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No. 116

GUIDANCE DOCUMENT 116 ON THE CONDUCT AND DESIGN OF CHRONIC TOXICITY AND CARCINOGENICITY STUDIES, SUPPORTING TEST GUIDELINES 451, 452 AND 453-FIRST EDITION INCLUDING THE GENERAL INTRODUCTION AND THE SECTION ON DOSE SELECTION

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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FOREWORD

This document is the first edition of the Guidance Document (GD) 116 on the Design and Conduct of chronic toxicity and carcinogenicity studies, supporting Test Guidelines 451, 452 and 453 (carcinogenicity, chronic toxicity and combined chronic toxicity/carcinogenicity studies). It includes the general introduction (Chapter 1) and the section on dose selection (Section 3.1).

All other parts are currently being developed and will be published as a second step, after approval of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) and Joint Meeting declassification.

The proposal for developing this GD was approved by the WNT in 1997. At that time the project only included the development of a GD on dose selection. In 2008, the objective of the project was revised. The WNT agreed that the GD should be developed in parallel with the update of the Test Guidelines 451, 452 and 453 as a supporting GD for these Test Guidelines. Thus, although the WNT agreed that the section on dose selection should be developed as a priority, work also started to develop other areas of guidance.

The proposal for this Guidance Document was first discussed, with the draft Test Guidelines 451, 452 and 453, at a workshop held in Washington D.C. in 2008. It was finalized at an expert meeting held in Paris on 7-8 October 2009. Comments on successive drafts were requested from the WNT in 2007, 2008, April 2009 and November 2009. Comments from the WNT have been addressed and the first edition of the GD was approved by the WNT at its meeting held in March 2010. The Joint Meeting of the Chemicals Committee and the working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of this document on 1 June 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the working Party on Chemicals, Pesticides and Biotechnology.

This Guidance Document includes Chapter 1 and Section 3.1 only. Other parts, still under development, are available as drafts on the public Website at:
http://www.oecd.org/document/12/0,3343,en_2649_34377_1898188_1_1_1_1,00.html
Details of Chapters and Sections not yet available and which will be published at a later stage, have not been included in the Table of Contents, only main heading of these sections have been presented here for information.

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1. GENERAL INTRODUCTION

1.1 Guiding principles and considerations

1. Chronic toxicity and carcinogenicity studies are intended to identify toxic effects and potential health hazards following prolonged, repeated exposure. This type of study is usually required if humans are likely to be exposed to a substance over a significant portion of their life span. In the 1960s, long-term animal bioassays (chronic toxicity and carcinogenicity studies) began to be routinely used for hazard identification, to assess the qualitative potential of a substance to cause chronic toxicity and cancer. In this Guidance Document "long-term bioassay" will be used to cover both chronic toxicity and carcinogenicity studies.

2. The objectives of the long-term bioassays have however expanded beyond hazard identification and are now focused primarily on hazard characterisation for use in the assessment of risk for humans. In addition there has been increasing pressure for the long-term bioassay designs to consider financial constraints and societal desires to minimise the number of animals needed for scientific interpretation of results. There is a growing desire for long-term bioassays to provide data that cover a number of objectives including characterisation of the nature of specific toxic responses, description of dose–response relationship, establishment of inflection points, and provision of insight into the roles of toxicokinetics and mechanisms of toxic action. In practice, it is likely that the bioassay design will be a compromise among a set of different purposes; to the extent that the ability to address one question is enhanced, the ability to address others may be diminished. For example, it may be necessary to achieve a balance between the power to detect toxicity and the ability to estimate the dose–response relationship of any observed effects. If information on carcinogenicity hazard identification is not available, this should be the main objective of the study.

3. The use of formal risk assessment procedures by government regulatory bodies began to emerge in the late 1970s and early 1980s bringing with it a strong interest in using data for quantitative as well as qualitative purposes. The need to gather data that allowed an understanding of the shape and slope of the dose-response curve focused attention on the number of doses in a bioassay and their spacing. Advances in knowledge of how chemicals perturbed or otherwise modulated biological processes in the development of tumours or other forms of toxicity provided bases for further improving the risk assessment process. Through meetings held primarily under the auspices of the International Programme on Chemical Safety (IPCS), a Mode of Action (MOA) framework was developed and refined (Sonnich-Mullin et al., 2001; Cohen et al., 2003; Meek et al., 2003; Holsapple et al., 2006; Boobis et al., 2006; EPA, 2005), as will be further developed in Chapter 2 of this guidance. The key purpose of this work was to introduce greater transparency into the process of assessing human relevance, and the goal was to use a broad array of relevant data to determine the predictive value of a bioassay tumour response to risk in humans.

4. The broadened range and complexity of scientific data used to evaluate chemical toxicity and carcinogenicity potential for humans highlighted the need to revise and update the following OECD Test Guidelines (TGs): TG 451 (Carcinogenicity Studies), TG 452 (Chronic Toxicity Studies), and TG 453–(Combined Chronic Toxicity/Carcinogenicity Studies), originally adopted in 1981. These TGs have therefore recently been revised in the light of scientific progress and the updating of related OECD Guidelines such as TG 408 (90-day oral toxicity study in rodents) and TG 407 (28-day oral toxicity study in rodents).

5. During the revision of the Test Guidelines, an emphasis was placed on providing guidance on factors that influence the selection of test doses, particularly for carcinogenicity studies. It was recognised that while general principles of dose selection should be contained in the Test Guidelines

themselves, there was a need for additional guidance on these principles. The revision took into account two publications by the International Life Sciences Institute (ILSI), "*Principles for the Selection of Doses in Chronic Rodent Bioassays"* (ILSI, 1997), and "*Issues in the Design and Interpretation of Chronic Toxicity and Carcinogenicity Studies in Rodents: Approaches to Dose Selection*" (Rhomberg et al., 2007) These reports provided theoretical and practical guidance on factors that influence dose selection in chronic bioassays.

6. A summary of the principles contained in these two publications, to underpin the texts on dose selection contained in the Test Guidelines, is provided in this guidance (Section 3.1, Appendix 1). During the development of this material, suggestions were made for additional guidance on specific aspects of study design in relation to core objectives of these studies, and how they might impact on other aspects of the study (e.g., designing for optimal collection of carcinogenicity data versus chronic toxicity data, design of studies for risk estimation rather than hazard assessment). It was generally agreed that the scope of the guidance should be wider than principles of dose selection, and should cover a number of key issues related to carcinogenicity and chronic toxicity testing.

7. This guidance therefore provides additional information on the conduct of studies performed using TG 451, 452 and TG 453. Its objective is to assist users of the TGs to select the most appropriate methodology to assess the chronic toxicity and carcinogenicity of a test substance so that particular data requirements can be met while reducing animal usage if possible/appropriate. It should be noted, however, that the basic principles for the conduct of chronic toxicity and carcinogenicity studies will differ, given that the endpoints are different. While the guidance provided in this document can be taken as generally applicable to the conduct of either a chronic toxicity or a carcinogenicity bioassay, or a combined chronic and carcinogenicity study, users of the guidance should be mindful of the primary objectives of the study.

