OECD DRAFT GUIDANCE DOCUMENT N° 116 ON THE DESIGN AND CONDUCT OF CHRONIC TOXICITY AND CARCINOGENICITY STUDIES, SUPPORTING TG 451, 452, 453
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To note: the following version of the draft GD 116 includes only sections 3.2, 3.3, 3.4, 3.5 and Chapters 5 and 6, currently under development by an OECD consultant. Chapter 2, Section 3.6 and Chapter 4 are under development as a separate exercise. Chapter 1 and section 3.1 have been adopted at the last WNT meeting in March 2010. The remaining Chapters 1, 2, sections 3.1 and 3.6 and chapter 4 have been retained in the Table of Contents for information

3.2 ROUTES OF EXPOSURE AND DOSE ADMINISTRATION CONSIDERATIONS

1. The three main routes of administration used in chronic toxicity and carcinogenicity studies are oral, dermal and inhalation, although other routes such as subcutaneous or intraperitoneal injection have been used, for example in experimental carcinogenicity studies (see paragraph 21). The choice of the route of administration depends on the physical and chemical characteristics of the test substance, its intended field of application and the predominant route of exposure of humans. For example, if human exposure to the test substance is likely to be through food or is a pharmaceutical intended to be taken by mouth, the relevant route of administration will be the oral route, while for a workplace gas, inhalable dust or volatile liquid, inhalation should be the route of choice. The dermal route may be chosen, e.g. for substances used in the workplace, where skin contact is likely, or for pharmaceuticals applied to the skin. The subcutaneous route has been used where the human route of exposure is intended to be intravenous, e.g. for pharmaceuticals. In choice of route of administration of the test chemical due consideration should be given to animal welfare (OECD GD 19).

2. Given the potential for oral exposure to a wide range of chemicals and also the practical experimental considerations associated with the long duration of chronic toxicity and carcinogenicity studies, the oral route is the route most commonly used in chronic toxicity and carcinogenicity studies. Route-to-route extrapolation may be considered for systemic effects when reliable data on ADME are available, rather than carrying out an additional study by a second route. For example, it may be possible to carry out an assessment of systemic effects via inhalation exposure based on the results of an oral chronic toxicity or carcinogenicity study (Gerrity and Henry, 1990). The use of route-to-route extrapolation should be decided on a case-by-case basis (Nielsen et al., 2008) and is not however relevant for the assessment of local toxicity.

3.2.1 The oral route of exposure

3. Test substances may be administered via the diet or drinking water, by oral administration in capsules or by gavage, normally in a vehicle, depending on the physical and chemical characteristics of the test substance, its intended field of application and the predominant oral route of exposure of humans. Each method has advantages and disadvantages. The animals are dosed with the test substance daily (seven days per week), normally for the entire duration of the study. Any other dosing regime, e.g., five days per week, needs to be justified. In the case of rodents, dosing of the animals should begin as soon as possible after weaning and acclimatisation and preferably before the animals are 8 weeks old.

4. As indicated in the Test Guidelines, in the interests of animal welfare, oral gavage should normally be selected only for those agents for which a bolus dose administration reasonably represents potential human exposure (e.g., administration of pharmaceuticals orally at one or more doses per day). In addition oral gavage sometimes is the option when sufficient dietary concentrations cannot be achieved due to e.g. physical, chemical properties of the test substance, or to its palatability in the diet. The practical experimental difficulties associated with long term gavage dosing must also be taken into consideration.

For dietary or environmental chemicals including pesticides, administration is typically via the diet or drinking water. When available, data from earlier toxicological studies including data on toxicokinetics will provide information on potential local, gastroenteral effects, and the extent of bioavailability of the test substance via the selected oral route, in order to demonstrate that systemic exposure is adequate (see also chapter 3.4).

5. Oral administration via the diet is the preferred route of administration if human exposure to the test substance is also likely to be via the diet. This route of administration may be appropriate if the objective is to establish an ADI or TDI, for example for substances deliberately added to food or for environmental contaminants entering the food chain, and the pattern of exposure is continuous ingestion of small doses. However oral gavage studies may also be used to derive an ADI or a TDI.

6. When using oral administration via the diet, the test substance is administered in the diet either as a constant dietary concentration (mg/kg diet), or as a constant dose level in terms of the animal's body weight. In the latter case the dietary concentration must be adjusted regularly based on anticipated food consumption and body weight of the animals. While doses are expressed in terms of mg/kg diet, food consumption must be monitored on a cage basis at least weekly in order to be able to derive the intake of the test substance on mg/kg body weight per day or mg/m^2 per day. The food intake e.g. in the rat decreases from above 100 g per kg. bw per day in early life (6-8 weeks, at the commencement of the study) to about little above 50 g per kg. bw per day for older females (e.g. 6 months or more) and below 50 g per kg. bw per day for older males. This will lead to a gradual decrease in intake of dietary administered test substance over age when keeping the dietary concentration of the test chemical constant. The concentration of the chemical in the feed should not normally exceed an upper limit of 5% of the total diet (FDA, 1982, Borzelleca, 1992), although higher levels are feasible (e.g. when testing carbohydrates or proteins) as long as the diet is adapted nutritionally adequately, e.g. the test substance is incorporated, at the expense of other components in a purified diet (Howlett et al, 2003).

7. Oral administration via the diet has the advantage that no handling of the animals is required. However, the palatability of the diet may be reduced at high dietary levels due to the taste or odour of the test substance, resulting in reduced food intake and thus reduced exposure to the test substance. This may require the introduction into the study design of an additional control group, pair fed (i.e. having matched food intake) in parallel with the high dietary level test group (see section 3.5.2 for further details). The substance should be stable during the preparation, storage and period of administration of the diet, for example it should not react chemically with dietary constituents, and analytical data must be provided to demonstrate this. It is also essential to ensure that the substance is mixed homogeneously in test diet at the desired level and, again, analytical data must be provided to demonstrate this, as required under Good Laboratory Practice (OECD, 1998).

8. Oral administration in drinking water is the method of choice if human exposure to the test substance is likely to be via drinking water (e.g. drinking water contaminants) or in liquids (e.g. for substances that are volatile, or reactive with feed components, or any case where drinking water has an advantage over diet administration such as for soft drinks or beverages). The test substance is normally incorporated at a fixed concentration in the

drinking water, at the approximate levels (in mg/ml water) required to provide the dose levels selected for the study (in mg/kg body weight per day), based on anticipated water consumption of the animals. While doses are expressed in terms of mg/ml water, water consumption must be monitored on a cage basis at least weekly in order to be able to derive the intake of the test substance on mg/kg body weight per day or mg/m^2 per day. Concerning possible adjustment of the concentration of the test substance in the drinking water e.g. when the dosing causes changes in water consumption similar measures as described for dietary dosing are appropriate (Sharp and Regina, 1998; Wolfensohn and Lloyd, 1998; Pool, 1999; Nielsen et al, 2008). The test substance should not affect the palatability of the drinking water or cause marked changes in the pH, and its content and stability must be demonstrated analytically, as required under GLP (OECD, 1998).

9. Oral administration by encapsulation or oral intubation (gavage) may be used if administration in the diet or drinking water is not possible, e.g. because of stability or palatability considerations, or if human exposure is expected to be through daily ingestion of a single, large bolus dose, as may be the case for some pharmaceuticals or diet supplements. Gavage dosing is experimentally more difficult than dietary administration, and also requires daily handling of the animals, which may interfere with experimental parameters e.g. if neurobehavioural assessments are carried out during the study. It should be kept in mind that the toxicokinetics of the test substance may be affected by the method of oral administration. For example, chloroform induces hepatocellular cytotoxicity, regenerative proliferation and liver cancer in mice when administered by gavage in corn oil at doses that do not cause these lesions when administered in drinking water (Larson et al., 1994).

10. If the test substance is administered by gavage, this should be done using a stomach tube or a suitable intubation cannula, at similar times each day. For larger animals, e.g. dogs, the test substance may be administered in capsules, dissolved or suspended in a suitable vehicle. Vehicles of choice include oil (e.g., corn oil) or aqueous solutions of thickeners such as carboxymethylcellulose, although other vehicles may be used. The maximum volume of solution that can be given by gavage in one dose depends on the size of the test animal. For rodents, the volume ordinarily should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100g body weight may be used (Diehl et al. 2001). If the gavage vehicle is oil, the use of a low-fat diet should be considered, and the volume administered should normally not exceed 0.5 ml/100 g body weight/day, since the administered oil may interfere with feed intake.

11. Normally a single dose will be administered once daily, but where for example a substance is a local irritant or the pattern of human dosing is multiple doses per day, the daily dose may be administered as a split dose e.g. twice a day , within a 6 hour period. Variability in dose volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. Potentially corrosive or irritant substances may however need to be diluted to avoid severe local effects, and testing at concentrations that are likely to be corrosive or irritant to the gastrointestinal tract should be avoided The frequency and length of time for which the animals in a chronic toxicity or carcinogenicity study are dosed can lead to irritation in the esophageal tissue and distress of the animals, potentially compromising the integrity of the study. If oral gavage is used, careful observation should

be conducted after dosing to watch for signs of distress such as laboured breathing, sudden lethargy, or poor mucous membrane colour.

3.2.2 The dermal route of exposure

12. The dermal route of exposure may be used in assessing the chronic toxicity and carcinogenicity of substances such as workplace chemicals, where skin contact is likely, or for pharmaceuticals applied to the skin, for which continuous dermal contact is anticipated. It has also been used in the assessment of a number of carcinogens such as polycyclic aromatic hydrocarbons, in skin painting studies generally carried out in the mouse. Assessment of systemic toxicity or carcinogenicity using the dermal route is only possible if it has been demonstrated that the test substance is bioavailable via the skin, i.e. it crosses the skin barrier.

13. The method is based on the repeated application of the test substance, generally at a defined concentration in mg/ml in a suitable vehicle, to a clipped or shaved area of skin of approximately 10% of the total body surface area, to provide the desired dose in mg/kg body weight per day. The site may be occluded with polyethylene sheeting and gauze patches or semi-occluded, in order to prevent dislodgement of material and oral ingestion, which could affect the validity or usefulness of the study. With volatile or semi-volatile materials, application and covering procedures should minimise the possibility of evaporation. Animals are normally treated with the test substance for at least 6 hours per day, 7 days per week, for a period of 24 months.

