

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 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 majority of chronic toxicity and carcinogenicity studies are carried out in rodent species, and this Test Guideline is intended therefore to apply primarily to studies carried out in these species. Should such studies be required in non-rodent species, the principles and procedures outlined may also be applied, with appropriate modifications, as outlined in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6).
3. The three main routes of administration used in chronic toxicity/carcinogenicity studies 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 an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6).
4. This Guideline focuses on exposure via the oral route, the route most commonly used in chronic toxicity and carcinogenicity studies. While long-term 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 chronic toxicity and 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 is available on the administration of test substances by the inhalation (6)(7) and dermal routes (6). The updated Guidelines TG 412, Subacute inhalation toxicity: 28 day study (8) and TG 413, Subchronic Inhalation Toxicity: 90-Day Study (9), together with the associated OECD Guidance Document on acute inhalation toxicity testing (7), should be specifically consulted in the design of longer term studies involving exposure via the inhalation route.
5. The objectives of chronic toxicity/carcinogenicity studies covered by this test guideline include:
 - the identification of the carcinogenic properties of a chemical, resulting in an increased incidence of neoplasms compared with concurrent control groups, and its chronic toxicity,
 - the identification of target organ(s) of chronic toxicity and carcinogenicity,
 - characterisation of the dose:response relationship,
 - identification of a no-observed-adverse-effect level (NOAEL) or point of departure for establishment of a Benchmark Dose (BMD),
 - extrapolation of carcinogenic effects to low dose human exposure levels,
 - prediction of chronic toxicity effects at human exposure levels,
 - provision of data to test hypotheses regarding mode of action (2)(6)(10)(11)(12)(13).

INITIAL CONSIDERATIONS

6. 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; short-term cancer initiation-promotion tests could also provide useful information. 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 (14)(15)(16)(17).
7. 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. 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 (18), in particular paragraph 62 thereof, should always be followed
8. 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 the design and conduct of chronic toxicity and carcinogenicity studies (6) as well as two International Life Sciences Institute publications (19)(20). The core dose selection strategy is dependent on the primary objective or objectives of the study (paragraph 5). 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.
9. 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 provides greater efficiency in terms of time and cost compared to conducting two separate studies, without compromising the quality of the data in either the chronic phase or the carcinogenicity phase. Careful consideration should however be given to the principles of dose selection (paragraphs 8 and 20-24) when undertaking a combined chronic toxicity and carcinogenicity study, and it is also recognised that separate studies may be required under certain regulatory frameworks. Further guidance on the design of the combined chronic toxicity and carcinogenicity study in order to achieve maximum efficiency of the study in terms of possibilities for reduction in numbers of animals used as well as via the streamlining of the various experimental procedures can be found in an OECD Guidance Document on Selection of Doses for Use in Chronic Toxicity and Carcinogenicity Studies (6)
10. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

11. The study design consists of two parallel phases, a chronic phase, normally of one year duration (see paragraph 31), and a carcinogenicity phase, normally of two years duration (see paragraph 32). The test substance is normally administered by the oral route although testing by the inhalation or dermal route may also be appropriate (paragraph 4). 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 12 months. 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 17). 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

12. This Guideline primarily covers assessment and evaluation of chronic toxicity and carcinogenicity in rodents (paragraph 2). 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 choice of species must be justified. The preferred rodent species is the rat, although other rodent species, e.g., the mouse, may be used. Although the use of the mouse in carcinogenicity testing may have limited utility (21)(22)(23), under some current regulatory programmes carcinogenicity testing in the mouse is still required. 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. The design and conduct of chronic toxicity/carcinogenicity studies in non-rodent species, when required, should be based on the principles outlined in this Guideline together with those in OECD TG 409, Repeated Dose 90-day Oral Toxicity Study in Non-Rodents. Additional information on choice of species and strain is provided in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6).
13. Young healthy adult animals of commonly used laboratory strains 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 normally criteria of survival for long-term studies (see OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6)), 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.

Housing and feeding conditions

14. Animals may be housed individually, or be caged in small groups of the same sex; individual housing should be considered only if scientifically justified (24)(25)(26). 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 the test substance is administered by the dietary route.

