

DRAFT OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Test Guideline 453: Combined Chronic Toxicity/Carcinogenicity Studies

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing assessment practices and animal welfare considerations. The original Test Guideline 453 was adopted in 1981. Development of a revised TG 453 was considered necessary, in order to reflect recent developments in the field of animal welfare and regulatory requirements (1)(2)(3)(4)(5). The updating of TG 453 has been carried out in parallel with revisions of the Test Guidelines 451, Carcinogenicity Studies and 452, Chronic Toxicity Studies, with the objective of obtaining additional information from the animals used in the study and providing further detail on dose selection.
2. The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends on the physical and chemical characteristics of the test substance and the predominant route of exposure of humans. Additional information on choice of route of exposure is provided in guidance (6). This Test Guideline focuses on exposure via the oral route, the route most commonly used in carcinogenicity studies. While long-term carcinogenicity studies involving exposure via the dermal or inhalation routes may also be necessary for human health risk assessment and/or may be required under certain regulatory regimes, both routes of exposure involve considerable technical complexity. Such studies will need to be designed on a case-by-case basis, although the Guideline outlined here for the assessment and evaluation of carcinogenicity by oral administration could form the basis of a protocol for inhalation and/or dermal studies, with respect to recommendations for treatment periods, clinical and pathology parameters, etc. OECD Guidance Documents are available on the administration of test substances by the inhalation (6)(7) and dermal routes (6). The updated Test Guidelines TG 412, Repeated Dose Inhalation Toxicity: 28-Day or 14-Day Study, and TG 413, Subchronic Inhalation Toxicity: 90-Day Study should be specifically consulted in the design of longer term studies involving exposure via the inhalation route.
3. The majority of chronic toxicity/carcinogenicity studies are carried out in rodent species. However, while this Test Guideline is primarily applicable to studies in rodents, the principles and procedures outlined may also be applied, with appropriate modifications, to studies in other species, as outlined in OECD Guidance Document (6).
4. The objectives of chronic toxicity/carcinogenicity studies covered by this Test Guideline include:
 - the identification of the carcinogenic properties of a chemical, namely its potential to induce neoplastic lesions, resulting in an increased incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms, and its chronic toxicity,
 - the identification of target organs,
 - characterisation of the dose:response relationship,
 - identification of a no-observed-adverse-effect level (NOAEL) or departure point for establishment of a Benchmark Dose (BMD),
 - the prediction of the health effects of a chemical at human exposure levels,
 - provision of data to test hypotheses regarding mode of action (2)(6)(8)(9)(10)(11).