14. TG 410 on Repeat Dose Dermal Toxicity: 21/28 day study (OECD, 1981) should be consulted in the case of testing carried out by the dermal route, and there is also useful information on dermal toxicity testing in the standard toxicology textbooks, *e.g.* Derelanko & Hollinger (1995) and Hayes (1994).

3.2.2 The inhalation route of exposure

15. If it is likely that humans may be exposed by inhalation to a test article, either as a gas, a vapour, or a liquid or solid aerosol (or a mixture thereof), then it is appropriate to use the inhalation route to evaluate the toxicity of this test article in animals. This also applies for test articles that may be used in mixtures for which inhalation exposure is possible or that are used under conditions which may affect the toxicological properties of the test article (e.g., when used as additive in petrol, as flame retardant etc.). In inhalation studies, animals are exposed to a time-weighted-average concentration of the test article in the air expressed as mg/L or mg/m^3 instead of a dose in mg/kg body weight as in oral and dermal studies. If concentration-dependent phase shifts from vapour to aerosol may occur, the relative percentage of each phase must be known. In any case, concentrations must be reported as aggregated concentration of mass from all phases.

16. An inhalation toxicity study of chronic duration should resemble Test Guideline 413 for subchronic inhalation toxicity: 90 day study (OECD, 2009a) in all respects except study duration. Particular emphasis must be directed toward technical problems that may arise from the large numbers of animals in inhalation chambers (e.g., time required to attain

inhalation chamber steady-state, heat and CO_2 production, and adsorption of test article on inhalation chamber walls and other surfaces. Further guidance on the performance of an inhalation toxicity study can be found in the Guidance Document 39 on Acute Inhalation Toxicity Testing (GD 39; OECD, 2009b) and the OECD Guidance Document on Histopathology for Inhalation Toxicity Studies. Although GD 39 was initially intended to provide guidance for acute inhalation studies, the technical aspects of exposing animals and generating and characterizing test atmospheres are similar for repeated exposures and single exposures and are therefore also applicable for chronic or carcinogenicity inhalation studies.

17. The nature of the test article and the object of the test should be considered when selecting an inhalation chamber. The preferred mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only). Nose-only exposure is generally preferred for studies of liquid or solid aerosols and for vapours that may condense to form aerosols. Special objectives of the study may be better achieved by using a whole-body mode of exposure, but this should be justified in the study report. To ensure atmosphere stability when using a whole-body chamber, the total volume of the test animals should not exceed 5% of the chamber volume. Principles of the nose-only and whole body exposure techniques and their particular advantages and disadvantages are addressed in GD 39.

18. Corrosive or irritating test articles may be tested at concentrations that will yield the desired degree of toxicity but in the absence of affecting longevity or undue stress to respiratory tract irritation (GD 39; OECD, 2009b). When exposing animals to these materials, the targeted concentrations should be low enough to not cause marked pain and distress, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations should be selected on a case-by-case basis, preferably based upon adequately designed range-finding studies that provide information regarding the critical location of irritation within the respiratory tract and endpoint for probing it. Adequately designed range-finding studies should demonstrate whether respiratory tract irritation depends on any irritation threshold (concentration-dependent) or on the total daily exposure intensity (concentration x time – dependent), and whether carry-over effects from one exposure day to another may lead to time-dependent exacerbations. Some irritant effects are instant in onset and others require time to accumulate. These factors need to be identified and may serve as justification for concentration selection.

19. The species selection needs to be carefully considered for test articles causing upper respiratory tract irritation with numerous secondary physiological responses making the extrapolation from small rodents to humans more difficult (GD 39; OECD, 2009b). Especially when using species other than rats for inhalation studies of irritant test articles, in depth justification for species-selection is needed.

20. Test substances that are irritating or corrosive should always be tested using methodology laid out in Test Guideline TG 413 because it provides the study director or principal investigator with control over the selection of target concentrations (OECD 2009b, Guidance Document 39). Dilutions of corrosive test articles may be tested at exposure concentrations sufficient to extend the concentration-response curve to levels that reach

the objective of the test and thus serve regulatory and scientific needs. Corrosive test substances should be assessed and tested following expert judgment on a case-by-case (OECD 2009b, Guidance Document 39). Testing corrosive and/or irritating test articles in long-term inhalation studies at concentrations that are expected to cause severe pain and/or distress should be avoided to the extent possible. The corrosive/irritating potential should be appraised by expert judgment using such evidence as human and animal experience (*e.g.,* from repeat dose studies performed at non-corrosive/irritant concentrations), existing in vitro data, pH values, information from similar substances or any other pertinent data.

3.2.3 Other routes of exposure

21. Other routes of exposure e.g. subcutaneous or intraperitoneal injection are generally only used in chronic toxicity or carcinogenicity studies when they mirror the anticipated route of administration in humans, although they have been used in experimental carcinogenicity studies, e.g. on insoluble materials such as fibres and plastics. For example subcutaneous or intramuscular injections may be used for pharmaceuticals and for materials designed to be used as implants or prostheses, while intravenous administration could be appropriate for substances that are administered by this route due to pharmacokinetic considerations such as lack of bioavailability by other routes. Intravenous administration may be via bolus injection, slow intravenous injection or intravenous infusion. The subcutaneous and intraperitoneal routes have been used in carcinogenicity bioassays for some solid-state, insoluble materials.

22. For substances administered parenterally, the dose volume used, stability of the formulation before and after administration, pH, viscosity, osmolality, buffering capacity, sterility and biocompatibility of the formulation are factors to consider (Diehl et al. 2001). The smallest needle size should be used for administration, taking into account the dose volume, viscosity of injection material, speed of injection and species. (Diehl et al. 2001). The use of parenteral injections in chronic toxicity and carcinogenicity studies is likely to result in local inflammation, and has significant animal welfare implications.

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3.3 CHOICE OF SPECIES AND STRAIN, STUDY DURATION, ALTERNATIVE *IN VIVO* MODELS

General issues

23. The choice of species to be used in a chronic toxicity or a carcinogenicity study is dictated by a number of factors, including the following:

    - physiological and metabolic similarity to humans, in order to provide a valid model for extrapolation of the findings,
    - familiarity with the species,
    - availability of existing data on the species chosen,
    - lifespan of the animals,
    - ease of handling under experimental conditions,
    - other issues such as cost of maintenance, litter size, and gestation period.

    Rodents have been used extensively, also dogs and primates. The choice of species may also be dictated by the purpose of the study (e.g. chronic toxicity or carcinogenicity) and by regulatory requirements.

24. With few exceptions, carcinogenicity and combined chronic toxicity/carcinogenicity studies are normally carried out in rodent species. Similarly, chronic toxicity studies are normally carried out in rodents. Chronic toxicity testing in non-rodents may however be required under certain regulatory regimes, e.g. requirements for pesticides and human medicines. The most commonly used non-rodent species in such studies is the dog, although there has been extensive debate about the need for, and added-value of, a chronic toxicity study in the dog as discussed further in section 3.3.2 (Box and Spielman, 2005; Doe et al. 2006; ESAC, 2006, EFSA, 2007).

3.3.1 Testing in rodents

25. As indicated, rodent species have been used in the majority of chronic toxicity studies and in almost all carcinogenicity testing. It is important to consider the general sensitivity of the test animals, their background pathology and hence the responsiveness of particular organs and tissues to the chemicals under test when selecting rodent species, strains or stocks for toxicity studies. In general the selected rodent strain or stock should be well-characterized preferably including data on e.g. body and organ weight, haematological and biochemical parameters and background pathology. Additionally, it is important that test animals come from healthy colonies. Normally Specific Pathogen Free (SPF) animals are used, being SPF derived at birth and maintained under barrier conditions.

26. The two rodent species most frequently used are rats and mice, given the (relatively) low cost of maintenance, their short lifespan, meaning that a lifetime study can be completed in 2 – 3 years, and the availability of a large amount of historical data on age-related biochemical, haematological and pathological changes including on spontaneous tumours at specific organ sites. Syrian golden hamsters have been used in studies of carcinogenesis

in the respiratory and urinary tract, particularly using parenteral routes such as intraperitoneal and intratracheal installation. Rodents have a number of metabolic pathways, physiological and pathological responses in common with humans. However, there are a number of instances where the chronic toxicity and carcinogenicity findings in rodents have been demonstrated not to be relevant to humans, because of toxicokinetic and toxicodynamic differences including species-specific pathways of metabolism, genetic differences, enzyme differences, differences in toxicologic pathways etc. If differences in toxicokinetics or toxicodynamics and/or other relevant parameters are suspected between the test species and humans, that may have an impact on the relevance of the outcome of the study, these should be explored to determine if another test species may be more appropriate for testing.

Rodent species and strain specificity

27. Assessment of chronic toxicity in rodents using the Test Guideline 452 is generally carried out in the rat, although other rodent species, e.g., the mouse, may be used. Since the duration of the study is normally 12 months, the potential for development of age-related background pathologies that may be influenced by the strain of rat is lessened, and in practice the strain commonly in use in the testing laboratory will be used, since the laboratory will have historical data that will aid in the interpretation of any test substance-related change.

28. Assessment of carcinogenic potential has traditionally been carried out in both rats and mice, particularly in the case of pharmaceuticals, pesticides and veterinary drugs, for which there is potential for widespread human exposure, either directly (pharmaceuticals) or via food and water. Common rat strains used in toxicological testing include the Fischer 344, Sprague-Dawley and Wistar rat, and genetic typing enables characterization of a range of different sub-strains within these main strains.

29. The Fischer 344 rat is a particularly well-characterised rat strain in carcinogenicity studies, since it has been the selected rat strain for the National Toxicology Programme (NTP) studies for over 20 years, together with the B6C3F1 mouse. However it has recently been reported (King-Herbert et al. 2010) that the NTP is currently evaluating the Harlan Sprague Dawley (Hsd: Sprague Dawley SD) as the primary rat model for NTP studies, due to a number of health issues and decreased fecundity inherent in the Fischer 344 rat, and has carried out a number of long-term studies with this strain. The B6C3F1 remains the mouse model used in the NTP cancer bioassay, but the use of multiple strains of mice is being explored by NTP (King-Herbert et al. 2010). The CD-1 mouse has been used by the US EPA OPP for chronic long term toxicity studies.