Preparation of animals

15. 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 8 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. If there are statistically significant differences, then the randomisation step should be repeated, if possible. 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

16. 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. Depending on the aim of the study, it may be possible to increase the statistical power of the key estimates by differentially allocating animals unequally to the various dose groups, with more than 50 animals in the low dose groups, e.g., to estimate the carcinogenic potential in low doses. 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 [ALTERNATIVE (1): For the chronic phase of the study, the high dose group should contain at least 20 animals of each sex, while the control group and the other treatment groups should contain at least 10 animals per group.].[ALTERNATIVE (2): An additional 2 satellite groups, one high dose group containing 20 animals of each sex and one control group containing 10 [20] animals of each sex, 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.] In studies involving mice, additional animals may be needed in each dose group of the chronic toxicity phase, to conduct all required haematological determinations. Further information on statistical design of the study and choice of dose levels to maximise statistical power is provided in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6).

Provision for interim kills, satellite group and sentinel animals

17. The study may make provision for interim kills, e.g. at 6 months for the chronic toxicity phase, to provide information on progression of non-neoplastic changes and mechanistic information, if scientifically justified. The animals used in the chronic toxicity phase of the study, normally of 12 months duration (paragraph 11) provide interim kill data for the carcinogenicity phase of the study, thus achieving a reduction in the number of animals used overall. [ALTERNATIVE: The animals used in the chronic toxicity phase of the study, normally of 12 months duration (paragraph 11) provide interim kill data for the carcinogenicity phase of the study, thus achieving a reduction in the number of animals used overall, although this will be limited to data on top dose and control.] Satellite groups may also be included in the chronic toxicity phase of the study, to monitor the reversibility of any toxicological changes induced by the chemical under investigation, these will normally be restricted to the highest dose level of the study plus control. An additional group of sentinel animals (typically 5 animals per sex) may be included for monitoring of disease status, if necessary, during the study (27). Further guidance on study design to include interim kills, satellite and sentinel animals, while minimising the number of animals used overall is provided in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6).
18. If satellite animals and/or interim kills are included in the study design, the number of animals in each dose group included for this purpose will normally be 10 animals per sex, and the total 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. Interim kill and satellite 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

19. At least three dose levels and a concurrent control should be used, for both the chronic and carcinogenicity phases [ALTERNATIVE: At least three dose levels and a concurrent control should be used for the carcinogenicity phase of the study, while for the chronic toxicity phase normally only a top dose level and control will be used (see paragraph 16)]. 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, but may be appropriate in the chronic toxicity phase of this combined chronic toxicity/carcinogenicity study. [ALTERNATIVE: For most chemicals, a limit test is not considered appropriate for an assessment of carcinogenicity.]
20. 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 21 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).
21. However, dependent on the objectives of the study (see paragraph 5), 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.

22. Dose level spacing should be designed to demonstrate a dose response and establish a NOEL or other intended outcome of the study, e.g a BMD (see paragraph 24). 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. 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. [ALTERNATIVE: Dose level spacing in the carcinogenicity phase of the study should be designed to demonstrate a dose response or other intended outcome of the study, e.g a BMD (see paragraph 24). 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. 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. The study design therefore only includes a top dose and a control group.]
23. 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 Guideline, but two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group [ALTERNATIVE: “...and addition of a fourth test group in the carcinogenicity phase of the study..”] 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 must be justified if used.
24. As outlined further in the OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (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 range of the anticipated BMD or a suspected threshold;
 - consideration of anticipated human exposure levels, especially in the choice of mid and low doses..
25. 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 (of the

order of 20% or more) due to the palatability of the diet, an additional pair-fed control group may be useful to allow for this.

Preparation of doses and administration of test substance

26. 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 OECD Guidance Document (6). The route and method of administration is dependent on the purpose of the study, the physical/chemical properties of the test substance, its bioavailability, and the predominant route and method of exposure of humans. A rationale should be provided for the chosen route and method of administration. In the interests of animal welfare, oral gavage should normally be selected only for those agents for which this route and method of administration reasonably represent potential human exposure (e.g., pharmaceuticals). For dietary or environmental chemicals including pesticides, administration should be via the diet or drinking water.
27. 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. The homogeneity of dosing solutions or diets containing the test article (as appropriate) should be confirmed analytically before the start of the study, and periodically (as appropriate) throughout the study, if the dose preparation procedure remains unchanged and if necessary, based on characteristics of the test article and the dosing vehicle.
28. 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 long-term toxicity studies using dietary administration, the concentration of the chemical in the feed should not normally exceed an upper limit of 5% of the total diet, in order to avoid nutritional imbalances. 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, calculated on a weekly basis, may be used; the alternative used should be specified.
29. In the case of oral or dermal administration, 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 31 and 32. 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.
30. 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 a local irritant, it may be possible to maintain the daily dose-rate by administering it as a split dose (b.i.d). 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 of 4 to 9.

