referred as “Integrated PK/TK”. The kinetic objectives of the regulatory and non-regulatory studies with integrated TK remains the same as those for the early repeated dose studies i.e., characterising the dose vs exposure relationship and the range of dose proportionality in parallel to toxicity assessment. With progression of product development, evaluations will focus progressively more on steady state toxicokinetics for longer treatment duration and extended to other species and to different physiological states (e.g., gestation, lactation and early life stage). Based on the overall weight of evidence of kinetic and dynamic effects, non-dose proportionality may be considered in the dose level selection. In particular, the top dose of long-term studies pivotal to hazard characterisation and risk assessment, could be chosen taking into account both toxicity and saturation of ADME processes that, in the long run, could severely confound with the evaluation of the intrinsic toxic properties of the compound.

It is recommended that a sufficient number of blood samples is taken within a day to calculate a robust AUC. The timepoint selection will be different depending on the exposure route and the individual molecule characteristics. Blood microsampling can be used in order not to deplete blood volume by conventional sampling methods and to avoid use of satellite animals. The volumes of blood less than 50 µL allows rapid and serial sampling from animals to generate kinetic profiles and this is an improvement to animal health and well-being and a reduction in animal use (Chapman et al., 2014). When using microsampling techniques, serial sampling is recommended, allowing collection of more than 2 blood samples within 24 h from an individual animal. This approach reduces the methodological variation, allows correlating the individual plasma concentration time profiles with the toxicity findings in the same animal, and, for non-dietary studies, allows robust TK modeling of the kinetic curve.

Usually, all animals (including control groups) are bled for TK analyses in the repeat dose-range finder studies in order not to introduce experimental bias. In the longer-term regulatory studies (i.e., 90-day studies, carcinogenicity, two-generation reproductive toxicity) blood samples from subsets (e.g., 4-6 animals) could be used for TK analyses. The day chosen for TK sampling should be sufficiently distant from the day of blood sampling for hematological examinations due to adaptive regenerative anemia potentially caused by the collected blood volumes which may become a confounding factor for hematology (typically a one-week interval is sufficient, when microsampling is employed in rodents; Diehl *et al.*, 2001). In addition to the analysis of blood or plasma, in reproductive studies urine and

milk concentrations can be used as a measure of systemic dose/internal dose in adults (see separate section on collection for different life stage).

3.4.3. Module 3 - Full ADME

Full ADME studies examine in detail the absorption, distribution, metabolism and excretion of a chemical substance and are typically conducted according to established design (OECD 417). These studies provide information on organ distribution and the potential for accumulation of the test substance in tissues and/or organs and the potential for induction of biotransformation as a result of exposure to the test substance.

Prior to full ADME studies, pilots/probes are conducted so that information is available for appropriate study design of the full ADME. These pilot/probes are an important first step in conducting integrated TK studies. The advantages of conducting early these pilot studies have been already described.

Full ADME studies and integrated TK repeat dose studies generate data that support each other.

3.4.4 Additional considerations for assessment of *in vivo* data

3.4.4.1. Dose effect

Dose effects characterise the relationship between systemic exposure and dose. In particular, focus should be on identifying the range over which increases in systemic exposure are proportional with the increase in dose.

Following single dose (bolus) administration, AUC_{inf} (AUC from time zero extrapolated to infinite time) should be used to assess dose proportionality. However, if all doses have been characterised to the limit of quantification and an argument can be made that the extent of extrapolated AUC would be small, especially at the higher doses, then an initial assessment based on AUC_{last} (AUC from time zero to the time of the last quantifiable concentration) will be acceptable.

Following repeat dosing to steady-state the interdosing interval AUC ($AUC_{\tau,ss}$) will be used to assess dose proportionality. In this case it is accepted that the dosing interval will usually be daily administration with the interdosing interval being 24 hours. In some cases, where the compound is

rapidly cleared and there is no accumulation to steady-state it may be necessary to use AUC_{last} in a similar way to that following single dose administration.

An initial assessment of dose proportionality can be made using the Power model (Gough et al., 1995). This model gives the proportional relationship between AUC and dose and is written as a power function

$$y = a \times dose^{b}$$

$$\underline{logy = loga + b \times logdose}$$

The exponent, b, is the estimated slope of the resulting regression line.
The relationship is considered dose proportional when b = 1.

The power model can be fitted to individual and/or mean AUC vs. dose data as appropriate and plotted to give an initial indication of evidence or lack of dose proportionality determined by commenting on the exponent and its closeness to 1. If there is evidence of sub (<1) or supra- (>1) proportionality of AUC with respect to dose, then further investigation may be conducted.
Other methods for assessment of proportionality can be conducted with or without statistical analysis. However, currently there is no international consensus on methodologies that are acceptable at the regulatory level.

3.4.4.2. Gender effect

Gender effects are usually assessed following dosing with the relevant route of administration (or intravenous dosing for certain sectors/specific needs), by comparing the male to female ratios of AUC obtained following the same dose. If the AUC at the same dose is not available then the AUC following dose normalisation may be used, providing the AUCs have been shown to be within the range of dose proportionality. As an empirical guide, a 2-fold difference in the male:female AUC ratio could be interpreted as demonstrating a gender difference with a coefficient of variation (CV%) of the mean AUC for each sex being equal to or less than 30%.

3.4.4.3. Time Effects and Accumulation Ratio (Rac)

To assess whether the TK of the compound changes with time and repeated dose administration, it is important to characterise the TK profile after the first dose (Section 3.4.4.1); sampling should follow the first dose (which acts as the reference point) and the last dose, or on a day close to the end of the study, where steady-state has been achieved. Potential changes in systemic exposure with time should be assessed by comparing estimates of AUC following the first and last dose.

Plots of concentration-time profiles associated with repeat dose administration are a good visual indicator of potential time effects and plots showing the relationship between the first and last dose and the rise to steady-state if pre-dose samples have been taken during the intervening days. The fold-change in exposure obtained on repeat dosing compared to the first dosing interval can also be quantified by calculation of an accumulation ratio (R_{ac}). This is based upon a comparison of the interdosing interval AUCs against using the first dose as the reference point and calculated as $AUC_{\tau\ Day\ (n)}/AUC_{\tau\ Day\ 1}$.

3.4.4.4. Differences between routes of exposure (oral gavage vs dietary route)

Gavage and dietary administration of comparable daily doses may result in quantitative differences of TK profiles in terms of AUC and C_{max} (Figure 4). Whilst the ideal is to use the same blood collection times irrespective of dose route, this is not always achievable due to feeding patterns. So as not to interfere with the dominant night-time feeding pattern of rodents in dietary toxicology studies it is accepted that TK data should only be obtained during daylight hours and as such only provides a snapshot of the TK profile during this time. However, important information can still be obtained relating to trends in exposure vs. dose following gavage administration and the relative bioavailability of dietary vs. gavage administration. This enables conclusions determined following gavage administration to be applied to longer term studies employing dietary administration.

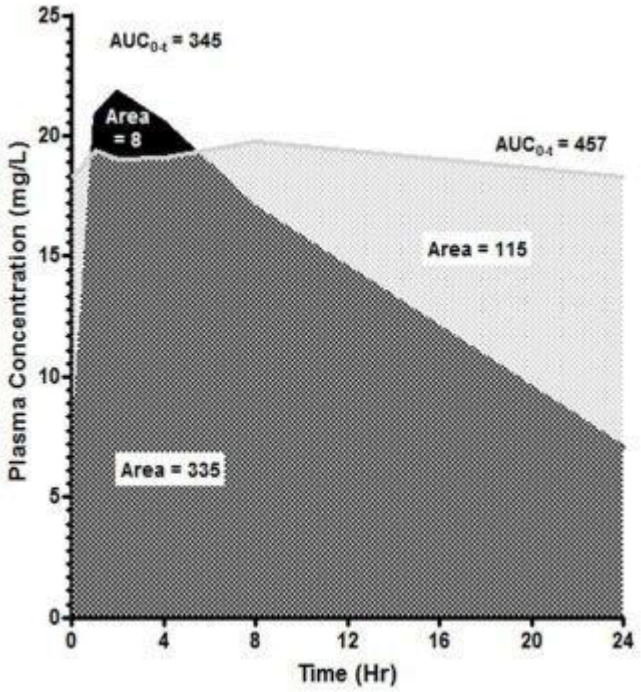


Figure 4: Oral gavage vs dietary exposures (AUC); black/light area represent diurnal plasma concentration of sulfoxaflor after 3 weeks of gavage/dietary administration to rabbits at comparable dose levels (adapted from Hannas B.R. et al., 2016).

Strategies to assess diurnal systemic exposures have been published in literature (see in example Saghir *et al.*, 2006 and Saghir *et al.*, 2012). Four blood samples over the twelve-hour light cycle are sufficient to generate a robust TK profile. The AUC_{24} at steady-state is typically calculated, as described by Jochemsen *et al.,* 1993, by taking the concentrations measured during the daylight hours and using the concentration measured at lights on again at 24 hours post the original time point to cover night-time exposure

The general approach with dietary studies is to look for trends to support findings that have been observed in gavage study data or to direct where to focus gavage studies used to set doses. The aim should be to calculate comparative parameters supported by the data, but keeping it simple so as not to over interpret.

3.4.4.5. TK differences based on altered physiological states

A variety of physiological alterations during pregnancy (e.g. total body water, plasma proteins, body fat, renal blood flow, transporters, fetoplacental compartment formation) can influence the kinetic behaviour of chemicals. Hence, selection of dose levels and route of administration are important study design considerations prior to the conduct of definitive embryo-fetal developmental toxicity studies. Preliminary TK data is obtained from an early toxicity study in non-pregnant animals, a dose-range finding study and/or a preliminary embryo-fetal developmental toxicity study. These data should be used to compare non-pregnant and pregnant TK profiles for dose level selection. Further guidance on the principles of dose selection in pregnant animals, including OECD TG 414, TG 416, TG 421, TG 422, TG 426 and TG 443, can be found in Table 5 and in relevant literature (Johnson et al., 2016).

3.4.5. Supporting evidence

3.4.5.1. Non-test methods and in silico tools

Prior to animal testing, information from non-test methods, such as data gathering and analysis, literature searches, and/or use *of in silico* toxicity tools or computational methods, provides a unique advantage of being able to predict toxicity.

In silico tools can aid in the prediction of ADME, which is often based on structure and/or physiochemical properties of the compound (Cronin and Madden, 2010). Integrating and applying this evidence could minimise late-stage study design failure and improve toxicity prediction and safety assessment. For example, information available on structural analogues or compounds of the same drug class, chemical or mode of action (MoA) may help speculate about the ADME profile of the compound of interest. In this case, certain kinetic information (e.g., AUC, C_{max}, T_{max}) may be anticipated to confirm any potential risk (e.g., toxic metabolites, bioaccumulation) and alternative compounds can be investigated. Although, non-testing TK methods may not always provide enough evidence to be used in dose level selection, information gathering can provide useful information for making biological justification(s), which may lead to further hypothesis-driven investigations.

Early use of *in silico* tools, such as with quantitative structure–activity relationship (QSAR) models summarise a supposed relationship between chemical structures and biological activity in a data-set of chemicals and can predict key information that can be useful, which includes: 1) identifying potential structural alerts and predictions of potential metabolites (e.g. epoxide formation, ester cleavage, putative oxidation, dealkylation, conjugation sites etc); 2) estimating certain physicochemical properties of the chemical (e.g. water solubility, log Pow, dissociation constant, pKa); and 3) modelling certain PK parameters such as AUC and fraction absorbed. *In silico* toxicology encompasses a wide variety of computational tools, and have been reviewed (Gleeson et al., 2012; Raies and Bajic, 2016).

Particularly for the chemical industry, the use of non-animal testing strategies is strongly encouraged by the European Union (EU), including the REACH legislation where it is stipulated to avoid animal experiments whenever possible (Annex XI of Regulation (EC) No. 1907/2006, REACH; EC, 2006)

3.4.5.2. In vitro tools

Particularly for the agrochemical industry, information from *in vitro* data is useful to confirm putative metabolic pathways, which includes metabolites that might be considered important for inclusion in the exposure assessment of subsequent *in vivo* TK studies, and to understand potential for fast clearance vs. bioaccumulation using relevant test systems. Some examples of commonly performed study types used are: 1) protein binding (to target, if known); 2) unbound fraction (plasma protein binding); 3) clearance of parent compound (typically rat microsomes); 4) metabolism of parent compound, study may employ S9, microsomes, hepatocytes from one species or from multiple species (Whalley, et al., 2017). Other *in vitro* studies that can provide useful early information are early penetration studies, particularly via the dermal route (human skin cells), or oral route (Caco-2 cells), and hypothesis-driven transporter or tissue partitioning assays characterise certain class specific effects on distribution, metabolic stability/clearance, excretion (Sambuy et al., 2005; Kleinstreur et al., 2018). Some of the parameters obtained in these assays may be useful as molecule specific input parameters in early physiologically based pharmacokinetic (PBPK) model building. For instance, some substances may have short half-lives, or the target tissue levels may be different from blood. This may be due to accumulation of a substance due to protein binding (e.g., total, free or bound plasma

fractions). All of these parameters should be considered when developing a multispecies PBPK model for more appropriate IVIVE (in vitro-to-in vivo extrapolation) studies (Martin et al., 2015).

Another application for dose level selection, is the use of high-throughput TK is reverse TK or "reverse dosimetry", which estimates the *in vivo* dose necessary to reach a tissue concentration that is active *in vitro* (Wetmore et al., 2012; Hartung, 2018; Honda et al., 2019). *In vitro* assays are usually conducted to elicit a dose-response and a concentration where half-maximal effects are observed (AC_{50}) and efficacy if data described by a Hill function are identified (Pearce et al., 2017). HTTK (High Throughput Toxico Kinetic) models can predict *in vivo* oral equivalent doses (i.e., NOAEL, LOAEL). When selecting the appropriate dose, consideration should be given to use a dose that will produce steady-state blood levels comparable to the *in vitro* assay concentration (i.e., AC_{50}) (Rotroff et al., 2010; Judson et al., 2011; Wetmore et al., 2012; Wambaugh et al., 2019). Therefore, caution should be taken when selecting *in vitro* concentrations because there are a number of factors that can alter the true exposure concentration of a substance and could introduce significant error into any nominal dose-based model (Gülden and Seibert, 2003; Kramer et al., 2012; Teeguarden and Barton, 2004; Truisi et al., 2015). Consideration should be given to critical assay components (e.g., percent serum in media, media volume, cell number) along with the physicochemical properties of the substance to calculate mass distribution of a chemical within the system (Armitage et al., 2014).

IVIVE is defined as the qualitative or quantitative transposition of experimental results or observations made in vitro to predict in vivo endpoint outcomes in biological organisms. Since in vitro experiments are not directly translatable to predict in vivo biological responses to chemical exposures in vivo, it is extremely important to develop a common biological scaffold (e.g., mode of action, key event, biological pathway, exposure) for which reliable in vitro to in vivo extrapolation can be applied (Yoon et al., 2014; Bell et al., 2018). IVIVE models can be used to assess pharmacokinetics or pharmacodynamics. For instance, a model of the dose-response relationship for a chemical observed *in vitro* can aid in the prediction of *in vivo* effects.

3.5. Conclusion

In summary, the use of TK in dose level selection highlights the important considerations that should be taken during early phases of toxicity study design. Kinetic endpoints are commonly used as a biological scaffold because there is a clear translational purpose; however, a biological justification should always be given. With a global push to align with the 3Rs, there is pressure to use alternative test methods. Hence there will be more integration of *in vivo* and non-animal test methods (e.g., data gathering, *in silico, in vitro* approaches). This exercise highlighted the discordance across industry sectors on the acceptability of how to apply these methods toward selecting dose levels for toxicity testing. Further investigations on how to better harmonise these methods are warranted as new approaches continue to be developed to improve study design and reduce animal usage.

4. TOXICODYNAMICS AND MODE OF ACTION

4.1. Approaches to investigating mode of action and working within defined mode of action groups

In certain circumstances there is no guidance on dose level selection. The purpose of this section is to illustrate case-by-case approaches in situations where insight into the mode of action (MoA) and/or the relevance of this MoA to humans is sought; and situations where alternative options for dose level selection can be identified within a well understood and defined MoA class. Although this approach illustrates some key scientific examples it is generally approached on a case-by-case basis, it is included in this report for completeness, but is generally out of the scope of most repeat studies conducted for registration purposes.

Appendix B contains several examples, in which the mechanistic factors and mode of action of chemicals can be used as a tool for justification of a suitable high dose level. This is important, since toxicological effects which are not relevant for human risk assessment can be avoided. However, it must be mentioned that a considerable amount of knowledge must be available to build up the framework of mode of action, before it can used to form a rationale for dose level selection and for judgements.

5. Cross Sector pre-requisites for dose selection

As covered in the Introduction and Background, different industry sectors have different levels of freedom to operate in dose level selection for repeat dose studies. However, there is some basic knowledge that may be taken into account prior to repeat dose studies regardless of the industry sector. The review of existing information is important for decisions on the route of administration, the choice of any dosing vehicle, the selection of animal species, dosages and potential modifications of the dosing schedule. Therefore, all relevant available information on the test chemical, i.e., physico-chemical, toxicokinetics (including species-specific metabolism), toxicodynamic properties, structure-activity relationships (SARs), in vitro metabolic processes, results of previous toxicity studies and relevant information on structural analogues should be taken into consideration in planning any repeat dose toxicity study. Limited predictions of absorption, distribution, metabolism and elimination (ADME) and bioaccumulation may be derived from chemical structure, physico-chemical data, extent of plasma protein binding or toxicokinetic (TK) studies, while results from toxicity studies give additional information, e.g., on NOAEL, metabolism or induction of metabolism (OECD 443, 2018).

Before selecting appropriate dose levels, the investigator should consider all available information, including:

5.1. Dosing information from previous studies

All the available toxicological data should be analysed before selecting dose in a new study.

5.2. Hazardous properties (e.g., irritant, corrosive, sensitisation)

Extensive irritation can cause a disruption in the natural barrier of the tissue system at the portal of entry, whether the skin, the gastric lining or the nasal epithelium, with inflammation, hyperkeratosis, ulceration and breakdown of epithelial integrity (cytotoxicity, venous access, scarring etc.). This can result in altered absorption of the material and unrealistic dosing and exposure scenarios. It is equally important to consider the animal welfare concerns of testing such chemicals. Approaches should be

taken to minimise potential pain and distress, which can be challenging to determine in laboratory animals.

5.3. Toxicokinetic data and bioavailability

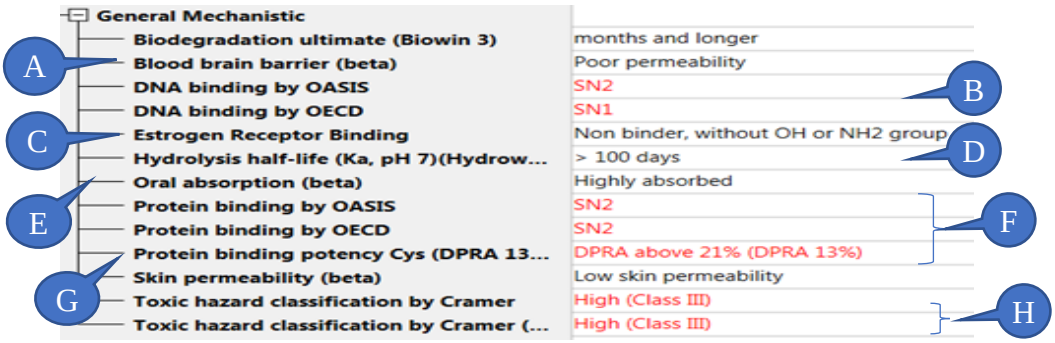
TK is not a toxicological endpoint and is not mandated as a specific requirement in regulatory guidelines or guidance (for example REACH), however the generation of TK information can be of great importance as a means to interpreting data, assisting with a testing strategy and to inform study design.

A knowledge of toxicokinetics may be considered as part of the basic information required to select the doses for repeated toxicity studies (see section 3). Toxicokinetic behaviour is normally assumed to be similar in animals and humans, at least from a qualitative perspective. Bioavailability is defined as the proportion of a drug or other substance which enters the circulation when introduced into the body and so has the ability to have an active effect. It is well documented within literature that LogKow, molecular weight (e.g., >500) and molecular flexibility, measured by number of rotatable bonds, low polar surface area or total hydrogen bond count are all important predictors of oral bioavailability (Veber et al., 2002) and therefore such information could be used to select the highest dose in experimental studies.

5.4. QSARs

Prior to starting any testing there is a plethora of computer-based models that can be used to obtain an understanding of a chemical structures, mechanisms and facilitate focus on key toxicological endpoints that should be considered. The most easily accessible and widely used is the OECD QSAR toolbox. Use of this software can identify structurally similar substances and identify opportunities for read across, but also the profiling module is a good early warning system to indicate toxicological mechanisms or endpoints that could be sensitive during in-vivo testing.

An example of how the General Mechanistic module of the QSAR toolbox can be used to create a toxicological profile for an epoxide is presented below:



A. Effects on Central nervous system and neural pathways unlikely

B. Structural alert identifies that substance is likely to be positive in an in-vitro Ames test **Consideration 1: Is this sufficient to not conduct and in-vitro Ames test and move directly to the in-vivo micronucleus?**

C. Based on the chemical structure (e.g ring structure, hydroxyl, amino groups and molecular weight there is no concern of the substance binding to the estrogen receptor

D. At pH 7 hydrolysis is likely to be slow. Within the rat digestive tract, the pH of the stomach is ca. 4.0 but from the duodenum to the colon is pH 6.0 to 7.0 (Eastman and Miller, 1935). Under the acidic conditions of the stomach the epoxide ring is attacked to create a trans-diol (see image below) and therefore

E. **Consideration 2:** what happens to hydrolysis DT_{50} at pH 4.0? Exposure to the stomach is less than the duodenum and intestines, emptying half time of the stomach in rats is ca. 25 min in the stomach and varies from 30 to >120 min through each quarter of the intestine (Purdon and Bass, 1973).

F. Oral absorption can be expected making the route suitable for testing

G. Substance is likely to be a strong skin sensitiser

H. Skin permeability is low and therefore systemic exposure via this route is unlikely

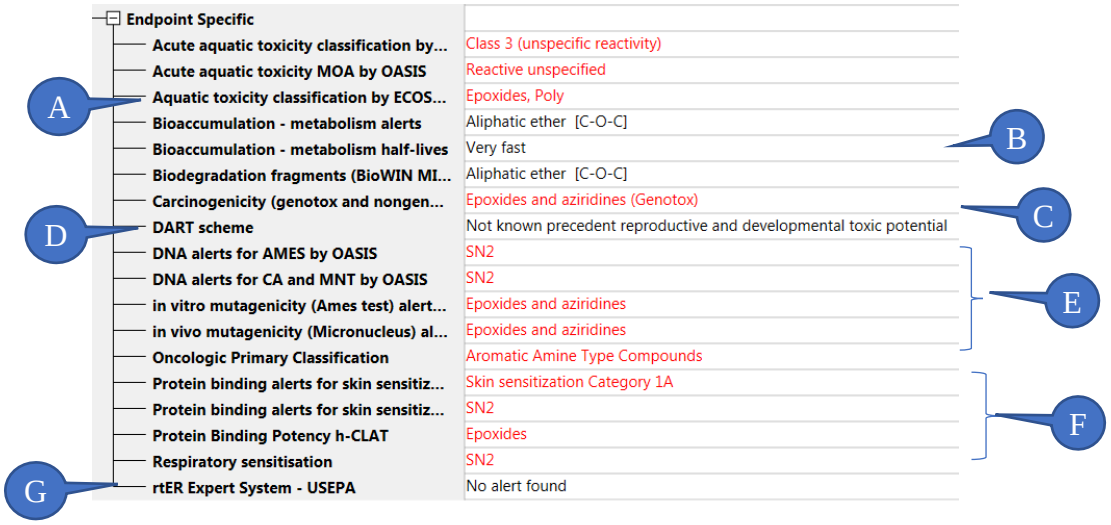
I. During experimental exposure toxicity is expected

Epoxides react with aqueous acid to create trans diols

It can therefore be assumed that following oral gavage there is a period of time (ca. 25 min) whereby an epoxide molecule is in aqueous acid conditions, whilst the transit rate is likely to be somewhat consistent, overload of this process could occur at excessively high doses leading to incomplete trans-diol formation and increasing concentrations of parent epoxide molecules entering the duodenum

from the stomach, this in turn results in site of contact effects due to the presence of highly reactive species, slower intestinal transit, increased contact time and cytotoxicity to epithelial membranes.

In addition to a mechanistic assessment, it is possible to conduct an endpoint specific profile:



A. Substance is an epoxide

B. Metabolism half-live will be very fast

C. Due to the large ring strain associated with the three-membered ring, epoxides are considered highly reactive molecules that could react (ring opening) with nucleophilic centres of DNA (Deoxy Ribose Nucleic Acid) molecules and result in alkylated products

D. Model was developed by Wu et al. (2013) based on the combination of known modes of action (MOA) and associated structural features, as well as an empirical association of structural fragments within molecules of reproductive or developmental toxic (DART) chemicals when MOA information was lacking. Based on this model reproductive or development effects from this substance is unlikely

E. Due to the reactive nature of epoxides and ring opening the Ames test results in positive findings. In this case an in-vivo micronucleus test has been conducted and the epoxide was determined to be negative thereby over-riding the *in silico* alert.

F. Sensitising properties expected

G. No binding to estrogenic nuclear receptor expected

Based on the results of the profiler a variety of assumptions of the profiled substance can be made to assist in test selection and dosing regime (1) the substance is a strong sensitiser (2) in-vitro mutagenicity tests designs are likely to yield positive results (3) Oral exposure would be a conservative method of dosing as oral absorption of the substance will take place (4) acidic conditions of the

stomach and increased intestinal transit time at pH7 could facilitate the residence of un-opened reactive epoxide structures in the intestinal tract leading to irritation and sensitisation at the site of contact. This could lead to false positive in in-vivo mutagenicity studies (5) Reproductive and developmental effects are not expected. However, damage to the digestive tract could result in compromised animal health during chronic exposure due to reduced consumption, reduced weight gain and other secondary effects associated with a compromised diet. It would be beneficial to conduct a hydrolysis study to better understand the impact of acidic pH on the DT_{50} of the substance e.g., a decrease in DT_{50} at pH 4 could indicate an increased rate of transformation of the epoxide to benign trans diols and protection of the duodenum and intestine. Exposure via the dermal route is not expected and neither is binding to estrogen receptors or effects on the CNS (Central Nervous System).

In conclusion, when selecting doses for toxicological testing damage to the gastrointestinal tract could be an apical endpoint for use in selecting an appropriate MTD and it would be beneficial to conduct histopathology of these tissues during range finding studies.

5.5. Data from structurally similar molecules

Prior to testing there should be consideration of the presence of data on structural analogues this could be based on a company's internal analytical expertise or by use of the OECD QSAR toolbox. This analysis could identify a suitable read-across opportunity to a substance which has available data which would negate the need for testing and in some circumstances whereby a specific read-across candidate is not identified it may highlight data for substances that are partially similar e.g., through the sharing of functional groups and provide understanding of possible modes of action, target organs and indications of data which may help in selecting dose groups for a target substance.

5.6. Estimate of human/worker exposure

Outside of the Pharmaceutical area where human exposure is intended, exposure in other industry sectors can be inferred. For example, during manufacturing, industrial and professional activities there are numerous Environmental, Health and Safety standards that have to be followed. In some cases, there may be availability of on-site air and workplace monitoring data, or dietary exposure data for

agrochemicals which could provide measurements of real-life exposure to certain substances, and availability of such data could be used to select the highest dose groups in experimental studies. For example, if monitoring data is available for all registered exposure scenarios during the manufacture of a substance and over the course of a working day it can be identified that a 100 kg worker without PPE (Personal Protective Equipment) could be exposed to 1 mg/kgbw/d (e.g., 100 mg human equivalent). In this situation is it really justifiable and in the interests of animal welfare to conduct an OECD 443 at 100 mg/kgbw/d (e.g., 10,000 mg human equivalent)?

5.7. Routes of relevance (oral gavage, dietary, dermal or inhalation)

The oral route is most often used for repeated toxicity studies. Repeated toxicity study by inhalation is appropriate in case of fine power, aerosol applications, volatile liquids or gas.

Considering repeated dose toxicity testing, the oral route is the default one because it is assumed to maximise systemic availability (internal dose) of most substances. However, the oral route generalises and does not differentiate between oral gavage and dietary, which creates inconsistency in experimentally derived endpoints as a dietary route of exposure could be influenced by different kinetics, physical chemical properties such as LogKow and LogKocC. On a case-by-case basis, the appropriateness of other routes of administration should also be assessed.