8. The guidance is intended to foster a common approach among those carrying out chronic toxicity and carcinogenicity studies, and thereby contributes to the harmonisation of activities undertaken by the OECD and other agencies, such as the WHO[1]. It should be consulted in addition to other guidance and requirements documents. It provides broad guidance on approaches to the execution of chronic toxicity and carcinogenicity studies. The text reflects current scientific understanding and standards. In time, the scientific community will gain a better understanding of the mechanisms of toxicity, and this may lead to changes in both methodology and interpretation of results, which should reflect scientific consensus at the time data are reviewed.

9. Two other OECD documents provide guidance on the analysis and evaluation of the results of repeat-dose toxicity studies and chronic toxicity and carcinogenicity studies, Guidance Notes for Analysis and Evaluation of Repeat-Dose Toxicity Studies (OECD, 2002a) and Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies (OECD, 2002b).

1.2 Scope of application of the guidance

10. The Test Guidelines 451, 452 and 453 and this Guidance Document are designed to be used in the testing of a wide range of chemicals, whatever their field of application, including pesticides, industrial chemicals and pharmaceuticals. However, as noted in the Test Guideline 451, some testing requirements may differ for pharmaceuticals. The International Conference on Harmonisation of Testing for Pharmaceuticals (ICH) has produced a series of safety guidelines for the testing of

[1] An example, already referred to, is the IPCS project *Harmonisation of Approaches to the Assessment of Risk from Exposure to Chemicals*, which has developed a *Conceptual Framework for Cancer Risk Assessment*. The framework is an analytical tool for judging whether the available data support a postulated mode of carcinogenic action.

pharmaceuticals, including guidelines on toxicokinetics (ICH, S3A), genotoxicity testing (ICH, S2), duration of chronic toxicity testing in animals (rodent and non-rodent toxicity testing) (ICH, M3(R2)), testing for carcinogenicity of pharmaceuticals (ICH, S1B) and dose selection for carcinogenicity studies of pharmaceuticals (ICH, S1C(R2)). These guidelines should always be consulted for specific guidance when testing pharmaceuticals using the approaches outlined in TG 451, 452 and 453. This guidance document provides, in various sections, examples of where the testing requirements may be different for pharmaceuticals.

1.3 Objectives of a chronic toxicity study

11. The objective of a chronic toxicity study, such as described by TG 452, is to characterise the toxicological response of a test substance in a mammalian species following prolonged and repeated exposure. The chronic toxicity study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time. Key objectives of the study are to provide information useful for classification and labelling and an estimate of a point of departure (e.g., the benchmark dose low level (BMDL) or the no-observed-adverse-effect level (NOAEL)) for any adverse effects, which can be used for establishing safety criteria for human exposure. The study will provide information on the toxic effects, and indicate target organs, progressive toxic effects and the possibility of delayed toxicity. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. Previous repeated dose 28-day and/or 90-day toxicity tests on a test substance may, among others, have indicated the potential to cause neurotoxic/neurobehavioural effects, or effects on the endocrine system, warranting further in-depth investigation as part of a chronic toxicity study.

1.4 Objectives of a carcinogenicity study

12. The objective of a long-term carcinogenicity study, such as described by TG 451, is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration. The carcinogenicity study may also provide information on the possible health hazards likely to arise from repeated exposure for a period lasting up to the entire lifespan of the species used. The study will provide information on potential carcinogenicity, and may indicate toxic effects, target organs, progressive toxic effects and the possibility of delayed toxicity. It can provide information useful for classification and labelling, and an estimate of a point of departure for toxic effects and, in the case of non-genotoxic carcinogens, for tumour responses, which can be used for establishing safety levels for human exposure. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. Such an assay requires careful planning and documentation of the experimental design, a high standard of pathology, and unbiased statistical analysis. These requirements are well known and have not undergone any significant changes in recent years.

1.5 Objectives of a combined chronic toxicity/carcinogenicity study

13. The objective of a combined chronic toxicity/carcinogenicity study, such as described by TG 453, is to determine the toxicological effects of a test substance (including carcinogenic potential) in a mammalian species following prolonged and repeated exposure. 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 test substance including potential carcinogenicity, and indicate target organs, progressive toxic effects and the possibility of delayed toxicity. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. The application of the TG 453 should generate data on which to identify the majority of chronic and carcinogenic effects and to determine dose-response relationships. Ideally, the design and conduct should allow for the detection of neoplastic effects and a determination of carcinogenic potential as

well as general toxicity, including neurological, physiological, biochemical, and haematological effects and exposure-related morphological (pathology) effects.

14. The design of the updated TG 453 recommends, for the chronic phase of the study, at least three dose groups and a control group, each group containing at least 10 males and 10 females. The reduction of the number of animals per sex in the updated TG 453 compared to the initial version (1981) is justified on the basis of further information being available from animals in the carcinogenicity phase of the study and a preference to minimise animal use while maximising tissue analysis. The design of the carcinogenicity phase of the revised TG 453 is identical to the revised TG 451. The study will thus provide similar information on chronic toxicity and carcinogenicity as TG 452 and TG 451. It will allow derivation of a point of departure (e.g. BMDL or NOAEL), and will offer 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. The data from both phases (chronic toxicity and carcinogenicity) will reinforce each other, as the animals used in the studies are drawn from the same stock and have similar characteristics at the start of the study. Measurements carried out on the animals in one phase will be relevant for the animals in the other phase, e.g. clinical signs, body weights, haematology and biochemistry (if carried out), pathology. The terminal kill of the chronic phase can act as an interim kill for the carcinogenicity phase.

1.6 Consideration of testing strategies

15. This section refers to testing strategies that may be applicable for certain regulatory authorities but have not been formally adopted by all. It does not recommend any particular testing strategy or approach, but suggests consideration of such approaches as part of an ongoing strategy to assess the toxic potential of a test substance in an intelligent and iterative manner. As new validated approaches become scientifically appropriate for use in chronic toxicity or carcinogenicity assessment, and accepted by the relevant regulatory authorities, the study sponsor is encouraged to implement them where possible.

16. A reasoned scientific approach to the assessment of substances for chronic toxicity or carcinogenicity must first include an assessment of all available information that has the potential to influence the study design. This can include the identity, molecular structure, class, and physico-chemical properties of the test substance; any information regarding mode of action; results of relevant *in vitro* or *in vivo* toxicity tests such as genotoxicity, subchronic toxicity and toxicokinetics studies; anticipated use(s) and potential for human exposure; available (Q)SAR modelling data; and relevant toxicological data on structurally-related substances. This analysis can focus the study parameters, but may also lead to the conclusion that a study can be refined in some way, or not conducted at all based on a weight of evidence (Carmichael et al., 2006; Doe et al., 2006; Barton et al., 2006; Cooper et al., 2006).