INITIAL CONSIDERATIONS

5. In the assessment and evaluation of the potential carcinogenicity and chronic toxicity of a chemical, all available information on the test substance should be considered by the testing laboratory prior to conducting the study, in order to focus the design of the study to more efficiently test for its toxicological properties and to minimize animal usage. Information that will assist in the study design includes the identity, chemical structure, and physico-chemical properties of the test substance; any information on the mode of action; results of any *in vitro* or *in vivo* toxicity tests including genotoxicity tests; anticipated use(s) and potential for human exposure; available (Q)SAR data, mutagenicity/genotoxicity, carcinogenicity and other toxicological data on structurally-related substances; available toxicokinetic data (single dose and also repeat dose kinetics where available) and data derived from other repeated exposure studies. The determination of chronic toxicity/carcinogenicity may be carried out after initial information on toxicity has been obtained from repeated dose 28-day and/or 90-day toxicity tests, also short-term cancer initiation-promotion tests. A phased testing approach to carcinogenicity testing should be considered, as part of the overall assessment of the potential adverse health effects of a particular chemical (12)(13)(14)(15).
6. The combined chronic toxicity/carcinogenicity study provides information on the possible health hazards likely to arise from repeated exposure over the majority of the entire lifespan (in rodents). The study will provide information on the toxic effects of the substance including potential oncogenicity, indicate target organs and the possibility of accumulation. It can provide an estimate of the no-observed-adverse effect level, which can be used for establishing safety criteria for human exposure (see paragraph 20 of this Test Guideline). The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. In conducting such a study, the guiding principles and considerations outlined in the OECD Guidance Document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (16), in particular paragraph 62 thereof, should always be followed
7. Detailed guidance on and discussion of the principles of dose selection for chronic toxicity and carcinogenicity studies can be found in an OECD Guidance Document on Selection of Doses for Use in Chronic Toxicity and Carcinogenicity Studies (6) as well as two International Life Sciences Institute publications (17)(18). The core dose selection strategy is dependent on the primary objective or objectives of the study (paragraph 4). In selecting appropriate dose levels, a balance has to be achieved between hazard screening on the one hand and characterisation of low-dose responses and their relevance on the other. This is particularly relevant in the case of this combined chronic toxicity and carcinogenicity study.
8. **In order to reduce the number of animals used**, consideration should be given to carrying out this combined chronic toxicity and carcinogenicity study, rather than separate execution of a chronic toxicity study (TG 452) and carcinogenicity study (TG 451). The combined test allows a modest reduction in animal use compared to conducting two separate studies, without compromising the quality of the data in either the chronic phase or the carcinogenicity phase. This reduction is made possible by combining the in-life measurements during the first year of the study for body weight, food consumption, and clinical observations. Careful consideration should however be given to the principles of dose selection (paragraphs 7 and 18-22) when undertaking a combined chronic toxicity and carcinogenicity study, and it is also recognised that separate studies may be required under certain regulatory frameworks.
9. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

10. The study design consists of two parallel phases, a chronic phase, normally of one year duration (see paragraph 29), and a carcinogenicity phase, normally of two years duration (see paragraph 30). The test substance is normally administered by the oral route although testing by the inhalation or dermal route may also be appropriate (paragraph 2). For the chronic phase, the test substance is administered daily in graduated doses to several groups of test animals, one dose level per group for a period of one year, and provision may also be made for interim kills (see paragraph 16). This duration is chosen to be sufficiently long to allow any effects of cumulative toxicity to become manifest, without the confounding effects of geriatric changes. The study design may also include one or more interim kills, e.g. at 3 and 6 months, and additional groups of animals may be included to accommodate this (see paragraph 16). For the carcinogenicity phase, the test substance is administered daily to several groups of test animals for a major portion of their life span. The animals in both phases are observed closely for signs of toxicity and for the development of neoplastic lesions. Animals which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are also killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

11. This Guideline primarily covers assessment and evaluation of carcinogenicity in rodents. **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 preferred rodent species is the rat, although other rodent species, e.g., the mouse, may be used. The use of the mouse in carcinogenicity testing may have limited utility (REF). The design and conduct of chronic toxicity/carcinogenicity studies in non-rodent species, when required, should be based on the principles outlined in this Test Guideline together with those in OECD TG 409, Repeated Dose 90-day Oral Toxicity Study in Non-Rodents.
12. In this Test Guideline, the preferred rodent species is the rat, although other rodent species, e.g., the mouse, may be used. Additional information on choice of species and strain is provided in Guidance Document (6), and the choice made should be justified. Rats and mice have been preferred experimental models because of their relatively short life span, their widespread use in pharmacological and toxicological studies, their susceptibility to tumour induction, and the availability of sufficiently characterised strains. As a consequence of these characteristics, a large amount of information is available on their physiology and pathology. Commonly used laboratory strains of young healthy adult animals should be employed. The combined chronic toxicity/carcinogenicity study should be carried out in animals from the same strain and source as those used in preliminary toxicity study(ies) of shorter duration, although, if animals from this strain and source are known to present problems in achieving the normally accepted criterion of 50% survival at 24 months, consideration should be given to using a strain of animal that has a acceptable survival rate for the long-term study. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are six weeks old.