Table 5: Dose selection recommendations and Toxicokinetics in OECD Test Guidelines for repeated dose toxicology studies

Recommended maximal dose	Additional information on dose selection	Toxicokinetics
TG OECD 407: Repeated dose 28-day oral toxicity study in rodents (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en		
1000 mg/kgbw/d	Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. In the presence of observed general toxicity (e.g. reduced body weight, liver, heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on immune, neurological or endocrine sensitive endpoints should be interpreted with caution.	Dose selection Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test compound or related materials. Route of administration The method of oral administration is dependent on the purpose of the study, and the physical/chemical/toxico-kinetic properties of the test material.
TG OECD 408: Repeated dose 90-day oral toxicity study in rodents (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en		
1000 mg/kgbw/d	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 chemical, 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	Dose selection 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.

	demonstrating any dosage related response and a NOAEL at the lowest dose level.	
OECD TG 409: Repeated dose 90-day oral toxicity study in non-rodents* (1998) https://www.oecd-ilibrary.org/fr/environment/test-no-409-repeated-dose-90-day-oral-toxicity-study-in-non-rodents_9789264070721-en		
1000 mg/kgbw/d	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.	Dose selection 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. Species choice The revised guideline allows for the identification in non-rodent species of adverse effects of chemical exposure and should only be used [..] where toxicokinetic studies indicate that the use of a specific non-rodent species is the most relevant choice of laboratory animal.
OECD TG 414: Prenatal developmental toxicity study# (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-414-prenatal-development-toxicity-study_9789264070820-en		
1000 mg/kgbw/d	Unless limited by the physical/chemical nature or biological properties of the test chemical, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death or severe suffering.	Dose selection Dose levels should be selected taking into account any existing toxicity data as well as additional information on metabolism and toxicokinetics of the test chemical or related materials.

		Interpretation of results The results of the study should be interpreted in conjunction with the findings of sub-chronic, reproduction, toxicokinetic and other studies
OECD TG 416: Two-generation reproduction toxicity study (2001) https://www.oecd-ilibrary.org/fr/environment/test-no-416-two-generation-reproduction-toxicity_9789264070868-en		
1000 mg/kgbw/d	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. In case of unexpected mortality, studies with a mortality rate of less than approximately 10 percent in the parental (P) animals would normally still be acceptable. Dose levels should be selected taking into account any existing toxicity data, especially results from repeated dose studies. Any available information on metabolism and kinetics of the test compound or related materials should also be considered.	Dose selection Any available information on metabolism and kinetics of the test compound or related materials should also be considered [for dose selection]. Interpretation of results The physico-chemical properties of the test substance, and when available, toxicokinetics data should be taken into consideration when evaluating test results. The results of the study should be interpreted in conjunction with the findings of subchronic, prenatal developmental and toxicokinetic and other available studies. T
OECD TG 421: Reproduction/developmental toxicity screening test (2016) https://www.oecd-ilibrary.org/fr/environment/test-no-421-reproduction-developmental-toxicity-screening-test_9789264264380-en		
1000 mg/kgbw/d	Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be	Dose selection Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available.

	chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects (NOAEL) at the lowest dose level. In the presence of observed general toxicity (e.g., reduced body weight, liver, heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g., reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.	
OECD TG 422: Combined repeated dose toxicity study with the reproductive/developmental toxicity screening test (2016) https://www.oecd-ilibrary.org/fr/environment/test-no-422-combined-repeated-dose-toxicity-study-with-the-reproduction-developmental-toxicity-screening-test_9789264264403-en		
1000 mg/kgbw/d	Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be chosen with the aim of inducing toxic effects but not death nor obvious suffering. In the presence of observed general toxicity (e.g., reduced body weight, liver, heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g., reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.	Dose selection Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available.
OECD TG 424: Neurotoxicity study in rodents (1997) https://www.oecd-ilibrary.org/fr/environment/test-no-424-neurotoxicity-study-in-rodents_9789264071025-en		
1000 mg/kgbw/d	Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or	Dose selection

	related materials. The highest dose level should be chosen with the aim of inducing neurotoxic effects or clear systemic toxic effects. Where there is a reasonable estimation of human exposure this should also be taken into account.	Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or related materials. Route of administration Considerations of the choice of the route of administration depend on the human exposure profile and available toxicological or kinetic information. Frequency of observation If kinetic or other data generated from previous studies indicates the need to use different time points for observations, tests or post-observation periods, an alternative schedule should be adopted in order to achieve maximum information.
OECD TG 426: Developmental neurotoxicity study (2007) https://www.oecd-ilibrary.org/fr/environment/test-no-426-developmental-neurotoxicity-study_9789264067394-en		
1000 mg/kgbw/d	Unless limited by the physico-chemical nature or biological properties of the substance, the highest dose level should be chosen with the aim to induce some maternal toxicity (e.g., clinical signs, decreased body weight gain (not more than 10%) and/or evidence of doselimiting toxicity in a target organ). The high dose may be limited to 1000 mg/kg/day body weight, with some exceptions. For example, expected human exposure may indicate the need for a higher dose	Dose selection Dose levels should be selected taking into account all existing toxicity data as well as additional information on metabolism and toxicokinetics of the test substance or related materials.

	level to be used. Alternatively, pilot studies or preliminary range-finding studies should be performed to determine the highest dosage to be used which should produce a minimal degree of maternal toxicity. If the test substance has been shown to be developmentally toxic either in a standard developmental toxicity study or in a pilot study, the highest dose level should be the maximum dose which will not induce excessive offspring toxicity, or in utero or neonatal death or malformations, sufficient to preclude a meaningful evaluation of neurotoxicity.	
TG OECD 443: Extended one-generation reproductive toxicity study (EOGRTS) (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-443-extended-one-generation-reproductive-toxicity-study_9789264185371-en		
1000 mg/kgbw/d	In the absence of relevant TK data, the dose levels should be based on toxic effects, unless limited by the physical/chemical nature of the test chemical. If dose levels are based on toxicity, the highest dose should be chosen with the aim to induce some systemic toxicity, but not death or severe suffering of the animals.	Dose selection When selecting appropriate dose levels, the investigator should consider all available information, including the dosing information from previous studies, TK data from pregnant or non-pregnant animals, the extent of lactational transfer, and estimates of human exposure. If TK data are available which indicate dose dependent saturation of TK processes, care should be taken to avoid high dose levels which clearly exhibit saturation, provided of course, that human exposures are expected to be well below the point of saturation. In such cases, the highest dose level should be at, or just slightly above the inflection point for transition to nonlinear TK behaviour. Considerations of TK data

		Although not required, TK data from previously conducted dose range-finding or other studies are extremely useful in the planning of the study design, selection of dose levels and interpretation of results. Of particular utility are data which: 1) verify exposure of developing fetuses and pups to the test compound (or relevant metabolites), 2) provide an estimate of internal dosimetry, and 3) evaluate for potential dose-dependent saturation of kinetic processes. Additional TK data, such as metabolite profiles, concentration-time courses, etc. should also be considered, if they are available. Supplemental TK data may also be collected during the main study, provided that it does not interfere with the collection and interpretation of the main study endpoints. As a general guide, the following TK data set would be useful in planning the Extended One-Generation Reproductive Toxicity Study: late pregnancy (e.g., Gestation Day 20, maternal blood and foetal blood), mid-lactation (PND 10 (Post Natal Day), maternal blood, pup blood and/or milk), early post-weaning (e.g., PND 28, weanling blood samples).
OECD TG 451: Carcinogenicity studies (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-451-carcinogenicity-studies_9789264071186-en		
1000 mg/kgbw/d	Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account	Dose selection Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take

	any existing toxicological and toxicokinetic data available for the test chemical or related materials. In the dose selection the investigator should also consider and ensure that data generated is adequate to fulfil the regulatory requirements across OECD countries as appropriate (e.g., hazard and risk assessment, classification and labelling, ED assessment, etc.) Unless limited by the physical-chemical nature or biological effects of the test chemical, the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. [..] the highest dose level should normally be chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%). However, [..] a top dose lower than the dose providing evidence of toxicity may be chosen, e.g., if a dose elicits an adverse effect of concern that nonetheless has little impact on lifespan or body weight.	into account any existing toxicological and toxicokinetic data available for the test chemical or related materials. [..] Points to be considered in dose selection include: - Toxicokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur. - Known or suspected nonlinearities or inflection points in the dose–response.
OECD TG 452: Chronic toxicity studies (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-452-chronic-toxicity-studies_9789264071209-en		
1000 mg/kgbw/d	Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test chemical or related materials. In the dose selection the investigator should also consider and ensure that data generated is adequate to fulfil the regulatory requirements across OECD countries as appropriate (e.g., hazard and risk assessment, classification and labelling, ED assessment, etc.).	Dose selection Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test chemical or related materials. [..] Points to be considered in dose selection include: - Toxicokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur.

	Unless limited by the physical-chemical nature or biological effects of the test chemical, the highest dose level should normally be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. [..] the highest dose level should be chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%). However, dependent on the objectives of the study, a top dose lower than the dose providing evidence of toxicity may be chosen, e.g., if a dose elicits an adverse effect of concern that nonetheless has little impact on lifespan or body weight. The top dose should not exceed 1000 mg/kg body weight/day (limit dose).	- Known or suspected nonlinearities or inflection points in the dose–response.
OECD TG 453: Combined chronic toxicity / carcinogenicity studies (2018) https://www.oecd-ilibrary.org/fr/environment/test-no-453-combined-chronic-toxicity-carcinogenicity-studies_9789264071223-en		
1000 mg/kgbw/d	Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test chemical or related materials. In the dose selection the investigator should also consider and ensure that data generated is adequate to fulfil the regulatory requirements across OECD countries as appropriate (e.g., hazard and risk assessment, classification and labelling, ED assessment, etc.). For the chronic toxicity phase of the study, a full study using three dose levels may not be considered necessary, if it can be anticipated that a test at one dose level, equivalent to at least 1000 mg/kgbw/d, is unlikely to produce adverse effects. This should be based on information from preliminary studies and a consideration that toxicity	Dose selection Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test chemical or related materials. [..] Points to be considered in dose selection include: - Toxicokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur. - Known or suspected nonlinearities or inflection points in the dose–response.

	would not be expected, based upon data from structurally related substances. A limit of 1000 mg/kgbw/d may apply except when human exposure indicates the need for a higher dose level to be used. Unless limited by the physical-chemical nature or biological effects of the test chemical, the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. The highest dose level should be normally chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%). However, dependent on the objectives of the study, a top dose lower than the dose providing evidence of toxicity may be chosen, e.g., if a dose elicits an adverse effect of concern, which nonetheless has little impact on lifespan or body weight.	
OECD TG 474: Mammalian erythrocyte micronucleus test (2016) https://www.oecd-ilibrary.org/fr/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264264762-en		
> 14 days exposure: 1000 mg/kgbw/d < 14 days exposure: 2000 mg/kgbw/d	Identify the maximum tolerated dose (MTD), defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study period (for example, by inducing body weight depression or hematopoietic system cytotoxicity, but not death or evidence of pain, suffering or distress necessitating humane euthanasia). Highest dose may also be defined as a dose that produces toxicity in the bone marrow (e.g., a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or	<u>Dose selection</u> Substances that exhibit saturation of toxicokinetic properties, or induce detoxification processes that may lead to a decrease in exposure after long-term administration, may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

	peripheral blood of more than 50%, but to not less than 20% of the control value). If toxicity is identified in a range finding test the MTD should be the highest dose administered and the dose levels used should preferably cover a range from the maximum to a dose producing little or no toxicity.	
OECD TG 488: Transgenic rodent somatic and germ cell gene mutation assays (2020) https://www.oecd-ilibrary.org/fr/environment/test-no-488-transgenic-rodent-somatic-and-germ-cell-gene-mutation-assays_9789264203907-en		
>14 days exposure: 1000 mg/kgbw/d <14 days exposure: 2000 mg/kgbw/d	Dose levels should be based on the results of a dose range-finding study measuring general toxicity that was conducted by the same route of exposure, or on the results of preexisting sub-acute toxicity studies. Non-transgenic animals of the same rodent strain may be used for determining dose ranges. The top dose should be the Maximum Tolerated Dose (MTD). The MTD is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Test chemicals with specific biological activities at low non-toxic doses (such as hormones and mitogens), and test chemicals which exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The dose levels used should cover a range from the maximum to little or no toxicity.	<u>Dose selection</u> Test chemicals which exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. <u>Administration period</u> In any case, all available information (e.g., on general toxicity or metabolism and pharmacokinetics) should be used when justifying a protocol. <u>Sampling time</u> TGR (Trans Genic Rodent) assays are well-suited for the study of gene mutation induction in male germ cells, in which the timing and kinetics of spermatogenesis have been well-defined.

		<u>Selection of tissues</u> The choice of tissues should be based on considerations such as: [..] pharmacokinetic parameters observed in general toxicity studies, which indicate tissue disposition, retention or accumulation, or target organs for toxicity.
OECD TG 489: In vivo mammalian alkaline comet assay (2016) https://www.oecd-ilibrary.org/fr/environment/test-no-489-in-vivo-mammalian-alkaline-comet-assay_9789264264885-en		
>14 days exposure: 1000 mg/kgbw/d <14 days exposure: 2000 mg/kgbw/d	If a preliminary range-finding study is performed because there are no suitable data available from other relevant studies to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study according to current approaches for conducting dose range-finding studies. The study should aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects relative to the duration of the study period (for example, clear clinical signs such as abnormal behaviour or reactions, minor body weight depression or target tissue cytotoxicity), but not death or evidence of pain, suffering or distress necessitating euthanasia. The dose levels used should also preferably cover a range from the maximum to one producing little or no toxicity. When target tissue toxicity is observed at all dose levels tested, further study at non-toxic doses is advisable.	<u>Dose selection</u> Substances that exhibit saturation of toxicokinetic properties, or induce detoxification processes that may lead to a decrease in exposure after long-term administration, may be exceptions to the dose setting criteria and should be evaluated on a case-by-case basis. <u>Sample time</u> The optimum sampling time(s) may be substance- or route specific resulting in, for example, rapid tissue exposure with intravenous administration or inhalation exposure. Accordingly, where available, sampling times should be determined from kinetic data (e.g., the time (Tmax) at which the peak plasma or tissue concentration (Cmax) is achieved, or at the steady state for multiple administrations).

* Includes dogs; # Includes rabbits

6. Sector specific requirements and approaches

6.1. Pharmaceuticals

6.1.1. Dose selection for pharmaceuticals

Before a potential new medicine can be administered to humans it is essential that its safety is adequately assessed. Safety assessment in animals forms an integral part of this process, and spans several disciplines (e.g., safety pharmacology, general toxicology, genetic toxicology, reproductive toxicology and immunotoxicology), from early drug discovery and initial candidate selection to mandatory regulatory tests under Good Laboratory Practice (GLP) in animals. Studies are conducted to provide greater understanding of the potential intrinsic hazard of the test item and to estimate safety margins, in order to determine an initial safe starting dose for clinical trials in humans, to support continued use in longer clinical trials and, ultimately, to gain marketing approval for use within the wider population.

There are many different guidance documents which provide considerations for dose selection for pharmaceuticals, including multiple guidelines from the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH), with varying approaches depending on the study type and scientific objectives (Table 6).

Table 6: References for ICH guidelines

ICH guideline
ICH M3(R2). Nonclinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals. In: International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use. Topic M3(R2): June 2009.
ICH S1C(R2). Dose Selection for Carcinogenicity Studies of Pharmaceuticals. Topic S1C(R2). October 1994. Revised March 2008.
ICH S2 (R1). Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended of human use. Topic S2(R1). November 2011.

ICH S5(R2). Detection of toxicity to reproduction for medicinal products & toxicity to male fertility. Topic S5(R2). Revised November 2005.
ICH S5(R3). Detection of toxicity to reproduction for medicinal products & toxicity to male fertility. Topic S5(R3). February 2020.
ICH S6 (R1). Preclinical safety Evaluation of Biotechnology-derived pharmaceuticals. Topic S6 (R1). June 2011.
ICH S7A Safety Pharmacology studies for Human Pharmaceuticals. In: International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use. Topic S7A. November 2008.
ICH S9. Nonclinical evaluation for anticancer pharmaceuticals. In: International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use. Topic S9. March 2010.

One special feature in the development of pharmaceuticals is that it usually does not start without knowledge. New molecules are often designed based on experience with compounds with the same mode of action. This makes dose-selection somewhat easier compared to general chemicals. Another important feature in this field is that pharmaceuticals are evaluated by a risk-assessment and not by a hazard assessment. The ICH guidelines for conducting preclinical safety studies are more flexible compared to OECD guidelines. Kinetic data in rodents and non-rodents are generated very early in development, namely during the preclinical development before phase I clinical trials.

6.1.2. General repeat dose toxicity studies

The Committee for Medicinal Products for Human Use (CHMP) guidance on repeated dose toxicity studies indicates that doses should be selected to establish a dose- or exposure-response to treatment (EMA, 2010). This can generally be achieved using three groups of animals receiving the test item, at low, intermediate, and high doses, plus a vehicle-only control group. Experience has shown that three appropriately chosen doses will usually cover the full range of the dose-response continuum, from the no observed adverse effect level (NOAEL) through to evidence of toxicity. However, there are exceptions, and on occasion additional dose levels may be required, or in very specific cases (e.g., as specified in ICH S6(R1) for some large molecules), fewer dose levels may suffice.

In terms of appropriate selection of the high dose there are five general criteria outlined in ICH M3(R2) (2009): (i) maximum tolerated dose (MTD), (ii) limit dose (1000 mg/kg/d or up to 2000 mg/kg/d in

specific circumstances) (iii) top dose based on saturation of exposure (i.e., plateau), (iv) maximum feasible dose (MFD) (i.e., dose restricted due to logistical considerations such as formulation concentration, dose volume, etc.) or (v) dose providing a 50-fold margin of exposure. However, ICH M3(R2) (2009) states that to support Phase III clinical trials for the United States, dose-limiting toxicity should be identified in at least one species when using the 50-fold margin of exposure as the limit dose. If there is no dose-limiting toxicity, a study of one-month or longer in one species conducted at the 1000 mg/kg limit dose, MFD or MTD (whichever is lowest), is recommended. However, if a study of a shorter duration identifies dose-limiting toxicity at doses higher than those resulting in a 50-fold exposure margin, this might not be warranted.

Determination of an appropriate dose through consideration of the above also requires relevant experience and judgement and should take the nature of the test item, its target pharmacology, and its intended therapeutic use in humans in to account. A*bsorption, distribution, metabolism, and excretion* (ADME) considerations, including species differences in metabolism and brain penetration can also play an important role, and may even limit exposures in certain circumstances, which can result in under- or over-estimation of the clinically efficacious plasma level.

Selection of inappropriate doses can have a negative impact on the clinical development program and can also cause unnecessary animal suffering. For example, selecting a dose that is too high or that does not produce toxicity may risk repetition of the study, thus requiring the use of additional animals. It may also prevent identification of target organ toxicity or early indicators that can be used to monitor potential effects in human studies. Careful consideration and design of studies can avoid the use of unnecessarily high doses and can reduce animal suffering without compromising scientific goals or human safety and improve the quality of scientific data. Practical advice on this topic has been developed for study directors and other toxicologists to maximise the implementation of refinement in dose level selection for regulatory toxicology studies (LASA/NC3Rs, 2009).

6.1.3. Maximum Tolerated Dose (MTD) Studies

Of the five criteria used as the basis for high dose selection (see above), determination of the MTD is the most subjective. In general, it is agreed that clinically relevant effects of a new drug can be sufficiently characterised by using a range of doses up to and including the MTD. However, though

there is debate around what exactly constitutes an MTD, it is agreed that it does not need to be determined in every study duration. By determining the MTD in the studies of shortest duration (e.g., up to 7 days) the information can be used to inform dose setting in subsequent toxicity studies, to avoid larger numbers of animals from being exposed to unanticipated pain and distress in later regulatory studies (LASA/NC3Rs, 2009) (see also section 2.2.3)

6.1.4. Body weight as an indicator of toxicity

Though body weight loss is an objective measurement and is often used as a primary endpoint in MTD studies, there is no industry or regulatory agreement on what level of body weight loss constitutes an MTD, and opinions vary on the impact and significance of body weight loss, which will vary depending on study duration

The OECD Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation recommend a weight loss of more than 20% body weight as a condition where humane killing may be appropriate for ethical reasons (OECD, 2000). The Federation of European Laboratory Animal Science Associations (FELASA) working group "Severity Classification of Procedures – Guidance on implementation of the process" classifies a body weight loss up to 20% as an upper moderate level of severity (LASA/NC3Rs, 2009).

See also section 2.2.3 and 2.3.

6.1.5. Specific guidance

Separate advice with considerations for dose selection for other study types and/or specific molecule types and therapy areas are provided in other ICH guidance documents and summarised below.

6.1.6. Biotherapeutics, ICH S6 (R1)

For biotherapeutics, ICH S6(R1) guidance states that dosage levels should provide information on dose-response relationship. However, due to the targeted mechanism of action of these molecules, it may not be possible to define a specific maximum dose due to little or no toxicity (though high doses

can elicit adverse effects which are apparent as exaggerated pharmacology). Here, it is recommended that the high dose is justified based on projected multiples of human exposures (e.g., approximately 10-fold exposure multiple over the maximum exposure to be achieved in the clinic) considering expected pharmacological/physiological effects, availability of suitable test material, the intended clinical use, as well as any species differences with regards to expected affinity and potency in the test species compared to humans. If toxicity is still not observed, the guidance states that additional toxicity studies at higher multiples of human dosing are unlikely to provide additional useful information.

6.1.6.1. Anticancer Pharmaceuticals

For anticancer pharmaceuticals, ICH S9 (2010) outlines some standard approaches. Nonclinical toxicology studies to determine a NOAEL or no effect level (NOEL) are not considered essential to support clinical use of an anticancer pharmaceutical. For small molecules, the general toxicology testing usually includes rodents and non-rodents. The establishment of a Severely Toxic Dose in 10% of the animals (STD 10) in rodents and a Highest Non-Severely Toxic Dose (HNSTD) in Non-Rodents is needed. The HNSTD is defined as the highest dose level that does not produce evidence of lethality, life-threatening toxicities or irreversible findings. This can generally be achieved using three groups of animals receiving the test item, at low, intermediate, and high doses, plus a vehicle-only control group. For biopharmaceuticals the number of species to be studied can differ and the criteria are defined in ICH Guideline S6 (in certain justified cases one relevant species may suffice e.g., when only one relevant species can be identified or where the biological activity of the biopharmaceutical is well understood). In addition, an assessment of the potential to recover from toxicity should be provided to understand whether serious adverse effects are reversible or irreversible, therefore recovery animals are included at least at the highest dose level, 'if there is severe toxicity at approximate clinical exposure and recovery cannot be predicted by scientific assessment'. In the development of anticancer drugs, clinical studies often involve cancer patients whose disease condition is progressive and fatal. In addition, the dose levels in these clinical studies often are close to or at the adverse effect dose levels. For these reasons, the type, timing and flexibility called for in the design of nonclinical studies of anticancer pharmaceuticals can differ from those elements in nonclinical studies for other pharmaceuticals.

6.1.6.2. Genotoxicity studies

ICH S2 (R1) suggests selection of three dose levels. For short-term genotoxicity studies (1-3 administrations) the high dose level is selected based on a limit dose of 2000 mg/kg (if tolerated), or an MTD is defined as the dose producing signs of toxicity such that higher doses would be expected to produce lethality. Suppression of bone marrow red blood cell production should also be taken into account. More information on how to monitor and account for bone marrow toxicity are provided in the guidance document.

For multiple administration studies, similar advice applies as for other studies. When the *in vivo* genotoxicity test is integrated into a multiple administration toxicology study, the doses are generally considered appropriate. When carrying out follow-up studies to address an indication of genotoxicity the following factors should be evaluated to determine whether the top dose is appropriate for genotoxicity evaluation: the high dose selection based on MFD, a limit dose of 1000 mg/kg/d (for studies of 14 days or longer, if tolerated), and/or plateau/saturation in exposure. For micronucleus tests, a top dose is suggested based on ≥ 50% of the top dose that would be used for acute administration dose, i.e., close to the minimum lethal dose, if such acute data are available - the high dose for acute administration micronucleus tests is currently described in OECD guidance as the dose above which lethality would be expected; similar guidance is given (e.g., Hartmann et al., 2003) for other *in vivo* assays. Selection of a high dose based only on an exposure margin (multiple over clinical exposure) without toxicity is not considered a sufficient justification.

6.1.6.3. Carcinogenicity studies

ICH S1C(R2) (2008) outlines criteria for selection of the MTD for carcinogenicity studies in rats and mice. Generally, the selection of the high dose is based on the results of 90-day studies, where the MTD is defined as the dose that produces minimal toxicity during the phase of the carcinogenicity study and not to interfere with the life-expectancy of the animals. Here, a 10% decrease in body weight gain, target organ toxicity and significant alterations in clinical pathology parameters are given as an example of an indication of the MTD. Similar to other studies mentioned, selection of the high dose can also be based on saturation of absorption, maximum feasible dose (based on practicalities and local tolerance), or a limit dose of 1500 mg/kg/d except when the maximum recommended human

dose exceeds 500 mg/kg and the compound is not genotoxic. For feeding studies, the limit dose is 50 000 ppm. Exposure in animals should be at least 10-fold higher than the human exposure at the maximum recommended dose.

For pharmacokinetic and pharmacodynamic endpoints, species differences in metabolism and protein binding should be taken into account.

6.1.6.4. Developmental and Reproductive Toxicity (DART) Studies

The ICH Guideline S5(R2, R3) (1994, 2020) deals with criteria for selection of the high dose level in reproductive toxicity studies. The high dose should produce minimal toxicity and some considerations to guide this decision include changes in bodyweight gain (reduced or increased), specific target organ toxicity, hematology and clinical chemistry, physico-chemical properties (maximum feasible dose), kinetics and exaggerated pharmacological response. However, the guidance does not include information on the amount of change that would indicate an appropriate level of toxicity had been reached. Kinetics can be particularly useful in determining high dose exposure for low toxicity compounds. For example, it is noted that there is little value in increasing the dose if it does not result in increased plasma or tissue concentration (i.e., past saturation). A dose providing a > 25-fold exposure margin to the maximum recommended human therapeutic dose is considered as an appropriate high dose. Where low toxicity is observed a limit dose of 1000 mg/kg/d is suggested.

6.1.6.5. Safety pharmacology studies

Factors concerning choice of dose levels for use within safety pharmacology studies are broadly described within the ICH S7A guideline (ICH S7A, 2008). In general, as with general repeat-dose studies, three dose groups would normally be sufficient to define the dose-response relationship of any adverse effect observed. These dose levels should provide exposure to the parent substance and its major metabolites that exceed the primary pharmacodynamic or therapeutic range achieved in humans. In practice, due to the timing of the safety pharmacology studies either early in discovery or within the package of studies for first-in-human (FIH) submission, the high dose level tends to be a dose near to the MTD (as already defined within the toxicity package). Some effects in the toxic range (e.g., tremors or fasciculation during ECG (Echo Cardio Gram) recording) may confound the

interpretation of the results and may also limit dose levels. Guidelines state that testing of a single group at the limiting dose may be sufficient in the absence of an adverse effect on safety pharmacology endpoints in the test species.

6.1.7. Conclusion and Approaches

The following key messages can be summarised for pharmaceuticals which differentiate this sector from the other ones:

- Exposure data to the drug candidate in animals are determined very early in the development, i.e., before Clinical Phase I studies
- Exposure at the NOAEL in rodents and non-rodents can be used for the calculation of the first dose in humans
- Exposure at the predicted therapeutic human dose can be used as justification for the dose levels in first toxicological studies
- Pharmaceuticals are evaluated by a risk-assessment and not by a hazard assessment
- ICH guidelines for conducting preclinical safety studies are more flexible compared to OECD guidelines

6.2. Agrochemicals

6.2.1. Relevant Regulations

The data requirements for active substances, the purposes of testing and the uses of test data are well exemplified by taking as an example the Commission Regulation (EU) No 293, 2013 which describes the approaches within the EU. In principle and indeed in practice the data requirements of other national authorities (such as EPA; 40 CFR (Code of Federal Regulations) 158.500 and Brazil; PC (Public Consultation) 484) are not inconsistent with these.

Agrochemical sector data requirements are generally well harmonised globally. Unlike other industry sectors (such as pharmaceuticals), there is little or no discretion or judgment applied, in that the requirements are laid out as a set of extensive core data needs, and defined study types, with a limited number of additional studies that may be triggered depending on the chemical type, mode of action

or adverse outcomes observed in the emerging toxicity database. Studies are conduced to the relevant test OECD guidelines. The purpose of testing is clearly stated and is expected to cover the needs of risk assessment but also to fulfil the requirements of hazard-based classification schemes. Currently the data generated, and the studies conducted to fulfil these needs are accepted globally under the OECD Mutual Acceptance of Data; there is no necessity to provide duplicate databases to meet regional variations.