17. Integrating a wide range of information to determine the potential toxicity of a substance is becoming more common as the gap between assessments that need to be conducted and the resources with which to conduct such assessments widens. Efforts are underway in many OECD countries to determine ways in which assessments of substances can be satisfactorily completed, and protection of public health and the environment achieved, while minimising costs in terms of time, money and animal use. However, the acceptability and use of testing strategies and weight-of-evidence approaches differ among OECD countries and regulatory sectors; thus, application of these approaches should always occur in consultation with appropriate regulatory authorities.

18. Shorter-term *in vitro* or *in vivo* tests may provide information regarding potency, mode of action, metabolism, interspecies differences with respect to biotransformation and/or target organ that can help refine the chronic toxicity study protocol parameters or priorities for observation. Tiered

approaches using a combination of *in silico*, *in vitro*, and *in vivo* tests have been proposed but are not yet widely implemented (Worth and Balls 2002; Becker et al., 2007).

19. A phased or tiered approach to the assessment of the carcinogenic potential of a substance should also be considered (Ashby, 1996). A number of shorter-term tests can be conducted which will provide useful information for determining whether and how a substance may be carcinogenic, including genetic toxicity assays, cell transformation or other cell-based assays, short-term cancer initiation-promotion tests which may or may not include toxicogenomic analyses (Ellinger-Ziegelbauer et al., 2005; 2008), and *in vivo* repeated dose 28- or 90-day toxicity tests (for a review on *in vitro* and *in vivo* short-term test see Maurici et al., 2005). (Q)SAR prediction models have been used in a regulatory context to predict the carcinogenic potential of substances for several decades. The OECD QSAR Application Toolbox contains a number of validated models to assess the carcinogenic potential of a substance. It is available at www.oecd.org/env/existingchemicals/qsar. There are also commercial (Q)SAR models available for predicting rodent carcinogenicity (Benigni et al., 2007). (Q)SAR models should be validated according to OECD principles (OECD 2007, GD No. 69).

20. A number of different strategies for assessing carcinogenicity have been proposed (Langley 2001; Worth and Balls 2002; Knight et al., 2006; Combes et al., 2008). All feature a stepwise process or decision tree that prescribes information analysis and stopping points where classification and labelling and/or risk assessment could be possible. However, specific approaches have not yet been optimised or validated.

21. Consideration of particular tests or approaches should always be made within the context of whether the results will contribute mechanistic information that will be useful in the weight-of-evidence assessment of carcinogenic potential (OECD, 2002b).

22. The US National Toxicology Program, along with institutes in other OECD countries, has had a longstanding interest in the use of transgenic or knockout mouse models for the assessment of carcinogenicity (Bucher and Portier, 2004). They consider that these models offer potential refinements, in terms of study duration and animal numbers, over the traditional long-term carcinogenicity study. At the time this guidance was prepared, some regulatory authorities in the pharmaceutical sector may accept studies with these models in combination with a full long-term rat carcinogenicity study in lieu of a long-term carcinogenicity study in a second rodent species (ICH, 1997). In the past, the predictive ability of the models, and any refinements or animal reductions, have been questioned (Goodman, 2001; van Zeller and Combes, 1999; RIVM, 2004). A detailed review paper (DRP) on transgenic rodent mutation assays prepared by Canada was published by OECD (OECD, 2009).

1.7 Animal welfare considerations

23. The principles of the "3Rs" (Replacement, Reduction, and Refinement), first articulated by Russell and Birch in 1959 (Russell and Birch, 1959), should be considered as integral to the assessment of carcinogenicity or chronic toxicity in mammals, in order to ensure sound science, maximise animal welfare, and minimise animal use. Animals in a condition of stress or distress have a documented effect on the outcome of the study (Olsson and Dahlborn, 2001; Reinhardt and Reinhardt, 2002). For these reasons the following principles should be implemented as much as practicably possible.

24. First and foremost, as discussed above, consideration of documented existing information from any reliable source that could provide a refinement in the testing protocol or procedure is recommended. Existing information could be used to inform dose spacing or selection, exposure route, observation priorities, potential modes of action or target organs of the test substance, and/or

study design. Use of this information to focus the study before it begins ensures that the study will meet the expectations of the study sponsor and/or regulatory authorities, decreasing the likelihood of repeat studies.

25. The use of the combined chronic toxicity/carcinogenicity study (TG 453) is also recommended, which can in most cases accomplish the objectives of both studies, and offers savings in the numbers of animals used. This is due to the use of 10 animals per sex per dose group for the chronic toxicity phase of the study instead of 20 animals per sex per dose group when the carcinogenicity and the chronic toxicity studies are performed separately.

26. Any studies involving animals should abide by the principles of humane euthanasia as detailed in the OECD Guidance Document 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation, and in particular paragraph 62 thereof (OECD, 2000). This paragraph states that "In studies involving repeated dosing, when an animal shows clinical signs that are progressive, leading to further deterioration in condition, an informed decision as to whether or not to humanely kill the animal should be made. The decision should include consideration as to the value of the information to be gained from the continued maintenance of that animal on study relative to its overall condition. If a decision is made to leave the animal on test, the frequency of observations should be increased, as needed. It may also be possible, without adversely affecting the purpose of the test, to temporarily stop dosing if it will relieve the pain or distress, or reduce the test dose." Close and frequent observations are recommended in order to determine the status of the animals, and any animals exhibiting clear signs of severe pain or distress should be humanely killed.

27. Animals may be housed individually, or be caged in small groups of the same sex; individual housing should be considered only if scientifically justified. Further detailed information on housing, feeding, and handling will be provided in Section 3.5.

28. As will be further discussed in Section 3.2, the route of administration will depend on the physical and chemical characteristics of the test substance, the major objective of the study (identifying hazard or characterising risk) and the expected route of human exposure. However, mixing the test substance into the diet or drinking water is normally recommended for rodent studies. If the oral gavage route is employed then its use should be justified. The testing of substances at potentially irritating or corrosive concentrations/doses should be avoided, as administering such substances could result in severe pain and tissue damage at point-of-entry, which would compromise both animal welfare and the integrity of the study.

29. Testing the chronic toxicity or carcinogenicity of inhaled substances can be achieved using either of two exposure conditions: whole-body or nose-only/snout-only. Because of the long-term nature of chronic toxicity and carcinogenicity tests, the preferred exposure method is whole-body. However, nose-only exposure is generally preferred for studies of liquid or solid aerosol and for vapour that may condense to form aerosol. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimise species-specific distress (GD 39).