Housing and feeding conditions

13. Animals may be housed individually, or be caged in small groups of the same sex; individual housing should be considered only if scientifically justified (19)(20)(21). 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°C (\pm 3°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. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. 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. Analytical information on the nutrient and dietary contaminant levels should be generated periodically, at least at the beginning of the study and when 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 and to meet the nutritional requirements of the animals when administered by the dietary route.

Preparation of animals

14. Healthy animals, which have been acclimated to laboratory conditions for at least 7 days and have not been subjected to previous experimental procedures, should be used. 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 6 weeks old. The test animals should be characterised as to species, strain, source, sex, weight and age. At the commencement of the study the weight variation of animals used should be minimal and not exceed $\pm 20\%$ 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. Each animal should be assigned a unique identification number, and permanently marked with this number by tattooing, microchip implant, or other suitable method.

PROCEDURE

Number and sex of animals

15. Both sexes should be used. A sufficient number of animals should be used so that a thorough biological and statistical evaluation is possible. Each dose group and concurrent control group intended for the carcinogenicity phase of the study should therefore contain at least 50 animals of each sex. In some cases disproportionate testing with more than 50 animals in the low dose groups might be considered as appropriate; e.g., to estimate the carcinogenic potential in low dosages. However it should be recognised that a moderate increase in group size will provide relatively little increase in statistical power of the study. **An additional 2 satellite groups of 20 animals of each sex, one high dose group and one control group, should be included for evaluation of chronic toxicity and non-neoplastic pathology at 12 months. An additional dose group may be useful to better define the dose response for chronic toxicity.** Depending on the aim of the study, it may be possible to increase the statistical power of the key estimates by differentially allocating animals to dose levels. **An additional group of sentinel animals (5[10] animals per sex) may be included for monitoring of disease status, if necessary, during the study (22).**
- 15bis A sufficient number of animals should be used so that a thorough biological and statistical evaluation is possible. Each dose group and concurrent control group intended for the carcinogenicity phase of the study should therefore contain at least 50 animals of each sex. In some cases disproportionate testing with more than 50 animals in the low dose groups might be considered as appropriate; e.g., to estimate the carcinogenic potential in low dosages. However it should be recognised that a moderate increase in group size will provide relatively little increase in statistical power of the study. Each dose group and

concurrent control group intended for the chronic toxicity phase of the study should contain at least 20 animals of each sex. Depending on the aim of the study, it may be possible to increase the statistical power of the key estimates by differentially allocating animals to dose levels. An additional group of sentinel animals (5 animals per sex) may be included for monitoring of disease status, if necessary, during the study (22).

Provision for interim kills and satellite groups

16. The study may make provision for interim kills, e.g. at 12 months in the carcinogenicity phase or 6 months for the chronic toxicity phase, to provide information on progression of non-neoplastic changes and mechanistic information. Satellite groups may also be included to monitor the reversibility of any toxicological changes induced by the chemical under investigation, and may also be required to conduct all required haematological determinations in mice. With the exception of additional satellite groups of mice required to conduct all required haematological determinations, satellite groups are normally restricted to the highest dose level of the study plus control. If interim kills or inclusion of satellite groups are planned, the number of animals included in the study design should be increased by the number of animals scheduled to be killed before the completion of the study. These animals should normally undergo the same observations, including body weight, food/water consumption, haematological and clinical biochemistry measurements and pathological investigations as the animals in the chronic toxicity phase of the main study, although provision may also be made (in the interim kill groups) for measurements to be restricted to specific, key measures such as neurotoxicity or immunotoxicity.