Duration of study

31. The period of dosing and duration of the chronic phase of this study is normally 12 months. All dose groups allocated to this phase will be terminated at 12 months for evaluation of chronic toxicity and non-neoplastic pathology. [ALTERNATIVE: The high dose and control satellite groups allocated to this phase will be terminated at 12 months 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.
32. The duration of the carcinogenicity phase of the study will normally be 24 months for rats and 18 months for mice or hamsters, representing the majority of the normal life span of the animals to be used. The following provides some guidance on duration, termination of the study and survival; further guidance, including consideration of the acceptability of a negative carcinogenicity study relative to survival in the study, is provided in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6)
 - 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.

OBSERVATIONS (CHRONIC TOXICITY PHASE)

33. All animals should be checked for morbidity or mortality, and for specific signs of toxicological relevance, in particular for neurofunctional and neurobehavioural signs (28), usually at the beginning and end of each day. Additionally animals should be checked at least once each weekend day and holiday. 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.
34. Detailed clinical observations should be made on all animals at least once prior to the first exposure (to allow for within-subject comparisons), at the end of the first week of the study and monthly thereafter. The protocol for observations should be arranged such that variations between individual observers is 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, stereotypies (e.g., excessive grooming, repetitive circling) or bizarre behaviour (e.g., self-mutilation, walking backwards) should also be recorded (28).
35. 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 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.

36. For chemicals where previous repeated dose 28-day and/or 90-day toxicity tests indicated the potential to cause neurotoxic effects, sensory reactivity to stimuli of different types (28) (e.g., auditory, visual and proprioceptive stimuli) (29), (30), (31), assessment of grip strength (32) and motor activity assessment (33) may optionally be conducted before commencement of the study and then 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.
37. For chemicals where previous repeated dose 28-day and/or 90-day toxicity tests indicated the potential to cause immunotoxic effects, further investigations of this endpoint may optionally be conducted at 12 months.

Body weight, food/water consumption and food efficiency

38. All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and at least monthly thereafter. Measurements of food consumption and food efficiency should be made at least weekly for the first 13 weeks and at least monthly thereafter. Water consumption should also be considered for studies in which drinking activity is altered, and should be measured at least weekly for the first 13 weeks and at least monthly thereafter, when the substance is administered in drinking water.

Haematology and clinical biochemistry

39. In studies involving rodents, haematological examinations should be carried out on at least 10 male and 10 female animals per group at 3, 6, and 12 months, using the same animals throughout. [ALTERNATIVE: In studies involving rodents, haematological examinations should be carried out on the 20 [from 10 of the 20] high dose and 10 control satellite animals comprising the chronic phase of the study, at 3, 6, and 12 months.] In mice, satellite animals may be required in order to conduct all required haematological determinations (see paragraph 16). In non-rodent studies, samples will be taken from smaller numbers of animals (e.g. 4 animals per sex and per group in dog studies). Measurements at 3 months need not be conducted if no effect was seen on haematological parameters in a previous 90 day study carried out at comparable dose levels. Samples should be collected at the end of the designated test period, namely from animals on study at 3 and 6 months, and at 12 months 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. The following list of parameters should be investigated (34): total and differential leukocyte count, erythrocyte count, platelet count, haemoglobin concentration, haematocrit (packed cell volume), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), prothrombin time, and activated partial thromboplastin time. In addition Heinz bodies or other atypical erythrocyte morphology and methaemoglobin should be investigated if there is any indication of anaemia or other haematopoietic disorders.. If the chemical has an effect on the haematopoietic system, reticulocyte counts and bone marrow cytology may also 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 10 male and 10 female animals per group, using the same animals throughout, at the same

time intervals as specified for the haematological investigations. [ALTERNATIVE: 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 the 20 [from 10 of the 20] high dose and 10 control satellite animals comprising the chronic phase of the study, at the same time intervals as specified for the haematological investigations.] In mice, satellite animals may be required in order to conduct all required clinical biochemistry determinations. Measurements at 3 months need not be conducted if no effect was seen on clinical biochemistry parameters in a previous 90 day study carried out at comparable dose levels. Overnight fasting of the animals (with the exception of mice) prior to blood sampling is recommended. The following list of parameters should be investigated (34): 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)(35), and at least two appropriate tests for hepatobiliary evaluation (alkaline phosphatase, gamma glutamyl transferase, 5'-nucleotidase, total bilirubin, total bile acids)(35). 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 10 male and 10 female animals per group [ALTERNATIVE: Urinalysis determinations should be performed on the 20 high dose and 10 control satellite animals comprising the chronic phase of the study,] on samples collected at the same intervals as for haematology and clinical chemistry. Measurements at 3 months need not be conducted if no effect was seen on urinalysis in a previous 90 day study carried out at comparable dose levels. The following list of parameters was included in a recent expert recommendation on clinical pathology studies (34): 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. It is generally considered that baseline haematological and clinical biochemistry variables need not be determined before treatment (26). However, if historical baseline data (see paragraph 56) are inadequate, consideration should be given to generating such data.