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3. STUDY DESIGN

3.1 Dose Selection

3.1.1 Introduction

30. The purpose of a long-term bioassay (chronic toxicity and/or carcinogenicity studies) is the detection of biological evidence of any toxic and/or carcinogenic potential of the substance being investigated. Protocols should therefore maximise the sensitivity of the test without significantly altering the accuracy and interpretability of the biological data obtained. The dose regimen has an extremely important bearing on these two critical elements. Since one of the objectives is determination of the dose–response relationship in respect to any endpoints, the OECD TGs 451 (Carcinogenicity Studies), 452 (Chronic Toxicity Studies) and 453 (Combined Chronic Toxicity/Carcinogenicity Studies) normally require at least three dose levels, as well as controls.

31. OECD TGs 451, 452 and 453 outline general principles for dose selection in their respective bioassays. Provision of in depth guidance and a strategy for dose selection is however beyond the scope of the Test Guideline texts. This section of the Guidance Document is designed to underpin and expand the principles of dose selection for chronic toxicity and carcinogenicity studies outlined in the Test Guidelines.

32. These principles of dose selection are generally applicable to a wide range of chemicals, whatever their field of application, e.g. pesticides, industrial chemicals and pharmaceuticals. However, although this document provides a number of references to specific requirements for dose selection for pharmaceuticals, the principles applied in studies on pharmaceuticals may differ from that for other agents (Rhomberg et al., 2007; ICH, 2008). More information is generally available on the pharmacodynamic effects of pharmaceuticals, including the results of controlled clinical studies, than for other types of chemicals. The intended systemic human exposure is known and detailed pharmacokinetic studies enable valid comparisons to be made between the systemic exposures in rodents at the chosen dose levels and those in humans under therapeutic administration of the drug, as measured by the comparative areas under the curve (AUC) of blood concentrations over time (Rhomberg et al., 2007). Users of the Guidance should therefore consult the Guideline S1C (R2) on dose selection for carcinogenicity studies of pharmaceuticals for specific information on testing of such chemicals (ICH, 2008).

33. General principles and guidance on dose selection for chronic toxicity and carcinogenicity studies in rodents are provided in two publications of the International Life Sciences Institute (ILSI). An initial 1997 report, entitled *Principles for the Selection of Doses in Chronic Rodent Bioassays* (ILSI, 1997), presented common views on the selection of doses for carcinogenicity and chronic toxicity studies while a second ILSI working group publication in 2007, entitled *Issues in the Design and Interpretation of Chronic Toxicity and Carcinogenicity Studies in Rodents: Approaches to Dose Selection* (Rhomberg et al., 2007) provides additional discussion of the factors that influence dose selection in chronic bioassays (Rhomberg et al., 2007). The latter publication incorporates concepts included in other documents prepared by national and international organisations (OECD, ECETOC, NTP and USEPA), and places emphasis on the influence of the objectives of a long-term bioassay on dose selection, as summarised in section 3.1.3 of this guidance. Users of this Guidance Document are recommended to consult these publications for more information on the factors influencing dose selection. The following sections provide guidance on (a) the principles for dose selection in the Test Guidelines 451, 452, 453, and (b) the influence of the objectives of a long term bioassay on dose selection.

34. The basic principles for the conduct of chronic toxicity and carcinogenicity studies will differ, given that the endpoints are different. However, given the drive to reduce the number of animals for welfare reasons and the cost of carcinogenicity bioassays, there is a need to maximise the results to assess non-cancer effects that may arise during the study, as these may be critical to the interpretation of any carcinogenic effects. The possibilities for considering non cancer effects in the interpretation of carcinogenic effects are maximised in the TG 453, Combined Chronic Toxicity/Carcinogenicity Study.

35. While the guidance provided in this chapter can be taken as generally applicable to dose selection for either a chronic toxicity or a carcinogenicity bioassay, or a combined chronic and carcinogenicity study, users of the guidance should be mindful of the primary objectives of the study in establishing the optimum study design in terms of dose selection.

36. In selecting appropriate dose levels for long-term bioassays (e.g. TG 451, TG 452, TG 453), a balance has to be achieved between hazard identification/characterisation on the one hand and characterisation of low-dose responses and their relevance on the other. This is particularly relevant in the situation where a combined chronic toxicity and carcinogenicity study (TG 453) is to be carried out.

3.1.2 Principles for Dose Selection

37. The general principles for dose selection laid down in the TGs are summarised as follows:

- Dose levels should 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 (Barton at al. 2006).
- The highest dose level should be chosen to identify toxic effects including the principal target organs while avoiding severe toxicity, morbidity, or death (OECD 2000, GD No.19).
  It should be noted that the severity of toxicity and survival in a two year study may be underestimated from the short-term study; for this reason, Test Guidelines indicate that a top dose lower than the dose providing evidence of toxicity in a short-term study may be chosen. When there is no toxicity in shorter-term studies it is recommended to consult with the relevant regulatory authorities.
- Dose level should be selected to reflect the purpose of the study. In most cases, dose levels and dose level spacing may be selected to establish a dose-response and to derive a point of departure (e.g., BMDL or NOAEL).

38. These principles for dose selection are broadly similar to the key principles for dose selection outlined in the ILSI publications (ILSI, 1997; Rhomberg et al., 2007), as listed in full in Appendix I. They are further discussed in the following sections.

3.1.2.1 Key information for the selection of doses in chronic toxicity and carcinogenicity studies

39. Identifying/characterising carcinogenic effect is the primary objective of the OECD TG 451 on Carcinogenicity Studies while identifying/characterising other toxic effects is the primary objective of the OECD TG 452 on Chronic Toxicity Studies. The OECD TG 453 Combined Chronic Toxicity/Carcinogenicity Studies combines the objective of OECD TG 451 and OECD TG 452. For all three studies the core minimum study design comprises at least one control group and three dose groups, each of which is exposed to different concentrations of the test substance.

40. The robustness of a carcinogenicity or chronic toxicity study, in particular the former, is dependent on a demonstration that the dose levels selected in the study are adequate to show an effect or effects of the test substance, without producing either false negative results (because the doses

selected were too low) or false positive results (because metabolic/homeostatic mechanisms are overwhelmed, etc), which may be problematic in assessing risk in humans.