Dose groups and dosage

17. **At least** three dose levels and a concurrent control should be used. Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test substance or related materials. For most chemicals, a limit test is not considered appropriate for an assessment of carcinogenicity.
18. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. While taking into account the factors outlined in paragraph 18 below, the highest dose level should be chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10 per cent).
19. However, dependent on the objectives of the study (see paragraph 4), a top dose lower than the dose providing evidence of toxicity may be chosen, e.g. if a dose elicits an adverse effect of concern that nonetheless has little impact on lifespan or body weight. The top dose should not exceed 1000 mg/kg body weight/day.
20. **Dose level spacing should be designed to demonstrate a dose response and establish a NOAEL or other intended outcome of the study**, e.g. a BMD (see paragraph 22) for the chronic toxicity phase. Factors that should be considered in the placement of lower doses include the expected slope of the dose–response curve, the doses at which important changes may occur in metabolism or mode of toxic action, where a threshold is expected, or where a point of departure for low-dose extrapolation is expected. It should be noted that in conducting a combined carcinogenicity /chronic toxicity study, the primary objective will be to obtain information for carcinogenicity risk assessment purposes, and information on chronic toxicity will normally be a subsidiary objective. This should be borne in mind when selecting dose levels and dose level spacing for the study.

21. The dose level spacing selected will depend on the objectives of the study and the characteristics of the test substance, and cannot be prescribed in detail in this Test Guideline, but two to four fold intervals are frequently optimal for setting the descending dose levels and **addition of a fourth test group** is often preferable to using very large intervals (e.g., more than a factor of about 6-10) between dosages. Further guidance on dose selection and dose level spacing is provided in the OECD Guidance Document (6), but in general the use of factors greater than 10 should be avoided, and should be justified if used.
22. As outlined further in the OECD Guidance Document (6), points to be considered in dose selection include;
- known or suspected nonlinearities or inflection points in the dose–response;
 - pharmacokinetics, and dose ranges where metabolic induction, saturation, or nonlinearity between external and internal doses does or does not occur;
 - precursor lesions, markers of effect, or indicators of the operation of key underlying biological processes;
 - key (or suspected) aspects of mode of action, such as doses at which cytotoxicity begins to arise, hormone levels are perturbed, homeostatic mechanisms are overwhelmed, etc.;
 - regions of the dose–response curve where particularly robust estimation is required, e.g., in the neighborhood of the anticipated BMD or a suspected threshold;
 - consideration of anticipated human exposure levels, especially in the choice of mid and low doses.
23. The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used among the dose groups. If a test substance is administered in the diet, and causes reduced dietary intake, an additional pair-fed control group should be included in order to allow for the effects of dietary restriction.

Preparation of doses and administration of test substance

24. The test substance is normally administered orally, by gavage or via the diet or drinking water. Additional information on routes and methods of administration is provided in Guidance Document (6). The method of administration is dependent on the purpose of the study, the physical/chemical properties of the test material and its bioavailability. In the interests of animal welfare, oral gavage should normally be selected only for those agents for which this route reasonably represents potential human exposure (e.g., pharmaceuticals). For dietary or environmental chemicals including pesticides, administration should be via the diet or drinking water. The choice of the route of administration depends on the physical and chemical characteristics of the test substance and the predominant route of exposure of humans. A rationale should be provided for the chosen route.
25. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: effects on the absorption, distribution, metabolism, or retention of

the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/ emulsion in oil (e.g., corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle should be known. The stability of the test substance under the conditions of administration (e.g. diet) should be determined, and the homogeneity of each batch of dosing solution or diet containing the substance (as appropriate) should be confirmed analytically.

26. For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. In chronic toxicity studies using dietary administration, the concentration of the chemical in the feed should not exceed an upper limit of 5% of the total diet (REF) unless the diet is adjusted accordingly to meet the nutritional requirements of the test animals. When the test substance is administered in the diet, either a constant dietary concentration (mg/kg or ppm) or a constant dose level in terms of the animal's body weight may be used; the alternative used should be specified.
27. The animals are dosed with the test substance daily seven days each week for a period of 12 months (chronic phase satellite groups) or 24 months (carcinogenicity phase), see also paragraphs 29 and 30. Any other dosing regime, e.g., five days per week, needs to be justified. Dosing by the inhalation route is carried out for 6 hours per day, 5 days per week.
28. When the test substance is administered by gavage to the animals this should be done using a stomach tube or a suitable intubation cannula, at similar times each day. Normally a single dose will be administered once daily, where for example a compound is locally irritant, it may be possible to maintain the daily dose-rate by administering it as a split dose (e.g. twice a day). The maximum volume of liquid that can be administered at one time depends on the size of the test animal. Normally the volume should be kept as low as practical, and should not exceed 1 ml/100g body weight, except in the case of aqueous solutions where 2 ml/100g body weight may be used. Variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. Potentially corrosive or irritant substances are the exception, and need to be diluted to avoid severe local effects. The pH of dosing solutions should normally lie in the range X to Y. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