6.2.2. Human Exposure

Direct high exposures are not expected for agrochemicals, except in rare and single spill or accident situations. There are a number of potential exposure scenarios for those applying agrochemicals in a professional setting, however training and the use of personal protective equipment means that repeat exposure is controlled and is short term or transient. Longer term exposure to very low levels of agrochemical residues or metabolites thereof can potentially occur though the dietary route (including water).

6.2.3. Existing Guidance for dose level selection

6.2.3.1. Repeat Dose Studies

The purpose of oral repeat dose toxicities studies (from 28 days to chronic duration) as described in data requirements and in Test Guidelines is primarily to provide a point of departure for a risk assessment. This can be combined with the additional purpose of providing information on hazard from specific target organ toxicity to carcinogenic potential (essentially by non-genotoxic mechanisms as in reality agrochemicals with the potential to cause genotoxicity will have been identified and discarded during the development process). This latter purpose therefore can provide the data necessary to classify based on hazard. Studies of shorter duration (28 days and 90 days) than chronic bioassays are also used for a classification purpose where the point of departure is used to assign substances to different hazard categories based on the degree of hazard (potency) observed, e.g., GHS Specific Target Organ Toxicity – Repeat dose (STOT. RE).

The general existing recommendations on dose level selection relating to repeat dose toxicity studies from 28 days to lifetime are provided in Section 1 of this report (Introduction, Background and

Principles) and can be confusing. The guidance on dose level selection is located across numerous OECD test guidelines and guidance documents, as well as other regional guidelines e.g., U.S. EPA Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005), can be inconsistent or unclear and this has resulted in differing approaches to dose level selection.

6.2.3.2. Reproductive and developmental toxicology (DART) studies

Developmental and reproductive hazard identification and risk assessment are accomplished within the agrochemical testing paradigm by the conduct of studies designed to assess effects of a test substance (i) on the developing conceptus and (ii) on the integrity and performance of the male and female reproductive systems and subsequent growth and development of the resulting offspring.

It is usual to conduct prenatal developmental toxicity studies (e.g., OECD 414) in two species (rat and rabbit) and a "multigeneration" study (e.g., OECD 416) in the rat. An important aspect of the conduct of such "definitive" studies is the selection of appropriate dose levels. If the dose levels are set too high and the resulting toxicity is excessive, the study may be uninterpretable and may need to be terminated or repeated. If dose levels are set too low, these may be deemed inadequate and the study rejected by regulatory authorities. Current guidance on dose level selection is summarised in Table 5 for developmental (OECD 414) and multigeneration studies (OECD 416).

In developmental toxicity studies the highest dose should be chosen with the aim to induce maternal toxicity (clinical signs or a 10-20% decrease in body weight gain) but not death or severe suffering. The use of toxicokinetic information is encouraged in regulatory guidance. It is important to note that rabbits have high sensitivity to reduced food consumption and therefore reduced weight gain (due to test compound-related toxicity), which can result in abortions, rendering a study useless for developmental toxicity identification.

Multigeneration studies (OECD 416) are generally undertaken later in an active ingredient development process and information, including toxicokinetic assessment, from repeat dose studies (28 and 90 day), as well as pregnant rat and rabbit studies is usually available to inform the dose selection process. It is noteworthy that the test guideline advice on dose level selection has remained unchanged since 2001 and is vague on the level of toxicity acceptable at the highest dose tested.

6.2.4. *In vivo* genetic toxicity studies

In vivo genetic toxicity studies represent the highest level of testing for adverse effects on genetic material in whole animals. It is clearly recognised in OECD test guidelines that a key part of the conduct of these studies is dose level selection. The guidance provided is covered in the summary of OECD test methods Table 5.

6.2.5. ECETOC Recommendations for dose level selection

- All dose level selection should be based on an understanding of toxicokinetics. A knowledge of the ADME of the test substance in the animal model is essential basic information and should always be used where appropriate to guide dose level selection. Where the TK data indicate that linearity in internal dose has been lost then this should be taken fully into account in dose level selection
- An understanding of TK should be combined with responsible and humane use of the 'minimally toxic dose' concept i.e., a top dose level based on no more than a 10% decrease in weight gain over the duration of treatment may be considered adequate. (Derelanko, 2000; OECD, 2002); the is consistent with a dose inducing slight toxic effects relative to the duration of the study period (for example, clear clinical signs such as abnormal behaviour or reactions, minor body weight depression or target tissue cytotoxicity), but not death or evidence of pain, suffering or distress necessitating euthanasia. As well as considering the 'classical' endpoints of body weight gain and clinical condition in selecting the high dose to be tested, consideration should also be given to a range of toxicities on a case-by-case basis; there is no reason why this approach should not form the basis for dose level selection approaches to sub-acute and sub-chronic evaluations. The use of the minimally toxic dose for chronic and carcinogenicity studies is well accepted for classification purposes and should be extended to shorter term studies. Other relevant classifications including those for specific target organ toxicity on repeat exposure (STOT/RE) can be obtained using this approach where the discriminating dose levels of 10mg/kg/d and 100mg/kg/d over 90 days are very likely to be covered in essentially all cases.

- Dose levels should be guided by a knowledge or a prediction of human dietary exposure using a margin of exposure approach
- In order that this approach is a credible option to guide dose level selection, accurate measured or predicted human exposure data are required.
- For dietary exposure which would be most relevant to dose level selection for longer term and chronic studies in rodents, there are several comprehensive databases of consumption data. In order to use these data, one would firstly need to identify the key diets in relevant target markets, then assume mean exposures would be relevant to lifetime. In addition, and in order to make this approach as accurate as possible crop residue levels would need to be generated earlier in a project in order to derive an experimental equivalent dose. Alternatively, in order to establish 'default' residue values for different indications/chemical/crops, existing industry data and metadata could be pooled and assessed.

A potential decision tree for an approach to repeat dose study dose selection is provided below. Margins of exposure between human exposures and the doses used in animal studies can be considered on a case-by-case basis and consistent with the protection goal, but would be designed to provide the most accurate and relevant data on the point of departure for risk assessment and the need to provide data on relevant hazard for classification and labelling purposes.

At the current time chronic dietary exposure is the most reliable data set in the absence of large, validated databases of cumulative, all-sources exposure. Dose level selection informed by chronic human exposure is a current or short-term reality (as illustrated by the example in Appendix D) is currently considered a possibility, dose levels based on human exposure for shorter term risk assessments requires further investigation.'

The recommended approaches outlined above and in Figure 5 will allow the dual needs of defining a point of departure for risk assessment and in providing data for potential hazard classification. Risk assessment and hazard-based classification can co-exist using these recommended approaches; they also have the benefit that animals are used in the most relevant and responsible way focusing dose level selection towards more relevance to human exposures. Two case studies illustrating how this approach could be used are shown in Appendix D.

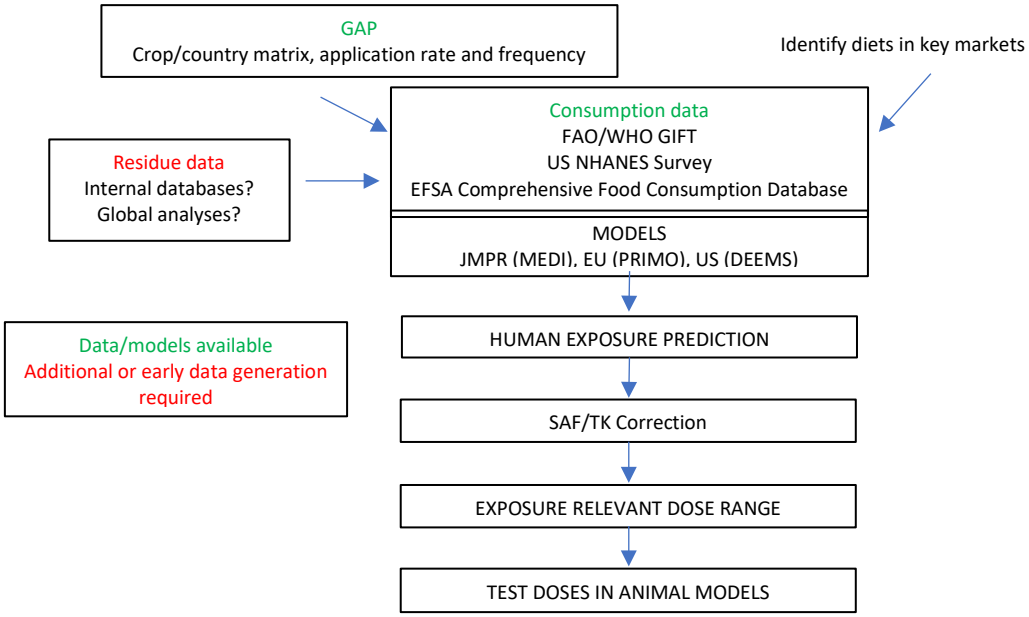


Figure 5: Dietary exposure assessment consideration tree.

DEEM, Dietary Exposure Evaluation Model; EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization; GAP, Good Agricultural Practice; GIFT, Global Individual Food consumption Tool; IEDI, International Estimated Daily Intake; JMPR, Joint FAO/WHO Meeting on Pesticide Residues; NHANES, National Health and Nutrition Examination Survey; PRIMO, Pesticide Residue Intake Model; SAF, Safety Assessment Factor; WHO, World Health Organization.

6.3 Industrial Chemicals

6.3.1 Relevant regulations (e.g., REACH and CLP)

The manufacture of industrial chemicals is regulated by two main European legislations (a) EC No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and (b) The Classification, Labelling and Packaging (CLP) Regulation (EC) No 1272, 2008 based on the United Nations' Globally Harmonised System (GHS).

Whilst REACH focusses on both "Hazard Characterisation" and "Risk Assessment", CLP is almost entirely "hazard" based and whilst there is some opportunity to consider "risk" this approach is seldom accepted during classification and labelling.

The REACH regulation (EU 1907, 2006) specifies toxicological data requirements that need to be fulfilled during substance registration. The data requirements are driven based on annual manufacture or import quantities and facilitate the identification of tests that should be conducted for hazard assessment and risk characterisation purposes.

6.3.2 Human exposure

Direct high exposures are not expected for industrial chemicals, except following accidents due to failure of process or misuse for example. Industrial chemicals are controlled either by closed automated processes, semi-closed processes or by introduction of personal protective equipment (PPE) or mechanical exposure control systems.

In addition to the use of PPE and mechanical controls human exposure is controlled by registered use patterns and exposure scenarios that fall into one of four exposure scenarios: Manufacture, Industrial use, Professional use and Consumer use.

6.3.3 Existing recommendations for dose selection

When assessing acute toxicity, ECHA guidance (R.7a) and OECD test guidelines propose to conduct the acute toxicity study following a stepwise procedure with the use of three animals of a single sex per

step. Testing should be conducted at doses up to 2000 mg/kgbw/d to determine is the substance tested should be classified or not for acute toxicity.

However, neither EU Regulation n°1907, 2006 (REACH) or ECHA (European Chemicals Agency) guidances make recommendations regarding the dose selection for repeated toxicity testing. Decisions related to dose selection must lean on the recommendations stated within the OECD test guidelines (TG) (Table 5).

A descending sequence of dose levels should be selected in order to demonstrate any dose related effect and to establish NOAELs or doses near the limit of detection that would allow for derivation of a benchmark dose for the most sensitive endpoints.

The OECD TG for repeated and reproduction toxicity studies give one major, but not detailed, recommendation regarding the top dose, which should induce toxic effects but not death or obvious suffering. The OECD TG for *in vivo* genotoxicity studies introduce the notion of MTD.

In Helsinki on 11 and 12 October 2018, ECHA held a joint workshop with the members of the Member State Committee (MSC) and of the Committee for Risk Assessment (RAC)[1]. The two committees comprised of stakeholders from industry, civil society, and observer organisations i.e. the Commission (namely DG Environment and DG Grow) the European Food Safety Authority (EFSA), the Joint Research Centre (JRC), the Organisation for Economic Co-Operation and Development (OECD).The aim of the workshop was to raise awareness regarding the possibilities and limitations faced by each of the two committees while executing their statutory tasks and, where possible, to align views on topical issues. A key topic of interest was **dose selection in systemic toxicology tests**. The aim was to promote a constructive dialogue about toxicity testing and dose level selection for regulatory purposes. The committees discussed on the resulting consequences of particularly low top dose selection in terms of human health protection, threshold derivation (DNEL, ADI (Acceptable Daily Intake), etc.), identification of target organ(s), ED assessment, triggering of tailor made higher tier studies and fulfilment of the CLP regulation.

[1] https://echa.europa.eu/documents/10162/26175471/msc-rac_ws_conclusions_20190131_en.pdf/0dd53ddd-69d0-7c8d-7d51-74efaf3233d9

The selection of appropriate dose levels in such studies is an essential requirement. Indeed, toxicological studies should be able to detect adverse effects in test animals and, preferably, to be able to establish dose responses and provide information on primary and secondary toxicological effects.

The workshop concluded that:

- There was consensus on an urgent need for the review of the MTD concept as some consider the MTD is reached when severe effects are seen whilst others consider marginal changes in body gain are enough to be considered as an MTD.
- Spacing between low, mid and high doses needs careful consideration to ensure dose-response curve adequately covered.
- Clear and thorough rationale for dose selection should be indicated in study reports as request in the OECD test guidelines but importantly the justification for dose selection should also be documented in both CLH (Harmonised Classification and Labelling) and REACH dossiers.
- For an optimal hazard assessment, the results of dose-range finding studies should be added in dossiers to justify the dose selection in the main studies.
- In view of the criteria used for classification and labelling and ED assessment it is essential that the data generated for the various regulatory frameworks are adequate to serve that purpose.
- The limitations of testing three dose levels have a huge impact on the power of a study.

6.3.4 ECETOC recommendations for dose selection

If there is not sufficient information to select the dose used in a new toxicological study, a dose-range finding (DRF) study should be performed before the definitive study in order to give key toxicity data and to facilitate definitive dose selection. No test guideline or guidance exists to describe the design of the DRF. Usually, the DRF study design (by gavage) contains three dose levels and a concurrent negative vehicle group. Each group contains between 3 and 10 animals, both sexes can be used in rodent studies. The duration of the study varies from 5 to 28 days, and may include such as clinical signs, bodyweight changes, food consumption, organ weights, macro- and microscopic examinations etc., depending on individual study needs?

Recommended design of the DRF study:

- One negative control group and at least three tested groups;

- Two sexes (except for developmental studies);
- Animals with the same age than in the main study;
- At least 14-day of exposure (except for rabbit developmental studies);
- Minimal observations during *in vivo* phase: Mortality, clinical signs, bodyweight, bodyweight gain, food consumption;
- Minimal observations after *in vivo* phase: Macroscopic examination of all animals, Organ weight of livers, kidneys, brain.

In the absence of previous repeat toxicity study, the results obtained in the oral acute toxicity can be used for dose selection for repeat toxicity study. If no toxicity was observed up to 2000 mg/kgbw/d in the oral acute toxicity study, the dose levels in the oral 14-day DRF study is often 100, 300 or 500 and 1000 mg/kgbw/d. When no adverse effects are observed in the DRF study up to 1000 mg/kgbw/d, the same dose levels are used in the main study. However, when severe adverse toxicity was showed at one or more dose levels, the selection of dose levels for the main study is challenging. For example, when there are no effects at the intermediate dose, but severe toxicity or mortality at 1000 mg/kgbw/d, it is nearly impossible to select the high dose levels for the main study. It assumes that the high dose should be between 300/500 and 1000 mg/kgbw/d but the gap is large. The best way would be to do a second DRF with an additional dose (between 300/500 and 1000 mg/kgbw/d) even if testing facility capacity, regulatory deadline pressure, escalating costs and animal welfare considerations all contribute to substantial pressure.

The range-finding study can also follow a stepwise procedure if virtually nothing is known about a substance, the first part DRF study (pilot study) may consist of a single administration of one dose to 2 animals (1 male and 1 female) and subsequently, depending on the reaction of the animals, with single administrations of lower or higher doses to additional animals. Thus, one gets some preliminary information on the acute toxicity of the substance. This WoE approach is primarily meant for cases where no acute toxicity study, nor repeated dose toxicity (28-day) study are available. A detailed description of this approach is presented in ECHA (2017).

The DRF study should result in the determination of a MTD up to the limit dose. The selection of the high dose should be taken with caution because toxic overload makes impossible the interpretation

of toxicity mode of action. Table 7 shows of the data recommended to inform dose selection in the future toxicological studies. Figure 6 outlines considerations for oral dose selection for chronic studies.

Table 7: Recommendations data for dose selection in OECD Test Guidelines.

Test guideline study	Recommended data to select the dose
OECD 407: 28-day oral repeated toxicity study (rodents)	14-day dose range finding study
OECD 408: 90-day oral repeated toxicity study (rodents)	14-day dose range finding study, 28-day repeated toxicity study to identify MTD and target organ toxicity or OECD 407, 421, or 422
OECD 421: Reproduction/ developmental screening test	14-day dose range finding study, or 28-day repeated toxicity study to identify MTD and target / primary organ toxicity, or OECD 407 or 408
OECD 422: Combined Repeated Dose Toxicity Study with the reproduction/ Developmental Toxicity Screening Test	14-day dose range finding study to identify MTD and target / primary organ toxicity, or OECD 407, OECD 408
OECD 443: Extended one-generation reproductive toxicity study	90-day dose range finding study and Reproduction/ developmental screening test to identify MTD and target / primary organ toxicity, screening test alone may be sufficient depending on the results. It may be necessary to conduct an additional dose range finding study if there are concerns about offspring survival through lactation or the effects in weanlings at the commencement of dosing.
OECD 414: Prenatal developmental toxicity study in rat	Preliminary tolerability study in non-pregnant rats if repeat dose data do not exist. Dose-range finding study with pregnant rats (except if no adverse effect was observed at 1000 mg/kg/d in the Reproduction/ developmental screening test)
OECD 414: Prenatal developmental toxicity study in rabbit	Preliminary tolerability study in non-pregnant rabbits if repeat dose data do not exist.

	and Dose-range finding study with pregnant rabbits, consider any acute data a confirm whether rabbit is likely to be a more sensitive species
OECD 474: In-vivo micronucleus test	Specific preliminary study in both sex, *in vitro* micronucleus test, obtain MTD from 28 day repeat dose study and consider target organ toxicity
OECD 488: Transgenic rodent (TGR) somatic and germ cell gene mutation assays	Positive *in vivo* micronucleus test, obtain MTD from 28-day repeat dose study, consider specific organ toxicity, TGR doses should not lead to site of contact or gross organ toxicity in the stomach, duodenum or liver. In addition, specific preliminary study in both sex due to use of a transgenic breed of rat.
OECD 489: In-vivo mammalian alkaline comet assay	Positive *in vivo* micronucleus test, obtain MTD from 28-day repeat dose study, consider specific organ toxicity, doses should not lead to site of contact or gross organ toxicity in the stomach, duodenum or liver. Specific preliminary study in both sex

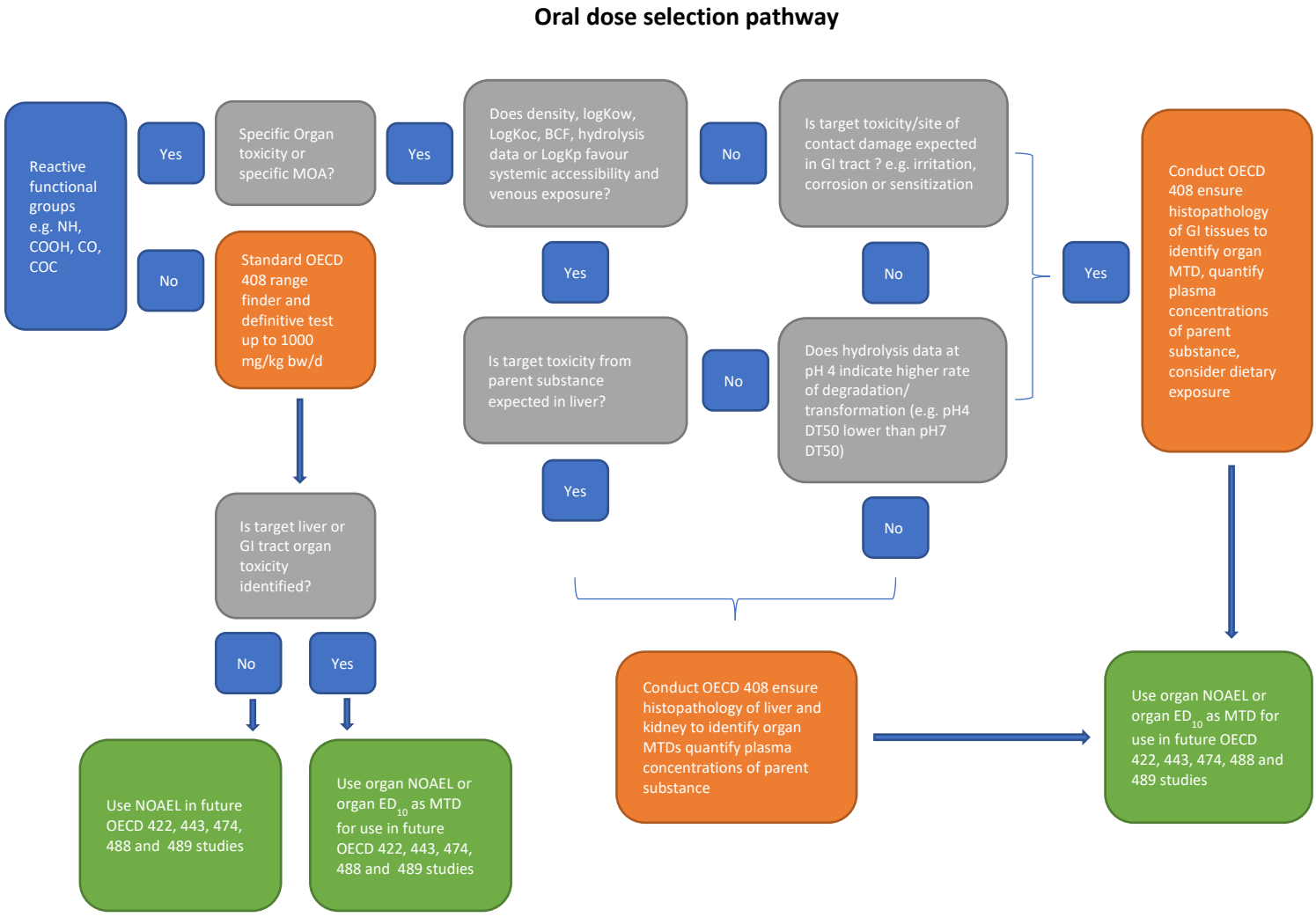


Figure 6: Considerations for oral dose selection for chronic studies

Note, this should be used as a guide and is not intended to take the place of expert judgement and experience

6.3.5 Specific cases

6.3.5.1 Irritating, corrosive and sensitising substances

Dose selection with irritating, corrosive and sensitising compounds is a significant challenge. With irritating substances, decreases in body weight and feed consumption may be present, but other clinical signs such as hunched posture and unkempt appearance of the animal can also be indicative. Each laboratory has their own guidance and approaches to testing such materials that requires

approval of their animal review board which follows national or international standards and regulations. If the material has a high acidity or alkalinity that can be buffered in an aqueous dose solution without affecting the composition of the material, then this approach should be considered. This can often allow for high dose setting based on systemic toxicity instead of point of contact irritation. In other cases, the irritating properties of the material will drive the high dose level selection. It is important to note that the progression of studies required for REACH is based on tonnage band as well as a tiered approach to testing. Therefore, studies are not always conducted in order of exposure duration. Depending on the type of irritation, exposure duration as well as the concentration of the chemical may be critical for dose setting.

The results of mammalian acute toxicity testing or *in vitro* assays can provide an indication of this potential and can help inform the design of repeat dose studies.

6.3.5.2 Reproduction and developmental toxicity studies

6.3.5.2.1 Developmental toxicity study

It is critical to determine the repeat dose toxicity potential prior to dosing in pregnant animals. Once an acceptable high dose level is determined in non-pregnant animals, that dose should be evaluated along with other doses in a preliminary study in pregnant animals prior to the definitive study. These two phases should not be combined. The dose spacing in the pregnant DRF should be fairly close together (1.5-2X) to refine the selection of the high dose level. The high dose level should strive to have a bodyweight gain reduction of 10-15% maximum compared to controls over one or more 3-day intervals during gestation. With effects beyond this driving fetal weight decreases in feed restriction studies when occurring in the last week of gestation.

Pregnant animals should never lose body weight (different than a reduction in body weight gain, or a reduction in body weight compared to controls) over an extended period of time, or to fail to have adequate food intake.

6.3.5.2.2 Extended one-generation reproductive toxicity study (EOGRTS)

Most of the time, data from previously conducted OECD 421 or 422 study are used to choose the dose levels in the EOGRTS because the exposure period of the parental generation is often similar. But sometimes later found to be inadequate for dose setting due to a lack of understanding of toxicity in late lactation and with the onset of dosing in weaning animals. Indeed, it is essential to assess the lactational transfer of the substance. The toxicokinetics of a chemical and the likelihood that it will enter the milk may be predicted on the basis of the physico-chemical properties of the chemical (e.g., using pKa, logP, water solubility, and molecular weight etc), or can be evaluated in a lactational transfer study. In the later study, a development of an analytical method in the milk and blood are needed.

6.3.5.3 Genotoxicity studies

The harmonised test guidelines of the OECD that utilised for genotoxicity studies propose limit doses of 2000 or 1000 mg/kgbw/d for exposures <14 days or > 14 days respectively. Based on a 70 kg human this equates to unrealistically high maximum human equivalent doses of 140 grams or 70 grams of pure test substance per day for <14 day or >14 days respectively.
In the context of industrial chemicals human exposure to such high doses is completely unrealistic and therefore it would be more relevant to use intelligent methods that consider “risk” and “exposure scenarios” when designing toxicological tests and selecting dose ranges. This would reflect a bottom-up approach that to set thresholds with the protection goals of ensuring worker, human and environmental safety under realistic conditions rather than the current top down approach that utilises high numbers of animals, no protection goal and a thirst for academic query.

This approach would minimise unnecessary pain and suffering to laboratory animals whilst ensuring a test is fit for purpose but also require improved understanding of how products are used downstream.

During the conduct of *in vitro* mutagenicity studies the tests are conducted at excessively high doses (ca. 5000 µg/mL). OECD guidance states that doses should result in 0 – 50% cell toxicity and therefore it can be expected that some positive findings could be a result of a true mutagenic response but also cytotoxicity or apoptosis and at no point should a positive *in vitro* result take precedence over an *in*

vivo study such as a micronucleus, comet or transgenic rodent assay (TGR). Prior to starting any of the afore mentioned *in vivo* studies it is important to identify the MTD_{animal} or more importantly the MTD_{organ} whereby organ specific toxicity may occur in the duodenum, stomach or liver which are the three main tissues analysed for mutagenicity. Ensuring that doses are set at or below the MTD will ensure the capture of findings that result from a purely mutagenic mode of action and not an artefact of site of contact effects or compromised organ epithelia.

Example case studies illustrating dose selection approaches for industrial chemicals can be found in Appendix E.

6.4 Food Ingredients

6.4.1 Relevant Regulations

Repeated dose oral toxicity studies (dietary or gavage) are designed to identify the potential for adverse effects that can occur following consumption of a substance, identify target organ(s), and to characterise the dose–response relationships for the adverse effects detected. The aim of hazard identification is to identify potential critical endpoints that may be of relevance for human health (EFSA, 2017). Data from repeated-dose toxicity studies are often required for the safety assessment of substances in most of the geographical areas covered by regulatory authorities such as the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA). Repeated dose toxicology studies are also used by scientific organisations such as the Flavor and Extract Manufacturers Association (FEMA) and the Joint Expert Committee on Food Additives JECFA for purposes of quantitative risk assessment. While different tests can vary in duration from single exposure to up to two years, the 90 day (subchronic) is often the test from which risk assessment values are derived.

Repeated dose toxicity studies with ingredients intentionally added to foods that are conducted for international regulatory agencies are usually in accordance with guidelines established by the Organisation for Economic Co-operation and Development (OECD, Table 5). However, just what constitutes a “food ingredient” can vary from substances that are added directly to foods for sensory

or functional purposes to substances that become incorporated unintentionally such as migrants from packaging materials to contaminants from agricultural sources.