30. Importantly, in selecting a suitable rat strain for carcinogenicity testing, test animals should be selected that are likely to survive for the recommended duration of the study (see section 3.3.2). A number of publications have indicated that survivability problems exist for certain strains, notably the Sprague-Dawley rat (Nohynek et al., 1993; Keenan, 1996). For strains with poor survival such as Sprague Dawley rats, higher numbers of animals per group may be needed in order to maximise the duration of treatment (typically at least 65/sex/group).

31. Britton *et al* (2004) reported that of the three rat strains studied (Harlan Hsd:Sprague-Dawley SD, Harlan Wistar Hsd:BrlHan:WIST, Charles River Crl:CD), Harlan Wistar strain survived in much greater numbers in 104-week carcinogenicity studies. The improved survival rate, according to the authors, appeared to be independent of body weight and food consumption and was reflected in the spontaneous pathology profile. Other authors believe this phenomenon to be attributable to a combination of obesity and genetic susceptibility and advocate dietary restriction as a method of extending survival in long-term carcinogenicity bioassays (Keenan, 1996).

32. As discussed further in section 3.5, and as reported by many investigators, dietary restriction results in a delay in age-related degenerative diseases such as nephropathy, which is commonly seen in all rat strains and has been shown to be diet-related. Dietary restriction may however result in a lower susceptibility of the animals to the development of tumours in carcinogenicity studies and to development of chemically-induced toxicity, thus presenting problems in extrapolation of the results of such studies to humans. (See paragraph 79, section 3.5 for further information).

33. Mouse strains used in carcinogenicity testing include the B6C3F1 mouse, as used by NTP, the ICR Swiss (CD-1), BALB/c. Notably, different mouse inbred strains show a variation in susceptibility to tumorigenesis in different organs. The commonly used strains, in particular the B6C3F1 mouse used by NTP, carry hepatocellular tumour susceptibility loci that result in a high susceptibility to chemically induced hepatocarcinogenesis (Gariboldi *et al.* 1993; Manenti *et al.* 1994), which has limited their usefulness in carcinogenicity testing, while CD-1, an outbred mouse line derived from the Swiss strain has a relatively high incidence of spontaneous lung tumours and a high susceptibility to chemically induced lung tumorigenesis (Manenti *et al.* 2003).

34. In recent years there has been considerable debate about the value of the two rodent species approach to carcinogenicity and about the continued use of the mouse as a second species, particularly within the ICH (ICH, Proceedings of the Third International Conference, 1995). This issue is also discussed in Chapter 2 on Guidance on Providing a Carcinogenic Mode of Action. A number of studies have assessed the relative individual contribution of rat and mouse carcinogenicity studies and whether the use of rats or mice alone would result in a significant loss of information on carcinogenicity relevant to human risk assessment. The main conclusions drawn from a detailed analysis carried out by ICH (Proceedings of the Third International Conference, 1995) were that:

    - Although very few instances have been identified of mouse tumours being the sole reason for regulatory action concerning a pharmaceutical, data from this species may have contributed to a “weight of evidence” decision and in identifying agents that caused tumours in two rodent species.

    - Of the compounds displaying carcinogenic activity in only one species, the number of "rat-only" compounds was about double the number of "mouse-only" compounds, implying in a simplistic sense that the rat is more "sensitive" than the mouse.

- As with other surveys accessible in the literature, the data for pharmaceuticals were dominated by the high incidence of rodent liver tumours. The high susceptibility of mouse liver to nongenotoxic chemicals has been the subject of many symposia and workshops. These have concluded that these tumours may not always have relevance to carcinogenic risk in humans and can potentially be misleading.

This debate has led to the suggestion that there may be no need for routine conduct of two long-term rodent carcinogenicity studies, however testing in both the rat and the mouse is still required by some regulatory authorities.

35. The ICH has recommended other experimental approaches to the evaluation of carcinogenic potential that may obviate the requirement to test in a second species. These approaches include short or medium-term in vivo rodent test systems providing insight into carcinogenic endpoints, such as models of initiation-promotion in rodents, or models of carcinogenesis using transgenic or neonatal rodents. However, testing in a second species is still acceptable. Thus, although the use of the mouse in carcinogenicity testing may have limited utility (Griffiths et al. 1994, Usui et al, 1996, Carmichael et al. 1997; Meyer, 2003, Doe et al., 2006), under some current regulatory programmes carcinogenicity testing in the mouse is still required or accepted

3.3.2 Testing in non-rodents

36. The use of non-rodent species may be considered when available data suggest that they are more relevant for the prediction of health effects in humans. The principles and procedures outlined in this Guidance Document, together with those outlined in OECD TG 409, Repeated Dose 90-day Oral Toxicity Study in Non-Rodents (OECD, 1998) should be applied, with appropriate modifications. The use of non-rodent species should be, in the main, restricted to special purpose studies, rather than for basic screening of chronic toxicity and carcinogenicity. As indicated in the Test Guideline, a second species should only be used:

- where effects observed in other studies indicate a need for clarification/characterisation in a second, non-rodent species, or
- where toxicokinetic/toxicodynamic studies indicate that the use of a specific non-rodent species is the most relevant choice of laboratory animal, or
- where other specific reasons justify the use of a non-rodent species.

The choice of species must be justified. It should also be noted that mechanistic studies should be performed on the same species and strain as the cancer/chronic toxicity studies (e.g., mechanistic studies in CD-1 mice or Syrian hamsters cannot be used to explain tumours in F344 rats).

37. The most commonly used non-rodent species for chronic toxicity testing is the dog, although there has been extensive debate about the need for, and added-value of, chronic toxicity studies in the dog (Box and Spielmann, 2005, Doe *et al.*, 2006, ESAC, 2006). As a result of analyses carried out by these authors and also by the US EPA (Baetcke *et al.*, 2005), it has been suggested that tests using typical non-rodent species, such as the dog, do

not have a substantial added value beyond a duration of 3 months. In contrast, for pharmaceuticals, an ICH review of studies of 1, 3, 6, 9, and 12 months in dogs found that that in about 5% of the cases, completely new findings (not increased severity) in dogs seen after 6 months seriously changed the course of clinical development of the product (e.g., cataracts, first seen at 7 months).

38. Dogs used for chronic toxicity and (rarely) carcinogenicity testing should be of a defined breed. Beagles are the most commonly used dog strain. The study design should minimise the numbers of animals used, and for a chronic toxicity study normally 4-6 animals per dose level are used. Dosing should begin preferably at four to six months and not later than nine months of age. Where the study is conducted as a preliminary to a long-term chronic toxicity study, the same species/breed should be used in both studies. Animal welfare considerations are of the utmost importance when using dogs for toxicity testing, including housing, exercise, the need for environmental enrichment and for social contact. These aspects are discussed further in Chapter 3.5.

39. Other non-rodent species used include mini-pigs, as their basic physiology is considered to be very similar to humans, and they may therefore provide a better model than e.g. dogs or rodents. Rabbits, although used in the area of skin and eye irritation testing and reproductive toxicity testing, are rarely if ever used as a second species for chronic toxicity and carcinogenicity testing, and their use is therefore not discussed further in this Guidance Document. The use of non-human primates is now contraindicated and in 2007 the European Parliament called for a phase out of all use of primates in medical research and toxicity testing. However, in Europe, the Scientific Committee on Health and Environmental Risks (SCHER) came to the conclusion that for many areas of biomedical research, there are no valid alternatives which would allow the complete discontinuation of the use of non-human primates at this time (SHER, 2009). Their use should however always be rigorously justified.

40. Minipigs used for chronic toxicity testing should be of a defined breed. Gøttingen Minipigs are the most commonly used minipig strain used. The study design should minimise the numbers of animals used, and for a chronic toxicity study normally 4-6 animals per dose level are used. Dosing should begin preferably at three to four months of age. Where the study is conducted as a preliminary to a long-term chronic toxicity study, the same species/breed should be used in both studies. Animal welfare considerations are of the utmost importance when using minipigs for toxicity testing, including housing, exercise, the need for environmental enrichment and for social contact. These aspects are discussed further in Chapter 3.5.

3.3.3 Study duration

41. The duration of the chronic toxicity study and of the chronic toxicity phase in the combined chronic toxicity/carcinogenicity study is normally 12 months, although longer or shorter periods may be used if scientifically justified, and for pharmaceuticals, chronic studies of 6 months duration in rats are required.

42. In the carcinogenicity study, mice are generally exposed to the test chemical for 18–24 months and rats for 24–30 months with exposure being longer for strains of greater longevity or with a lower spontaneous tumour rate The Test Guideline 451 specifies that the duration of the study will normally be 24 months for rodents, representing the majority of the normal life span of the animals to be used. Shorter or longer study durations may be used, dependent on the lifespan of the strain of the animal species in the study, but should be justified. For specific strains of mice, e.g., AKR/J, C3H/J or C57BL/6J strains, a duration of 18 months may be more appropriate, but should be justified by reference data from historical controls. Many carcinogenicity studies in mice are conducted for 18 months; therefore, there is limited historical control data available at 24 months. The study may also make provision for interim kills, e.g., at 12 months, to provide information on progression of neoplastic changes and mechanistic information, if scientifically justified. Where such information is already available from previous repeat dose toxicity studies on the substance, interim kills may not be scientifically justified.

43. Termination of the study should be considered when the number of survivors in the lower dose groups or the control group falls below 25 per cent, considering the survival of each sex separately. The US EPA Health Effects Test Guidelines 870.4200 (US EPA, 1998b) specify that survival in any group should not fall below 50% at 15 months in the case of mice and 18 months in the case of rats, or below 25% at 18 and 24 months respectively. In addition, the WHO (1990) recognises a further type of carcinogenicity study that continues until mortality in the most susceptible group reaches a fixed level, usually 80%.

44. The study should not normally be extended beyond the point when the data available from the study are no longer sufficient to enable a statistically valid evaluation to be made. However, in the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination. While the validity of the study may be prejudiced by early mortality, e.g. in the high dose group, valuable information will still be obtained from it, and a decision to terminate the study in its entirety must be carefully weighed against the animal welfare implications of having to repeat the study. The lower dose groups still may be used for the evaluation.