41. The data provided by shorter-term repeated dose or range finding studies, including 28-day or 90-day studies, are important in selecting the dose levels for a longer-term chronic toxicity or carcinogenicity study. The dose levels used in such studies and the NOAELs established can be used as a starting point for dose selection, both in relation to the highest dose level to be chosen in the study and possibly (but not necessarily) to the lower dose levels. Considerations that should be taken into account in determining whether similar, lower or higher dose levels than those used in a short-term study should be selected for a chronic toxicity or carcinogenicity study include (Rhomberg et al., 2007):

- whether the effect is an adaptive response (e.g., liver hypertrophy in the absence of any other evidence of hepatotoxicity);
- potential of the toxic effect(s) observed in repeat dose toxicity studies of shorter duration to progress to neoplasia. A dose that induces a marked effect in such study should not be excluded from a carcinogenicity study if the effect or effects can reasonably be anticipated to be a precursor event in the development of neoplasia (e.g., a key event for the mode of action of the test substance). However, care should be taken that selection of a dose level that induces such effects will not result in excessive toxicity in the carcinogenicity study;
- the potential that an effect may limit the sensitivity of the chronic/carcinogenicity study to detect tumours (e.g. haemolytic anemia may limit the duration of the study due to an increase in mortality or to severe toxicity that may compromise the health of the animals);
- the duration of the short-term study (e.g., repeated dose 90-day study, repeated dose 28-day study, two-generation reproduction study) and the potential for a toxic effect to progress in severity (e.g., progression from focal to multifocal necrosis);
- evidence of transitory effects that may be life-threatening: if prechronic studies revealed transitory effects that may last during some days or weeks until metabolic capacity (e.g. by liver enzyme induction) is adapted, testing of high dose is limited by transitory effects of life-threatening nature (e.g., sedation);
- use of gavage for administration of the test substance in studies of shorter duration. A dose that induces overt toxicity in a gavage study may be tolerated if a dietary route of administration is selected for a carcinogenicity study because of the differences in toxicokinetics and toxicodynamics resulting from the two methods of administration.

42. Additional evidence on the extent to which dose levels should be increased or decreased in a long-term study relative to a short-term study or studies may be provided by dose–response data from the latter studies. For example, a marked reduction in dose levels would be warranted if results from short-term studies show that a minor increase in dose is associated with a pronounced increase in severity or incidence of a lesion (i.e., a steep dose–response). It is recommended that all the information from such short-term studies, (rather than the use of an arbitrary factor e.g. one-tenth the highest dose tested in a short-term study that induced a severe toxic effect) should be used when selecting the high dose (or mid and low dose levels) for a proposed carcinogenicity study.

43. Available toxicokinetic data (ADME) should always be taken into account when selecting dose levels for a chronic toxicity or carcinogenicity study, although such data may not be readily available for all chemicals, as they are not required under all regulatory schemes. Many toxicokinetic processes influencing absorption, distribution, elimination and metabolic activation or detoxication may become saturated at higher doses, resulting in systemic exposures to parent compound or metabolites that would not be expected in the real life human exposures for which risk assessments are needed. The effect of repeated exposures on the pattern of absorption, metabolism, detoxification, and clearance of a compound will provide information on the internal dose achieved during chronic exposure under conditions of the bioassay. The importance of having data on toxicokinetics in

reaching a decision on the design most suitable for a chronic toxicity or carcinogenicity study is stressed in this guidance and the use of such data will be discussed in more detail in Chapter 3, Section 3.4 of this Guidance Document.

44. Physiologically-based toxicokinetic (PBTK) modelling is also a valuable tool for defining doses where non-linear toxicokinetics may occur, thus allowing this to be considered in selecting the highest and other dose levels in the study. The use of PBTK modelling will be explored in more detail in Section 3.4 of Chapter 3. Finally, specific mechanistic studies (where available) may provide useful information regarding target tissues affected by the test substance and the doses associated with effects on key events, and should be taken into account when selecting doses for a chronic toxicity or carcinogenicity study.

45. Additional considerations in selecting dose levels for chronic toxicity or carcinogenicity studies arise as a result of practical constraints such as the physicochemical characteristics of the substance to be tested (e.g., solubility, vapour pressure), palatability of the compound in food or drinking water, and other factors such as the potential for the test substance to cause adverse effects such as irritancy at the site of administration (Rhomberg et al., 2007). Further guidance on these aspects is provided in the ILSI publications (ILSI, 1997; Rhomberg et al., 2007) and will be published in Chapter 3, Section 3.5 of this guidance.

46. Information on, and consideration of, the mode of action (MOA) of a suspected carcinogen is particularly important, since the dose selection may differ depending on the known or suspected mode of action (Sonnich-Mullin et al., 2002; Cohen et al., 2003; Meek et al., 2003; Holsapple et al., 2006; Boobis et al., 2006; EPA 2005). In selecting dose levels for such a study, doses will need to be placed carefully, to yield observations of subtle precursor effects or other biomarkers of toxicity without inducing confounding effects related to frank toxicity. This approach requires some previously gathered information on potential modes of action, e.g. from genotoxicity studies. Further guidance on these aspects will be discussed in Chapter 2 of this Guidance Document.

3.1.2.2 Selection of the top dose

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

48. The selection of the highest dose level to be used in a chronic toxicity or carcinogenicity study has long been a matter of controversy. At the time when long-term animal bioassays began to be routinely used to assess the qualitative potential of a test substance to cause chronic toxicity and cancer, the emphasis was on testing at high levels in order to maximise the potential of such studies to detect effects. The concept of the Maximum Tolerated Dose (MTD), conventionally defined as the highest dose to produce toxic effects without causing death and to decrease body weight gain by no more than 10% relative to controls (OECD 2002, GD No. 35) became well established. The MTD is often used in the assessment of a chronic toxicity or a carcinogenicity study to decide whether the top dose tested was adequate to give confidence in a negative result. This Guidance Document focuses on the selection of the top dose, rather than attempting to define an MTD.

49. While some regulatory bodies or organisations interpret an adequate high dose to be a minimally toxic dose, others emphasize the need to select a dose level that is a maximally tolerated dose (i.e., more severe toxicity should be demonstrated). Thus, because of differences in views regarding the severity of toxic effects that are interpreted as providing evidence that an adequate high dose has been attained or exceeded, a completed carcinogenicity bioassay may be considered to be

acceptable by one organisation but not by another. Many carcinogenicity studies can be challenged on the basis of selection of a top dose that is too high, particularly if there is a large interval to the next highest dose. This results in data that are difficult to interpret and cannot be used for regulatory purposes. Appendix 2 of Rhomberg (Rhomberg *et al*., 2007) provides detailed guidance on criteria that can be applied in order to assess the acceptability of the high dose level or MTD (Rhomberg *et al*., 2007).

50. If the main objective of the study is to identify a cancer hazard, there is broad acceptance that the top dose should ideally provide some signs of toxicity such as slight depression of body weight gain (not more than 10%), without causing e.g., tissue necrosis or metabolic saturation and without substantially altering normal life span due to effects other than tumours. Excessive toxicity at the top dose level (or any other dose level) may compromise the usefulness of the study and/or quality of data generated. Criteria that have evolved for the selection of an adequate top dose level include: (in particular) toxicokinetics; saturation of absorption; results of previous repeated dose toxicity studies; the MOA and the MTD.