6.4.2 Human Exposure

For components intentionally added to food, incorporation levels are known and controlled, and accurate assessments can be made of the likely human consumption of that foodstuff and therefore of the ingredient. Similarly, analysis of levels of any contaminants will give an assessment of the potential for and the extent of any human exposure.

6.4.3 Existing Guidance on Dose Level Selection

It is often the case that little or no information is available to guide the appropriate doses of a test substance to be administered for a repeated dose toxicology study.

6.4.3.1 Repeat Dose Studies

The general existing recommendations on dose level selection relating to repeat dose toxicity studies from 28 days to lifetime are provided in Section 1 of this report (Introduction, Background and Principles) and can be confusing. The guidance on dose level selection is located across numerous OECD test guidelines and guidance documents, can be inconsistent or unclear and this has resulted in differing approaches to dose level selection.

- It is critical to emphasise that limit dose studies are not required in food regulation and in fact repeated dose toxicology studies conducted at lower doses based on possible human exposure are often used for purposes of a quantitative risk assessment. Doses chosen should provide information on both the lower and higher part of the dose–response relationship to characterise the full dose–response relationship (EFSA, 2017).

- The process of selecting the high dose is not consistent with testing requirements among different regulatory agencies and scientific authorities. In contrast to the OECD guidelines which clearly define the upper limit of exposure, the US FDA Redbook does not specify a top

(i.e., limit) dose for repeated doses toxicology studies. Rather, it indicates that the high dose should produce an adverse effect and the low dose should not (FDA, 2007).

- In chronic/carcinogenicity studies where the test substance is administered in the diet, it is important to limit the concentration of the chemical in the feed to avoid nutritional imbalances. Most testing guidelines establish an upper limit of 5% of the total diet (OECD, 2002). When oral doses (gavage and diet) are maintained at a fixed level based on body weight, the upper limit has generally been set at 1000 or 1500 mg/kgbw (Rhomberg, 2007). It is recognised that the MTD is difficult to predict accurately. A weight-of-evidence approach should be used to determine whether the clinical pathology and pathology findings represent or support attainment of an MTD.

- Human exposure should also be considered in dose selection, particularly for selection of the middle and lowest doses to characterise the shape of the dose-response curve as much as possible. In food regulation, this assumption makes sense because humans are exposed daily to small amounts of substances from different foods. As related to doses administered in a repeated dose toxicology study, this principal would allow for the metabolic elimination of the test substance at dietary exposure levels that would be much closer to anticipated patters of human consumption compared with the potentially overwhelming effects that are more likely to occur following exposure to extremely high doses (e.g., the limit dose) when delivered as a bolus. Some ingredients that will be incorporated into foods at high concentrations will not be tolerated and would be expected to produce adverse effects when administered to animals in repeated dose toxicology studies. As an example, consider high intensity sweeteners that are hundreds and even thousands of times sweeter than sucrose (Whitehouse et al., 2008). These substances can be added to foods at very low concentrations to achieve their technical purpose however when they are added to rodent diets at high concentrations, they can produce an adverse sensory effect which can cause the animals to reduce or even stop eating (WHO 1987; Mann et al., 2000a and 2000b; Flamm et al., 2003; Mayhew et al., 2003; Magnuson et al., 2017). This could easily appear to be a substance related adverse effect when in fact it is simply not palatable owing to the excessive amounts that are present in the food.

- It is clear that feeding studies with contaminants such as mycotoxins and organophosphate insecticides well below the limit dose have been extremely useful for purposes of risk

assessment. In many cases such as two year carcinogenicity assays, these studies were conducted by government agencies such as the National Toxicology Program (NTP). In these carcinogenicity studies, the doses administered are often based on effects observed from shorter term studies rather than the limit dose approach to ensure that the animals will survive the duration of the studies (See https://ntp.niehs.nih.gov). Scientific authorities such as the Joint Expert Committee on Food Additives (JECFA) have also routinely conducted quantitative risk assessments for food ingredients that were tested with repeated dose toxicology studies with the highest doses being well below the limit high dose. These types of studies take into consideration the body of knowledge about the safety and potency of the particular substance rather than the default approach where any substance would automatically be incorporated into the diets at concentrations that would administer limit dose or by oral gavage. Many long-term repeated dose toxicology studies have been conducted at doses that are far below the limit dose owing to the combined input of individual chemical potency (i.e., would be expected to cause premature death if administered at greater doses) and good information about potential human exposure (Benford et al., 2010).

- A review of some of the published literature of repeated dose toxicology studies that have been conducted with food ingredients demonstrates that the nature of the substance being investigated can also impact the doses that are selected. For example, numerous repeated dose toxicology studies have been conducted with dietary ingredients intended for addition to foods as non-caloric sweeteners such as rebaudioside (steviol glycoside). In these subchronic and reproductive repeated dose toxicology studies the highest dose administered was well in excess of the limit dose (Curry and Roberts, 2008 [4161 and 4645 mg/kg/d]; Curry et al., 2008 [2048 and 2273 mg/kg/d]); Nikiforov and Eapen, 2008 [2000 mg/kg/d]). Based at least in part to the corpus of previously conducted studies it was appropriate that the actual study used by EFSA to determine the ADI (a 2 year carcinogenicity study) included groups that were exposed to up to 5% of the total dietary intake even though, in this case, the longer term study itself was conducted prior to the shorter term studies (Toyoda et al., 1997). From the 2 year study, EFSA determined that the ADI for humans was 4 mg/kgbw/d based on the NOAEL being the group that consumed 2.5% in the diet. That dose corresponds to 967 mg stevioside/kg/day or 388 mg steviol equivalents/kg/day. When uncertainty factors were applied, EFSA determined that the ADI was 4 mg steviol equivalents/kg/day (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2010.1537).

6.4.3.2 Reproductive and developmental toxicology (DART) studies

Developmental and reproductive hazard identification and risk assessment are accomplished within the testing paradigm by the conduct of studies designed to assess effects of a test substance (i) on the developing conceptus and (ii) on the integrity and performance of the male and female reproductive systems and subsequent growth and development of the resulting offspring.

It is usual to conduct prenatal developmental toxicity studies (e.g., OECD 414) in two species (rat and rabbit) and a "multigeneration" study (e.g., OECD 416, 2001) in the rat. An important aspect of the conduct of such "definitive" studies is the selection of appropriate dose levels. If the dose levels are set too high and the resulting toxicity is excessive, the study may be uninterpretable and if often terminated. If dose levels are set too low, these may be deemed inadequate and the study rejected by regulatory authorities. Current guidance on dose level selection is summarised in Table 5 for developmental (OECD 414, 2018) and multigeneration studies (OECD 416, 2001).

In developmental toxicity studies the highest dose should be chosen with the aim to induce maternal toxicity (clinical signs or a 10-20% decrease in body weight gain) but not death or severe suffering. The use of toxicokinetic information is encouraged in regulatory guidance.
Mutigeneration studies (OECD 416, 2001), are generally integrate information, including toxicokinetic assessment, from repeat dose studies (28 and 90 day), as well as pregnant rat and rabbit studies is usually available to inform the dose selection process. It is noteworthy that the test guideline advice on dose level selection has remained unchanged since 2001 and is vague on the level of toxicity acceptable at the highest dose tested.

6.4.3.3 *In vivo* genetic toxicity studies

In vivo genetic toxicity studies represent the highest level of testing for adverse effects on genetic material in whole animals. It is clearly recognised in OECD test guidelines that a key part of the conduct of these studies is dose level selection. The guidance provided is covered in the summary of OECD test methods (Table 5).

6.4.4 ECETOC Recommendations for Dose Level Selection

- It is essential to carefully select the high dose for repeated toxicity studies to be conducted with food ingredients in order to identify the potential risks relative to chemical exposure.

- The entire body of information related to the safety of individual food ingredients should be considered before determining the doses to be administered in repeated dose toxicology studies. The information could include in silico methodology, in vitro studies, range finding studies, toxicokinetic data, and, whenever possible, repeated dose toxicology studies with structurally similar substances. Importantly, it is critical to distinguish between the testing goals for food ingredients and the testing of industrial and other types of chemicals.

- In the case of food ingredients, it is often possible to achieve the technical purpose for their use with very low concentrations and often in a narrow range of different foods. It can normally be demonstrated that humans are exposed to very low doses so when considering repeated dose toxicology studies it is appropriate to consider a risk-based approach rather than the hazard-based approach where limit doses would be more applicable. This is not to suggest that the testing standards for food ingredients should be any less rigorous than for other types of chemicals. It indicates that applying a hazard-based approach to food ingredients is likely to "identify" hazards that are often many orders or magnitude in excess of any possibility of human exposure.

Example case studies illustrating dose selection approaches for industrial chemicals can be found in Appendix F.

7. Conclusions and Recommendations

It is recognised that different sectors have differing degrees of freedom to operate in dose level selection and this is reflected in the overall recommended approaches. The recommendations of this report represent pragmatic approaches to selecting dose levels that allow for accurate risk assessment but also enable hazard-based classification based on identification of relevant hazards. These can be applied within the current regulatory frameworks, but also cover some forward-looking future options and approaches to dose level selection.

- As recommended in test guidelines and guidance documents, wherever practically possible an understanding of internal exposure should be developed, through the deployment of toxicokinetic approaches, and used to guide dose level selection. In the great majority of cases and situations internal exposure (blood and tissue) will be linear with applied external dose. Knowing this will provide reassurance that the biological effects, including toxicities that are observed, represent true responses to increasing exposure. In a minority of cases a less that proportional increase in internal exposure may be demonstrated. In such a situation this knowledge is vital in shaping approaches to dose level selection where plateaus of exposure or less than proportional exposure with increasing applied dose can be taken into account. This information must come from appropriate and rigorous TK approaches. Figure 7 summarises approaches that include sampling for TK information.

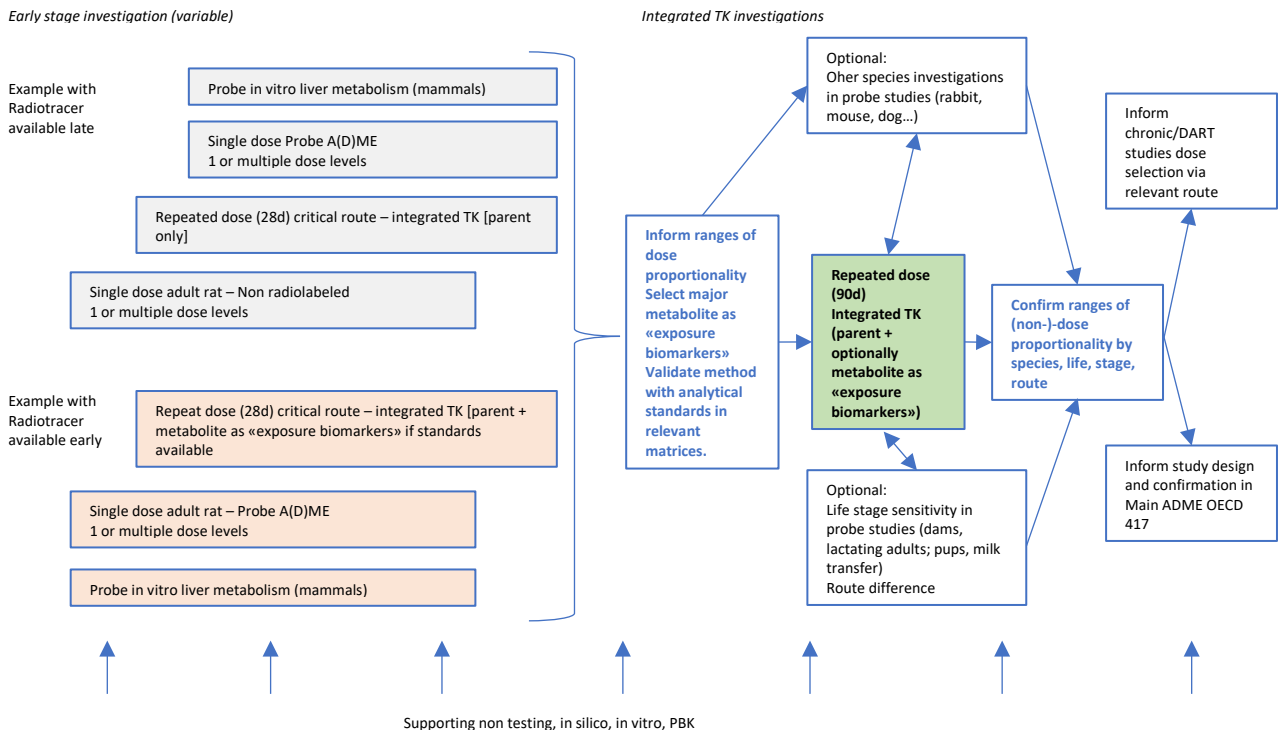


Figure 7. Integration of TK: possible data development framework

- Where there are no or little data to make a dose selection decision based on internal dose, or where internal dose is linear with applied dose then signs of toxicity remain the main source of knowledge for selecting appropriate dose levels. With the possible exception of early dose range-finding studies and in the absence of any clear prior information on mode of action or structural class to guide dose level selection, then in repeat dose studies the highest dose level should be limited to a reasonable level such as that which causes evident but minimal toxicity (as an example existing guidance often points to effects such as a 10% reduction in body weight gain). There should be no situation in 21st century toxicology practice where there is any scientific justification for selecting the high dose in repeat dose studies with the intention of causing pain, distress, suffering, significant toxicity or lethality in any of the experimental subjects. In practice, laboratories and investigators conducting studies have very clear local guidance and legislation aimed to limit or prevent the intentional use of doses that cause such effects. As an example, if any repeat dose study causes lethality, peri-lethal effects or a sustained period of reduced weight gain or weight loss at the high dose, this dose would very

likely be terminated without further evaluation and further studies needed at more appropriate dose levels.

- As the science of predictive human exposure further develops and matures, this will provide exciting and novel opportunities for more relevant approaches to dose level selection. In some sectors this approach is well understood and is currently used in dose level selection (pharmaceuticals); in other sectors such as agrochemicals, the knowledge and understanding needed to support a margin of exposure approach is developing.

- In circumstances where the mode of toxic action is well understood and described, and where a material can be clearly assigned to such a class, then different opportunities exist and should be considered in approaches to dose level selection. It is however recognised that in order to base doses on MoA, one would need a quite extensive and existing knowledge-base. Similarly, the paradigm for dose level selection in studies designed to elucidate a mode of action (usually based on the findings from more traditional regulatory toxicity studies), can be very different and designed on a case-by-case basis.

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Appendix A – Acronyms, glossary and abbreviations

Acronyms

3Rs	Replacement, Reduction and Refinement
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism Excretion
AI	Active Ingredient
ALAT	Alanine Amino Transferase
ALH	Amplitude of Lateral Head Displacement
ALT	Alanine Amino Transferase
ASAT	Aspartate Amino Transferase
AST	Aspartate Amino Transferase
AUC	Area Under the Curve
AUD	Area Under the Data
BADGE	Bisphenol A Diglycidyl Ether
BrdU	Bromodeoxyuridine
BUN	Blood Urinary Nitrogen
BW	Body Weight
BWL	Body Weight Loss
C&L	Classification and Labelling
CAR	Constitutive androstane Receptor
CFR	Code of Federal Regulations
CHDA	Cyclohexanedicarboxaldehyde
CHMP	The Committee for Medicinal Products for Human Use
CLH	Harmonised Classification and Labelling
CLP	Classification, Labelling and Packaging
CNS	Central Nervous System
CODEX	Codex Alimentarius
CYP2E1	Cytochrome P-450
DART	Developmental and Reproductive Toxicity
DEEM	Dietary Exposure Evaluation Model
DNA	Deoxy Ribose Nucleic Acid

DRF	Dose Range Finder
EB	Ethyl Benzene
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECG	Echo Cardio Gram
ECHA	European Chemicals Agency
ED10	Effective Dose 10 (Dose causing a 10% effect)
EFSA	European Food Safety Authority
EG	Ethylene Glycol
EH	Epoxide Hydrolase
EOGRTS	Extended One- Generation Reproductive Toxicity Study
EPA	Environmental Protection Agency
ETBE	Ethyl tertiary-butyl ether
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FELASA	Federation for Laboratory Animals Science associations
FEMA	Flavor and Extract Manufacturers Association
FIH	First-In-Human
GAP	Good Agricultural Practice
GD	Gestational Day
GEMS	Global Environment Monitoring System
GHS	Globally Harmonised System
GIFT	Global Individual Food Consumption Tool
GLP	Good Laboratory Practice
GSH	Glutathione
GSTT1	Glutathion-S-transferase
Ha	Hectare
HDL	High Density Lipid
HNSTD	Highest Non-Severely Toxic Dose
HPPD	Hydroxyphenylpyruvate Dioxygenase
HTTK	High Throughput Toxico Kinetic
ICH	International Consortium of Harmonisation
IEDI	International Estimated Daily Intake

ILSI-HESI	International Life Sciences Institute – Health and Environment Science Institute
IND	Investigational New Drug
IVIVE	In Vitro-to-In Vivo Extrapolation
JECFA	Joint Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
JRC	Joint Research Centre
KMD	Kinetically-derived Maximum Dose
LATAM	Latin America
LCMS	Liquid Chromatography Mass Spectroscopy
LD	Limit Dose
LDH	Lactate Dehydrogenase
LOAELs	Low Observed Adverse Effect Levels
LOQ	Limit of Quantitation
MAF	Maximum Aerobic Function
MFD	Maximum Feasible Dose
MoA	Mode of Action
MSC	Member State Committee
MTD	Maximum Tolerated Dose
N/A	Not Applicable
NAPQI	N-acetyl-p-benzoquinone imine
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NCI	The National Cancer Institutes
NHANES	National Health and Nutrition Examination Survey
NOAEL	No Observed Adverse Effect Level
NoAm	North America
NOEL	No Effect Level
NQ	Not Quantifiable
NTP	National Toxicology Program
OAT	Organic Anion Transposter
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office for Prevention, Pesticides and Toxic Substances
OSR	Oil Seed Rape
PBPK model	Physiologically Based Pharmacokinetic model

PC	Public Consultation
PHI	Pre Harvest Interval
PK	Pharmacokinetics
PND	Post Natal Day
PNDT	Pre-Natal Developmental Toxicity
PoD	Point of Departure
PPE	Personal Protective Equipment
PPRA	Peroxisome Proliferator-Activated Receptor
PRIMO	Pesticide Residue Intake Model
PXR	Pregnane X Receptor
QSAR	Quantitative Structure–Activity Relationship model
RA	Risk Assessment
RAC	Committee for Risk Assessment
RDT	design of Repeated Dose Toxicity
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAF	Safety Assessment Factor
SARs	Structure Activity Relationships
SCE	Sister Chromatid Exchange
SCLs	Specific Concentration Limits
STD	Severely Toxic Dose
STMR	Supervised Trial Median Residue
STOT. RE	Specific Target Organ Toxicity – Repeat dose
TAT	Tyrosine Aminotransferase
TBA	Tertiary-Butyl Alcohol
TG	Test Guidelines
TGR	Trans Genic Rodent
TK	Toxicokinetics
TRPV	Transient Receptor Potential Vanilloid
TRR	Total Radioactive Residue
UK	United Kingdom
UVCB	Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials
VAP	Average Path Velocity
VCL	Curvilinear Velocity or Track Speed

VSL	Progressive or Straight-Line Velocity
WHO	World Health Organization

Glossary

Parameter	Description of parameter
AUC	Area Under the Concentration-time curve
AUC_{last} or AUC_t AUC_{0-t}	AUC from time zero to the time of the last quantifiable concentration (C_{last})
AUC_{inf}	AUC from time zero extrapolated to infinite time
C_{last}	Last quantifiable concentration
C_{max}	The maximum observed blood/plasma/tissue concentration
$t_{½}$	Apparent terminal phase half-life.
t_{max}	Time of the observed maximum blood/plasma/tissue concentration.

Appendix B – Mode of Action case studies

Mechanistic factors

Case study: Methylene Chloride

Methylene chloride is used e.g., in aerosols, as paint remover and as a metal cleaning agent. Furthermore, it is used as solvent in the chemical synthesis of chemicals and pharmaceuticals. Production volume is greater than 500000 tons per year. It is metabolised by two pathways. One pathway is mediated by Cytochrome P-450 (CYP2E1). After an initial hydroxylation, formyl chloride is formed by rearrangement, which then decomposes to carbon monoxide: This pathway has a high affinity, but a low capacity. The second pathway involves Glutathion-S-transferase (GSTT1), which forms S-Chloromethylglutathione. S-Chloromethylglutathione decomposes rapidly to chloride and formaldehyde. Formaldehyde is oxidised to formic acid, which is oxidised further to carbon dioxide. This pathway has a low affinity, but a high capacity (ECETOC, 1988; Green, 1997).

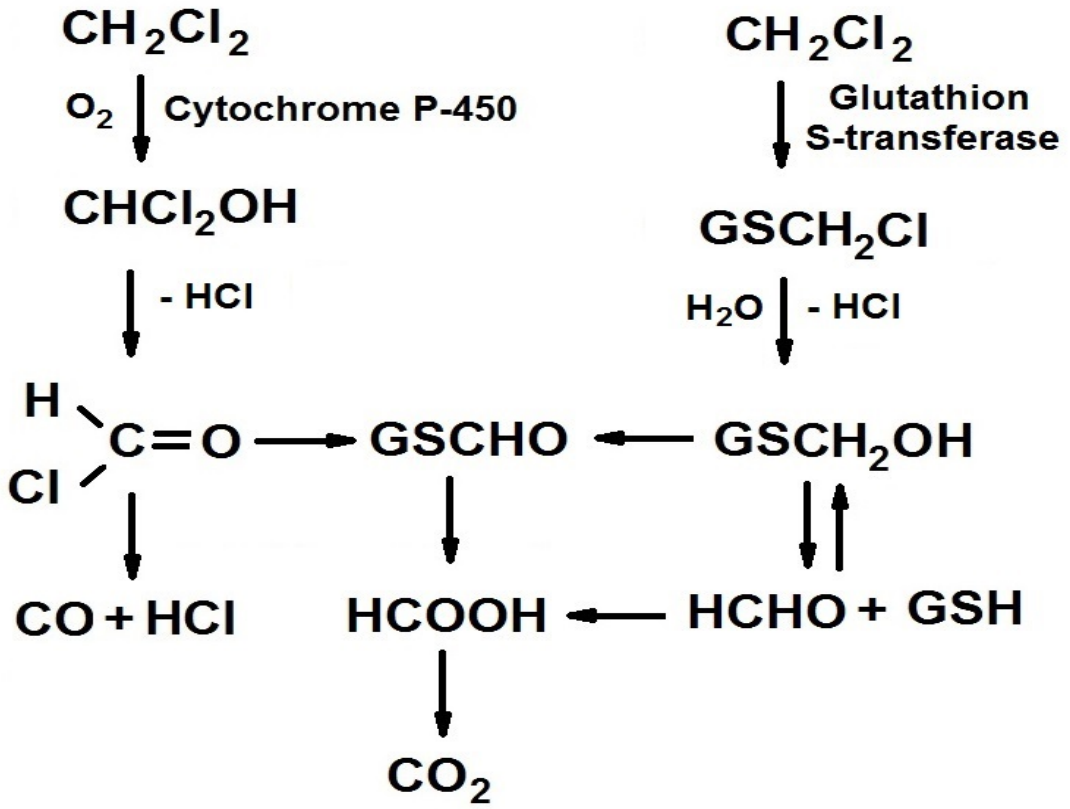

Figure B1 Metabolism of methylene chloride

Methylene chloride is mutagenic in prokaryotic systems and in chromosome aberration tests, but most tests in eukaryotic systems and chromosome aberration tests in vivo were negative. Testing for unscheduled DNA synthesis was negative. There was no evidence for DNA binding in vivo.

Several carcinogenicity studies are available with methylene chloride. In hamsters, methylene chloride showed no evidence for carcinogenicity after inhalation up to 3500 ppm. In a rat inhalation carcinogenicity bioassay, increased incidences of mammary tumours were observed at 1500 and 3500 ppm. Increased incidences of sarcomas of the salivary gland were observed in males at 3500 ppm, which were considered to be secondary related to a viral salivary gland infection (sialodacryoadenitis). In a second rat inhalation bioassay, which was conducted at 50, 200 and 500 ppm, increased incidences of mammary gland tumours were observed. These mammary adenomas were later shown to be related to increased prolactin levels; a mechanism not relevant for humans. No evidence for carcinogenicity was evident in a drinking water study at doses up to 250 mg/kgbw/d.

The last carcinogenicity studies were conducted by the National Toxicology Program in rats and mice. Rats were exposed to 0, 1000, 2000 or 4000 ppm. The formerly observed increased incidences were confirmed in the experiments. Mice were exposed to 0, 2000 or 4000 ppm. In both sexes, a dose-dependent increase in adenoma and carcinoma of the liver and of alveolar/bronchial adenoma and carcinoma were observed.

Mechanistic studies showed, that the Clara cells in the lung were the primary target in the mouse at > 2000 ppm, but not in the rat. Furthermore, damage of Clara cells was accompanied by suppression of Cytochrome P-450 metabolism, whereas the Glutathion-S-transferase activity remained unchanged. Furthermore, an increased number of cells in the S-phase was present in the bronchiolar and alveolar epithelium. Comparative metabolism studies showed, that the Cytochrome P-450 pathway was saturated in rats and mice at 500 ppm in vivo, quantitatively similar in rats and mice in vivo and quantitatively similar in liver fractions of rats, mice, hamsters and humans. In contrast, it turned out that the Glutathion-S-transferase pathway was the major pathway only in mice. The activity at 4000 ppm was one order of magnitude higher than in rats, and the activity in human liver fractions were lower than in rats. The metabolism of methylene chloride is dose-dependent: The Cytochrome-P 450 pathway was the major source of carbon dioxide at 100 ppm, whereas the Glutathion-S-transferase was the major source of carbon dioxide at 4000 ppm and was 10-12 times more active in mice when compared to rats at 4000 ppm. PB-PK modelling showed that humans are adequately protected from a carcinogenic effect by the current hygiene standards, which are based on the

formation of carboxyhaemoglobin (ECETOC, 1988). Follow-up studies revealed that high concentrations of GST T1-1 were present in mouse liver and lung samples, GST T1-1 is present in the nucleus of the cells (proximity to DNA). High concentrations of GST T1-1 were not identified in human or rat liver tissue. The increases in lung and liver tumours in mice exposed to methylene chloride are therefore unique to that species (Green, 1997).

Taken together, the low dose used in the NTP study of 2000 ppm in mice was clearly too high, since the Cytochrome P 450 pathway was already saturated. Based on the facts outlined above, considerably lower dose levels would have yielded more realistic results. Therefore, more work on kinetics should be performed before initiating carcinogenicity studies to avoid use of too high dose levels.

Case Study: Paracetamol (Acetaminophen)

Paracetamol is a widely used over the counter analgesic drug. Although it is considered safe when used at recommended doses (Prescott et al., 1983), hepatotoxicity is the major side effect. Paracetamol is also hepatotoxic in animals at higher dose levels (Bergman et al., 1996).

Paracetamol is metabolised by several pathways (Fig. 2). At lower (in humans therapeutic) doses sulfatation and glucuronidation are the primarily pathways and yield non-toxic metabolites, which are renally excreted. In addition, CYP 450 2E1, CYP 450 2A2 and CYP 450 3A4 (Zaher et al., 1998) form a small fraction of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) in small amounts, which can bind to macromolecules. NAPQI is deactivated by conjugation with Glutathione (GSH). At higher doses, sulfatation and glucuronidation pathways become saturated, resulting in a higher fraction of NAPQI. Due to the conjugation with GSH, the GSH levels decrease and more unconjugated NAPQI becomes available and can exert toxicity.