45. If the current dosing regime results in severe animal toxicity and the study must be terminated before the full duration of exposure, the study sponsor needs to contact the regulatory authority immediately. All data should be compiled and all available tissues preserved for further evaluation. While this study may not meet all guideline requirements for long term/carcinogenicity testing, the results may be useful and considered in the overall risk assessment. The determination of a retest will be made on a case-by-case basis by the regulatory authorities.

Consideration of the acceptability of a negative carcinogenicity result relative to survival in the study.

46. For a negative result to be acceptable in a rat carcinogenicity bioassay, survival in [all] [the lower dose] group[s] in the study should ideally be no less than 50% at 24 months. It is the responsibility of the study director to use rat strains that would ensure adequate survival at 24 months. In a mouse study, survival in all groups in the study should be no less than

50% in all groups at 18 months. Additionally, no more than 10% of any group should be lost due to autolysis, cannibalism, or management problems. Survival of less than 50% of animals in the top dose group need not disqualify the evaluation of a negative study outcome, provided that the higher mortality in this group can be clearly attributed to another toxic effect, such as chronic undernutrition or malabsorption resulting from gastrointestinal irritation by too high a dietary concentration of the test substance. Evaluation of a negative study outcome may be based on calculation of the power of the test for groups with lower mortality. A broad general guideline is that at interim sacrifice, survival should not be below 50%, while at study termination, survival should not be less than 25%.

3.3.4 Alternative *in vivo* models for carcinogenicity testing, including testing in transgenic animals

47. Some of the medium-term tests for carcinogenicity involve the development of proliferative lesions in a single tissue, *e.g.* foci of alteration in the liver (Williams *et al.*, 1982; Goldsworthy *et al.*, 1986; Ito *et al.*, 1989). Others use tumour end-points, such as induction of lung adenomas in the A-strain mouse (Maronpot *et al.*, 1986) or induction of tumours in initiation–promotion studies using various organs, including the skin, bladder, intestine, liver, lung, mammary gland and thyroid (see reviews by Enzmann *et al.*, 1998a & 1998b; IARC, 1992 & 1999). A further category of study is the "start/stop" protocol. Here, an agent is administered for a limited period to induce particular effects or lesions; the progression or reversibility of these is then observed in the absence of further treatment (Todd, 1986; Marsman & Popp, 1984).

48. Transgenic assays in genetically engineered rodents have also been developed following the identification of genes, such as proto-oncogenes and tumour-suppressor genes, that are highly conserved across species and associated with a wide variety of human and animal cancers. They involve activated oncogenes that are introduced (transgenic) or tumour suppressor genes that are deleted (knocked out). If appropriate genes are selected, these assay systems may provide information on mechanisms of tumour formation or serve as selective tests for carcinogens. The modified transgene is expected to accelerate carcinogen-induced cancer development without interfering with other relevant genetic and/or epigenetic steps. High spontaneous tumour incidence in control animals is a major confounding factor of the conventional bioassay; the presence of the transgene itself does not induce high spontaneous tumour incidence in the short time span of the assay. These assays have been extensively reviewed in publications, including a single-theme issue of Toxicological Pathology (26 (4), 1998) and others (Tennant *et al.*, 1995; Contrera & DeGeorge, 1998; Eastin, 1998; Bucher, 1998; Eastin &Tennant, 1998).

49. The transgenic mouse model has not yet been fully validated or accepted by most national and international validation organizations (e.g., Scientific Advisory Committee on Alternative Toxicological Methods of the Interagency Coordinating Committee on the Validation of Alternative Methods) or testing laboratories. Although at this time, there is no large repository of historical control data to establish baseline parameters, work is currently on-going towards validation and the development of an OECD Test Guideline on Transgenic Rodent *in vivo* Gene Mutation Assays based on a Detailed Review Paper that

was published in 2009 (OECD, 2009). This document provides a comprehensive review of the transgenic rodent mutation assay literature and assesses the potential use of these assays in a regulatory context.

50. Transgenic mouse models appear to have usefulness as hazard identification screening models as part of an initial phase of the risk assessment process. However, they are not definitive proof of potential human carcinogenicity, and they are not proof of a specific mechanism of action. It appears that they could readily serve in place of, rather than merely in addition to, the mouse 2-year bioassay. However, like the 2-year bioassay, the results from tests in these models need to be incorporated into an overall integrated, weight of evidence evaluation for a given compound that takes into account genotoxicity, particularly DNA reactivity, structure activity relationships, results from other bioassays, and the results of other mechanistic investigations including toxicokinetics, metabolism, and mechanistic information (ICH, Proceedings of the Third International Conference,1995; Meyer, 2003; NAS, 2007; EFSA, 2009).

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3.4 TOXICOKINETICS

51. Studies examining the toxicokinetics (TK) of a chemical substance are conducted to obtain adequate information on its absorption, distribution, biotransformation (i.e. metabolism) and excretion, to aid in relating concentration or dose to the observed toxicity, and to aid in understanding its mechanism of toxicity (OECD, 2010). Basic TK parameters determined from these studies will also provide information on 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 (OECD, 2010). Toxicokinetic studies may provide useful information for determining dose levels for toxicity studies (linear vs. non-linear kinetics), route of administration effects, bioavailability, and issues related to study design.

52. The specific objectives of toxicokinetic studies include the following (ICH, 1994):

- to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study.

- to relate the exposure achieved in toxicity studies to toxicological findings and to contribute to the assessment of the relevance of these findings for other species i.e. humans/extrapolation.

- to provide information which, in conjunction with the toxicity findings, contributes to the design of subsequent toxicity studies.

53. For the purpose of dose selection, TK studies are informative in indicating whether there is a “point of saturation" or saturation kinetics evident in the dose response curve. They may also indicate a dose at which all biochemical changes plateau and doses above which such changes are not informative to the toxicity profile.

54. The kinetics of absorption will determine the internal exposure dose achieved. The absorption and clearance of the compound and its metabolites will determine the systemic and target organ exposure resulting from a single dose and can be used to design the treatment regimen required to achieve a desired internal dose. The effect of repeated exposures on absorption, metabolism, biotransformation, and clearance of a compound will provide information on the internal dose achieved during chronic exposure under conditions of the bioassay. The nutritional status of animals exposed chronically to a test substance may be affected during the experimental period; thus, information on potential interactions between the test chemical and nutritionally important compounds may be of value in the interpretation of the final results of the chronic study.

55. As indicated in OECD TG 417 on Toxicokinetics (OECD, 2010), there are numerous studies that might be performed to evaluate the TK behaviour of a chemical for regulatory purposes. However, depending on particular regulatory needs or situations, not all of these possible studies may be necessary for the evaluation of a chemical. Flexibility, taking into consideration the characteristics of the substance being investigated, is needed in the design of toxicokinetic studies. In some cases, only a certain set of questions may need to

be explored in order to address chemical-associated hazard and risk concerns. In some situations, TK data can be collected as part of the evaluation in other toxicology studies. For other situations, additional and/or more extensive TK studies may be necessary, depending on regulatory needs and/or if new questions arise as part of chemical evaluation.

56. In order to be of maximum utility in planning the design of a chronic toxicity or carcinogenicity study, particularly in the selection of dose levels, TK studies should be carried out, or data should be available, in the same species used in the long-term study and should preferably be performed using the same route and, where appropriate, the same vehicle as that used in the other toxicity studies. It should be noted however that such data may not be readily available for all chemicals, as they are not required under all regulatory schemes.

57. While single dose TK studies may provide useful information on absorption, distribution, metabolism and excretion of the test substance, the information most relevant in the planning and the execution of a chronic toxicity or carcinogenicity study will come from a repeat-dose toxicokinetic study over an extended period. As noted in OECD TG 417, repeated administration of the test substance may be needed to address more fully the potential for accumulation and/or persistence or changes in TK, or as required by a competent authority.

58. Information on repeat-dose toxicokinetics may be generated as part of a chronic toxicity (TG 452) or carcinogenicity (TG 451) study, or the combined chronic toxicity/carcinogenicity study (TG 453). This will require inclusion of additional animals, typically satellite animals included in the study design for the purpose of providing excreta and blood samples for toxicokinetic analysis. Quantity of test substance excreted in urine, feces, and expired air should be measured on at least two time points on day 1 of collection (one of which should be at 24 hours post-dose), and daily thereafter until the experiment is terminated. Blood samples should be taken from the satellite animals (and also in the case of an independent TK study) at suitable time points. The volume and number of blood samples which can be obtained per animal may be limited by potential effects of repeated sampling on animal health and/or physiology and the sensitivity of the analytical method. Comparison of the area-under-curve (AUC) on Day 1 and the last day is used to indicate accumulation, or not.

59. Guidance on toxicokinetic investigations following administration of test substance by the dermal or inhalation route(s) is given in the OECD TG417.

60. With respect to plasma levels of the test chemical measured in toxicity studies, an important point to note is that in rats there is a marked influence of sex hormones on liver biotransformation processes (see e.g. Chhabra & Fouts, 1974). In general, male rats metabolise xenobiotics (as well as endogenous substrates) faster than females, a finding not generally seen in other species. Thus rat studies may exhibit sex differences in plasma kinetics and in clinical and toxicological effects of the test chemical. These findings may not be relevant to human exposure.

61. As indicated in OECD TG 417 on Toxicokinetics (OECD, 2010), all available information on the test substance and relevant metabolites and analogs should be considered by the testing laboratory prior to conducting a toxicokinetic study in order to enhance study quality and minimise animal usage. This could include data from other relevant test methods (*in vivo* studies, *in vitro* studies, and/or *in silico* evaluations). Physicochemical properties, such as octanol-water partition coefficient (expressed as log P_{OW}), pKa, water solubility, vapour pressure, and molecular weight of a chemical may be useful for study planning and interpretation of results. They can be determined using appropriate methods as described in the relevant OECD Test Guidelines.

62. The draft revised TG 417 also provides guidance on use of supplemental approaches in addition to the *in vivo* studies described in the preceding paragraphs, that can provide useful information on absorption, distribution, metabolism and excretion (OECD, 2010). For example, use of freshly isolated or cultured hepatocytes and subcellular fractions (e.g. microsomes and cytosol or S9 fraction) from liver can provide useful information on metabolism of the test substance. Local metabolism in the target organ, e.g. lung, may be of interest for risk assessment. For these purposes, microsomal fractions of target tissues may be useful. Studies with microsomes may be useful to address potential gender and life-stage differences and characterize enzyme parameters (Km and Vmax) which can aid in the assessment of dose dependency of metabolism in relation to exposure levels. In addition microsomes may be useful to identify the specific microsomal enzymes involved in the metabolism of the substance which can be relevant in species extrapolation.