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 18). These animals need not be subjected to necropsy and the subsequent procedures described in the following paragraphs. Sentinel animals may require necropsy on a case-by-case basis, at the discretion of the study director.
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, 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. In the case of paired organs, e.g., kidney, adrenal, both organs should be weighed separately. In a study using mice, weighing of the adrenal glands is optional.

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 (36): 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, [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. In the case of paired organs, e.g., kidney, adrenal, both organs should be preserved. 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 studies involving the dermal route of administration, the list of organs as set out for the oral route has to be examined, and specific sampling and preservation of the skin from the site of application is necessary. 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. Guidance is available on best practices in the conduct of toxicological pathology studies (36). 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,
 - in the case of paired organs, e.g., kidney, adrenal, both organs should be examined.
- [ALTERNATIVE (replacing from "The minimum histopathological...): All tissues from the 20 high dose and 10 [20] control animals comprising the chronic phase of the study should be examined, including all tissues from animals dying or killed during the study.]

OBSERVATIONS (CARCINOGENICITY PHASE)

47. All animals should be checked for morbidity or mortality and for specific signs of toxicological relevance (see paragraph 34),, 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.

Body weight, food/water consumption and food efficiency

48. All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks and at least monthly thereafter. Measurements of food consumption and food efficiency should be made at least weekly for the first 13 weeks and at least monthly thereafter. Water consumption should also be considered for studies in which drinking activity is altered, and should be measured at least weekly for the first 13 weeks and at least monthly thereafter, when the substance is administered in drinking water.

Haematology, clinical biochemistry and other measurements

49. In order to maximise the information obtained from the study, especially for mode of action considerations, blood samples may be taken for haematology and clinical biochemistry, although this is not obligatory and is at the discretion of the study director. Urinalysis may also be appropriate. Further guidance on the value of taking such samples as part of a carcinogenicity study is provided in an OECD Guidance Document on the design and conduct of chronic toxicity and carcinogenicity studies (6). If blood samples are taken, these should be collected at the end of the test period, just prior to or as part of the procedure for killing the animals. They should be taken from a named site, for example by cardiac puncture or retro-orbital sinus, and stored, if applicable, under appropriate conditions. Blood smears may also be prepared for examination, particularly if bone marrow appears to be the target organ, although the value of such examination of blood smears in the carcinogenicity phase for the assessment of carcinogenic/oncogenic potential has been questioned (28). 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

50. All animals in the study except sentinel animals and other satellite animals (see paragraph 17) 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. Sentinel animals and other satellite animals may require necropsy on a case-by-case basis, at the discretion of the study director. 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. If they are part of a satellite study, they should be collected at no later than one year after initiation of the study.
51. 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. In the case of paired organs, e.g., kidney, adrenal, both organs should be preserved. 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 studies involving the dermal route of administration, the list of organs as set out for the oral route has to be examined, and specific sampling and preservation of the skin from the site of application is necessary. 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

52. 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,
 - In the case of paired organs, e.g., kidney, adrenal, both organs should be examined.

DATA AND REPORTING (CARCINOGENICITY AND CHRONIC TOXICITY)

Data

53. Individual animal data should be provided for all parameters evaluated. 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.
54. 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. This is particularly the case 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 historical control data should be separated by sex and malignant and benign lesions should be presented separate and combined, where appropriate, and preferable by individual study. The use of historical data should be restricted to data generated during the five years preceding the study in question..
55. 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.

Test report

56. 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, calculations of food efficiency, if made, and water consumption if applicable;
- toxicokinetic data if available;

Clinical findings

- Include signs of toxicity;
- Incidence and, if scored, severity of any abnormality;
- Nature, severity, and duration of clinical observations ((whether transitory or permanent));

Ophthalmoscopy (chronic toxicity phase animals only)

Haematology (chronic toxicity phase animals only)

Clinical biochemistry (chronic toxicity phase animals only)

Urinalysis (chronic toxicity phase animals only)

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,
- Report of any peer review of slides;

Statistical treatment of results, where appropriate.

- body weights,
- organ weights,
- feed consumption (or water consumption)
- Tumour incidences

Discussion of results including:

- Discussion of any modelling approaches
- Dose:response relationships
- Historical control data
- 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.

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

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