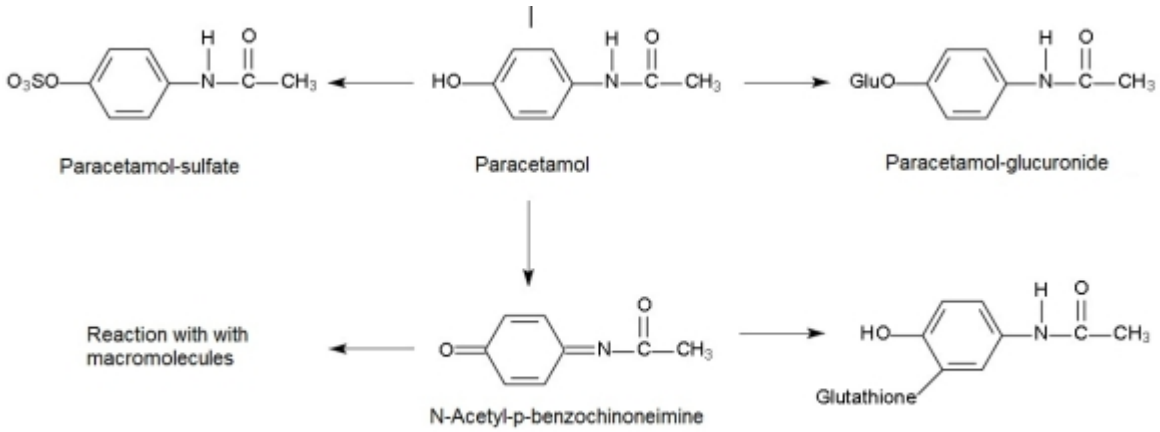

Figure B2: Main metabolism pathways of paracetamol

Paracetamol did not induce point mutations in bacteria of mammalian cells. One mouse lymphoma assay showed a positive result, but this may have been attributed to aberrations rather than point mutations. In contrast, paracetamol induced chromosome aberrations in vitro and in vivo. In vitro, the effect was dependent on both incubation time and concentration and occurred only at cytotoxic concentrations (Bergman et al., 1996). In vivo, there was one recent micronucleus test, which investigated the correlation of micronucleus formation, organ toxicity and metabolite profiles (Baumeister et al., 1994; Bergman et al., 1996). Rats received paracetamol at doses of 0, 25, 50, 100, 175, 250 or 500 mg/kgbw/d three times in a 4 h interval or once at 1500 mg/kgbw/d. Micronucleus induction was observed at 500 and 1500 mg/kgbw/d. Liver toxicity (centrilobular necrosis) was evident from 175 mg/kgbw in males and 500 mg/kgbw in females, accompanied by increases in ASAT (Aspartate Amino Transferase), ALAT (Alanine Amino Transferase), LDH (Lactate Dehydrogenase) and creatinine. GSH content in the liver was decreased from 100 mg/kgbw. Increases in BUN (Blood Urinary Nitrogen) indicated nephrotoxicity at 500 mg/kgbw in males and at 1500 mg/kgbw. Investigation of biotransformation showed that sulfatation and glucuronidation were relevant pathways at 100 mg/kg (approximately human levels at therapeutic doses) and were saturated at 500 mg/kgbw/d. In conclusion, weak mutagenic effects occurred at doses >500 mg/kgbw, where sulfatation and glucuronidation pathways were saturated and organ toxicity was present.

Paracetamol induced liver tumours in IF mice when given at a at hepatotoxic dose. In other studies, no tumourigenic effects were observed in B6C3F_1 mice at non-hepatotoxic and in NIH mice at hepatotoxic doses. No tumourigenic effects were observed in rats (Bergman et al., 1996).

Case Study: Afidopyropen

Afidopyropen is an insecticide, which's mode of action is modulation of Transient Receptor Potential Vanilloid (TRPV) channels in chordotonal organs of insects. In carcinogenicity studies, afidopyropen was administered to rats at dietary concentrations of 0, 100, 300 or 1000 ppm (corresponding to 0, 5, 15 or 50 mg/kgbw/d, respectively) for two years. In a second study a higher dose of 3000 ppm (corresponding to 150 mg/kgbw/d) was tested. An increased incidence of uterus tumours was observed at 1000 and 3000 ppm via the diet. No tumours were induced in mice. Hence, kinetic and mechanistic studies were undertaken to evaluate the possible relevance of these findings for humans. Kinetic data showed that the excretion of the test compound and its metabolites were saturated at doses > 15 mg/kgbw/day. Thus, tumours occurred only at dose levels, where the excretion was saturated, and the organism was unproportionally overloaded with the test compound. Furthermore, mechanistic examination showed that the test compound acts as dopamine agonist at these high doses, a mode of action not relevant to humans (Van Cott et al., 2018).

Case Study: Chemical X

Assessment of subchronic toxicity and developmental toxicity

Chemical X is an industrial chemical. For the purpose of chemical registration two studies were to be performed: subchronic toxicity study according to OECD TG 408 and developmental toxicity study according to OECD TG 414.

The present case study illustrates the process of dose level setting for the main studies and the evaluation of the appropriateness of the selected top doses after having obtained the outcome of the main studies.

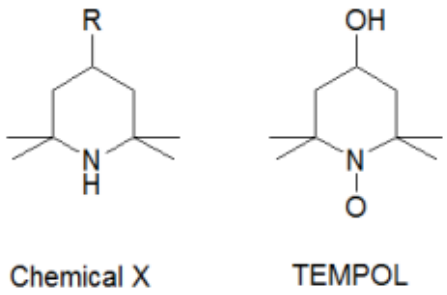

Figure B3: Chemical Structure of HA and structurally related compound TEMPOL

Issues related to dose selection:

A 28-day dose ranging finding study was performed.

Rats were treated via gavage at dose levels of 0, 15, 62.5, 250 and 1000 mg/kgbw for up to 28 days.

Mortality occurred at 1000 mg/kgbw for males and females and at 250 mg/kgbw for females. Remarkable emaciation occurred prior to death. Upon necropsy the animals had swollen intestine, thymus- and spleen shrinkage and adrenals enlargement.

Findings for males at 250 mg/kgbw comprised reduced food consumption, reduced body weight gain (55% of control), reduced spleen and increased testes- and epididymides weights.

No effect was observed for animals treated with 62.5 and 15 mg/kgbw.

A dose ranging finding study for the developmental toxicity study was performed.

Pregnant rats were treated via gavage at dose levels of 0, 15, 45 and 135 mg/kgbw from gestation day 6 to 19.

Dams receiving 135 mg/kgbw test substance were found dead/moribund on gestation day 14 onwards. These animals exhibited emaciation and total resorption. Dams receiving 45 mg/kgbw showed reduced absolute-weight-gain (body weight gain subtracted by gravid uterus weight).

No effects in litter parameters as well as in external examination of fetuses were noted at dose levels of 45 and 15 mg/kgbw.

The most characteristic structural feature of Chemical X is the 2,2,6,6-tetramethylpiperidine moiety which belongs to 'hindered amines'. This moiety spontaneously undergoes piperidine nitroxide formation, thereby reacting as radical scavenger in polymers. The hindered amines are considered as a structural alert for the toxicity to male reproductive system (DEREK, ToxCast). A similar structurally related compound induced microcytotic anemia in 90-day toxicity study that was persistent also after the recovery phase, so that a concern of hematoxicity for Chemical X was given.

Further, the piperidine nitroxide moiety is also the active site of heterocyclic nitroxide drugs that are primarily used as antihypertensive agent. The underlying mechanism is related to the superoxide dismutase mimic action on the sympathetic nervous system, further related to vasodilatative effect. Among the various effects attributed to nitroxide drugs, body weight decreasing effects as well as sex-specific and/or hormone statues dependent antihypertensive effects are known. Nitroxide drugs are

reported to induce substantial reductions in arterial blood pressure when applied at doses well above therapy range.

Dose Selection for the 90-day toxicity according to OECD TG 408 study and the outcome.

In the dose-ranging finding study males and females exhibited remarkably deviating sensitivity. The top dose selection based on male toxicity would lead to excessive toxicity in females, certainly not reconcilable with animal welfare practice, while the top dose based on female toxicity would lead to failure of achieving the MTD in males, thereby failing to identify the potential concern of toxicity on the male reproductive system.

Therefore, different top doses were used:

Dose levels for males: 0, 25, 75 and 225 mg/kgbw

Dose levels for females: 0, 10, 25 and 75 mg/kgbw

Males treated with 225 mg/kgbw exhibited decreased body weight gain (84% of control), reduced spleen and increased testes weights.

The changes in hematology were indicative of slight leucopenia in females of 75 mg/kgbw, comprising decreased values of all leucocytes.

Dose Selection for the developmental toxicity study according to OECD TG 414 and the outcome

In the dose-ranging finding study the dose 135 mg/kgbw was associated with 100% mortality/moribund of dams, whereas the dose 45 mg/kgbw (corresponding to 1/3-fold of 135 mg/kgbw) did not induce apparent maternal toxicity.

The main study was performed using the dose levels of 0, 10, 25 and 62.5 mg/kgbw. The top dose corresponded to ½-fold of dose associated with mortality in the dose-ranging finding study.

Maternal toxicity: In the top dose group general toxic effects such as hunched posture, emaciation, piloerection, and reduced food consumption up to 50% were observed. Further, terminal body weight was reduced by approximately 20% compared to control as well as the body weight gain throughout

pregnancy by about 50% compared to controls. Upon necropsy small thymus was found in five animals out of 24. In mid and low dose groups the food consumption was reduced by up to 20%. No other effects were found in these two treated groups.

In all groups, the parameters related to reproduction performance was not affected.

Fetal toxicity: For high dose animals the mean fetal weight was reduced by ca. 20 % with 82 fetuses out of 364 exhibiting fetal weight less than 2g. In high dose group one acauda and one anus imperforation were found. Further, increased incidences of incomplete or no ossification of the skeleton were observed in most parts of the skeleton.

No treatment related effect was observed for mid and low dose animals upon skeletal as well as visceral examinations.

Evaluation of the appropriateness of the selected dose levels

The purpose of the performed studies was to fulfill the regulatory requirement such as classification and labelling as well as to understand the toxicity profile for the potential use such as application in food contact material. For the latter purpose it is of high importance that the study outcome delivers clear information about the dose-response relation, which sometime allows to derive possible underlying mode of action.

The top dose selected in the 90-day (225 mg/kgbw) for males was associated with body weight gain decrease (84% of control) and absence of any other significant effect, mitigating the concern of toxicity on the male reproductive system. The top dose for females (75 mg/kgbw) verified the blood system as the potential target organ, whereas the expected body weight gain reduction was not evident. The question how severe the blood system would be affected at higher doses cannot be answered, but less likely to be of hazard assessment relevance due to the severe toxicity found at doses of 250 and 135 mg/kgbw in the dose-range-finding studies.

The animal treatment duration in the developmental toxicity study is far less than in the subchronic toxicity study, for which reason higher doses are normally used in the developmental toxicity study. In the given case the top dose in the developmental toxicity study (62.5 mg/kgbw) was lower due to the observed sensitivity of pregnant animals in the dose-ranging finding study. Still, the outcome of the main study is clearly indicative of having exceeded the currently valid MTD.

Taking together all the observation in the dose-range finding and main studies, a non-linearity of dose response relation (or a noticeably steep dose-response relation) can be established in regard with clinical signs, body weight and clinical pathology.

It can be reasonably derived that vasodilation became the determining mode of action above certain dose, resulting in the impairment of cardiovascular system. The observed higher susceptibility of females would be then comparable to the observed gender different response to nitroxide drug application in animal studies. Even more enhanced susceptibility of pregnant animals could be attributed to the changed physiological conditions of pregnancy such as decreased total peripheral resistance.

Dose level sections approaches when working with known modes of Action

Knowledge of the mode of action of a chemical can often provide important information regarding the selection of the high dose level.

Peroxisome Proliferator-Activated Receptor (PPAR) Agonist Therapeutics

PPARα-agonists (Fenofibrate, Clofibrate) are used for treatment of dyslipidemia. They increase HDL (High Density Lipid) and decrease triglycerides. PPARγ-agonists (Troglitazone, Rosiglitazone and Pioglitazone) were used for treatment of type of type II diabetes. They act via an insulin-sensitising mode of action. Dual PPARα/γ-agonists (Glitazars) were developed for combined treatment of dyslipidemia and type II diabetes.

The first PPARγ-agonist Troglitazone was withdrawn from the market due to drug-induced liver failure. After FDA approval of the PPARγ-agonists Rosiglitazone and Pioglitazone in 1999, interest emerged in the evaluation of dual PPARα/γ-agonists. The idea behind was that a combination of lipid-lowering and insulin-sensitising properties would allow a lower dose of the drug and therefore induce less side effects as the traditional treatment with pure PPARα- or pure PPARγ-agonists. In the time period of 7 years after the last approval, more than 50 INDs were filed for dual PPARα/γ-agonists (El-Hage, 2006).

Most of the developments of dual PPARα/γ-agonists were terminated due to safety issues, which occurred both in animals and in clinical trials. Since many chronic toxicity and carcinogenicity studies

were conducted with PPARα/γ-agonists (same mode of action) it became evident which specific toxic effect after 13-week treatment is dose-limiting for the subsequent carcinogenicity studies. Dose levels, which led to a greater than 25% increased heart weight in rodents after 3 months caused a premature mortality due to cardiovascular failure in the carcinogenicity bioassay. Therefore, a 20% to 25% increase in heart weight was accepted as the maximum tolerated dose in rats and mice for this class of compounds (El-Hage, 2006). The same applied for a doubling of liver weights after 3-months treatment, which was also accepted as MTD. Greater effects on liver weight resulted in life-threatening necrosis of the liver capsule in the carcinogenicity study.

HPPD Inhibitor Herbicides and Ocular Toxicity

The enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) is essential for carotenoid biosynthesis in plants. Inhibition of HPPD leads to destruction of chlorophyll due to the lack of protecting carotenoids. Therefore, many HPPD inhibitors were developed as herbicides. The enzyme HPPD is also present in mammalian species where it is involved in the catabolism of the amino acid tyrosine. Most of phenylalanine and tyrosine is absorbed via the diet and excess is removed by an efficient catabolic process. This involves the oxidation of tyrosine right down to acetoacetate and fumarate which are used for extrahepatic oxidation and for incorporation into the citric acid cycle (Lewis & Botham, 2013).

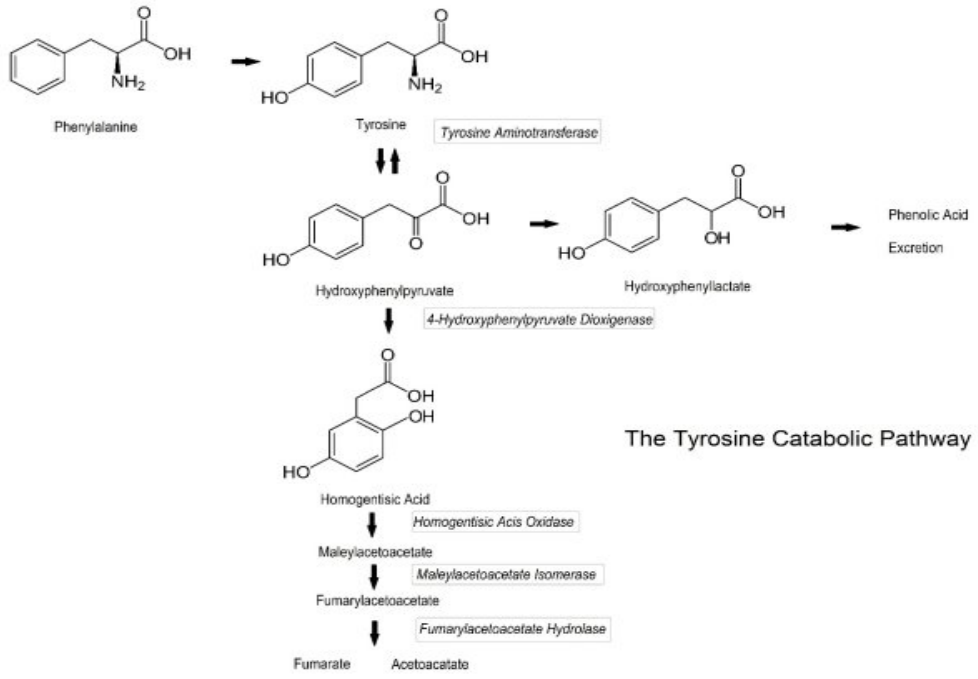

Figure B4: The tyrosine catabolic pathway

Tyrosine is not excreted into urine due to efficient reabsorption by the kidneys. Hydroxyphenylpyruvate can be excreted directly into urine, however this process is negligible under normal conditions.

Tyrosine aminotransferase (TAT) the first enzyme of this biochemical process of tyrosine catabolism. This is the rate limiting step for catabolism of tyrosine. When HPPD is inhibited, the catabolism of tyrosine is shifted towards the phenolic acid pathway and excreted into urine. Removal of excess tyrosine by this process is less efficient than the normal route leading to fumarate and acetoacetate. The consequence is an increase in circulating levels of tyrosine – tyrosinemia.

An Adverse Outcome Pathway has been presented by the US-EPA: 1. Inhibition of HPPD. 2. Increase in plasma 4-hydroxyphenylpyruvic acid (and excretion of this in urine). 3. Induction of tyrosinemia. 4. Tyrosine-mediated ocular effects. Steps 1 to 3 occur in all species, but step 4 is not seen in all species. For example, ocular effects were observed in rats, but not in mice.

There is no significant difference in pharmacokinetic parameters of the HPPD inhibitor Mesotrione between male and female rats. However, the extent of the tyrosinemia in male rats is about 2-fold greater than in female rats and about 4-fold higher than in male or female mice:

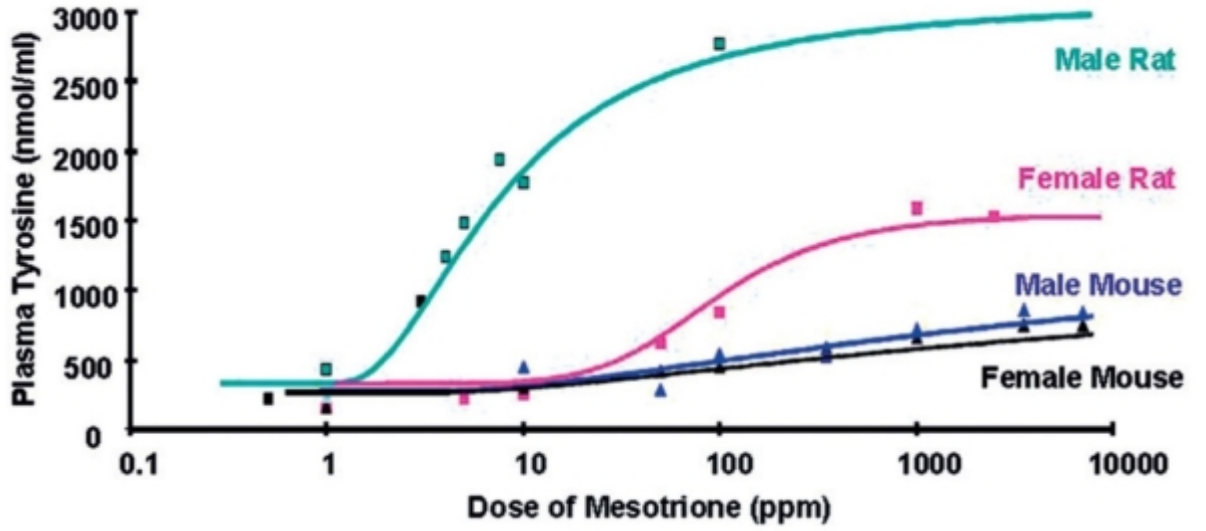

Figure B5: Plasma tyrosine levels at different Mesotrione doses in rats and mice

The reason for these species differences are different activities of the rate-limiting enzyme TAT. In male rats the TAT activity is approximately half of that of the female rat. Male and female mice have a steady-state tyrosinemia about 4-fold lower than male rats because the TAT activity in male/female mice is about 4-fold higher. Therefore, after inhibition of HPPD, the extent of the developing tyrosinemia across species can be predicted predicted from their TAT activity and this information is applicable to ocular toxicity of all HPPD inhibitors.

When HPPD is not inhibited excess tyrosine is metabolised by the normal catabolic process to fumarate and acetoacetate with the rate-limiting step being TAT. The phenolic acid excretion pathway is negligible and may be considered as a low affinity pathway. When HPPD is inhibited the flow is diverted to the phenolic acid excretion pathway. The systemic tyrosine levels rise with inhibition of HPPD and the phenolic acid excretion pathway becomes the dominant pathway for removal of excess tyrosine.

If HPPD is totally inhibited in mice, excess tyrosine is diverted to the phenolic acid excretion pathway. This diversion is very efficient in mice producing only a moderate increase in steady-state systemic tyrosine concentrations. In contrast, rats have much lower TAT activity, and the excretion via the phenolic acid is limited. Therefore, rats develop a considerably more severe steady state tyrosinemia than mice.

Threshold for Ocular Toxicology: In order to investigate the dependency of corneal lesions on tyrosine plasma levels many triketone structures were taken into account. Based on these data, there is evidence that there appears to be a threshold at about 1000 nmol/ml tyrosine (in plasma), which must be exceeded for long periods of time before ocular lesions result. Therefore, a pre-requisite for ocular toxicity is maintenance of steady-state plasma tyrosine of at least 1000 nmol/mL.

In-line with this data are the facts, that ocular lesions are observed in male rats at lower dose levels than in female rats, and why mice do not develop ocular lesions. The threshold of 1000 nmol/mL tyrosine is simply not achieved in mice.

After comparison of the hepatic activity of TAT in rats, mice and humans, it appears that humans have similar a TAT activity as mice. Therefore, humans are not expected to develop tyrosine plasma levels greater than 1000 nmol/mL, which are the threshold for ocular toxicity.

These example shows how knowledge of the mode of action could be used for selection of the high dose. In case of HPPD-inhibitors, dose levels producing tyrosinemia above 1000 nmol/mL should be avoided in rats in order to prevent toxicity which is not relevant for other species including humans.

However, significant research is needed to establish these frameworks.

Toxicodynamic factors and Dose level selection in carcinogenicity studies

Body weight. As classical definition, a decrease in body weight gain by 10% after 13 weeks should be considered as MTD. Within this context, it should be considered that lower body weights per se result in decreased tumour incidences and the animals are more resistant towards age-related toxicity.

Clinical pathology parameters. Although changes in hematology and clinical chemistry parameters are usually discussed together with other endpoints, guidance can be given for some parameters alone as outlined below:

Hematology parameters. Anemia, as evidenced e.g., decreased blood cell count, can be dose limiting for the rodent bioassay, if red blood cell counts are decreased by 20% after 13 weeks treatment. In case of methaemoglobinemia-inducing agents, formation of 10 to 20% should be considered as adverse and dose-limiting.

Clinical chemistry parameters. Clinical chemistry parameters alone are rarely dose-limiting and usually evaluated together with pathology findings. Increases in ALT (Alanine Amino Transferase) by 2 to 4-fold, together with other changed could be used as justification of the MTD. Likewise, increased in blood urea nitrogen and creatinine by > 1.5-fold are indicative for kidney damage.

Organ weights. When the mode of action and target organs are known, organ weights can serve as dose-limiting. For example, FDA had issued criteria for an MTD in case of dual PPARα/γ agonists. Doubling of liver weigh after 13 weeks treatment was considered as MTD, since stronger effects were considered most likely to induce necrosis of the liver capsule during chronic administration. Likewise, an increase in heart weight by 20 to 25% were initially proposed to represent an MTD, since otherwise the animals would die in the second half of cancer bioassay due to cardiovascular failure. Later it turned out that this increase in heart weight was still too high.

Histopathological findings. The following histopathological findings in liver and kidney are considered as indicator for an MTD (as described in Rhomberg at al., 2007).

Liver

- Hepatocellular hypertrophy, when associated with >2 to 4-fold increase in ALT/AST (Aspartate Amino Transferase)
- Hepatocellular regeneration as reaction to necrosis
- Fatty change, when associated with >2 to 4-fold increase in ALT/AST
- Hydropic change, when associated with >2 to 4-fold increase in ALT/AST
- Degeneration, necrosis
- Cirrhosis indicates that the MTD is exceeded
- Bile duct necrosis
- Bile duct, severe oval cell hyperplasia
- Proliferation of Ito cells, associated with fibrosis or necrosis
- Cholestasis

Kidney

- Hyaline droplet nephrosis, when accompanied by increased blood urea nitrogen and/or creatinine
- $\alpha_{2\mu}$-Globulin nephropathy when accompanied chronic progressive nephropathy
- Lipofuscin accumulation because of a toxic mechanism
- Tubular necrosis
- Tubular dilatation or cystic tubules due to degeneration and regeneration
- Tubular hyperplasia
- Tubular basophilia and/or regeneration
- Papillary necrosis

Beside these classical pharmaco-/toxico-dynamic endpoints other factors should be considered. Irritating compounds should not be administered at doses causing chronic irritation and consequently inflammation, increased cell turnover with a higher probability of fixation of mutations. The same applies to chronic, persistent toxicity and for mitogenic compounds as well as compounds, which change the methylation level of the DNA. Generally, disturbances of the physiology and homeostasis. In case of compounds, which are administered orally, effects on the microbiome of the gut, vitamins, and nutrition can occur, Likewise, effects on the intestinal transit time can have significant nutritional effects.

In general, all the assessments regarding toxicodynamic factors above should be taken together in the dose setting process, thus looking at the whole picture. Furthermore, toxicokinetic data have to be taken into account in this process.

Dose level selection in reproductive toxicity studies

In an ILSI-HESI workshop dealing with maternal toxicity (Beyer et al., 2011) some participants considered a 5% decrease in body weight gain as possibly adverse in general toxicity studies, whereas others favoured 10% as threshold. Regarding developmental and reproductive toxicity studies, there was no consensus. However, a 20% decrease in body weight gain was judged as too much.

Based on these discussions, a decrease in body weight gain (corrected by uterus weight) during the treatment period in the dose-range finding study of 10 to 15% should justify this dose as a suitable high dose in reproductive toxicity studies.

When hematology and clinical chemistry parameters are examined in maternal toxicity dose range finding studies, anemia in the range of 10 to 15% should be judged as dose limiting. In case of histopathological examinations, changes indicating impairment of liver function (necrosis, elevated ALAT, ASAT) or kidney (necroses, degeneration/regeneration, increased BUN, increase creatinine) can be considered as dose-limiting.

Appendix C – TK case studies

Saturation of metabolism (Ethylene Glycol)

Summary: PK information along with an understanding of the mode of action (in this case metabolism to a toxic metabolite) can be used to inform dose selection and risk assessment. Saturation of metabolism of both parent and toxic metabolite leads to non-proportional (supra linear) increase in plasma levels (Pottenger LH et al., 2001; Corley RA et al., 2005; Carney EW et al., 2011; Fowles J et al., 2017).

ADME study results in pregnant rats: With a single gavage treatment to GD 10 animals at doses of 10, 150, 500, 1000 and 2500 mg/kg; n=5 per dose), Glycolic acid (metabolite) blood levels increase disproportionately compared to ethylene glycol blood levels in rats. Glycolic acid urinary excretion increases disproportionately at 500 and 1000 mg/kg (Table C1 and Figure C2). The metabolism saturation for glycolic acid occurs in the range 150-500 mg/kg and the metabolism saturation for ethylene glycol in the range 1000-2500 mg/kg.

Table C1*: ADME study results.*

Ethylene Glycol (EG) external dose		Blood level (AUC)				Urinary Excretion			
Dose (mg/kg bw/day)	*Fold increase*	Ethylene Glycol		Glycolic acid		Ethylene Glycol		Glycolic acid	
		[µg·h/g]	Fold increase	[µg·h/g]	Fold increase	[% EG applied]	Fold increase	[% EG applied]	Fold increase
10	***1x***	23	***1x***	NQ	N/A	14.95	***1x***	0.88	***1x***
150	***15x***	292	***13x***	84	N/A	27.86	***1.9x***	1.18	***1.3x***
500	***50x***	1208	***53x***	641	N/A	41.92	***2.8x***	12.43	***10.5x***
1000	***100x***	2928	***127x***	1829	N/A	39.64	***2.7x***	20.13	***22.9x***
2500	***250x***	11638	***506x***	4031	N/A	37.64	***2.5x***	32.79	***37.3x***

EG (ethylene glycol); NQ (not quantifiable); N/A (not applicable)

Toxicity profile: Ethylene glycol is a developmental toxicant in rats when administered by gavage, through metabolism to glycolic acid, which has been identified as the developmental toxicant. The

NOEL and LOEL in rats are 500 mg/kg/d and 1000 mg/kg/d respectively. Ethylene glycol does not induce developmental toxic effect at comparable doses following dietary, dermal or respiratory exposures.

Metabolism: The elimination pathway of ethylene glycol occurs mainly via the urinary excretion of ethylene glycol and via oxidation to glycolic acid. Glycolic acid undergoes urinary excretion or further degradation/incorporation.

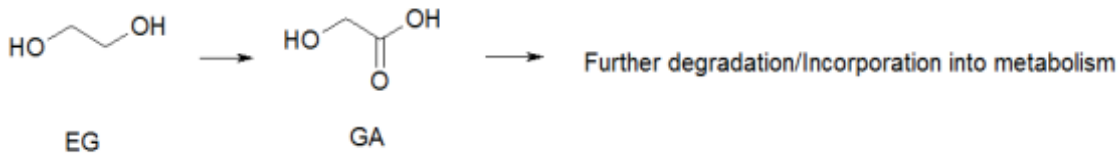

Figure C1: metabolic pathway

Mode of action: The metabolite, glycolic acid, is identified as the developmental toxicant.