Duration of study

29. The period of dosing and duration of the chronic phase of this study is normally 12 months. **The high dose and control satellite groups** will be terminated at this stage for evaluation of chronic toxicity and non-neoplastic pathology. Satellite groups included to monitor the reversibility of any toxicological changes induced by the chemical under investigation should be maintained without dosing for a period not less than 4 weeks and not more than one third of the total study duration after cessation of exposure.
30. The duration of the carcinogenicity phase of the study will normally be 24 months for both rats and mice, representing the majority of the normal life span of the animals to be used. Considerations on appropriate survival rates for the control group(s) or the lower dose groups to achieve this objective are provided in Guidance Document (6). Survival of each sex should be considered separately. In the case where excessive and/or premature mortality is observed only in the high dose group, this should not trigger termination of the study. The following guidelines are recommended:

- 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.
- In the case where only the high dose group dies prematurely due to toxicity, this should not trigger termination of the study.
- Survival of each sex should be considered separately.
- The study should not 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.

31. In order for a negative carcinogenicity study to be considered acceptable, it should meet the following criteria:

- (1) No more than 10 per cent of any group should be lost due to autolysis, cannibalism, or management problems.
- (2) Survival in each group in the study should be no less than 50 per cent at 24 months.

[Alternative to paragraph 31: Proposal from consultant: either delete paragraph 31 completely and address this issue in the guidance document, or replace it by the old TG 453 text.]

31 bis In order for a negative carcinogenicity study to be considered acceptable, it should meet the following criteria:

- (1) No more than 10 per cent of any group is lost due to autolysis, cannibalism, or management problems.
- (2) Survival of all groups is no less than 50 per cent at 18 months for mice and hamsters and at 24 months for rats.]

OBSERVATIONS (CHRONIC TOXICITY PHASE)

32. General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded. All animals should be checked for morbidity or mortality, and for specific signs of toxicological relevance, usually at the beginning and the end of each day. Additionally, animals should be checked at least once each weekend day and holiday.
33. At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. The protocol for observations should be arranged such that variations between individual observers are minimised and independent of test group. These observations should be made outside the home cage, preferably in a standard arena and at similar times on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Efforts should be made to ensure that variations in the observation conditions are minimal. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behaviour (e.g., self-mutilation, walking backwards) should also be recorded (23).

34. Ophthalmological examination, using an ophthalmoscope or other suitable equipment, should be carried out on all animals prior to the first administration of the test substance. At the termination of the study, this examination should be preferably be conducted in all animals but at least in the high dose and control groups. If treatment-related changes in the eyes are detected, all animals should be examined. If structural analysis or other information suggests ocular toxicity, then the frequency of ocular examination should be increased.
35. For chemicals for which previous repeated dose 28-day and/or 90-day toxicity tests may have indicated the potential to cause neurotoxic effects, sensory reactivity to stimuli of different types (23) (e.g., auditory, visual and proprioceptive stimuli) (24), (25), (26), assessment of grip strength (27) and motor activity assessment (28) may optionally be conducted at 3, 6, 9 and 12 months. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used. If oral gavage is the method of administration, all clinical observations, detailed clinical and neurological functional tests should be carried out prior to dosing.
36. For chemicals for which previous repeated dose 28-day and/or 90-day toxicity tests may have indicated the potential to cause immunotoxic effects, further investigations of this endpoint may optionally be conducted at 12 months.