63. The potential for induction of biotransformation can also be examined by using liver subcellular fractions (e.g., microsomes and cytosol) of animals pretreated with the substance of interest, *in vitro* via hepatocyte induction studies or from specific cell lines expressing relevant enzymes (OECD, 2010). In certain circumstances and under appropriate conditions, subcellular fractions coming from human tissues might be considered for use in determining potential species differences in biotransformation. Primary cell cultures from liver cells and fresh tissue slices may be used to address similar questions as with liver microsomes. In certain cases, it may be possible to answer specific questions using cell lines with defined expression of the relevant enzyme or engineered cell lines. In certain cases, it may be useful to study the inhibition and induction of specific cytochrome P450 isozymes (e.g., CYP1A2, 2A1, and others) and/or phase II enzymes by the parent compound using *in vitro* studies. Information obtained may have utility for similarly structured compounds (OECD, 2010).

64. The results from *in vitro* investigations may also have utility in the development of PBTK models (Loizou et al., 2008), see also paragraphs 65 and 66. *In vitro* dermal absorption studies may provide supplemental information to characterize absorption (OECD, 2004).

65. Toxicokinetic models such as PBTK modelling may have utility for various aspects of hazard and risk assessment as for example in the prediction of systemic exposure and internal tissue dose. A PBTK model comprises an independent structural mathematical model, comprising the tissues and organs of the body with each perfused by, and connected via, the blood circulatory system. PBTK modeling may be used to predict the target tissue dose of the parent chemical or its reactive metabolite. Information derived

from PBTK modeling experiments may aid in the comparison of biotransformation and pharmacokinetics of a test substance and/or its metabolites and may provide a basis for extrapolation across species or dosing patterns. Such experiments may also provide estimates of relevant internal tissue dose which might be important to the hazard or risk assessment process (Andersen, 2003; US EPA 2006; Nielsen et al. 2008, Clewel and Clewel, 2008). Furthermore, specific questions on mode of action (see chapter 2) may be addressed, and these models can provide a basis for extrapolation across species, routes of exposure or dosing patterns. Use of the approach must however be adequately validated against experimental data and must be justified.

66. Data useful for developing PBTK models for a chemical in any given species include 1) partition coefficients, 2) biochemical constants and physiological parameters, 3) route-specific absorption parameters and 4) *in vivo* kinetic data for model evaluation (e.g. clearance parameters for relevant (> 10 %) excretion pathways, Km and Vmax for metabolism) (OECD, 2010). The experimental data used in model development should be generated with scientifically sound methods and the model results validated. Chemical- and species-specific parameters such as absorption rates, blood-tissue partitioning and metabolic rate constants are often determined to facilitate development of non-compartmental or physiologically-based models (IPCS, 2010).

67. The ICH guidance on the Assessment of Systemic Exposure in Toxicity Studies provides additional guidance on the value of TK data in dose selection in carcinogenicity studies (ICH, 1994). The ICH guidance emphasises the need to estimate systemic exposure to the parent compound and/or metabolite(s) at appropriate dose levels via TK studies and at various stages of a carcinogenicity study, in order to ensure that the findings of the study can be interpreted in relation to the comparative exposure for the animal model and humans. The guidance notes that increases in exposure may arise unexpectedly as a result of non-linear kinetics due to saturation of a clearance process. Increasing exposure may also occur during the course of a study for those compounds which have a particularly long plasma half-life. Careful attention should also be paid to compounds which achieve high Cmax values over comparatively short time periods within the dosing interval. Conversely, unexpectedly low exposure may occur during a study as a result of auto-induction of metabolising enzymes.

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3.5 HOUSING, FEEDING, HANDLING OF ANIMALS AND EXPERIMENTAL PROCEDURES

68. Many national and international standards have been developed for animal care including housing, feeding health and handling, e.g. NRC (1995), NRC (1996), Council of Europe (2006), the European Community (EEC, 1986) GV-SOLAS (1988), Dept. Primary Industries (2004). The general principles outlined in these guidelines are similar, and in conducting a chronic toxicity or carcinogenicity study, those guidelines applicable at a national level should be followed.

69. An overarching principle is that the particular needs of given species and strains must take precedence and that adherence to guidelines should never replace close observation of the particular animals involved, continued throughout their lives (Council of Europe, 1997). Provision of exhaustive guidelines for all species and strains is difficult to achieve and local initiatives for improving housing conditions should be taken whenever possible. Appendix A of the Council of Europe Convention "Guidelines for accommodation and care of animals", does however provide detailed guidance on these issues, including aspects such as design and maintenance of the test facilities (Appendix A, Council of Europe, 2006). It should be consulted for in-depth information.

3.5.1 Housing

70. Taken as an example, the Council of Europe Convention (Appendix A, 2006) states on housing that special relevance should be given to the enrichment of the environment of the respective species according to their needs for social interaction, activity-related use of the space, appropriate stimuli and materials. In a review of laboratory environments and rodents' behavioural needs, Balcombe (2006) notes that there is growing recognition of the inherent problems of depriving rodents the space and resources to carry out natural behaviours, such as exploring, foraging, running, escaping hiding and hygiene maintenance. The author reports a recent survey of animal facilities at the US National Institutes of Health which indicates that a slight majority of rats and mice at these facilities are now being provided with nesting and structural (shelter) enrichment (Hutchinson et al. 2005). Other indicators that rodent housing conditions are improving include the availability of commercially produced resources for nesting, shelter, gnawing and play (Key 2004), and a sharp rise since the late 1980s in the number of citations using keywords 'environmental enrichment' and 'rodent' (Hutchinson et al. 2005). Considering that two decades ago environmental rodent enrichment was scarcely being discussed, the author notes that these are laudable trends (Balcombe, 2006).

71. The Council of Europe Convention Appendix A (2006) recommendations on housing for rodents are as follows:

    - Rodent species other than guinea pigs should be kept in cages rather than pens. The cages should be made of easy to clean material and their design should allow proper inspection of the animals without unnecessarily disturbing them;

- The cages should be provided with solid floors with bedding instead of grid floors, unless there is good reason to have alternatives;
- Gregarious species should be group-housed. Although it may be difficult to achieve stable and harmonious groups of male mice, and also female hamsters, it is possible and should be attempted;
- Where the experimental procedures or welfare requirements make group-housing impossible, consideration should be given to accommodating animals of the same species within sight, sound or smell of one another;
- Encouragement should be given to break up the interior space of a cage by introducing objects such as platforms, tubes, boxes, etc. and attempts should be made to provide environmental enrichment with objects to explore, carry or transform, unless negative effects are observed on welfare or on the intended scientific use;
- High hygiene standards should be maintained. However, it may be advisable to maintain odour patterns left by the animals;
- Special attention should be paid to ensuring that the lighting intensity particularly on the top row of cages is not too high. Maximum light intensity should not exceed 350 Lux measured 1 metre from the floor. Provision should be made for shaded areas within the cage to allow the animals to withdraw.

72. The Convention makes specific recommendations for size of caging and stocking densities, dependent on the size/weight of the animals. In relation to environmental conditions, the Convention provides specific recommendations for temperature, humidity and ventilation for each species of laboratory animal covered in the guidelines. Those for rodents are in line with those indicated in the OECD Test Guidelines, as outlined in the next paragraph.

73. The Test Guidelines make some specific recommendations for housing of rodents only, including the recommendation (in line with that of the Convention) that animals may be housed individually, or be caged in small groups of the same sex; individual housing should be considered only if scientifically justified. Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g., morbidity, excitability) may indicate a need for individual caging. Rodents should be housed individually in dermal studies and during exposure in inhalation studies.

74. The Test Guidelines also specify that cages should be arranged in such a way that possible effects due to cage placement are minimised. The temperature in the experimental animal room should be 22^{o}C (± 3^{o}C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. These recommendations, together with those of the Convention should be applied in any rodent chronic toxicity or carcinogenicity study conducted according to the Test Guidelines.

75. As indicated in Chapter 3.3, although rodents (rats or mice) are the main species used in chronic toxicity or carcinogenicity studies, other species, dogs in particular, may be used on some occasions. As for rodents, specific guidelines for the care of dogs including housing, feeding health and handling have been developed, e.g. Council of Europe (2006). In relation to the housing of dogs, the Council of Europe Convention recommends that:

- Dogs should be housed in socially harmonious groups, unless the experimental procedures or welfare requirements make this impossible;
- Dogs should be exercised at least daily. Under no circumstances should dogs be caged without exercise for more than 14 days. Preferably, dogs should be exercised with other dogs.
- Dog pens should allow some privacy for the animals. They should include playthings and structures, including elevated platforms.
- Solid floors should be used for dogs. The materials, design and construction of slatted or perforated floors should provide surfaces which do not produce welfare problems such as irritation or injury of the feet or toes, blistering, etc. (these must be prevented at all times), and should supply a solid resting area.
- Temperature in dog studies should be held within a range of 15-21^{o}C, light period between 10 and 12 hours a day, and humidity 45-70%.

3.5.2 Feeding

76. In both humans and laboratory animals, diet has a direct bearing on health, and many neoplastic and non-neoplastic diseases are caused (or prevented) by dietary factors, including variations in the composition and amount of feed consumed. The association in rats of caloric consumption, the spontaneous formation of tumours and life span is well established. Although the zero-dose group may be expected to control for the influence of diet, dietary constituents may still profoundly affect the outcome of an experiment (OECD, 2002).

77. A nutritionally-balanced diet is important both for the welfare of laboratory animals and to ensure that experimental results are not biased by unintentional nutritional factors (NRC 1995). The US National Research Council provides detailed guidance on the nutritional requirements of a wide range of laboratory animals, with detailed information on essential nutrients and other considerations for each species (NRC, 1995). The NRC guidance emphasizes that feed palatability and intake, nutrient absorption and utilization, and excretion can be affected by physicochemical characteristics of feeds such as physical form, sensory properties, naturally-occurring refractory or anti-nutritive substances, chemical contaminants, and conditions of storage (NRC, 1995). Many biological factors also affect nutritional requirements, including genetic differences between species and strains, stage of life of the animals, environmental influences (e.g. diurnal rhythms, temperature etc.), housing and microbiological status (NRC, 1995). Detailed information is also given on diet

formulation for natural-ingredient diets, purified and chemically-defined diets, and on manufacture and storage procedures and other considerations (NRC, 1995).