A B

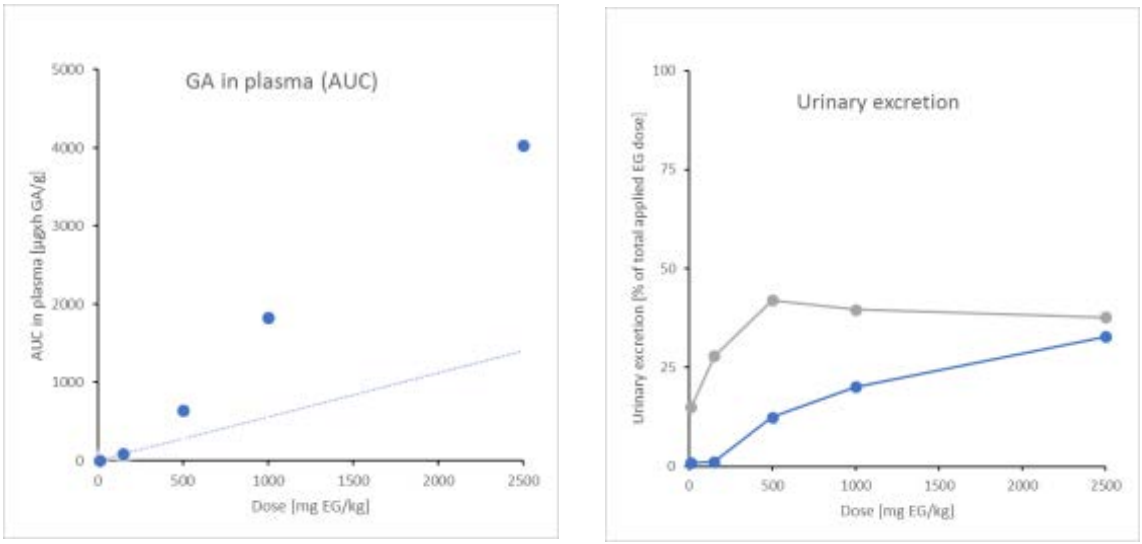

Figure C2: ADME study results demonstrating metabolic saturation of glycolic acid. A) Glycolic acid plasma levels (AUC). The dashed line is the extrapolated linear response for glycolic acid based on administration of 150 mg/kg ethylene glycol. B) Urinary excretion of ethylene Glycol (grey circle, upper curve) and glycolic acid (blue circle).

Relevance to human risk assessment: Comparison studies in rats treated either via high bolus gavage or continuous infusion demonstrates the saturation of glycolic acid metabolism is not only dose but also dose rate dependent. Only the high bolus exposure is associated with the saturation of glycolic acid metabolism and developmental toxicity in rats. Further, the PBPK models on the glycolic acid systemic burden predicts low level burden for continuous exposure. Considering that the exposure pattern for consumers and workers is more of continuous nature, the developmental toxicity associated with saturation of glycolic acid metabolism is of limited relevance for the risk assessment for consumers and workers.

Saturation of Metabolism (Afidopyropen)

Summary: At doses exceeding 15 mg/kg bw afidopyropen in the 2-year rat study, non-dose proportional kinetic properties (saturation of excretion) were observed in concentrations of afidopyropen in rat plasma. Noteworthy is that rat tumors (uterine adenocarcinomas) were only observed at high doses where non-dose proportional kinetics were observed (Van Cott et al., 2018).

ADME: At high saturated doses, both the elimination profile and the metabolic profile shifted. Urinary excretion was significantly increased at the high dose of 300 mg/kg bw and fecal and biliary excretion were decreased as compared to the lower dose of 3 mg/kg bw. Based on PK parameters the KMD is between 3 and 15 mg/kg bw/d.

Table C2 Plasma PK parameters for F344 rats administered dietary doses for 14 days followed by a radiolabeled dose on day 15. Table 19 from Van Cott et al., 2018. (Note: AUD (Area Under the Data) is a conservative estimation of AUC, used when LCMS (Liquid chromatography mass spectroscopy) detection at a low dose was below LOQ (Limit of Quantitation) for a normal AUC calculation).

External dose		Plasma parameters				
Dose		Cmax		AUC	AUD	
mg/kg bw/d	*Fold increase*	(ng/mL)	*Fold increase*	ng*h/mL	ng*h/mL	*Fold increase*
3	***1x***	24.7	***1x***	n/c	104	***1x***
15	***5x***	1500	***61x***	4480	4530	***44x***
50	***16.7x***	4759	***192x***	20700	20700	***199x***

n/c not conducted

Toxicity profile: A primary MoA leading to uterine adenocarcinomas in F344 rats is dopamine agonism and its subsequent inhibitory effect on prolactin release from the pituitary gland.

Metabolism: The major low dose metabolic pathway is the N-oxidation of afidopyropen to metabolite M4401017, is saturated at high doses. The alternate metabolic pathway is esterase hydrolysis from afidopyropen to M4401001 and this metabolite is excreted in urine disproportionately.

Mode of action: The MoA demonstrated for afidopyropen is consistent with the well-known MoA for dopamine agonists and the associated formation of uterine adenocarcinomas in rats.

Carcinogenicity study in rats: Afidopyropen was administered at dietary concentrations of 0, 100, 300 or 1000 ppm (equal to 0, 4.4, 12.9 and 42.7 mg/kg bw/day for males and 0, 5.3, 15.5, and 50.8 mg/kg bw/day for females) for 104 weeks to groups of 50 rats/sex/dose level. A second cancer study was conducted with Afidopyropen doses of 0, 1000 or 3000 ppm (equal to 0, 41.6 and 128.2 mg/kg bw/day for males and 0, 50.4 and 146.9 mg/kg bw/day for females) for 104 weeks.
Results from the 1st study: Adenocarcinoma of the uterus in females increased significantly in the 1000 ppm group.
Results from the 2nd study: Adenocarcinoma of the uterus in females increased significantly in both the 1000 and 3000 ppm group.
Based on PK parameters, dose non-proportionality occurs between 3 and 15 mg/kg bw/d. Rodent tumors were increased significantly at dose levels above 15 mg/kg bw/d.

Relevance to human risk assessment: Analysis of the mechanistic data indicates that the uterine adenocarcinomas induced by afidopyropen are quantitatively (pharmacokinetics) and qualitatively (dopamine agonist mechanism) not relevant to humans.

Saturation of Metabolism (Ethylbenzene)

Summary: Rat and mouse tumors were observed only at inhalation concentrations of 750 ppm in 2-year rodent bioassays conducted at 0, 75, 200 and 750 ppm (Chan *et al*., 1998*;* Tox Lett, 1998). Based on pharmacokinetics studies in mice AUC of EB (Ethyl benzene) in blood are disproportionately higher at 750 ppm than at 75 ppm.

Pharmacokinetics: Inhalation pharmacokinetics of ethylbenzene (EB) in B6C3F1 mice following single and repeated exposures was characterised (Charest-Tardif et al., 2006). Male and female mice were exposed for 4 h to 75, 200, 500, or 1000 ppm to determine potential non-linearity in the kinetics of EB. In addition, groups of male and female mice were exposed for 6 h to 75 ppm and 750 ppm (corresponding to the NTP cancer study exposures) for 1 or 7 consecutive days.
Single and repeated exposure pharmacokinetic data suggest that kinetics is saturable at exposure concentrations exceeding 500 ppm (and therefore at 750 ppm used in the NTP mouse cancer bioassay) but is in the linear range at the lower concentration used in the bioassay (75 ppm). The AUC of EB are disproportionately higher at 750 ppm than at 75 ppm.

Table C3: AUC based on concentration ethylbenzene in blood

Inhalation concentrations		AUC
Ppm	*Fold increase*	*Fold Increase*
75 ppm	**1x**	**1x**
200 ppm	**2.7x**	**4.7x**
500 ppm	**6.7x**	**41x**
1000 ppm	**13x**	**216x**

Charest-Tardif et al., TAAP 210: 63-69, 2006

EB kinetics is saturable at exposure concentrations exceeding 200 ppm.

Toxicity profile: Chronic inhalation exposure in animals is associated with liver hypertrophy and necrosis, kidney hyperplasia and nephropathy and thyroid and pituitary hyperplasia.

Metabolism: The principal urinary metabolites include mandelic, phenylglyoxylic, and benzoic acids which result from 1-phenylethanol, the product of a-carbon oxidation of ethylbenzene.

Mode of action: Existing MoA studies, while supporting lack of human relevance, are insufficient to exclude alternative MoAs.

Carcinogenicity studies: Studies in B6C3F1 mice resulted in renal tubular adenoma and carcinoma in the Fischer 344 rat and alveolar/bronchiolar, hepatocellular adenoma or carcinoma at inhalation concentrations of 750 ppm (NTP, 1999).

Relevance to human risk assessment: Human population-level exposures are very low, generally < 0.1 ppm. Human exposure is 7,500X lower than peak dose used in the 2-year bioassays.

Saturation of Metabolism (Ethyl tertiary-butyl ether, ETBE)

Summary: Increased incidence of hepatocellular adenomas in male rats occurred following inhalation exposure to 5000 ppm; under conditions of non-linear kinetics. PBPK TK analysis provided evidence that ETBE metabolism is saturable at exposure concentrations exceeding 2000 ppm (Borghoff et al., 2016).

Pharmacokinetics: ETBE metabolism is saturable at inhalation exposure concentrations exceeding 2000 ppm.

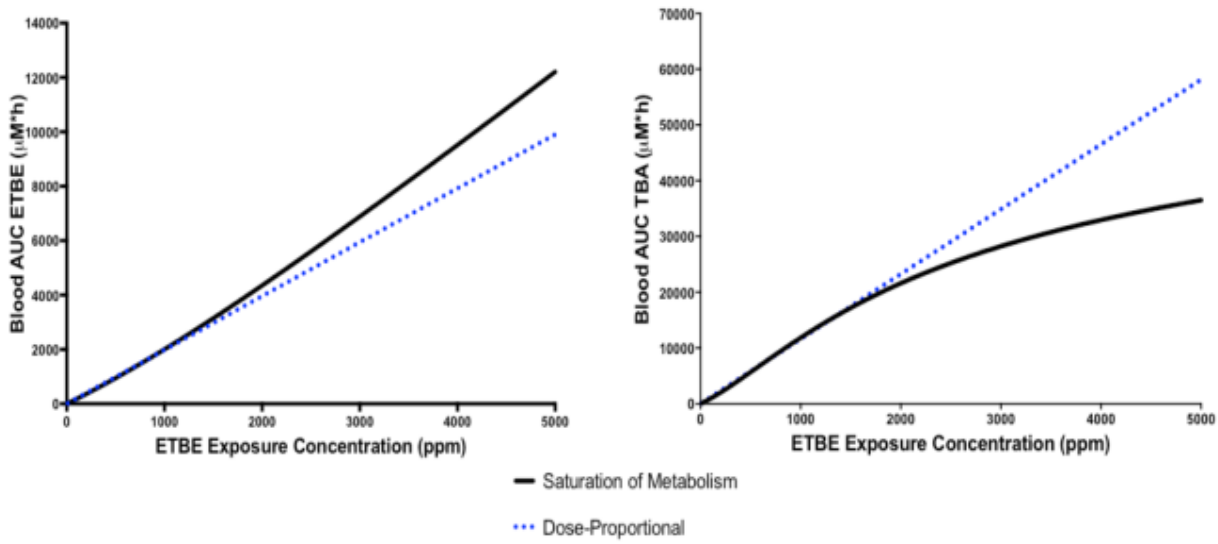

Figure C3: (Borghoff et al., 2016) PBPK model simulations of blood AUCs of ETBE and TBA following inhalation exposure to ETBE.

Toxicity profile: Kidney toxicity and liver tumors in rats.

Metabolism: ETBE metabolised to tertiary-butyl alcohol (TBA).

Mode of action: There is evidence for a role of oxidative stress via activation of CAR (Constitutive androstane Receptor), PXR (Pregnane X Receptor) and PPAR signaling pathways (Kakehashi et al., 2013).

Carcinogenicity studies: Inhalation exposures of 5000 ppm increased hepatocellular adenomas in male rats.

Relevance to human risk assessment: Human ETBE exposure levels are low compared to the range for onset of non-linear kinetics (~ 1750-2000 ppm) in animal studies. General human exposure is ~0.0091 ppm and occupational exposure ranges 0.02-0.28 ppm (Eitaki et al., 2011)

Saturation of renal clearance (2,4-D)

Summary: 2,4-D is eliminated from the body by kidney organic anion transposter-1 (OAT-1) that is expressed in rats and humans. OAT-1 is not expressed in dogs. Renal OAT-1 transporter is subject to saturation. KMD was determined to be < 26 mg/kg/day based on saturation of renal clearance in a 28-day rat study.

Pharmacokinetics:

Table C4: Plasma Cmax and AUC for 2,4-D in rats and dogs following a single oral gavage administration. Van Ravenzwaay et al., Xenobiotica 33: 69-98, 2003

TK Parameter	5 mg/kg		50 mg/kg	
	Male	Female	Male	Female
Rat				
Cmax (µg eq./g)	10	14	190	267
$AUC_{(0-t)}$ (µg eq.h/g)	21	57	1222	2358
Dog				
Cmax (µg eq./g)	32	34	233	224
$AUC_{(0-t)}$ (µg eq.h/g)	2579	2853	18310	19956

Toxicity profile: Toxicity in animal studies is observed at high doses estimated to be above saturation of 2,4-D renal clearance. Dose non-proportionality due to renal saturation has been shown in dietary studies in rats.

Metabolism: In the rat, 2,4-D is unmetabolised and excreted in urine as parent compound. In the dog, 2,4-D is excreted following metabolism; conjugated and excreted in the urine.

Mode of action: 2,4-D is actively secreted by the proximal tubules of the kidney, and toxicity appears to result when renal clearance capacity is exceeded.

Relevance to human risk assessment: worst-case human exposure is 13,000X below low dose.

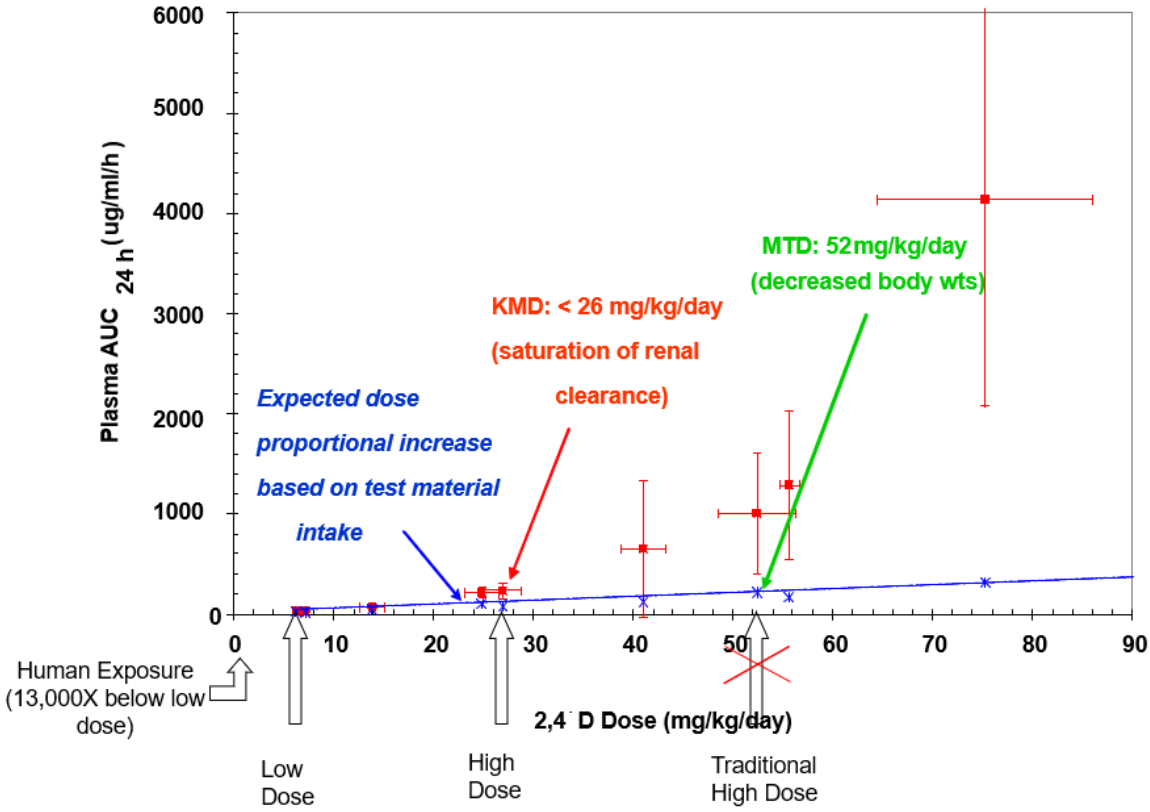

Figure C4: Modified from: Saghir *et al.,* Reg. Tox. Pharm. 63: 321-332 (2012). Plasma AUC of 2,4-D in female rats after 28 days of exposure where dose non-proportionality was determined to be less than 26 mg/kg bw /day.

APPENDIX D - AGROCHEMICALS CASE STUDIES

Dose level selection based on predicted human exposure

Exposure extrapolation for pesticides from preliminary data

The regulatory requirements for the registration of pesticide active ingredients drives risk assessment approaches which are highly data-rich. However, at the point at which dose level setting for long term and reproductive mammalian studies is being considered, the exposure data package is typically, at best, partial, and dose level setting is traditionally driven by findings in prior sub-chronic studies.

It is possible to make robust assessments of human exposure from preliminary data sources, using conservative evaluations of available data (primary and/or public) and making relevant extrapolations across both primary crop groups and secondary areas of dietary exposure. By considering potential areas of uncertainty (such as future expansion of the Good Agricultural Practice (GAP) or global market for a given AI) dose-level estimates can be made suitably robust and future-proof from increasing exposure estimates.

Data sources

In order to be able to set dose levels for chronic toxicity studies based on exposure estimates, an approximate understanding of exposure is required. This can be approximated from any of three different bodies of data, depending on the data available to the project in question.

The preferred and most reliable data source would be preliminary magnitude of residue data, generated on crops which could be extrapolated across the proposed GAP in line with regulatory accepted crop groupings. Ideally, these data (crop specific and extrapolated within crop group) should represent ≥80% of the proposed GAP. From these data, a median residue found in supervised trails (an STMR) can be derived which can be directly applied in the subsequent exposure assessment model.

Another option would be to use relevant total radioactive residue (TRR) data generated from metabolism studies in place of the STMR. It is commonly accepted that TRR data would be expected to represent a conservative overestimate of the STMR (typically considered to be approx. 3x higher), firstly as they represent the total labelled residue (irrespective of metabolism) and secondly as the studies are typically overdosed compared to GAP application rates. In the case that the active

ingredient has multiple labels for the generation of the metabolism data, the highest value for any single label should be used as representative of the worst-case TRR.

A third option would be to apply a read-across from relevant established publicly-available CODEX (Codex Alimentarius) STMR data for relevant, established and registered active ingredients. If read-across is to be considered, the degree of chemical similarity to the existing data should be ascertained. For example, a new active ingredient falling within a well-characterised family with known mode of action, for example HPPD inhibitors, could be subject to a read-across within the chemical group. If a number of related data are available (5+ related materials), a mean of the STMR data could be reliably taken as a relevant point of departure for exposure assessment. If fewer related data are available, (1-4 comparative chemistries) a 75th percentile could be considered. Should the new active ingredient fall into a novel chemical space and/or novel or undefined MoA, a read-across assessment could be considered by taking a 90th percentile of the STMR data from a broad spectrum of exposure data on relative crops for any AIs within the indication of interest with similar uses.

Data extrapolation and analysis

The data (primary or derived) would then need to be extrapolated to crops representing >80% of the intended crop/country market. This extrapolation should include linear correction for application rate, number of applications and differences in pre-harvest interval (PHI). If the intended uses include crops which are key contributors through the animal dietary burden, consideration should be made of exposure via the indirect food chain. As milk can be a key contributor to dietary exposure for infant, the most relevant consideration would be data from a cow feeding study. However, if these data were not available, an extrapolation could be made using a transfer factor calculation.

Analysis of the resultant residue data should then be converted into an exposure calculation using the most conservative relevant dietary exposure model (PRIMo3, DEEM, GEMS (North America)), relative to the intended crop/country markets (e.g., for intended uses in NoAm (North America) and EU, assessment would be conducted using PRIMo3 as the more conservative exposure tool).

Uncertainties

A range of further uncertainties would then need to be considered, including potential future expansion of the GAP. As the preliminary data was extrapolated to >80% of the intended market, a minimum uncertainty factor of 1.5x should be considered, to allow for further market expansion of the product. However, consideration should be made of the intended uses of the product in the initial

analysis. If the preliminary data includes crops which are known to be significant contributors to the food chain (e.g., wheat, apples), the potential impact of any future market expansions would be expected to be minimal. Similarly, if the initial data included post-harvest uses (which can give rise to higher exposures than pre-harvest uses) the expected impact of any scope increase would again be minimal. However, if the initial intended uses are limited to less key crops, with a small geographic market, the potential impact of future market expansion could be greater. It is considered that a doubling of potential exposure due to market expansion would typically be considered an unusually high increase, however in very niche products a potential exposure increase (and therefore uncertainty factor) of up to 5x may need to be considered.

Extrapolation to hazard point of departure and dosing

Following identification of the worst-case exposure value, an uncertainty factor of 100x (10x for both inter and intra-species differences) should be reverse applied to achieve a 'target' NOAEL for the study. Using these data as a guide to the low dose in a chronic study, relevant mid and high doses can be set at conservative intervals (e.g., 10x).

These approaches are summarised in Figure 1 and case studies are provided to illustrate the concept in use:

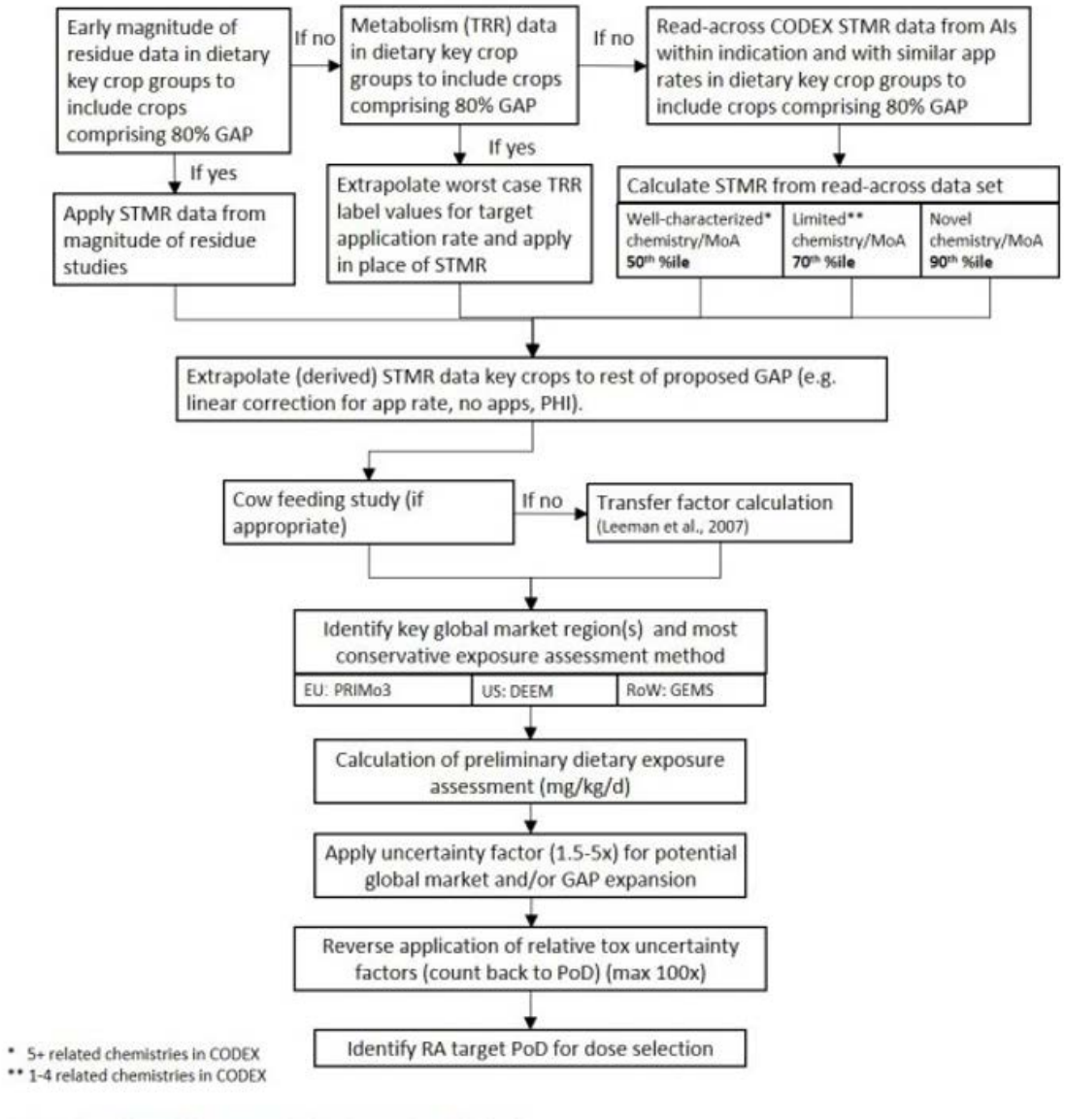

Figure D1: Decision tree for exposure assessment and dose level setting

Case study A

This case study outlines a retrospective analysis where the dose levels used in chronic and oncogenicity studies, set using traditional approaches is compared with that derived from exposure data available at the time of dose level selection.

Fungicide A has robust metabolism data available in three key crops but no magnitude of residue data. These data could be extrapolated to the rest of the GAP, including corrections for application rate and PHI as appropriate (table 1).

Table D1: Worst case TRR data for Fungicide A (single label, 1 day after application)

Crop	TRR	Application rate
Wheat	0.465 (Forage); 1.391 (Hay); 1.527 (Straw); 0.057 (Grain) mg/kg/d (TRR)	2x125 g/ha
OSR (Oil Seed Rape) seed	0.02 mg/kg/d (TRR)	1x150 g/ha
Tomato	0.630 mg/kg/d (TRR)	1x 400 g/ha

These data were extrapolated across other crops in the intended GAP, within the EU crop groups, and for different potential application rates. As the worst-case TRR data used in the analysis were samples from 1 day after application, no further extrapolation was considered necessary to correct for variances in PHI across the GAP.

No data were available to calculate the animal dietary burden, therefore by calculating exposure using transfer factors (Leeman *et al.,* 2007), extrapolated values were ascertained using the EFSA dietary burden calculator[2] and included in the dietary exposure assessment.

Based on the intended markets in EU, North and South America, the most conservative dietary exposure model was considered to be EU PRIMo3.

[2] https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_animal_model_2017.xls

Table D2: PRIMo3 input values

Crop/commodity	Input value
Solanacea	
Tomatoes	0.630
Sweet peppers/bell peppers	0.630
Aubergine/eggplant	0.630
Oilseeds	
Rapeseed	0.020
Cereals	
Barley	0.057
Maize/corn	0.114
Wheat	0.114
Products of animal origin	
Bovine muscle/meat	0.04
Bovine fat	0.05
Bovine liver	0.05
Bovine kidney	0.06
Bovine milk	0.05

Using the extrapolated input data, the output from the PRIMo3 modelling (figure 2), demonstrates that the most conservative predicted human dietary exposure is the NL toddler cohort, with an exposure of 5 mg/kg/d. As the AI has a broad GAP, and the preliminary TRR data includes key contributor crops (wheat) a worst-case extrapolation of 2x for market expansion would equate to 10 mg/kg/d. Recent calculations of dietary exposure to support EU registration have demonstrated that using the full GAP, and based on robust magnitude of residue data generated in accordance with EU requirements, the overall exposure to Fungicide A has been demonstrated to be 5.4 mg/kg/d, which indicates the robustness of the method proposed.

Table D3 EFSA PRIMo3 output values

MS Diet	Expsoure (µg/kg bw per day)
NL toddler	5.00
GEMS/Food G06	3.88
RO general	2.91
UK infant	2.65
FR child 3 15 yr	2.36
DE child	2.31
FR toddler 2 3 yr	2.21
GEMS/Food G15	2.17
NL child	2.16
UK toddler	1.94
ES child	1.93
GEMS/Food G10	1.91
SE general	1.85
GEMS/Food G08	1.79
IT toddler	1.76
GEMS/Food G07	1.70
DK child	1.68
GEMS/Food G11	1.59
DE women 14-50 yr	1.47
DE general	1.39
IT adult	1.32
ES adult	1.21
PT general	1.19
IE adult	1.04
FR infant	1.03
NL general	1.02
UK vegetarian	0.86
FR adult	0.85
DK adult	0.82
LT adult	0.75
UK adult	0.68
PL general	0.61
FI 3 yr	0.56
FI 6 yr	0.46
FI adult	0.42
IE child	0.36

To extrapolate back to a relevant hazard point of departure, reverse application of the 10x uncertainty factors for inter- and intra-species correction (total of 100X) results in a relative hazard endpoint of 1 mg/kg/day.