51. Toxicokinetic non-linearity should also be considered in the selection of the top dose to be used. Although top dose selection based on identification of inflection points in toxicokinetic non-linearity may result in study designs that fail to identify traditional target organ or body weight effects, it must be appreciated that metabolic saturation in fact represents an equivalent indicator of biological stress. In this case, the stress is evidenced by appearance of non-linear toxicokinetics rather than appearance of histological damage, adverse changes in clinical chemistry, haematology parameters or decrease in body weight gain (Toxicokinetics will be discussed in Section 3.4).

52. For compounds that are (or potentially are) genotoxic, conventional considerations of top dose given above would apply. For compounds that are not genotoxic, the top dose should be informed by considerations of MOA (see Chapter 2). For a given compound, for which the mode of action is known or suspected, establishing a point of departure based on precursor key events, may also be protective against any carcinogenic effect. This is because non-genotoxic carcinogens produce cancer by perturbing normal physiology or biochemistry. The long-term assay should be designed to identify and characterise these key events and not necessarily cancer *per se*.

53. Nutritional effects, physiological factors, physical-chemical factors and compound bioavailability can influence selection of the top dose level to be used in a long-term bioassay. For nutritional and possibly other physiological reasons a maximum level is imposed, commonly 5% concentration in the diet (Sontag et al., 1976; Chhabra *et al*., 1990).

54. Palatability of a compound in either feed or water can also lead to perturbation of physiological homeostasis or nutritional status. A compound's solubility limit or vapour pressure may constrain selection of the top dose level. Irritation at the site of compound deposition may constrain dose or otherwise confound cross species extrapolation. Inhalation of doses that overwhelm pulmonary clearance may lead to tissue responses that are specific to the species being tested; however, this does not apply to asbestos-like substances. These limitations may influence selection of the top dose.

55. The top dose used in the study may be based on a defined level of the target population's exposure of interest and multiples of that exposure (e.g. 100 times or 150 times higher based on dose ratios expressed in terms of body weight). If toxicokinetic data are available, dose levels based on internalised doses (e.g., AUC) can be used. It has been shown that the relative systemic exposure corresponds better with dose ratios expressed in terms of body surface area (AUC ratios) rather than rations expressed in terms of body weight. It should be noted that the use of systemic exposure comparison between rodents and humans to derive the top dose may be useful in the case of pharmaceuticals testing (ICH, M3(R2), S1C(R2)) but is not likely to be useful for testing plant

protection products or commodity chemicals, given the uncertainties regarding exposure levels in scenarios where these are used, and given the need for these chemicals that the top dose be sufficiently high for the purpose of classification for carcinogenic effect, i.e. an inherent potential of the substance in question to cause cancer irrespective the dose should be demonstrated, if appropriate.
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56. The relevance of the top dose level recommended to be used in the study to potential human exposures can also be debated. Mechanistic information gleaned from this type of study may be irrelevant. If the top dose level is set lower, to ensure relevance, the power to detect effects may be compromised. In short, positives may be difficult to interpret vis-à-vis low exposure levels, because they may reflect a high-dose-only phenomenon.

3.1.2.3 Dose level spacing

57. Selection of dose intervals is influenced by the study objectives (see section 3.1.3) and the available information. Dose levels and dose level spacing may be selected to establish a dose-response and to derive a point of departure (e.g., BMDL or NOAEL). The dose level spacing does not need to be regular. It may be reduced in regions of the dose-response curve where particularly robust estimation is needed, e.g. in the range of the anticipated BMD or a suspected threshold. The increasing emphasis on consideration where the lower dose levels used in the study are placed, and the number of such dose levels, reflects the changing purposes of lifetime bioassays.

58. If the primary purpose is identification of hazard, whether this is chronic toxicity or carcinogenicity, the focus of dose selection should be on maximizing the power of the study and on the top doses tested. As the risk assessment process becomes increasingly concerned with characterisation of human risk, there has been a corresponding need to characterise whether and how high- dose effects extend to responses at lower exposure levels as well, with a consequent interest in how the lower dose levels are placed in bioassays (Rhomberg et al., 2007).

59. Dose selection and dose level spacing need to be based, where possible, on the following considerations:

- known or suspected non-linearities or inflection points in the dose–response;
- toxicokinetics, and dose ranges where metabolic induction, saturation, or non-linearity 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 neighbourhood of the anticipated point of departure;
- consideration of anticipated human exposure level;
- a suspected threshold.

60. Dose levels should be selected to reflect the purposes of the study, and they should use available knowledge on how dose-dependent biological and impacted physiological factors may affect study outcomes. The Test Guidelines (TG 451, Par. 24, TG 452, Par. 24, and TG 453, Par. 26) indicate that "The dose level spacing selected will depend on the characteristics of the test substance, and cannot be prescribed in this Guideline, but two to four fold intervals frequently provide good test performance when used 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. In general, the use of factors greater than 10 should be avoided, and must be justified if used".

61. If prior evidence allows, it may be possible to optimise the design in terms of the location of the dose levels. A design often applied uses a mid dose that is half of the top dose, or the geometric mean of the low and high dose. This will ensure that the power and sensitivity of the assay is maximised and that at least one dose is unlikely to have a carcinogenic or other effect. This approach minimises the chance of a false negative (failing to detect an effect that actually exists) at some increased risk of a false positive (finding a high-dose effect that is an artefact of excessively high doses and is not relevant to the dose range of interest).

62. Limited information may be obtained regarding the shape of the dose–response curve, particularly if non-linearity is seen in the middle of the dose range. The power of the assay at lower dose levels will also be limited if the incidences of the responses of interest are low (e.g., rare tumours) and not markedly different from the controls. Information on the dose-response relationship would depend on how well the dose range of interest is anticipated.

63. The issue of where to place the lowest dose should receive comparable attention as to the placement of the top dose. If the lowest dose is too low, it may be insufficiently powerful and therefore uninformative; if too high, it may lose opportunities to characterise effects as near as possible to environmental exposure levels. For example, for pharmaceuticals, a dose sufficient to produce a pharmacodynamic effect or result in systemic exposure comparable with that expected at the intended clinical use is normally selected for the low dose level (see also ICH guideline S1C(R2)).

64. It may be possible to place adjacent dose levels somewhat above and below the levels at which a key transition in underlying biological actions, including considerations of the mode of action, is believed to lie, thereby revealing its influence on response. Transitions need not be sharp; typically, there are ranges of doses over which an underlying biological factor, such as metabolic saturation or cytotoxicity, comes increasingly into play.

65. When evaluating threshold effects in a chronic toxicity bioassay, the doses selected will include at least one dose high enough to show toxicity, at least one dose low enough to show lack of toxicity, and usually one but occasionally more than one in between to help characterise the shape of the curve near the point where the threshold appears to lie (Rhomberg et al., 2007). These dose placement concerns differ from those in the carcinogenicity bioassay for substances where genotoxicity is known or suspected; however, this difference disappears if the BMD approach is used. The same dose range is preferred for both phases of a combined chronic toxicity/carcinogenic study, particularly if the MOA is under investigation.