78. The Test Guidelines state that rodents should be fed and watered ad libitum with food replaced at least weekly. Conventional laboratory diets may be used. The diet should meet all the nutritional requirements of the species tested and the content of dietary contaminants, including but not limited to pesticide residues, persistent organic pollutants, phytoestrogens, heavy metals and mycotoxins, that might influence the outcome of the test, should be as low as possible. Control and test animals should be fed from the same batch and lot. Analytical information on the nutrient and dietary contaminant levels should be generated periodically, at the beginning of the study and whenever there is a change in the batch used, and should be included in the final report. Analytical information on the drinking water used in the study should similarly be provided. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance in the diet and to meet the nutritional requirements of the animals when the test substance is administered by the dietary route.

79. As noted in section 3.2.1, the concentration of the test substance in the feed should not normally exceed an upper limit of 5% of the total diet (FDA, 1982, Borzelleca, 1992), although higher levels are feasible (e.g. when testing carbohydrates or proteins) as long as the diet is adapted to be nutritionally adequately, e.g. incorporated, at the expense of other components, in a purified diet. Section 3.2.1 also discusses the problems associated with the palatability of diet (or drinking water) containing test substances affecting the organoleptic characteristics of the food. If this is marked, it may be necessary to introduce into the study design an additional control group, pair fed (i.e. having matched food intake) in parallel with the high dietary level test group.

80. An important aspect of the feeding regime used in chronic toxicity and carcinogenicity is the recognized effect on study outcome of feeding *ad libitum*. Traditionally, maximal growth and reproduction have been used as criteria for the evaluation of laboratory animal diets (NRC, 1995). However, evidence from a number of studies indicates that restricting the caloric intake of laboratory animals may have beneficial effects on life span, the incidence and severity of degenerative diseases, and the onset and incidence of neoplasia (Weindruch and Walford, 1988; Yu, 1994; Keenan et al. 1997). Based on these results, allowing animals to eat *ad libitum* to produce maximum growth and reproduction may not be consistent with objectives of long-term toxicological and aging studies (NRC, 1995). Overfeeding by *ad libitum* food consumption is generally considered to be the most significant, uncontrolled variable affecting the outcome of the current rodent bioassay, and in particular, the correlation of food consumption, the resultant adult body weight and the 2-year survival in Sprague-Dawley rats is highly significant. (Keenan et al., 1997). Species and strain differences in survival are discussed further in chapter 3.3.

81. At a practical (experimental) level however, restriction of the caloric intake of laboratory animals is not straightforward. It may disrupt normal diurnal eating rhythms and is not compatible with group housing. It is also important to achieve caloric restriction of test animals without producing unintended nutrient deficiencies (NRC,

1995). Elevation of nutrient concentrations in the diet may be necessary to ensure that the nutrient intake of animals whose eating is restricted is comparable to that of animals allowed to eat *ad libitum.* There is, however, relatively little information available about the extent to which caloric restriction affects nutrient requirements (NRC, 1995). Since rats regulate their food intake according to caloric intake, the mineral and vitamin etc. content of the diet should be adjusted to "caloric density".

82. As already noted, the US National Research Council provides detailed guidance on the nutritional requirements of a wide range of species other than laboratory rodents, including dogs and rabbits (NRC, 1995). In the case of a chronic toxicity or carcinogenicity study involving animals other than rodents, this guidance should be consulted for information regarding feeding.

3.5.3 Handling, Health Surveillance and Experimental Procedures

83. The quality of care provided in the laboratory may influence not only growth rate and welfare, but also the quality and outcome of experimental procedures (Council of Europe Convention 2006). The animals should be accustomed to competent and confident handling during routine husbandry and procedures; this will reduce stress both to animals and personnel. In particular, for non-rodent species such as dogs, animals should be handled or be in social contact with humans on a regular basis. The behaviour of an animal during handling and the performance of experimental procedures depend to a considerable extent on the confidence and competence of its handler. Good technique should be unhurried, sympathetic and gentle but firm and safe for the animal and operator. All personnel should be appropriately educated and trained, and records of training maintained.

84. A strategy should be in place in all establishments to ensure that an appropriate health status is maintained, which safeguards animal welfare and meets scientific requirements (Council of Europe Convention 2006). This strategy should include a microbiological surveillance programme, plans for dealing with health breakdowns, and should define health parameters and procedures for the introduction of new animals, e.g. quarantining. Supervision of the accommodation and care by a veterinarian or other competent person is essential.

85. In relation to the experimental phase of a chronic toxicity or carcinogenicity study, as indicated in the Test Guidelines, the animals selected for the study should have been acclimated to laboratory conditions for at least 7 days and should not have been subjected to previous experimental procedures. A period of acclimatisation is needed to allow animals to recover from transport stress, to become accustomed to a new environment and to husbandry and care practices, and to ensure that their health status is sound. The test animals should be characterised as to species, strain, source, sex, weight and age. Each animal should be assigned a unique identification number, and permanently marked with this number by tattooing, microchip implant, or other suitable method. The method chosen should be reliable and cause the minimum pain and discomfort to the animal when applied and in the long-term. Staff should be trained

in carrying out the identification and marking techniques, and sedatives or local anaesthetics and analgesics should be used if necessary.

86. At the commencement of the study, the weight variation for each sex of animal used should be minimal and not exceed ± 20 % of the mean weight of all the animals within the study, separately for each sex. Animals should be randomly assigned to the control and treatment groups. After randomisation, there should be no significant differences in mean body weights between groups within each sex. If there are statistically significant differences, then the randomisation step should be repeated, if possible.

87. The animals should be inspected regularly throughout the study, at least daily by a trained person, to ensure that all sick or injured animals are identified and appropriate action taken. Regular health monitoring should be carried out. The Test Guidelines specify that all animals should be checked for morbidity or mortality, usually at the beginning and the end of each day. Animals should additionally be checked once a day following dosing in the case of gavage studies, for specific signs of toxicological relevance, taking into consideration the peak period of anticipated effects after dosing in the case of gavage administration. Particular attention should be paid to tumour development. The time of tumour onset, location, dimensions, appearance, and progression of each grossly visible or palpable tumour should be recorded. Body weights and food/water consumption and food efficiency should be assessed and recorded at the intervals specified in the guidelines.

88. At the end of the study, for interim kills and in the case of animals found sick or moribund during the study, the animals should be humanely killed. For non-scheduled killing i.e. for animals showing clinical sign of pain, suffering or distress, OECD Guidance Document 19 on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoint for Experimental Animals Used in Safety Evaluations should be followed (OECD, 2000). All humane methods of killing animals require expertise, which can only be attained by appropriate training. Animals should be killed using a method that adheres to the principles set by the European Commission Recommendations for the euthanasia of experimental animals (Part 1 and Part 2) (EEC, 1986). A deeply unconscious animal can be exsanguinated, but drugs which paralyse muscles before unconsciousness occurs, drugs with curariform effects and electrocution without passage of current through the brain, should not be used without prior anaesthesia. Disposal should not be allowed until death has been confirmed.

89. Records of source, use and final disposal of all animals bred, kept for breeding, or for subsequent supply for use in scientific procedures should be used not only for statistical purposes but, in conjunction with health and breeding records, as indicators of animal welfare and for husbandry and planning purposes.

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5. DATA ADEQUACY, RELIABILITY AND RELEVANCE, REPORTING, USE OF HISTORICAL CONTROL DATA

5.1. General

90. The scientific evaluation of, and conclusions to be drawn from, any chronic toxicity or carcinogenicity study is dependent on the documentation submitted. This chapter of the Guidance Document covers the documentation of data by the test laboratory in a test report, in order to be able to demonstrate (normally to a regulatory agency) the reliability, relevance and adequacy of the data, and hence its validity and the conclusions that can be drawn from it. It also covers guidance on the use of historical control data as an adjunct to the internal controls of the study in question and on the reporting of the study.

91. Assurance of the quality, integrity, and completeness of the experimental data from chronic toxicity and carcinogenicity studies is essential to the subsequent independent analysis and evaluation of the study report. Evaluation of these data will initially be carried out by the laboratory conducting the studies and by the sponsor, and ultimately by a regulatory agency to whom they are submitted. A qualitative assessment of the acceptability of study reports and the underlying data is therefore an important part of the process of evaluation. In order to be acceptable, studies must be of an adequate standard.

5.2. Reliability, relevance and adequacy

92. The process of determining the standard or quality of the data takes into consideration three aspects – reliability, relevance and adequacy. These terms were defined by Klimisch et al. (1997) as follows:

- **Reliability** - evaluating the inherent quality of a test report or publication relating to preferably standardised methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings;

- **Relevance** - covering the extent to which data and tests are appropriate for a particular hazard identification or risk characterisation; and

- **Adequacy** - defining the usefulness of data for hazard/risk assessment purposes.

93. The reliability of a chronic toxicity and/or carcinogenicity study is judged in terms of the test method used, any deviations from the procedures laid down in it, the competence of the laboratory carrying out the study, and the way that the conduct of the study and the results are described. Factors to be considered in assessing the reliability of a study include the observational and experimental methods used; frequency and duration of exposure; the species, strain, sex and age of the animals used; the numbers of animals used per dosage group; dose, route and frequency of

dosing; and the conditions under which the substance was tested. All of these are part of Good Laboratory Practice considerations (OECD, 1998).

94. The relevance of the data generated in a chronic toxicity and/or carcinogenicity study is based on the appropriateness of the species studied and the route of exposure used (see chapters 3.2 and 3.3 of this guidance), relative to the population likely to be exposed to the chemical under test. The test chemical should also be representative of that to which the population is or will be exposed, and it is essential therefore that a full identification and characterisation of the chemical and any significant impurities contained in it is available (EC, 2008).