Body weight and food/water consumption

37. All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and monthly thereafter. Measurements of food consumption should be made at least weekly for the first 13 weeks, and monthly thereafter. Water consumption may also be considered for studies during which drinking activity may be altered, and should be measured at least weekly when the substance is administered in drinking water. For a study in which the substance is administered by oral administration, measurements of water consumption should be undertaken if previous studies have indicated an effect on this parameter.

Haematology and Clinical Biochemistry

38. At the end of the test period, samples are collected just prior to or as part of the procedure for killing the animals. Blood samples should be taken from a named site, for example by cardiac puncture or retro-orbital sinus, and stored, if applicable, under appropriate conditions.
39. Haematological examinations should be carried out in **at least [5] [10] male and [5] [10] female animals per group**, at 3, 6, and 12 months, using the same animals throughout. In mice, satellite animals may be required in order to conduct all required haematological determinations. The following list of parameters should be investigated (29): Total and differential leukocyte count, erythrocyte count, platelet count, haemoglobin concentration, haematocrit (packed cell volume), methaemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), prothrombin time, and activated partial thromboplastin time. If the chemical has an effect on the haematopoietic system, reticulocyte counts and bone marrow cytology may be indicated, although these need not be routinely conducted.
40. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from **at least [5] [10] male and [5] [10] female animals per group** at the same time intervals as specified for the haematological investigations, using the same animals throughout. In mice, satellite animals may be required in order to conduct all required haematological

determinations. Overnight fasting of the animals prior to blood sampling is recommended¹. The following list of parameters should be investigated (29): glucose, urea (urea nitrogen), creatinine, total protein, albumin, calcium, sodium, potassium, total cholesterol, at least two appropriate tests for hepatocellular evaluation (alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, total bile acids)(30), and at least two appropriate tests for hepatobiliary evaluation (alkaline phosphatase, gamma glutamyl transferase, 5'-nucleotidase, total bilirubin, total bile acids)(30). Other clinical chemistry parameters such as fasting triglycerides, specific hormones and cholinesterase may be measured as appropriate, depending on the toxicity of the substance. Overall, there is a need for a flexible approach, depending on the observed and/or expected effect from a given substance.

41. Urinalysis determinations should be performed on **at least [5] [10] male and [5] [10] female animals per group** on samples collected at the same intervals as for haematology and clinical chemistry. The following list of parameters was included in a recent expert recommendation on clinical pathology studies (29): appearance, volume, osmolality or specific gravity, pH, total protein, and glucose. Other determinations include ketone, urobilinogen, bilirubin, and occult blood. Further parameters may be employed where necessary to extend the investigation of observed effect(s).
42. If historical baseline data are inadequate, consideration should be given as to whether haematological and clinical biochemistry variables need to be determined before dosing commences; it is generally not recommended that this data be generated before treatment (29). See also paragraph 56.

Pathology

Gross necropsy

43. All animals in the study shall be normally subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. However provision may also be made (in the interim kill or satellite groups) for measurements to be restricted to specific, key measures such as neurotoxicity or immunotoxicity (see paragraph 16). These animals need not be subjected to necropsy and the subsequent procedures described in the following paragraphs.
44. Organ weights should be collected from all animals, other than those excluded by the latter part of paragraph 43. The adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid (weighed post-fixation, with parathyroids), and uterus of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to prevent drying.
- 45.. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination (31): all gross lesions, adrenal gland, aorta, brain (including sections of cerebrum, cerebellum, and medulla/pons), caecum, cervix, coagulating gland, colon, duodenum, epididymis, eye

¹ For a number of measurements in serum and plasma, most notably for glucose, overnight fasting is preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. All animals should be assessed in same physiological condition and preferably detailed or neurological assessments should therefore be scheduled for a different day than clinical biochemistry sampling.