3.1.3 Integration of the objectives of a long-term bioassay

66. The ILSI publications (ILSI, 1997; Rhomberg et al., 2007) provide practical guidance on factors that influence dose selection in long-term bioassays, with particular emphasis on how the varying objectives of a chronic toxicity/carcinogenicity bioassay influence dose level selection.

67. Test Guideline 453 (combined chronic toxicity/carcinogenicity studies) identifies nine possible objectives:

- The identification of the carcinogenic properties of a chemical, resulting in an increased incidence of neoplasms, increased proportion of malignant neoplasms or a reduction in the time to appearance of neoplasms, compared with concurrent control groups;
- The identification of the time to appearance of neoplasms;
- The identification of the chronic toxicity of a chemical;

- The identification of target organ(s) of chronic toxicity and carcinogenicity;
- Characterisation of the dose:response relationship;
- Identification of a point of departure (e.g., BMDL or NOAEL);
- 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 (EPA 2005; OECD 2009, GD No.116; Boobis et al., 2006; Cohen et al., 2003; Holsapple et al., 2006; Meek et al., 2003).

68. Various study designs have been proposed to address these objectives, as described by Rhomberg et al., 2007. The core study design for a long-term bioassay as laid out in TGs 451, 452, 453 primarily addresses the objective of identification/characterisation of carcinogenic substances or those causing other toxic effects, while seeking to integrate the other objectives as far as possible. Modifications of the core study design in TGs 451, 452, 453 in order to optimise data for the other objectives may compromise the Mutual Acceptance of Data and should be discussed with the relevant regulatory authorities before the commencement of the study. More generally, it is recommended to ensure regulatory acceptance of the study design before performing any long-term bioassay to be submitted to a competent authority.

69. The different objectives outlined above seek to maximise the statistical power of the study at different points on the dose-response curve. The focus may be on a level of response or on the shape and slope of the overall curve. The situation is also complicated by the fact that, below a certain dose, attempts to increase statistical power by increasing animal numbers in particular dose groups become futile.

70. For the majority of bioassays, there will be one primary objective (typically the identification of carcinogenic potential and/or chronic toxicity) and several subsidiary objectives such as characterising the dose-response curve, extrapolating to low doses, or identifying a point of departure. The nature of the subsidiary objectives will be contingent on the intended outcome. If a valid negative result is obtained in a carcinogenicity study (e.g., OECD TG 451), and this was the only objective of the study, there may be no further questions to be answered. If a positive result is obtained, however, a number of issues arise regarding the nature of the carcinogenic responses and their relevance to the levels of exposure of target populations, requiring further investigation into the nature and interpretation of the effects seen. Consideration of the mode of action framework before embarking on a long-term bioassay will provide guidance on optimising the design to collect the information necessary to the interpretation (see Chapter 2).

71. The choice of dose levels for the identification of a point of departure will depend on the type of point of departure sought. For a NOAEL, a dose without effects is required but ideally at the highest dose at which this can be observed. For a BMDL, the data from all dose levels is used but it is important that the responses differ at the different doses.

72. The study design selected at the outset should include dose levels that combine several objectives. One approach to achieve this is to include additional dose groups in such a way that the optimal doses for a number of different objectives are all included (Rhomberg et al., 2007). Some doses would be optimised for some objectives and others for other objectives, essentially running several bioassays in tandem.

73. However, this is not feasible, given animal welfare, economic and time constraints. When attempting to combine these various objectives into a single study, selection of dose levels must be done in a way that does not compromise the primary objective while still allowing a secondary objective to be pursued in an acceptable albeit suboptimal manner. There may be embellishments to

the core design based on study objectives but it would be a rare event when an erosion of the core minimum would be acceptable.

74. Based on Rhomberg et al., 2007, four core selection schemes are presented below:

1. **Hazard Identification/Characterisation Plus Dose–Response:** The top dose is chosen to increase the study's statistical power to detect effects that may be rare. A second dose combines two functions: (1) hedging against the top dose being found to have been too high in retrospect, and (2) providing the opportunity for dose–response characterisation of any effects found. The lowest dose level or, if considered necessary, other lower dose levels can be placed so as to inform dose–response, no-effect levels, or other purposes. Key challenges will be balancing statistical power and toxicological relevance of the top dose level and compromising among subsidiary objectives while accounting for relevant dose-related physiological changes when setting lower dose levels.

2. **NOAEL/BMDL-Seeking for Threshold Effects:** The main aim is to identify no-effect (or low-effect) levels for the more sensitive adverse threshold effects. The top dose should aim at engendering an adverse effect, the lowest dose should aim at constituting a NOAEL/BMDL, and the intermediate dose or doses should be set so as to identify the dose levels at which the top dose responses begin to manifest.

3. **Assessment of Safety of Human Exposure Levels:** This is modeled on safety assessment studies for nutrients and pharmaceuticals. For agents that are not genotoxic, show low toxicity, and evince no known difference in metabolic profile between rodents and humans, one can test multiples of anticipated human exposure. Lack of adverse effects at doses sufficiently above human exposure (and the perceived implausibility of non-threshold effects) gives evidence supporting the safety of the anticipated exposures. The bioassay exposures should be selected on an appropriate basis for animal:human comparison; for instance, the application to pharmaceuticals is typically based on area under the blood concentration-time curve that results from anticipated human exposures.

4. **Special-Purpose Bioassays:** this case would be beyond the OECD Test Guidelines and therefore is not developed in this document.

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APPENDIX 1: ILSI PRINCIPLES FOR DOSE SELECTION IN CHRONIC RODENT BIOASSAYS[2] (RHOMBERG ET AL., 2007)

Principle 1

Dose selection for chronic studies must be based on sound toxicologic principles. Within a reasonable dose range, increasing the dose can increase the ability to detect an effect; therefore, doses for chronic rodent bioassays should be selected within this range to maximize the sensitivity of a chronic bioassay. However, trying to increase study sensitivity by increasing doses into ranges that do not reflect application of sound toxicologic principles could lead to results that are inappropriate for human risk assessment.

Increasing the highest dose in a chronic bioassay may increase sensitivity within some defined dose range, but the potential exists that different mechanisms of toxicity or chemical mode of action are active at higher doses, which may not be relevant to humans exposed to lower doses. In this case, selection of the highest dose may be influenced by consideration of the mechanism/ mode of action and other factors discussed in Principle 4. However, when the highest dose in a carcinogenicity assay is limited by effects (e.g., a mode of action in one organ system) that are thought not to occur in humans, one must be aware that it still is possible that a higher dose of the chemical may be carcinogenic in other animal/organ systems.