If this were to be considered as part of dose-level setting in a carcinogenicity study for example, a conservative strategy that would be suitably broad to ensure any future market expansion would be adequately covered, could be to use the predicted human exposure (with an uncertainty factor of100X as described above) as the lowest dose, and 10x extrapolations to set the medium and high doses. In this case, the resultant doses would be 1, 10 and 100mg/kg/d; these doses represent 100, 1,000 and 10,000 fold human exposure and should therefore be considered highly health protective.

In comparison, as part of the final registration package, a chronic rat study was conducted on Fungicide A, in accordance with current OECD requirements. Doses were achieved at 10.2/9.9, 51/31 and 319/102 mg/kg/day (male/female). In this study, a clear NOEL was demonstrated at the low-dose of 10.2/9.9 mg/kg/d, and a clear NOAEL was demonstrated at the mid-dose of 31 mg/kg/d in females and 51 mg/kg/d in males.

For Fungicide A, we can conclude that predicting the exposures from early TRR data can be used to establish a suitable paradigm for dose-level setting in chronic studies which would robustly address both the NOAEL and avoid unnecessary high-dose testing.

Case study B

This case study presents a prospective analysis and illustrates how human exposure data could be used to select doses for long term studies yet to be conducted.

Fungicide B has limited preliminary magnitude of residue and metabolism data. Parent residues were assessed for 15 other fungicide compounds on same crop with broadly similar use patterns. Residue values adjusted to match a 2 x 200 g AI (Active Ingredient)/ha (Hectare), 7d int, 7d PHI use pattern (MAF (Maximum Aerobic Function) + scaling approach). The preliminary magnitude of residue and metabolism data was compared to the resultant data extracted from CODEX to assess the veracity of the read-across comparison. Assuming the mean of the 90th %ile assessment of the CODEX STMR data to be a conservative exposure estimate, relevant inputs for modelling can be calculated (table 3).

Table D4: Fungicide B extrapolated residue table

Crop	Extrapolated STMR
Tropical tree fruits	0.41 mg/kg/d
Root veg	0.03 mg/kg/d
Soybean	0.05 mg/kg/d
OSR	0.36 mg/kg/d
Leafy veg	2.86 mg/kg/d
Pome fruit	0.45 mg/kg/d
Rice	0.37 mg/kg/d
Maize	0.03 mg/kg/d

Wheat/Barley	0.12 mg/kg/d
Bulb veg	1.48 mg/kg/d
Legume veg	0.62 mg/kg/d
Berries/small fruit	1.94 mg/kg/d
Fruiting veg	0.26 mg/kg/d
Grape	0.92 mg/kg/d

Based on the intended markets in EU, NoAM and LATAM (Latin America), the most conservative dietary exposure model was considered to be EU PRIMo3.

An estimate of potential transfer from feed into edible animal commodities was calculated using the structure-property study of Leeman *et al*. 2007, using median transfer factor values, resulting in predicted residues as listed in table 4.

Table D5: Transfer of residues into animal commodities

Animal Commodity	Transfer Factor (median)	High Predicted Residue (mg/kg)
Whole Milk	0.002	0.148
Mammalian meat	0.0024	0.177
Mammalian fat	0.0033	0.244
Mammalian offal	0.005	0.369
Eggs	0.0049	0.055
Poultry meat	0.0024	0.027
Poultry fat	0.0033	0.037
Poultry offal	0.005	0.056

Using the PRIMo3 model, using these data for both primary dietary residues and animal dietary burden levels, the resultant systemic exposure is predicted to be between 0.0019 mg/kg/d and 0.0112 mg/kg/d. Using the higher exposure level, to extrapolate back to a relevant hazard point of departure, reverse application of the 10x uncertainty factors for inter- and intra-species correction results in a relative hazard endpoint of 1.12 mg/kg/day.

As in case study A, if this were to be considered as part of dose-level setting in a carcinogenicity study for example, a conservative strategy that would be suitably broad to ensure any future market expansion would be adequately covered, could be to use the predicted human exposure (with an uncertainty factor of100X as described above) as the lowest dose, and 10x extrapolations to set the medium and high doses. In this case, the resultant doses would be 1, 10 and 100mg/kg/d; these doses represent 100, 1,000- and 10,000-fold human exposure and should therefore be considered highly health protective.

Appendix E - Industrial Chemicals Case Studies

Case study 1: OECD 422 – N-Nitrosodiphenylamine, CAS # 80-30-6

In order to define the dose levels of the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD 422), a 14-day repeated toxicity study was performed with N-Nitrosodiphenylamine in Sprague-Dawley rats. The test item was administered daily by gavage to five males and five females at dose levels of 100, 300 or 1000 mg/kg/d.

The dose level of 1000 mg/kg/d was associated, in both sexes, with mortality, premature euthanasia of the surviving animals and signs of poor clinical condition (i.e. thin appearance, hunched posture, piloerection, pallor of extremities, half-closed eyes, hypoactivity, hypotonia, staggering gait, decreased grasping reflex and/or dyspnea), appetite loss leading to a lower body weight, markedly reduced food consumption and macroscopic changes in the stomach (white, black or red discolouration, gas distension), forestomach (white or red discolouration), intestine (distension with feces or with gas and/or thick or liquid brown content), spleen (enlargement), ureters (dilatation), kidneys (enlargement, pelvis dilatation, gelatinous hilus and perirenal adipose tissue and/or yellow discolouration), pancreatic lymph nodes (enlargement) and liver (enlargement and/or yellow discolouration). No microscopic observation was performed.

The dose level of 300 mg/kg/d was associated with poor clinical condition (i.e. hunched posture and piloerection in one male and one female), body weight loss in males at the beginning of the treatment period, moderate reduced food consumption in both sexes during the first week of the treatment period, organ weight changes in the liver (absolute and relative liver weights moderately increased day in both sexes), the spleen (marked increases in the mean absolute and relative spleen weights in both sexes), and in the kidneys and the heart (mean absolute and relative kidney or heart weights minimally increased in females reaching statistical significance for the absolute weight), macroscopic changes in the spleen (enlargement in both sexes) and ureters (dilatation in males) and microscopic changes in the liver (slight to moderate hepatocellular hypertrophy and minimal hematopoiesis in both sexes), the spleen (increased severity of hematopoiesis and congestion along with increased hemosiderin in both sexes suggestive of hemolysis) and the kidneys (eosinophilic granules in renal tubules in males suggestive of hemolysis).

The dose level of 100 mg/kg/d was associated with slight reduced food consumption in females during the study, macroscopic changes in the spleen (enlargement in both sexes) and microscopic changes in the liver (minimal hepatocellular hypertrophy in males and minimal hematopoiesis in both sexes), and the spleen (increased severity of hematopoiesis in both sexes and congestion along with increased hemosiderin in females suggestive of hemolysis).

The doses of 300 and 1000 mg/kg/d are considered to be higher than the Maximum Tolerable Dose (MTD). So, 150 mg/kg/d was selected as the high-dose level in the main OECD 422 study. The low-dose and mid-dose were selected using a ratio representing approximately a 3-fold interval (i.e., 15 and 50 mg/kg/d).

In the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test, the No Observed Adverse Effect Level (NOAEL) for parental systemic toxicity was considered to be 50 mg/kg/d based on the microscopic findings observed at 150 mg/kg/d in the urinary bladder (i.e., hyperplasia and single cell necrosis of the urothelium) of some males and females and in heart (i.e., cardiac atrial thrombosis and hypertrophy) of one female. The NOAEL for reproductive performance (mating, fertility and delivery) was considered to be 150 mg/kg/d in the absence of adverse findings at this high-dose level, and the NOAEL for toxic effects on progeny was considered to be 15 mg/kg/d (based on clinical signs and live birth and viability indexes at 50 and 150 mg/kg/d).

Case study 2: High dose setting with an irritant in oral gavage rat studies 1,3- and 1,4- cyclohexandicarboxyaldehyde, EC # 482-020-3

The following studies of 1,3 and 1,4-cyclohexandicarboxyaldehyde provide an example of the progression of repeat dose oral gavage studies in the rat where the high dose level was determined based on point of contact irritation in the gastric lining.

14 Day Oral Gavage Dose Range Finding Study in Rats

This repeated dose toxicity study was conducted to evaluate the toxicity potential of the test substance, 1,3 and 1,4 Cyclohexanecarboxaldehyde, when administered orally by gavage to Wistar rats for 14 consecutive days and in order to select dose levels for a further screening test on Reproduction/Developmental toxicity. The test substance was administered at an equivolume of 4 mL/kg/day to groups of rats at the dose levels of 250, 500 and 1000 mg/kgbw/d, respectively. Concurrently, vehicle control group animals were administered the vehicle alone (corn oil) at the same dose volume. Each group in the study was comprised of 3 rats per sex. All the rats in the study were observed for clinical signs and body weights and feed consumption were recorded. Kidney and liver weights were recorded from all the terminally sacrificed rats after gross pathology examination.

The daily oral gavage of 1,3 and 1,4 Cyclohexanecarboxaldehyde for 14 consecutive days at the dose of 250 mg/kgbw/d had no test substance-related effects on the general health of the animals, body weights, net body weight gains, feed consumption, organ weights of the liver and kidneys and gross pathology. However, at 1000 mg/kgbwt/d, treatment related mortalities in one male and one female were observed, and one male rat was also euthanised moribund. Clinical signs of hypo activity and salivation were observed at 500 and 1000 mg/kgbwt/d. The mean and net body weight gains and feed consumption were lower at 1000 mg/kgbwt/d compared to the concurrent control group. Percent body weight gain was also decreased in males (32%) and females (20%) at 500 mg/kgbwt/d. The treatment resulted in an increase in the absolute and relative liver weights in females at 1000 mg/kgbwt/d. There was also a marginal increase in absolute (15%) and relative (16%) liver weights in females at 500 mg/kgbwt/d. Gross pathological findings included erosions and thickening of the non-glandular and/or glandular stomach and intestines of some animals in the 1000 mg/kgbwt/d group. Raised foci were present in the non-glandular stomach of 3 males and 2 females in the 500 mg/kgbwt/d group.

The results of this range-finding study included body weight effects as well as point of contact irritation in the stomach at 500 mg/kgbwt/d, and death and moribundity at 1000 mg/kgbwt/d. Since the purpose of this study was to assist in determining dose levels for an upcoming definitive study, 500 and 1000 mg/kgbwt/d are both judged to be too high of a dose for the further screening test on Reproduction/Developmental toxicity (OECD 421), due to the longer duration of exposure, as well as the additional stressors of gestation and lactation phases on the dams.

Reproduction and Developmental Toxicity Screening Study in Rats (OECD 421)

This Reproduction/Developmental toxicity screening test was conducted in Wistar Rats to evaluate the possible toxicity of the test substance, 1,3 and 1,4 Cyclohexanecarboxaldehyde, on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition.

The test substance was administered in corn oil at an equal volume of 4 mL/kg/day groups of rats at the dose levels of 50, 150 and 400 mg/kgbwt/d. The vehicle control group animals were administered concurrently with corn oil alone at the same dosing volume. Each group in the study was comprised of 10 rats per sex. The males were dosed for a period of at least six weeks (which included 2 weeks prior mating, 2 weeks during mating and 2 weeks post mating), up to and including the day before sacrifice. Females were dosed throughout the treatment period. This included two weeks prior to mating (with the objective of covering at least two complete oestrous cycles), the variable time to conception, the duration of pregnancy and four days after delivery.

All the rats in the study were observed for clinical signs once daily and for morbidity and mortality twice daily. Parental body weights, gains, and feed consumption were recorded according to study guidelines. Pups from each litter were observed for total numbers, individual body weight, and survival during lactation day 0-4. All male rats were sacrificed two weeks after completion of the mating process and all the littered female rats were sacrificed on LD 5. For all adult animals, gross necropsy was performed and liver, kidneys, testes and epididymides were collected and weighed. Histopathological examination was carried out on all the preserved organs and tissues (including gross lesions) of control and high dose group rats with special emphasis on stages of spermatogenesis in male gonads and interstitial testicular cell structure. Stomach from low and mid-dose males and females were examined as treatment-related changes were observed in high dose groups. Salient findings of the study are as follows: The treatment-related clinical sign of slight salivation was observed during and after gavage administration in the 400 mg/kgbwt/d dose group males (9/10) and females

(2/10). This persisted for 10-15 minutes post dosing and rats returned to normal thereafter. All adult animals survived until termination. Treatment with the test substance 1,3 and 1,4 Cyclohexanecarboxaldehyde did not cause any test substance related effects on general health, body weights, feed consumption, mating and fertility, mean number of corpora lutea and implantations, live birth index, mean litter size, growth and development of pups. There were no test substance related changes in terminal fasting body weights and organ weights at all the doses tested. Multiple raised foci and/or diffuse thickening was observed grossly in non glandular stomach in 400 mg/kg/d dose males and females. Microscopically these observations were associated with ulcers and epithelial hyperplasia/hyperkeratosis and were considered test substance-related adverse changes. In view of the results observed: Under the conditions of this study, based on the adverse effects which were limited to the non-glandular stomach of males and females at 400 mg/kg/d, the No Observed Adverse Effect Level (NOAEL) for local effects was determined to be 150 mg/kg/d, while the NOAEL for systemic effects was 400 mg/kg/d. As there were no adverse effects on reproduction and fertility parameters up to and including 400 mg/kg/d, the NOAEL for reproductive toxicity was determined to be 400 mg/kg/d, the highest tested 1,3 and 1,4 Cyclohexanecarboxaldehyde dose level.

Developmental Toxicity Dose Range Finding Study in Rats

The purpose of this study was to make a preliminary evaluation of the maternal toxicity and embryo/fetal lethality potential of 1,3- and 1,4-Cyclohexanedicarboxaldehyde in Crl:CD(SD) rats following repeated gavage administration. Results from this study were used to set dose levels for a subsequent developmental toxicity study in Crl:CD(SD) rats. Groups of five time-mated female Crl:CD(SD) rats were administered 0, 250, 500, or 750 mg/kg/d 1,3- and 1,4-Cyclohexanedicarboxaldehyde in corn oil by oral gavage at a dose volume of 4 ml/kg on gestation day (GD) 6 through 20. In-life parameters evaluated for all groups included clinical observations, body weight, body weight gain, and feed consumption. On GD 21 all surviving dams were euthanised and examined for gross pathologic alterations. Liver and kidney weights were recorded, along with the number of *corpora lutea*, implantations, resorptions, and live/dead fetuses. Histopathological examination of the stomach was conducted on all control and 250 mg/kg/d animals.

Treatment-related noisy respiration was observed in one dam in each of the 500 and 750 mg/kg/d groups. There were no treatment-related clinical observations noted on animals in the 250 mg/kg/d group. In the 500 and 750 mg/kg/d groups, treatment-related decreases in body weight were present at GD 18-21. For both of these dose groups, there was a treatment-related decrease in maternal body

weight gain and feed consumption beginning at the GD 9-12 interval and continuing through the GD 18-21 interval. Body weight gain of animals in the 250 mg/kg/d group showed a treatment-related decrease at the GD 9-12 interval; however, body weight gains were similar to control for all other measured intervals, and body weight was similar to controls throughout the study.

There was a primary treatment-related increase in absolute and relative liver weights in the 750 mg/kg/d group. In addition, a treatment-related increase in relative kidney weights was observed in the 750 mg/kg/d group that was deemed secondary to decreased body weight. There were no treatment-related liver or kidney weight effects in the 250 or 500 mg/kg/d groups.

At all dose levels, point of contact irritation of the stomach was observed. In the 500 and 750 mg/kg/d groups, stomach gross pathology findings in all animals included multifocal or focally extensive thickening of the non-glandular mucosa. Additional gross findings in some animals given 500 or 750 mg/kg/d included glandular and nonglandular mucosal ulceration, glandular mucosal hyperemia, and gastric wall abscesses. A single animal in the 250 mg/kg/d group had a gross focal thickening of the non-glandular stomach. Microscopically, this focal observation was associated with epithelial ulceration, subacute inflammation, hyperkeratosis, and hyperplasia. Histological findings (without a gross pathology correlate) of very slight gastric epithelial hyperkeratosis and hyperplasia at the limiting ridge were present in four and three of five animals, respectively in the 250 mg/kg/d group.

Oral gavage administration of 1,3- and 1,4-Cyclohexanedicarboxaldehyde to time-mated Crl:CD(SD) rats resulted in maternal toxicity at all dose levels tested but no indication of embryo/fetal lethality at any dose level tested. Based upon the treatment-related increase in stomach epithelial ulceration observed at 250 mg/kg/d in this study, 1,3- and 1,4-Cyclohexanedicarboxaldehyde oral gavage dose levels less than 250 mg/kg/d are appropriate for a full prenatal developmental toxicity study in Crl:CD(SD) rats.

Developmental Toxicity Study in Rats (OECD 414)

The purpose of this study was to evaluate the maternal and developmental toxicity of 1,3- and 1,4-cyclohexanedicarboxaldehyde in Crl:CD(SD) rats following repeated gavage administration. Groups of 24 time-mated female rats were administered 1,3- and 1,4- cyclohexanedicarboxaldehyde by gavage in corn oil on gestation day (GD) 6 through 20 at dose levels of 0, 25, 75, or 225 mg/kg/d. These dose levels were selected based upon a treatment-related ulceration of the stomach in a single dam at 250 mg/kg/d along with gastric hyperkeratosis and hyperplasia in three of five dams in a preceeding

developmental toxicity dose range finding study. The stomach ulceration observed at 250 mg/kg/d in the developmental toxicity dose range finding study showed that this dose level exceeded an acceptable high dose level for the full developmental toxicity study.

In-life maternal study parameters included clinical observations, body weight, body weight gain and feed consumption. On GD 21, all rats were euthanised and examined for gross pathologic alterations. Liver, kidneys and gravid uterine weights were recorded, along with the number of corpora lutea, uterine implantations, resorptions and live/dead fetuses. Histopathological examination of the stomachs from all pregnant dams was conducted. All fetuses were weighed, sexed and examined for external alterations. Approximately one half of the fetuses were examined for visceral alterations while skeletal examinations were conducted on the remaining fetuses.

Gavage administration of 1,3- and 1,4-cyclohexanedicarboxaldehyde resulted in no treatment-related effects on clinical observations, body weight, body weight gain, feed consumption, organ weights, or gross pathology in dams in any treated groups. Histopathological examination of dams revealed treatment-related very slight or slight hyperkeratosis and hyperplasia of the nonglandular mucosa of the stomach at the limiting ridge with associated chronic active inflammation of the underlying submucosa at dose levels of 75 and 225 mg/kg/d. In addition to hyperkeratosis and hyperplasia, a single animal given 225 mg/kg/d 1,3- and 1,4-cyclohexanedicarboxaldehyde had focal moderate ulceration of the nonglandular mucosa at the limiting ridge and moderate focal chronic-active inflammation within associated submucosal tissues. Administration of 1,3- and 1,4-cyclohexanedicarboxaldehyde via gavage at dose levels up to and including 225 mg/kg/d produced no indications of embryo/fetal toxicity or teratogenicity.

Due to point of contact irritation resulting in hyperkeratosis and hyperplasia in the stomach at dose levels ≥75 mg/kg/d, the no-observed-effect level (NOEL) for maternal toxicity was 25 mg/kg/d. Based on the absence of systemic effects up to and including 225 mg/kg/d, the NOEL for systemic maternal toxicity was 225 mg/kg/d. The embryo/fetal no-observed-effect level (NOEL) was 225 mg/kg/d, the highest dose level tested.

Multigeneration Reproduction Study in Rats (OECD 416)

The purpose of this oral gavage two-generation reproduction toxicity study was to evaluate the potential effects of 1,3- and 1,4-cyclohexanedicarboxaldehyde (1,3- and 1,4-CHDA) on male and female reproductive function, as well as the survival, growth and development of the offspring.

Groups of 25 male and 25 female Crl:CD(SD) rats were administered the test material seven d/wk via oral gavage at dose levels of 0, 10, 50, and 150 mg 1,3- and 1,4-CHDA /kg of body weight/d (mg/kg/d, mkd) for approximately ten weeks prior to breeding and continuing through breeding, gestation and lactation for two generations. In-life parameters included clinical observations, feed consumption, body weights, estrous cyclicity, reproductive performance, pup survival, pup body weights, and puberty onset. In addition, post-mortem evaluations included gross pathology, histopathology, organ weights, oocyte quantitation and sperm count, motility and morphology in adults, and gross pathology and organ weights in weanlings.

Treatment of rats with 1,3- and 1,4-CHDA for two generations did not result in treatment related effects on any in-life parameter or any parameter of reproductive function or offspring survival, growth or development.

Treatment-related effects on organ weight were limited to increased kidney weights in P1 females and P2 males and females administered 150 mg/kg/d. At this dose level, absolute and relative P1 female kidney weights were increased 6.8% and 4.8%, respectively, compared to control. In the P2 generation at 150 mg/kg/d, absolute and relative kidney weights were increased in males (6.3 and 6.5%, respectively) and females (6.9 and 6.4%, respectively) compared to control. There was no treatment-related effect on absolute or relative kidney weights in any parental generation at 10 or 50 mg/kg/d. There were no treatment-related effects on F1 or F2 weanling organ weights in either generation.

Treatment-related gross pathologic and histologic observations were limited to the stomach of P1 and P2 male and female rats. At 150 mg/kg/d, P1 and P2 males and females had thickened squamous mucosa at the junction of the glandular and non-glandular portions of the stomach (i.e., stomach limiting ridge). Histopathologically, this gross observation corresponded to very slight to slight hyperplasia and hyperkeratosis at the stomach limiting ridge in most P1 and P2 males and females administered 150 mg/kg/d. At 50 mg/kg/d, gross pathology was limited to one P2 male with thickening of the stomach limiting ridge, and histopathological changes were limited to very slight hyperkeratosis and very slight to slight hyperplasia of the stomach limiting ridge in P2 males and females. No treatment related gross pathologic or histologic changes were observed in any parental generation at 10 mg/kg/d. No treatment-related gross pathologic change was observed in F1 or F2 weanlings at any dose level.

Under the conditions of the study and based on the increased P1 and P2 kidney weights at 150 mg/kg/d, the no-observed-effect level (NOEL) for systemic toxicity was 50 mg/kg/d. Based on the

stomach histologic change at 50 mg/kg/d, the NOEL for point of contact toxicity was 10 mg/kg/d. Due to the lack of any treatment-related effects on reproductive performance or offspring survival, growth and development, the NOEL for reproductive toxicity was 150 mg/kg/d, the highest dose level tested.

Summary of rat oral gavage studies with 1,3 and 1,4 Cyclohexanecarboxaldehyde

The table below summarises the dose levels, exposure duration and effect levels for systemic, point of contact irritation, and reproductive and developmental effects of selected oral gavage studies with 1,3 and 1,4 Cyclohexanecarboxaldehyde as they were conducted chronologically. This material causes irritation of the stomach (primarily the forestomach, but also the glandular stomach and small intestines) that increases in severity and/or incidence with increasing dose concentration or exposure duration. This example shows how one study can inform the next for dose level selection. One challenge with the order of these studies, is the high dose level selection of the OECD 414 study when the OECD 421 results are available can be difficult. In this scenario, the dose levels are selected based on a longer duration study going to a shorter duration study. For this purpose, the 14 day DRF study can be used, but it is not always clear if the results of stomach irritation in non-pregnant animals will be predictive of those in pregnant animals. In this case, pregnant animals were slightly more susceptible to the irritating effects of the material, as shown by the different effects between the two DRF studies, with grossly observed ulcerations in pregnant animals. In addition, the studies were performed at different laboratories and in different strains of rats. Therefore, to ensure that an MTD was determined, the DRF for the OECD 414 also included dose levels of 500 and 750 mg/kg/d. As a NOAEL for stomach irritation was not determined in the DRF, the high dose level for the full OECD 414 was set slightly below the lowest dose level, but still resulted in ulceration in the forestomach of one animal. From the results of all the previous studies, it was possible to set the dose levels for a multigeneration study, taking into consideration that stomach irritation would occur at dose levels ≥ 75 mg/kg/d. Across all studies, there were no indications of reproductive or developmental toxicity, and other systemic effects occurred only at doses that produced an unacceptable level of point of contact irritation. The aim of the dose setting for the longer term studies, therefore, was to induce irritation, but not to a level that would result in ulcers/erosions of the forestomach which would be contrary to animal welfare principles.

Table E1

STUDY CHRONOLOGICAL	DOSE LEVELS	NOAEL (SYSTEMIC)	NOAEL (POINT OF CONTACT)	NOAEL (REPRO/DEV)
DRF for OECD 421 **14 days exposure**	0, 250, 500 or 1000 mg/kg/d	250 mg/kg/d based on decreased BW (Body Weight) and organ weight changes at higher doses	250 mg/kg/d based on gross erosions and ulcerations in the stomach and intestines at higher doses	Not applicable
OECD 421 **About 6 weeks in both sexes; until LD 4 in females**	0, 50, 150, or 400 mg/kg/d	400 mg/kg/d	150 mg/kg/d based on adverse effects in the non-glandular stomach at 400 mg/kg/d	400 mg/kg/d
DRF FOR OECD 414 **15 days exposure (GD 6-20)**	0, 250, 500, or 750 mg/kg/d	250 mg/kg/d based on decreased BW gains at higher doses	Could not be determined based on adverse effects in the non-glandular stomach at all dose levels, including ulcers/erosions	750 mg/kg/d
OECD 414 **15 days exposure (GD 6-20)**	0, 25, 75, or 225 mg/kg/d	225 mg/kg/d	25 mg/kg/d based on adverse effects in the non-glandular stomach at higher doses, including ulcers/erosions in one animal at 225 mg/kg/d	225 mg/kg/d
OECD 416 **For two generations; about 18-20 weeks duration each**	0, 10, 50, or 150 mg/kg/d	50 mg/kg/d based on increased kidney weights at 150 mg/kg/d	10 mg/kg/d based on adverse effects in the non-glandular stomach at higher doses	150 mg/kg/d

Case study 3: Developmental toxicity study in rabbits – 1,3(4)-bis(tert-butylperoxyisopropyl) benzene, CAS # 25155-25-3

In a dose-range finding study (DRF) performed with 1,3(4)-bis(tert-butylperoxyisopropyl) benzene, roups of eight mated-female rabbits were administered to 0, 50, 250 and 500 mg/kg/d during the gestation period, starting from Day 6 through Day 28 post coitum at the dose volume of 2.5 mL/kg using a mixture of corn oil and CMC 2% as vehicle.

Maternal toxicity observed was summarised in the following table:

Table E2

	50 mg/kg/d	**250 mg/kg/d**	**500 mg/kg/d**
Clinical signs	Reduction in faeces (1/7)	Reduction in faeces (3/6)	Reduction in faeces (6/7) + Soft feces (1/7)
Body weight	-	-	Lower on Day 15 p.c. until termination (- 8%)
Body weight gain	-	Reduced on Days 18 (-13%) and 21 (-9%) p.c.	**Reduced on Day 12 (-4%), Day 15 (-19%), 18 (-25%) p.c.**
Food consumption	-	**Reduced on Day 18 (-38%)**	Reduced from Day 12 **(-17% on Day 12, -54% on Day 15, -51% on Day 18, -48% on Day 21)**
Macroscopic examination	-	**-**	-
Changes when compared to the control group; - : No relevant change or observation; In bold: statistical changes			

Developmental toxicity was observed at 500 mg/kg/d only with reduced uterus weight (-31%) associated with reduced litter weight (-31%) and a high post implantation loss incidence (17.73% versus 1.39%) when compared to the control group. No abnormal finding was recorded during the examination of foetuses.

In conclusion, the treatment with the test item at dose level of 500 mg/kg/d caused maternal and developmental toxicity and at 250 mg/kg/d signs of maternal toxicity were also evident. Based on these outcomes, the highest dose level for the subsequent main reproductive toxicity study should be lower than 250 mg/kg/d.

Then, the main pre-natal developmental toxicity (PNDT) study in rabbits was performed by gavage according to test guideline OECD 414. The doses used in the study were 25, 100 and 200 mg/kgbw/d. There was no maternal or developmental toxicity in the study and a NOAEL for maternal and developmental toxicity was considered to be equivalent to the highest dose tested (200 mg/kg).