(including retina), [femur with joint], gall bladder (for species other than rat), Harderian gland, heart, ileum, jejunum, kidney, lacrimal gland (exorbital), liver, lung, lymph nodes (both superficial and deep), female mammary gland, [nasal tissue], oesophagus, [olfactory bulb], ovary, pancreas, parathyroid gland, peripheral nerve, pituitary, prostate, [rectum], salivary gland, seminal vesicle, skeletal muscle, skin, spinal cord (at three levels: cervical, mid-thoracic, and lumbar), spleen, [sternum], stomach (forestomach, glandular stomach), [teeth], testis, thymus, thyroid gland, [tongue], trachea, urinary bladder, uterus (including cervix), [ureter], [urethra], vagina, and a section of bone marrow and/or a fresh bone marrow aspirate). Tissues in square brackets are optional. The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved. In inhalation studies, the list of preserved and examined tissues from the respiratory tract should follow the recommendations of Test Guideline 412. For other organs/tissues (and in addition to the specifically preserved tissues from the respiratory tract) the list of organs as set out for the oral route has to be examined.

Histopathology

46. A recent publication provides guidance on best practices in the conduct of toxicological pathology studies (31). The minimum histopathological examinations should be:
- all tissues from the high dose and control groups;
 - all tissues from animals dying or killed during the study;
 - all tissues showing macroscopic abnormalities;
 - target tissues, or tissues which showed treatment-related changes in the high dose group, from all animals in all other dose groups.
47. If treatment-related histopathological changes are observed in the high dose group, examination should be extended to the tissues from animals in all other dose groups.

OBSERVATIONS (CARCINOGENICITY PHASE)

48. All animals should be checked for morbidity or mortality, usually at the beginning and the end of each day. Additionally, animals should be checked at least once each weekend day and holiday. Particular attention should be paid to tumour development; the time of onset, location, dimensions, appearance, and progression of each grossly visible or palpable tumour should be recorded. Animals should be checked routinely for specific signs of toxicological relevance.

Body weight and food/water consumption

49. All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks and monthly thereafter. Measurements of food consumption should be made at least weekly when the test substance is administered in the diet. Water consumption may also be considered for studies during which drinking activity may be altered, and should be measured at least weekly when the substance is administered in drinking water. For a study in which the substance is administered by dietary admixture, measurements of water consumption should be undertaken if previous studies have indicated an effect on this parameter.

Hematology

50. Examination of blood smears in the carcinogenicity phase does not usually provide any valuable information for the assessment of carcinogenic/oncogenic potential (29). Conducting blood smears is thus left to the discretion of the study pathologist, but is not

strongly recommended. If in preliminary studies bone marrow appears to be the target organ, it may be useful to collect blood smears for future examination if appropriate.

Pathology

Gross Necropsy

51. All animals in the study except sentinel animals (see paragraph 15) and other satellite animals (see restrictions in paragraph 16) shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Organ weights are not normally part of a carcinogenesis study, since geriatric changes and, at later stages, the development of tumours confounds the usefulness of organ weight data. They may, however, be critical to performing a weight of evidence evaluation and especially for mode of action considerations. Should they be part of a satellite study, they should be collected at no later than one year after initiation of the study.

52. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination (31): all gross lesions, adrenal gland, aorta, brain (including sections of cerebrum, cerebellum, and medulla/pons), caecum, cervix, coagulating gland, colon, duodenum, epididymis, eye (including retina), [femur with joint] gall bladder (for species other than rat), Harderian gland, heart, ileum, jejunum, kidney, lacrimal gland (exorbital), liver, lung, lymph nodes (both superficial and deep), female mammary gland, oesophagus, [olfactory bulb], ovary, pancreas, parathyroid gland, peripheral nerve, pituitary, prostate, [rectum], salivary gland, seminal vesicle, skeletal muscle, skin, spinal cord (at three levels: cervical, mid-thoracic, and lumbar), spleen, [sternum], stomach (forestomach, glandular stomach), [teeth], testis, thymus, thyroid gland, [tongue], trachea, urinary bladder, uterus (including cervix), [ureter], [urethra], vagina, and a section of bone marrow and/or a fresh bone marrow aspirate). Tissues in square brackets are optional. The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved. In inhalation studies, the list of preserved and examined tissues from the respiratory tract should follow the recommendations of Test Guideline 412. For other organs/tissues (and in addition to the specifically preserved tissues from the respiratory tract) the list of organs as set out for the oral route has to be examined.