95. Chronic toxicity and/or carcinogenicity studies on a chemical under investigation are carried using animal models, and there will often be no data on the metabolism, toxicokinetics or toxicity of the chemical in humans. Under these circumstances, adverse effects observed in animal studies will normally be assumed to occur in humans, even if the threshold level of exposure is unknown. Clear and well documented scientific evidence of a species-specific effect / response would therefore be required before an animal study was deemed irrelevant to humans.

96. Examples of effects in chronic toxicity and/or carcinogenicity studies that have been generally accepted by the scientific community as not being relevant to humans include light hydrocarbon-induced renal nephropathy in male rats and liver tumours in certain species of susceptible mice. Other tumours have shown to be generally not relevant to humans (see chapter 2), e.g. rat urinary bladder tumours; rat pituitary gland tumours; mouse liver and mammary tumours; forestomach tumours in rats and mice; thyroid tumours in rats; interstitial tumours or leydic cell tumours in rats (F 344); tumours (sarcomas) in spleen in F 344; kidney tumours in male rats (α2u globulin); pheochromocytomas (adrenal medulla) in rats mediated via effects on calcium metabolism; liver, rodents, peroxisome proliferation; pancreatic adenomas in F 344 rats administered corn oil by gavage; leukaemia: Large Granular Lymphocyte (LGL) also named as synonym: Fischer rat leukaemia. In certain cases where human data are available on the test chemical or a close structural analogue, it may be possible to judge the relevance of animal data on the basis of comparative metabolism and toxicokinetics, or clinical experience.

97. The adequacy of a chronic toxicity and/or carcinogenicity study is judged in terms of its reliability and relevance, being a composite term covering both aspects. A reliable and relevant study is likely to be considered useful for hazard/risk assessment purposes. The reliability of the data is a key consideration, and an approach to evaluating the reliability of a study has been developed by Klimisch et al. (1997), as follows:

1 = reliable without restrictions: "studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline

(preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method."

2 = reliable with restrictions: "studies or data from the literature, reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable."

3 = not reliable: "studies or data from the literature/reports in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g., un-physiologic pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgment."

4 = not assignable: "studies or data from the literature which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.)."

98. The concepts of relevance and reliability and the application of the Klimish code are primarily used in the assessment of existing studies that may not have been carried in accordance with current test guidelines such as TGs 451, 452 and 453 on carcinogenicity and chronic toxicity testing. The assumption can be made that a study carried out in accordance with these guidelines, together with the additional guidance provided in the current document, is inherently reliable, provided that the laboratory has the competence and experience to carry out such a study. The latter can be judged, in part, by the documentation (study report) that is produced detailing the results of the study. Obviously, there should be no major deviations from the requirements of the guidelines, unless well justified and recorded.

5.3. Quality assurance and good laboratory practice

99. In addition to the Test Guidelines themselves and this Guidance Document, there are many other sources of information on the generation of scientifically valid data, concerning good experimental design and reporting, and accepted codes of Good Laboratory Practice, or GLP (OECD, 1998; US EPA, 2007*)*. The International Programme on Chemical Safety has produced detailed guidance on Quality Management for Chemical Safety Testing (WHO, 1992), while OECD also provides extensive guidance in its Series on Principles of Good Laboratory Practice and Compliance Monitoring (OECD, 1998). It is essential that the standard or quality of the data reported is verified by a quality management (assurance) system involving independent monitoring of laboratory management and personnel, facilities and equipment, methods records and controls (WHO, 1992).

100. While implementation of a quality management system is primarily the responsibility of the testing laboratory and guidance on the principles of Quality

Assurance or Good Laboratory Practice is beyond the scope of the current document, implementation of these standards are a prerequisite in demonstrating the competency of the laboratory and the reliability of the data generated. Quality assurance should be integrated within the entire process in order to ensure that the results are valid and that the final study report accurately reflects these results (WHO, 1992).

5.4. Reporting

101. The final study report will normally be the main documentation on which assessors (in regulatory agencies and/or in companies originally commissioning the study in question) will base their judgement on the reliability and relevance of the study. The report must therefore be comprehensive and sufficiently detailed to allow assessors to arrive at their conclusion and should be structured to allow for ready access to all significant and relevant points arising out of the assessment. (OECD, 2002a and 2002b).

102. These qualitative considerations establish the acceptability not only of specific reports but also the acceptability of the eventual evaluation, interpretation, judgments, and risk assessments made by toxicologists. The acceptability of reports and other technical information submitted to regulatory agencies is primarily a scientific judgment, although ultimately the submitters of the information should know the reasons underlying the rejection of a study.

103. The Test Guidelines 451, 453 and 453 provide an outline of the information that should be included in the study report. This includes information on the test substance, the vehicle, the test species, the test conditions and the details of the results to be included in the report. Further guidance on the reporting of the study is included in the following paragraphs.

5.4.1. Study Identification

104. Any report of a chronic toxicity or carcinogenicity study should include the following information to enable clear identification of the study. This information is important for the identification of the study, in the event that the report is referred to or resubmitted by the sponsor company at a later date, or submitted by another company. It can be incorporated into the heading and/or the first paragraph of the evaluation:

a. Title of study (should identify species/dose-route/study duration)

b. Report/study number

c. Laboratory report/project number

d. Study sponsor (usually the registrant)

e. Testing laboratory and brief address

f. Authors' names (if available/appropriate)

g. Date of report

h. Period over which the study was conducted

i. Test Guideline/protocol followed

j. GLP status (or QA statement) and relevant authority

k. Indication of whether the report is published or unpublished

5.4.2. Level of Study Reporting

105. The methodology used in the investigation and the study findings should be presented in sufficient detail for the evaluator in order to reach to an independent conclusion. Ideally, reports should obviate the need, during a subsequent review of the chemical, to have to refer back to the original raw study data.

106. It should be made clear in the report whether observed changes/differences are considered to be treatment related or not. Findings in the study and changes/differences in treated animals compared with controls must be reported and assessed for the relevance to treatment i.e. a conclusion must be drawn as to whether these are treatment-related or regarded as coincidental findings. However, clearly unrelated, coincidental findings should only be listed/reported without further comments. In case of equivocal/unclear findings, where there is a concern that an effect could possibly be related to dosing, such findings should be reported, with a comment about the lack of any dose-relationship or other unequivocal evidence. Tabulation of the data in question is essential in this situation - it enables a reviewer to examine relevant data to determine a level of concern, without the need to return to the raw data in the original study.

5.4.3 Information to be included

107. Within the body of the evaluation report the following (minimum) information should be recorded:

Introduction

- A brief statement of the objective of the test or study (if there is a special or unusual reason for conducting the study).

Test substance

- The identity (including batch no.) and purity of the test material, including its common (generic) name.

- The chemical names of the compound (IUPAC, CAS and common names) and synonyms, as well as the CAS number, company code names/numbers, any trade names, the empirical formula, the structural formula, the molecular weight and all available physicochemical data should be included at some point in the evaluation report. Information about identified impurities, isomer ratios and stability of the pure compound should also be included.

Vehicle (in the case of gavage administration)

- Justification for and details of the composition of solvents or dosing excipients.

Administration of test substance in the diet

- For compounds included in the diet, a description of the diet preparation should be included, including information on any vehicle used and frequency of preparation.

- Analyses for stability, homogeneity and concentration of the test compound in the dietary admix should be reported. Any deviation in these parameters during the study shall be reported and their potential influence on the study should be considered/discussed (OECD GLP 1998, WHO, 1992)

Administration of test substance in drinking water

- Analyses for stability and concentration of the compound in the drinking water should be reported.

- Details of the quality and analysis of the water for standard contaminants should be provided.

Test animals

- Species, strain and source of test animal used. Information about strain and source is essential, particularly in the event of the need to establish historical control incidences of pathological findings or to check baseline physiological or biochemical parameters.

- Number of animals per sex and per group, as well as numbers of animals in any additional subgroups or recovery groups.

- age of animals at start of test.

- individual weights of animals at the start of the test.

Environmental conditions and housing

- information on animal housing, environmental conditions including temperature, humidity, air changes and light/ dark daily periods etc. and the animal acclimation period should be included (see WHO, 1992; OECD 1998).

- Type and source of the animal diet among others including analysis for contaminants and similar information on drinking water e.g. tap water or acidified tap water including information on contaminants should be provided.

Test conditions: dosing

- The report should state the rational for dosage route and doses used (including the vehicle used for negative controls).
- Dietary levels should be quoted in mg/kg diet (ppm) with measured or estimated daily intakes of the substance in mg/kg bw/d including conversion factor from diet/drinking

water ppm to actual dose in mg/ kg bw/day or mg/m^2 per day if applicable also being reported.

- Details about dosing methods, especially for dermal and inhalational studies, should be recorded.
- For dermal studies, descriptions of the application procedure including site, vehicle used, manner in which the skin was treated (shaved, abraded, or non-abraded), whether the site was occluded (and method of occlusion), and the amount of body surface covered should be reported. Normally, for such studies, the test substance should be spread evenly over an area that is approximately 10% of the total body surface (OECD, 1981a and 1981b).
- For inhalational studies, the following minimum information should be recorded: (1) methods for generation of the test atmosphere and description of the test chamber, including whether whole-body or nose-only exposure; (2) time to equilibration of the test atmosphere; (3) test atmosphere concentration; (4) particle size determination, size distribution and consistency over the course of the study.
- Duration and timing of dosing should be reported.

Test conditions: scheduled investigations

- These have been detailed in chapter 3.6 of this Guidance Document. The observations made, parameters measured, and the frequency of their investigation should be fully reported in the study report, including among others any sign of toxicity, survival, including time of death and scheduled sacrifice times.

Results

The study report should include a detailed description of treatment-related effects on:

- mortalities (with examination for cause of death)
- gross observations for behaviour and appearance ("clinical signs of toxicity")
- food and water consumption if applicable. Water consumption is not specifically requested under OECD Test Guidelines 451, 452 and 453, unless the substance is administered in the drinking water, although it should be noted that changes in water consumption can give an indication of treatment-related effects.
- body weights/body weight changes
- functional investigations (e.g. ECG, motor activity, neurological tests) nature, severity, and duration of clinical observations (whether reversible or not)
- ophthalmology,
- blood coagulation and haematology (*)
- blood biochemistry (*)
- urinalyses (*) (not specifically required for chronic toxicity studies in rodents in US EPA or OECD Test Guidelines)
- results of any toxicokinetic analysis (if carried out)

- necropsy findings/ macroscopic pathology,
- organ weights (absolute and relative),
- histopathology (see section 3.6),
- any other special investigations

(*at intervals during the study and at term as prescribed in the TGs)

108. If one or more of the parameters listed have not been included in the study then these omissions should be noted and justified, and if necessary a statement included on the adequacy of the study in the light of the parameters assessed.