The European Authorities (ECHA) conclude that given the above DRF findings the doses used in the pre-natal developmental toxicity study were not selected with view to the principles of EU Test Method B.31, OECD TG 414 .i.e. “the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death or severe suffering”. They considered that there is still a concern over developmental toxicity and requested to perform a new study in the draft decision of a new Compliance Check.

Case Study 4: EOGRTs (OECD 443) - Reaction mass of bis(2,3-epoxypropyl) terephthalate and tris(oxiranylmethyl) benzene-1,2,4-tricarboxylate, EC # 940-592-6

Reaction mass of bis(oxiran-2-ylmethyl) terephthalate and tris(oxiran-2-ylmethyl) benzene-1,2,4-tricarboxylate is classified as skin sensitiser Cat 1, STOT RE2 (CNS, epididymides), skin irritant Cat 2 and eye corrosive Cat 1.

On 21st November 2016 ECHA published a final decision requesting the conduct of an OECD 414 Pre-natal developmental toxicity study in the rat and an Extended one-generation reproductive toxicity study using the oral route in rats with a 10-week premating exposure duration including Cohorts 1A, 1B, 2A and 2B. The request also specified that dose level setting shall induce some toxicity at the highest dose level. It was concluded by ECHA that an OECD 407 28-day repeated dose study identified adverse effects on reproductive tissues and central nervous system.

During the 28-day repeated dose study the test item was administered daily in graduated doses (40, 80 and 240 mg/kgbw/d) to 3 groups of test animals, one dose level per group for a treatment period of 28 days. Animals of an additional control group were handled identically as the dose groups but received corn oil, the vehicle used in this study. The 4 groups comprised of 5 male and 5 female Wistar rats Crl: WI(Han). During the period of administration, the animals were observed precisely each day for signs of toxicity. Body weight and food consumption were measured twice weekly. At the end of the treatment period, all animals were sacrificed and subjected to necropsy. The wet weight of a subset of tissues was determined and a set of organs/tissues was preserved. Full histopathological

evaluation of the tissues was performed on high dose and control animals. Organs showing gross alterations were also examined histopathologically. Stomach, liver and spleen were also examined in the mid and low dose groups.

During the weekly detailed clinical observation, no significant changes or differences between the groups were found. No relevant effects were observed in any of the parameters of the functional observation battery before and at the end of the treatment period. There were no ophthalmoscopic findings in any of the animals of this study.

On the basis of the present study, the 28 Days Repeated Dose Oral Toxicity study with bis (2,3-epoxypropyl) terephthalate in male and female rats, with dose levels of 40, 80 and 240 mg/kg/d the following conclusions can be made:

No effects of bis (2,3-epoxypropyl) terephthalate were found at dose levels of 40 and 80 mg/kgbw. The NOEL of bis (2,3-epoxypropyl) terephthalate in this study is considered to be 80 mg/kgbw.

At a dose level of 240 mg/kgbw slight clinical symptoms occurred in few more animals than in control animals and a tendency towards an attenuated body weight gain and food intake were observed. A slightly lower heamoglobin level in male animals was associated with a slight compensatory increase in reticulocytes. These effects are not considered to be in the respective toxic range. Thus, the NOAEL in this study is considered to be 240 mg/kgbw.

Diffuse minimal hyperkeratosis of the nonglandular part of the stomach was found in the majority of female rats dosed with 240 mg/kgbw. This effect might be related to a local irritant effect of the test item formulation when administered repeatedly by oral gavage and was therefore not considered relevant for humans.

The GLP OECD 407 28-day repeat dose study indicated the following effects:

1) No observed adverse effect level: 75 mg/kgbw/d
2) Effects on central nervous system
3) Effects on male reproductive system (epididymides)
4) Effects on red blood cells in females

EOGRTS Range finding 1

A 14-day range finding study was conducted at 50, 100 and 200 mg/kgbw/d, under the conditions of this test all female animals failed to litter with no signs of implantation at necropsy. Analysis of sperm identified a dose related reduction in sperm motility, morphology and cauda epididymal sperm numbers and sperm motion parameter at all doses. As a NOAEL could not be determined an additional Range Finding study was conducted

EOGRTS Range finding 2

A second 14-day range finding study was conducted at 3, 15 and 30 mg/kgbw/d. Again, in this second range finding study there was a reduction in sperm motion parameters and concomitant increases in percentage static sperm. In both the 15 and 30 mg/kgbw/d groups there was statistically significant decreases in testicular spermatids. At 3 mg/kgbw/d low testicular sperm count/concentration was observed however, with no decrease in epididymal sperm numbers or effects on mating performance and fertility, these seminology findings were of uncertain relationship to treatment.

Main EOGRTs

Based on the results of the two range finding studies the main OECD 443 EOGRTs test was conducted with dosing for 10 weeks at 2.5, 6.0 and 15 mg/kgbw/d. There were no test item related changes in clinical condition or signs related to the administration of any dose level investigated. No effects on body weight, food consumption, estrous cycle or haematology. Blood chemistry identified a significant increase in creatinine concentrations in all groups of treated males. A slight but not significant increase in urine output was evident in males receiving 15 mg/kgbw/d. This increase in urine also aligns with statistically significant increases in body weight relative kidney weights.

Among males given a 10-week pre pairing treatment period there were no observed effects on sperm motility, cauda epididymal and testicular spermatid counts or sperm morphology parameters. The assessment of sperm motion revealed, when compared to Controls, a statistically significant but non dose-dependent decrease in the rapid motion parameter in males given 6 or 15 mg/kg/d, with a slight increase in the associated motion parameters of medium, slow and static, although these differences from Control did not attain statistical significance. Due to the lack of dose dependence these findings are unlikely to have biological significance.

For males given the test item at 15 mg/kg/d for 2 weeks prior to pairing, there were no observed effects on sperm motility, cauda epididymal and testicular spermatid counts or sperm morphology

parameters. When compared to Controls, statistically significant slight increases were apparent in the motion parameters VAP (average path velocity), VSL (progressive or straight-line velocity), VCL (curvilinear velocity or track speed) and ALH (amplitide of lateral head displacement). In addition, there were slightly more normal and slightly less abnormal sperm than in Controls, with fewer sperm showing flat head and looped tail abnormalities. This additional group raises some doubt over the findings identified in the preliminary studies.

There was no effect of treatment on group mean litter size, offspring survival to Day 21 of age or sex ratio at any dose level investigated. There was also no discernible difference between litters derived from parental animals given 15 mg/kg/d for 10 weeks before pairing and litters derived from untreated females/parental males given 15 mg/kg/d untreated females for 2 weeks before pairing. There was no effect of treatment at any dose level investigated on group mean birth weights and subsequent body weight gain of male and female offspring. There was no effect of treatment on the ano-genital distance of F1 offspring at any dose level investigated.

Based on the findings of this study the highest tested dose of 15 mg/kgbw/d is concluded to be the NOAEL and representative of the MTD for this substance.

Case study 5: Mutagenicity - 4,4'-methylenebis [N, N-bis(2,3-epoxypropyl) aniline], CAS # 28768-32-3

Toxicity, absorption and distribution:

The low molecular weight (i. e., <500 g/mol), moderate log Pow value (i. e., between -1 and 4), and slight water solubility (i. e., around 10 mg/L) of epoxy resins favour their absorption from the gastrointestinal tract. However, due to a viscous liquid state, the absorption of substances is slow following oral exposure. In organs such as the duodenum and stomach this slow absorption can lead to increased residence time of the substances at the epithelial interface. The increased duration is likely to be important in local organ toxicity and manifest in site of contact effects particularly in cases where a substance is highly reactive and has hazardous properties such as irritation, corrosivity and sensitisation. The slow absorption is supported by the low systemic toxicity observed in acute oral, dermal and inhalation toxicity studies [1].

No signs of potential CNS effects were observed on any day of oral exposure of rats to epoxy resins at doses of up to 200 mg/kgbw/d for 90 days. Clinical signs decreased mean body weight, haematology

and clinical biochemistry findings at 200 mg/kg/d were observed in rats following oral administration for 90 days. No other statistically significant, compound-related systemic effects were observed. In addition, a 28 day repeat dose study by oral gavage the overall NOAEL was deemed to be 100 mg/kgbw/d although at this dose clinical signs included Ptylalism, piloerection and liver hepatocytic hypertrophy. Based on review of the 28-day and 90-day data presented in the table below it can be concluded that the maximum threshold dose based on the available 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] data-set is within the range of 50 – 100 mg/kgbw/d.

The viscous state, water solubility and log Pow value do not favour dermal absorption, since these values indicate that epoxy resins may be too hydrophilic to cross the stratum corneum. In addition, the high surface tension (i. e., above 10 mN/m) does not favour dermal absorption. Although dermal irritancy, corrosion or sensitisation may enhance dermal absorption by compromising the integrity of the epidermal barrier during chronic exposure, no corrosion or systemic effects were observed in the acute dermal toxicity study available. Thus, considering the physicochemical properties of the substance, and the lack of observed systemic effects following dermal exposure, absorption via the skin is not significant compared to the oral route, although it should be noted that the dermal route represents the highest probability of exposure based on worker activity and exposure scenarios in the operational and application landscape if PPE and safety recommendations are not followed.

Metabolism:

Once absorbed the substance may be metabolised by two different enzymatic routes: conjugation of the epoxide moiety with the endogenous tripeptide glutathione (GSH) catalysed by glutathione S-transferase (GST) or hydrolysis of the epoxide moiety catalysed by epoxide hydrolase (EH), the second way being the most efficient way of detoxification of epoxy compounds. The epoxide hydrolases are a class of proteins that catalyse the hydration of chemically reactive epoxides to their corresponding dihydrodiol products. Simple epoxides are hydrated to their corresponding vicinal dihydrodiols, and arene oxides to trans-dihydrodiols. In general, this hydration leads to more stable and less reactive intermediates that can be readily conjugated and excreted. In mammalian species, there are at least five epoxide hydrolase forms, microsomal cholesterol 5,6-oxide hydrolase, hepoxilin A(3) hydrolase, leukotriene A(4) hydrolase, soluble epoxide hydrolase, and microsomal epoxide hydrolase. Although highly concentrated in the liver, epoxyde hydrolases are also found in other organs like brain, adrenal gland or skin.

Investigation of epoxide hydrolysis and alkylation potency of various glycidyl compounds in vitro showed that half-life of the glycidyl compounds was between 7.3 minutes and 1 and a half hour in Mouse liver homogenate [2] however, the rate of this process is likely to be determined by molecular complexity and the number of epoxide functional groups on individual molecules. Epoxide hydrolases in mammals are similar, and humans are the species with the highest epoxide hydrolase activity compared to rodents, dogs or hamsters [3], Therefore it can be concluded that human can metabolise epoxides even faster than laboratory animals.

The epoxide hydrolase converts epoxides to trans-dihydrodiols, which can be conjugated and excreted from the body. Like for bisphenol A diglycidyl ether (BADGE) which is transformed after oral ingestion by hydrolytic ring-opening of the two epoxide rings to form diols [4], this metabolite (the bis-diol of BADGE) is excreted in both free and conjugated forms and is further metabolised to various carboxylic acids, the same scheme can be applied to the substance.

Elimination:

Trans-dihydrodiols formed during metabolisation can be conjugated and excreted from the body in the urine or faeces. Based on the above data, log Pow value, and water solubility, the substance is not expected to bioaccumulate.

Mutagenicity:

Using the current suite of in-vitro mutagenicity studies available it is well documented, that when tested most if not all epoxy based substances and potentially other reactive chemistries (e.g. amines, amides etc.) will trigger a positive result in at least one of the in-vitro test designs required for registration purposes this subsequently stimulates further in-vivo mutagenicity studies via extraordinary proposals for either of two tests that are not comparable as they designed to assess different exposure scenarios.

In the case of 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] a total of six in-vivo mutagenicity assays were performed: Two studies in male germinal tissues, a “nucleus anomaly” (micronucleus) test in bone-marrow and a recent micronucleus study were clearly negative. A sister chromatid exchange test came out ambiguous and was slightly positive at high dose levels (3000 and 5000 mg/kgbw, oral, gavage). A bone-marrow test in rodents was positive at the limit dose of 5000 mg/kgbw. Historic evidence indicates that mutagenic effects in vivo are restricted to high and excessive oral exposure levels.

The different outcome of in-vitro and in-vivo studies is explained by the presence of epoxide hydrolases in most tissues (both microsomal and cytosolic). This enzyme cleaves epoxide substituents efficiently and thereby detoxifies 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline]. For example, it has been demonstrated in metabolic studies with another glycidyl substituent containing substance (Bisphenol A glycidyl ether, BADGE) that only the overload of the epoxide hydrolase pathway leads to the formation of a genotoxic metabolite (glycidaldehyde). The overload effect is – similar for 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] and positive results are only observed at very high doses of BADGE. As a result, BADGE is approved by EFSA for food contact and is considered to be non-genotoxic in vivo.

Altogether, it is considered by weight of evidence that 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] has no significant mutagenic potential in-vivo in case of high human and environmental exposure. This is due to the fast detoxification in vivo by epoxide hydrolases. It also explains the positive outcome of the micronucleus test where effects have been observed at the limit dose of 5000 mg/kgbw and the slight positive result of the SCE (Sister Chromatid Exchange) study in vivo at doses>3000 mg/kgbw.

Altogether, during the REACH registration it was judged by the registrants as a conservative approach that 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] as monoconstituent or UVCB (Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials) substance is mutagenic in vitro, however, not mutagenic in in-vivo based on a weight-of-evidence approach the EU authorities requested the conduct of and OECD 489 Comet Assay or an OECD 488 Transgenic rodent assay.

Referring to the data matrix presented below the epoxy-based substance used for this case study has been tested in both the OECD 488 and OECD 489 testing regimes under GLP conditions. The Comet assay is a short-term exposure (2 days) which relies on the use of high doses to trigger a mutagenic response and the OECD 488 is a repeat dose study with at least a 28-day dosing regime and the highest dose should be the Maximum Tolerated Dose (MTD). The MTD is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. The dose levels used should cover a range from the maximum to little or no toxicity. The reference to the MTD raises questions as within the regulatory arena there are numerous claims that dose response is not relevant in mutagenicity studies. If this is the case then why not reduce the number of animals used and conduct all TGRs for all REACH substances at a pre-determined human

relevant limit dose (e.g. 100 mg/kgbw/d) , if dose response and thresholds are not relevant to mutagenicity why do we test multiple dose groups (e.g low, mid and high)?

In the case of 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] earlier review of the traditional toxicology data-set a clear MTD of 50 - 100 mg/kgbw/d[3] could be determined based on either a 90-day or 28-day exposure (see data matrix). However, when we look at the requested data set for the mutagenicity studies there is a requirement to test up to maximum doses of 1000 mg/kgbw/d and 2000 mg/kgbw/d in the OECD 488 and OECD 489 studies respectively.

The table below presents the data obtained following the conduct of the two studies and the following conclusions can be made:

1) Dose response is evident following the conduct of the OECD 488 and 489 studies
2) Positive mutagenicity results were only observed at doses which exceeded the MTD of 50 mg/kgbw/d based on the 90-day study and 100 mg/kgbw/d based on the 28-day repeated dose
3) Currently based on the REACH and CLP regulations this substance would require labelling as a Cat 2. Mutagen and further testing to assess germs cell has been requested
4) Mutagenicity only occurred at doses significantly above the MTD that which can be derived in traditional toxicology tests

Table E3

Tissue	Dose level (mg/kg bw/d)					
	Transgenic Rodent Assay			Comet Assay		
	10	100	300/200	500	1000	2000
Liver	-	-	-	-	+	+
Stomach	-	-	-	-	+	+
Duodenum	-	-	+	+	-	-
	28-day exposure			2-day exposure		

Outcome

European authorities concluded that MTD was irrelevant in mutagenicity studies, site of contact effects and evidence of gut sensitivity is irrelevant, and the substance should be considered mutagenic in somatic cells and further germ cell testing should be conducted even though effects are only evident

[3] Based on a human of 70kg this is a daily human equivalent of 7.0 grams per day for ≥90 days direct oral gavage

above the MTD of 100 mg/kgbw/d. Clearly, the request for confirmation of germ cell mutagenicity is not within the interests of animal welfare as mutagenicity only occurs above the MTD and at doses which will never be achieved under human relevant scenarios. In addition, situations like this will lead to the unnecessary classification and labelling of substances as mutagens based on extremely conservative experimental conditions.

Data matrix

Table E4

4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline]	**Acute oral toxicity (OECD 401)**	**Comet Assay (OECD 489)**	**28-day repeat dose**	**Transgenic rodent (OECD 488)**	**90-day**
Doses mg/kgbw/d					
	5000 - - -	500 1000 2000 -	100 300 400/750/1000 -	10 50 100 200	10 50 200 -
Duration (d)					
Exposure Observation	14 14	2 2	28 28	28 28	90 90
Overall NOAEL	**5000**	**500**	**100**	**100**	**50**
Mutagenicity NOAEL					
Duodenum Stomach Liver	- - -	2000 500 500	- - -	200 >200 >200	- - -
Histopathology					
Duodenum Stomach Liver	- - -	2000 2000 2000	- - -	- - -	- - -
Toxicity (LOAEL) Ptyalism Piloerection Hunched posture Weight loss/weight gain Reduced food consumption Blood chemisty Liver hepatocytic hypertrophy Minimal hypertrophy of bile duct cells Venous endothelial cells in portal tracts	**-** - 5000 5000 - - - - - -	 - - - - - - - - -	 100 100 300 400/750/1000 300 300 100 400/750/1000 400/750/1000	- - 100 - 200 - - - - -	 200 200 200 >200 200 - - -

Minimal fusion of villi	-	-	400/750/1000	-	-
Duodenum Increased number of nuclei	-	-	400/750/1000	-	-
Focal eosinophilic material fibrin (Duodenum/Jejunum)	-	-	400/750/1000	-	-
Superficial erosion (Stomach)	-	-	400/750/1000	-	-
Edema in the pancreas	-	-	400/750/1000	-	-
Lymphoid atrophy (thymus and spleen)	-	-	400/750/1000	-	-
Reflux at dosing	-	-	-	-	200
Loud breathing/Dyspnea	5000	-	-	-	200
Seminology	-	-	-	-	>200
Increased liver weight (females)	-	-	-	-	200
Distented colon	-	-	-	-	50
Discolouration of mesentric lymph node	-	-	-	-	50
Liver- hepatocellular hypertrophy	-	-	-	-	200
Stomach decreased goblet cell formation	-	-	-	-	200
Duodenum basophilia and atrophy of villi	-	-	-	-	200
Diarrhea	-	-	-	100	-
Exophthalmus	5000	-	-	-	-

Case study 6: Mutagenicity – Theobromine, CAS # 83-67-0

During the REACH registration process there has been a requirement to conduct an increasing number of in-vivo toxicological tests and by doing so it is becoming increasingly apparent that there is a disconnect in the maximum doses proposed in mutagenicity studies versus traditional toxicological tests one of many examples that can be used to illustrate this disconnect is the case of theobromine[4].

The oral acute toxicity of the theobromine was determined following a method similar to OECD Guideline 401 without GLP. Theobromine was tested in mouse and rat and several hemodynamic changes were observed. The LD50 of theobromine was determined to be 837 ± 175 mg/kgbw and

[4] Theobromine is a bitter alkaloid of the cacao plant, it is found in cocoa powder (2-10%), as well as in several other foods including chocolate, tea and coffee

1265± 178 mg/kgbw for mouse and rat respectively (ECHA dissemination tool). The oral acute toxicity of the test substance in the rat is also reported as 950 mg/kgbw by a secondary source (IARC Monograph, 1991).

Feeding high levels of theobromine to rats produced not only marked changes in the morphology of certain organs (thymus and testes) but also marked decreases in food intake and bodyweight. The NOAEL was considered to be 110 mg/kgbw/d as histopathological examination revealed signs of dysfunction at approximately 349 mg/kgbw/d (ECHA dissemination tool).

In the short-term toxicity study conducted according to OECD guideline 407, theobromine was given to mature and immature, male and female rats in concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 % in diet. The diets contained 10 or 22% casein and were fed for 28 days. After the exposure-period animals were killed and different organs were weighed and prepared to histological evaluation. The prominent effects of increasing concentration of dietary theobromine were anorexia (except female rats given 22% casein diet) and atrophy of the thymus gland and testes which became prominent at the 0.6% dietary theobromine level. Many histopathological changes at the thymus gland and testes were detected, including necrosis at high theobromine levels. The low protein diet enhanced the severity of theobromine effects. The daily dose of theobromine which produced retrogressive changes in weight pattern and in the morphology of the thymus in both sexes and of the testes in males was approximately 250 – 300 mg/kgbw/d (IARC Monograph, 1991) in mature rats and approximately 500 mg/kgbw/d (0.4%) in immature rats (IARC Monograph, 1991). Decreases in body weights, food consumption and thymus weights were seen in some mature and immature rats groups given 22 and 10% casein diets with theobromine at 0.2 %, which corresponds to 110 -144 mg/kgbw/d (mature rats) and 200 mg/kg/day (immature rats), thus the LOEL can be considered 110 mg/kgbw/d. Histopatological evaluation of the thymus showed adverse findings at 0.6 % (approximately 349 mg/kgbw/d in mature rats). Given that theobromine produced decreases in food intake, the changes in organ weights and structure could reflect decreased food intake rather than any specific effects of theobromine.

Multiple in-vivo mutagenicity studies are presented within the REACH registration of theobromine, the one key in-vivo study is a mammalian erythrocyte micronucleus test (OECD 474) whereby equal doses of 666, 1000 and 1333 mg/kgbw/d were administered suspended in corn oil by oral gavage to groups of 6 animals per dose with equal number of males and females, 30 and 6 hours before the animals were euthanised. Theobromine caused positive results compared to the control but only at a

dose of 2 x 1333 mg/kgbw/d. The second key study presented is a sister-chromatid exchange test conducted in accordance with EPA OPPTS (Office for Prevention, Pesticides and Toxic Substances) 870.5915 guideline. Theobromine at doses of 83, 167, 333, 500 and 667 mg/kgbw/d was suspended in corn oil and administered by oral gavage as a single dose 2 hours after BrdU (Bromodeoxyuridine) implantation. 24 hours after exposure animals were euthanised and bone marrow cells prepared for histological examination (SCE frequencies). Theobromine produced significant increases in sister chromatid exchanges in a dose-dependent manner in the tested system, the results were considered positive at 333, 500 and 667 mg/kgbw/d. As a result of uncertain mechanistic understanding and implications for this study type the sister chromatid exchange test guideline has subsequently been removed by the OECD.

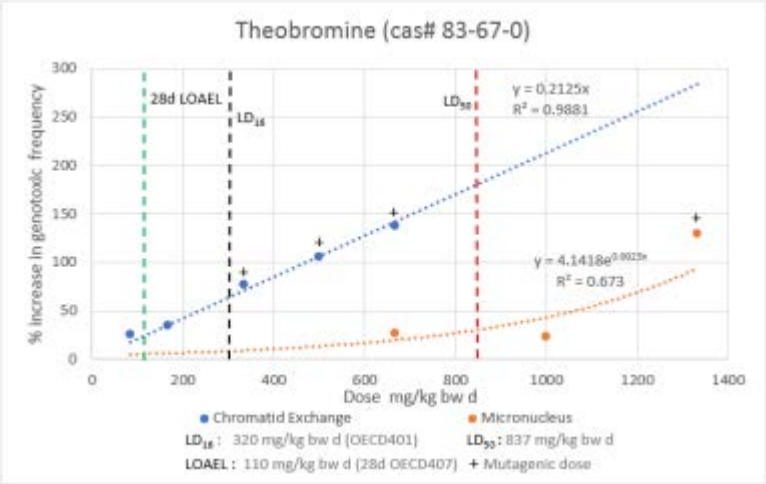

Figure E1: Comparison of the results of an in vivo micronucleus and sister-chromatid exchange assay

Experimentally derived LD16/LD50 and NOAEL values from an OECD 401 acute oral toxicity study and an OECD 407 28-day repeat dose study conducted with theobromine.

Figure 1 compares the results of an in-vivo micronucleus and sister-chromatid exchange assay with the experimentally derived LD16/LD50 and NOAEL values from an OECD 401 acute oral toxicity study and an OECD 407 28-day repeat dose study conducted with theobromine. When presented in this way it is clearly apparent that the two mutagenicity studies were conducted at excessive doses. The in-vivo micronucleus test was conducted at doses of 666, 1000 and 1333 mg/kgbw/d two of which exceed the determined acute LD_{50} of 837 mg/kgbw/d and although the low and mid dose were considered

negative the overall study was concluded positive for mutagenicity due to the positive findings at 1333 mg/kgbw/d which greatly exceeds the acute oral LD_{50}. A similar issue is also visible with the in-vivo sister-chromatid exchange study which utilised a dose range of 83, 167, 333, 500 and 667 mg/kgbw/d which span the LD_{16} derived in the acute oral study and exceed the NOAEL derived during the repeat dose study. The results of the sister-chromatid exchange study identified genotoxicity at 333, 500 and 667 mg/kgbw all of which exceed the acute LD_{16} and 28-d NOAEL.

This disconnect between dose setting has significant consequences and raises several concerns in relation to regulatory interpretation:

1) The in-vivo sister-chromatid exchange and micronucleus studies are reported as positive for genotoxicity; however, these unfavourable findings are only evident at doses which exceed intrinsic acute and subacute toxicological thresholds. What is the relevance of this? What is the need for identifying mutagenicity at doses above the NOAEL/LD_{10} or MTD?
2) Conducting mutagenicity studies at high doses identifies substances as potentially having mutagenic hazards at doses which will never occur in reality, an organism is likely to be in poor health, compromised by organ damage or even be deceased before manifestation of genetic change or mutation. Importantly, the apparent genotoxic hazard may be secondary to toxicity and unrelated to the genotoxic potential of a given chemical. Further, it is well-established in all in vitro and in vivo genotoxicity studies that the positive control chemicals also induce some level of cytotoxicity and must be considered for an accurate assessment of genotoxicity/mutagenicity.
3) High dose testing in mutagenicity studies triggers overly conservative hazard concern resulting in unnecessary classification and labelling, a saturation of the regulatory environment with substances carrying unjustified labelling and subsequently an attenuation of how the workforce/population perceive hazard labels which intern may encourage negligence. California's Proposition 65 approach is a classic example of over labelling which makes it difficult to identify scenario's whereby real hazards exist and require risk mitigation.

Appendix F - Food Ingredients Case Study

Components tested well above the 'Limit dose'

The sweetener D-xylose was administered at up to 5% of the diet in a carcinogenicity study based on results from a previous subchronic study that found no evidence of toxicity when administered at that dose (Imizawa et al., 1999; Kuroiwa et al., 2005). Similarly, the non-caloric sweetening substance sucralose was administered to rats in a subchronic feeding study at doses exceeding 6000 mg/kd/d and administered at concentrations of up to 3% of the diet in a two-year carcinogenicity study (Goldsmith, 2000; Mann et al., 2000). In the carcinogenesis study, the LOAEL was 4500 mg/kgbw/d which the authors estimated to be 4500x higher than the intake by humans. In cases like these where the doses administered in repeated dose toxicology studies were well in excess of the limit dose it has been useful in establishing human acceptable daily intake (ADI) values that are 100s or even 1000s of times in excess of possible human exposure. Similar results were also observed with non-sweetening dietary ingredients from natural sources such as lauric acid (NOAEL > 6000 mg/kg/d) and palmitic acid (NOAEL > 5000 mg/kg/d; JECFA, 1997) and corn starch fiber (NOAEL = 10,000 mg/kg/d; Crincoli et al., 2016) where doses administered to laboratory animals were well in excess of the limit dose and and still produced no evidence of adverse effects.

In contrast, some substances intentionally added to foods were tested at doses well below the limit dose including isoeugenol methyl ether (high dose = 200 mg/kg/d; Akagi et al., 2019), b-myrcene (high dose = 300 mg/kg/d; Bastaki et al., 2018), 2,4-decadienal (high dose = 800 mg/kg; Chan et al., 2011), and 5-hexenyl isothiocyanate (high dose = 48 mg/kg/d; Akagi et al., 2018) were tested at doses well below the limit dose because prior evidence suggested that the animals were not likely to tolerate greater doses and that the NOAEL being sought was going to be much lower than the limit dose. In the case of contaminants such as such as 3-monochloropropane-1,2-diol that can form in foods as a consequence of cooking even lower doses were administered by drinking water to identify a NOAEL that was considerably lower than the aforementioned substances (NOAEL =~3.5 mg/kg/d; Toyoda et al., 2017). What is important about these studies is that they still provide a margin of exposure when the NOAELs and subsequent ADIs are compared to human exposure from their intended uses in foods.

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www.ecetoc.org
D-2021-3001-262

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