Histopathology

53. A recent publication provides guidance on best practices in the conduct of toxicological pathology studies (31). The minimum tissues examined should be:
 - All tissues from the high dose and control groups;
 - All tissues of animals dying or killed during the study;
 - All tissues showing macroscopic abnormalities including tumours;
 - When treatment-related histopathological changes are observed in the high dose group, those same tissues are to be examined from all animals in all other dose groups.

54. If treatment-related histopathological changes are observed in the high dose group, examination should be extended to the tissues from animals in all other dose groups.

DATA AND REPORTING (CARCINOGENICITY AND CHRONIC TOXICITY)

Data

55. Individual animal data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.
56. 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, particularly in cases where there are indications that the data provided by the concurrent controls are substantially out of line compared to recent data from control animals from the same test facility colony. Historical control data 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. Historical control, if evaluated, should be submitted from the same laboratory, strain, species and specific ranges should be provided. The use of historical data should be restricted to data generated during the three [five] years preceding the study in question..
57. When applicable, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods and the data to be analysed should be selected during the design of the study. Selection should make provision for survival adjustments, if needed.
58. Any tumours found at the one-year phase may be statistically analysed if justified by their occurrence, but this analysis should not be included with the analysis of the lifetime findings. Combining these data is unacceptable as the two phases do not have the same tumour risk.

Test report

59. The test report should include the following information:
 - Test substance:
 - physical nature, purity, and physicochemical properties;
 - identification data;
 - source of substance
 - batch number.
 - Vehicle (if appropriate):
 - justification for choice of vehicle (if other than water).
 - Test animals:
 - species/strain used and justification for choice made;
 - number, age, and sex of animals at start of test;
 - source, housing conditions, diet, etc.;
 - individual weights of animals at the start of the test.
 - Test conditions:
 - rationale for route of administration and dose selection;

- when applicable, the statistical methods used to analyse the data;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- route of administration and details of the administration of the test substance;
- for inhalation studies, whether nose only or whole body;
- actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (mg/kg or ppm) to the actual dose, if applicable;
- details of food and water quality.

Results:

General

- survival data;
- body weight/body weight changes;
- food consumption, and water consumption if applicable;
- toxicokinetic data if available;

Clinical findings

- Include signs of toxicity;
- Incidence and severity of any abnormality;
- Nature, severity, and duration of clinical observations (whether reversible or not);

Necropsy data

- Terminal body weight;
- Organ weights and their ratios, if applicable;
- Necropsy findings; Incidence and severity of abnormalities.

Histopathology

- Non neoplastic histopathological findings,
- Neoplastic histopathological findings,
- Correlation between gross and microscopic findings
- Detailed description of all treatment-related histopathological findings including severity gradings;

Statistical treatment of results, where appropriate.

- Tumour incidences

Discussion of results including:

- Discussion of any modelling approaches
- Dose:response relationships
- Consideration of any mode of action information
- Relevance for humans

- Conclusions

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ANNEX

DEFINITIONS

Dose: is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g., mg/kg), or as constant dietary concentrations (ppm).

Dosage: is a general term comprising of dose, its frequency and the duration of dosing.

Hormesis: is the term for generally-favorable biological responses to low exposures to toxins and other stressors. A toxin showing hormesis thus has the opposite effect in small doses than in large doses.

NOAEL: is the abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.

To be expanded as appropriate