109. In reporting the findings of the study, the change relative to controls (absolute and percentage/incidence), the dose relatedness of the changes, the biological and statistical significance of findings, historical control ranges (if available, with information on the source of the historical data and how closely or otherwise it matches the study being evaluated), and suspected mechanisms of action should be covered. The NOEL/NOAEL for each treatment-related effect should be recorded in the relevant part of the study report.

110. Tabulation of data is essential and incidences of findings should be given in sufficient detail to allow independent assessment from the report. Narrative accompanying such tabular data should address the toxicological significance of the results and not just repeat what is presented in the table. If possible, compound-related changes in biochemical, haematological or urinalysis parameters should be linked with organ weight, gross pathology and/or histopathological changes (Tyson and Sawhney, 1985).

111. The report should identify the statistical method used to evaluate each parameter and, in the case of a carcinogenicity study, provide details of modelling approaches used to characterise a carcinogenic response, e.g. Bench Mark Dose or Linear Extrapolation. Statistical analysis should follow the guidance provided in chapter 4 of this document. Deficiencies in statistical testing may result in requests to conduct a re-analysis or provide further comment and/or analysis.

Discussion

112. The results of the study should be comprehensively discussed, including a discussion of target organs, dose:response relationships and mechanism(s) of action of the chemical under investigation. The first paragraph should give a brief description of the experimental design, incorporating all essential details i.e. species and strain, number of animals/sex/group, doses used, route and method of administration, duration of compound administration, the frequency of dosing, the vehicle and, if applicable, the duration of any recovery period and the number of recovery animals.

113. Dietary levels should be quoted as mg/kg of the test substance in the food together with measured and/or estimated mean intake in mg/kg/day over the course of the study.

The discussion should include some mention of extent of absorption and bioavailability of the test substance, from toxicokinetic data or other available information.

114. Comment should be made on the adequacy of the study. Any deficiencies should be discussed in detail and comment made on the regulatory relevance of the study.

115. The discussion should also address any potential influence of modifying factors (for example differences in food consumption due to palatability of the diet) which may result in major deviations in parameters in treated animals compared with control values). This qualitative consideration has more to do with the evaluation and interpretation of data than with acceptability of documentation. It is placed here because determination of the factors which may have a major influence on toxicological data needs to be made prior to the analysis of the data.

116. There are many factors influencing the responses of experimental animals to chemical substances; some of these are discussed by Nielsen et al., 2008. Circadian rhythms and seasonal physiological variations can subtly influence experimental results. Such factors influencing animal responses can be problematic for the assessment of the data and potentially leading to misinterpretation as toxic responses to treatment.

117. Findings should be considered on the basis of both statistical significance and likely biological significance. The variability of biological data must be considered when assessing a statistically-significant result. Conversely, a finding which is not statistically significant may have biological significance when considered in the light of the likely toxicological or pharmacological action of the compound, or when combined with results from other studies. Thus, trends or transient changes in parameters should be reported and discussed for possible relation to treatment. This information may be useful when comparing results across studies and in the consideration of the overall significance or relevance of an observed effect i.e. in one study an effect may be only a trend whilst in another study it may be very clearly treatment-related.

118. The overall evaluation of the study e.g. derivation of NOEL/NOAEL (for chronic toxicity or threshold carcinogenicity) or BMD (POD) or other analysis (for non-threshold carcinogens) should be presented with a statement for its basis provided (so that the LOEL/LOAEL is clearly apparent), and the discussion should end with a detailed analysis of the overall conclusions to be drawn from the data reported.

5.4.4 Use of historical control data

119. The Test Guidelines 451, 452, 453 state, in relation to use of historical controls, that in addition to data obtained from the concurrent controls used in the study, the use of historical control data may be valuable in the interpretation of the results of the study, where there are indications that the data provided by the concurrent controls are substantially out of line with recent data from control animals from the test facility colony.

120. Historical control data may be useful when evaluating the acceptability of the "normal" data obtained from control groups (Haseman et al, 1984; Paynter, 1984; Sumi et al, 1976; Tarone, 1982). However, they should be used only if concurrent controls appear to be significantly different; the priority should be placed on use of concurrent control over historical control data. Any departure from the norm by the control groups should be discussed in the evaluation document and taken into consideration, especially during the conduct of any statistical analysis. The finding of consistent departures from the norm in control groups could necessitate investigation of the source of the animals.

121. Historical control data submitted for consideration should be taken from the same laboratory, utilising the same strain, age and sex of animals obtained from the same supplier, and only include those studies ideally conducted within a five years span on either side of the study under review. The historical control data should be separated by sex and, for carcinogenicity studies, malignant and benign lesions should be presented separate and combined, where appropriate, and preferably by individual study. Specific ranges for the data should be quoted, and identification of methodology of the studies from which the data are derived, which could have affected the results, should be provided. This should include pre-sampling conditions such as fasting or non-fasting, assay methodology for study parameters, histopathological criteria for lesion identification, time of terminal sacrifice etc. Where historical data are used in an assessment, it should be clearly identified.

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6. DEFINITIONS/GLOSSARY

To note: this is a work in progress. Definitions/glossary will be added from Chapter 2, Chapter 3.1 and as gaps are identified by the WNT and Expert Group.

ADI/TDI: Acceptable daily intake/Tolerable daily intake: the amount of a test article in food or drinking water that can be ingested (orally) over a lifetime without an appreciable health risk.

Adverse: Any treatment-related alteration from baseline that diminish an organism's ability to survive, reproduce or adapt to the environment (US EPA, 1994).

AUC: Area Under the Curve (Area under the plasma concentration-time curve): Area under the curve in a plot of concentration of substance in plasma over time.

Benchmark dose (BMD): The statistical lower confidence limit of the dose corresponding to a small increase in effect over the background level. Typically, a 1% or 10% response level above the background is selected.

Bioaccumulation: the accumulation of the test article in an exposed organism. Bioaccumulation occurs when an organism absorbs a test article at a rate greater than that at which it is excreted.

Bioavailability: Fraction of an administered dose that reaches the systemic circulation or is made available at the site of physiological activity.

BMDL: Benchmark Dose Lower confidence limit.

Carcinogenicity: Substances are defined as carcinogenic if they induce tumours (benign or malignant), increase its incidence or shorten the time of tumour, when inhaled, ingested, dermally applied or injected.

Chronic Toxicity: Toxicity (adverse effect) after an exposure period of 12 months or longer due to a test article that has been ingested inhaled, dermally applied or injected.

Detoxification pathways: Series of steps leading to the elimination of toxic substances from the body, either by metabolic change or excretion.

Dose: Total amount of a test article administered to, taken up by, or absorbed by an organism, system, or (sub) population.

Dose-response: Relationship between the amount of an agent administered to, taken up by, or absorbed by an organism, system, or (sub) population and the change developed in that organism, system, or (sub) population in reaction to the agent.

Extrapolation: Inference of one or more unknown values on the basis of that which is known or has been observed.

Exposure: Concentration or amount of the test article that reaches a target organism, system, or (sub) population in a specific frequency for a defined duration.

Genotoxic/genotoxicity: A deleterious action on a cell's genetic material affecting its integrity.

GLP: Good Laboratory Practice

Induction/Enzyme induction: Enzyme synthesis in response to an environmental stimulus or inducer molecule;

Hazard: The inherent property of a test article to cause adverse effects when an organism, system, or (sub) population is exposed to that test article.

Hazard identification: The identification of the type and nature of adverse effects that an agent has as inherent capacity to cause in an organism, system or (sub) population.

Mechanism of Action: The individual biochemical and physiological events leading to a toxic effect).

MOA: Mode of Action: the processes by which a chemical induces toxicity. A MOA can inform about relevance of observed effects in laboratory animals to humans and the variability of response within the human population.

Local effect: Adverse effect at the site of first contact (e.g. skin, eye, mucous membrane/gastro-intestinal tract, or mucous membrane/respiratory tract).

MTD: Maximum Tolerated Dose

MTC: Maximum Tolerated Concentration

NOAEC: No-Observed- Adverse-Effect-Concentration. The highest concentration of a test article to which an organism is exposed, that does not cause any observed and statistically significant adverse effects on the organism compared with the controls.

NOAEL: No-Observed- Adverse-Effect-Level. The highest level of a test substance to which an organism is exposed, that does not cause any observed and statistically significant adverse effects on the organism compared with the controls.

NOEL: No-Observed-Effect-Level

POD: Point of Departure: The dose-response point that marks the beginning of a low-dose extrapolation. This point is most often the upper bound on an observed incidence or on an estimated incidence from a dose-response model.

QSAR: Quantitative Structure Activity Relationship

Read-across: The endpoint information for one or more chemicals is used to make prediction of the endpoint for the target chemical.

Reference dose: An estimate of a daily exposure to a chemical that is unlikely to cause harmful effects during a lifetime.

Route of administration (oral, IV, dermal, inhalation, etc.): Refers to the means by which substances are administered to the body (e.g., orally by gavage, orally by diet, dermal, by inhalation, intravenously, etc).

Route-to-route extrapolation: The prediction of an equivalent dose and dosing regime that produces the same toxic endpoint or response as that obtained for a given dose and dosing regime by another route.

SAR: Structure Activity Relationship

Systemic effect: A toxicological effect that affects the entire body or many organs.

Systems Modeling: (Pharmacokinetic-based, Physiologically-based Pharmacokinetic, Biologically-based, etc.): Abstract model that uses mathematical language to describe the behaviour of a system.

Target tissue: Tissue in which the principal adverse effect of a toxicant is manifested.

Threshold: Dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur.

Toxicity: Inherent property of an agent to cause an adverse biological effect.

Toxicodynamics: the processes of interaction of toxicologically active substances with target sites, and the biochemical and physiological consequences leading to adverse effects.

Toxicokinetics (Pharmacokinetics): A term describing the processes of chemical absorption, distribution, metabolism, and excretion in the organism (ADME).