To address these issues, the ILSI working group encourages an approach to dose selection that incorporates all relevant information from prechronic studies and other sources, uses toxicologic tools associated with an understanding of the mechanisms or mode of action by which a chemical produces an effect (e.g., genotoxicity, cell proliferation, etc.), and uses good scientific principles to enhance the accuracy of judgments of potential human risks. In the case of negative studies (particularly where the highest dose is chosen based on a full characterization of the chemical's toxicity in prechronic studies), use of sound scientific principles as well as all available chemical, physical, and toxicologic data will lessen concern that the result may be a false negative. Similarly, in positive carcinogenicity studies, this approach will lessen concern that the result may be a false positive. In both cases, the predictiveness of the bioassay for human health effects will be improved.

Principle 2

A chronic bioassay requires a major investment in resources and time, and the objective of such a study should be broader than hazard identification. Scientists who conduct chronic bioassays and those who use data from bioassays, including regulatory agencies, should encourage innovative approaches to dose selection by considering appropriate study designs, mechanistic data, and other information in the design and interpretation of studies. Use of additional endpoints and other

[2] The terminology used in the Guidance Document regarding "long term bioassay" designing both chronic toxicity and carcinogenicity studies doesn't apply to this annex.

information must be based on sound scientific rationale, and such designs should be evaluated on the basis of their individual merits.

A goal of high-dose selection in carcinogenicity bioassays is, in the context of hazard identification, to reduce the likelihood of a false-negative result. However, it is recognized that the qualitative nature of the hazard (e.g., carcinogenic response) may itself be dose dependent. This principle encourages approaches to dose selection that incorporate consideration of mechanistic and other toxicologic information. Such approaches should improve the scientific basis for dose selection and aid in interpretation of data generated from chronic bioassays.

Principle 3

Human exposure should be considered in dose selection, particularly for selection of the middle and lowest doses. Further, the middle and lowest doses should be selected to characterize the shape of the dose response curve as much as possible. Selection of the middle and lower doses should take into account factors such as the mechanism or mode of action, toxicokinetics, and others listed in Principles 4 and 5 and should not be based solely on a fraction of the highest dose.

Issues that should be considered when incorporating potential human exposure in dose selection include the human exposure route and mode, the dose range in the chronic bioassay in relation to human exposure, and the duration and frequency of human exposure, if known. Subpopulations that may be more highly exposed than the general population, or that are genetically more susceptible, also should be considered. The relationship between external and delivered (internal) dose (e.g., ingested dose versus dose delivered to the target organ, toxicokinetics) in both humans and test organisms may influence dose selection. Further, for substances expected to exhibit a toxicity threshold, or if the evaluation of carcinogenic potential is being combined with an evaluation of chronic toxicity, the study should be designed to include one dose that does not elicit adverse effects; that is, one dose should be a NOAEL. Of course, caution must be exercised to ensure that the NOAEL is not simply an artifact of small sample size or poor study design.

Principle 4

The [ILSI] working group has recommended the use of innovative approaches, additional endpoints, and other information in the selection of doses for chronic rodent bioassays. The following endpoints, generally determined in prechronic studies, should be considered in dose selection for chronic rodent bioassays. Further, it is recognized that endpoints other than those listed below may provide important information for dose selection, and use of those endpoints, where they are based on sound toxicologic principles, is encouraged. Such endpoints may be available presently, or they may be developed as the science of toxicology advances.

- **Histopathology**

  The site, morphology, and severity of the treatment-related effects observed in the pre-chronic study should be taken into account in setting dose levels for the chronic study. Histopathological examination of tissues, especially the liver, kidneys, gastrointestinal tract, urinary tract, respiratory tract, skin, spleen/bone marrow/blood, and endocrine tissues derived from properly designed pre-chronic studies, often provides information that is crucial for dose selection in chronic bioassays.

- **Toxicokinetics**

  Studies to determine the effect of dose (or exposure concentration) on absorption, tissue distribution, metabolism, and clearance of a compound

are helpful in selecting appropriate doses for the chronic bioassay. The kinetics of absorption will determine the internal exposure dose achieved. The absorption and clearance of the compound and its metabolites will determine the systemic and target organ exposure resulting from a single dose and can be used to design the treatment regimen required to achieve a desired internal dose. The effect of repeated exposures on the pattern of absorption, metabolism, biotransformation, and clearance of a compound will provide information on the internal dose achieved during chronic exposure under conditions of the bioassay. The nutritional status of the chronically exposed animals may be affected during the experimental period which is why adequate information on interactions between the exposure chemical and nutrionally important compounds may be of great value in the interpretation of the final results of the chronic study.

- **Cell Proliferation**

In the process of chemical carcinogenesis, events related to induced cell proliferation might be critical in fixing mutations and in providing a selective growth advantage to pre-cancerous cells. Considerations may be different for direct mitogenic stimulation of organ growth versus regenerative cell proliferation, and these modes of action should be distinguished for the test agent. Further, apoptosis can be a strong determinant of normal and pre-cancerous cell turnover kinetics and should be considered. Information on the dose dependence of regenerative cell proliferation is a useful adjunct to histological observations in determining the shapes of organ-specific toxic response curves. This information, when available, can be of value in selecting high, middle, and low doses and in interpreting the results of the study.

- **Physiological Functions**

Disturbances of physiology or homeostasis that would compromise the validity of the study should be considered in the dose-selection process. Examples include hypotension, inhibition of blood clotting, overwhelming normal pulmonary clearance mechanisms, immune system effects, and in some cases hormonal imbalance. Such disturbances, and their effects on the validity of a study, may be difficult to determine and may apply differentially to different categories of chemicals (e.g., pharmaceuticals in which the desired pharmacological action is a physiological effect).

- **Body Weight**

It is suggested that body-weight changes are the primary factor in the selection of the highest-dose group (that is, when no other toxic effects are observed), a decrement in body-weight gain of no more than 5–10% in pre-chronic studies should be used in the selection of the highest dose for chronic assays of carcinogenicity.

Historically, scientists have adopted a 10% decreased body-weight gain at the end of pre-chronic studies (typically 90 days duration) as the target that should not be exceeded in chronic (carcinogenicity) studies. It is now recognised that there is a positive correlation between body weight and

the occurrence of certain tumours in rodent species and strains used in safety assessment or for hazard identification; i.e., the higher the body weight between 6 and 18 months on test, the higher the probability that the animal will develop some tumours. Moreover, the lower the body weight, the less sensitive the animal may be to agent-induced toxicity, including cancer. A significant decrease in body-weight gain therefore could reduce the animal's ability to respond to compound-induced toxicities.

- **Clinical Chemistry, Haematology, Urinalysis**

  Clinical chemistry and urinalysis results are best used to support dose-selection decisions based on other criteria/parameters. Changes in serum clinical chemistry in the absence of histopathological observations may not affect high-dose selection but may complement dose-selection decisions based on toxicokinetics, cell proliferation, and other parameters. Haematology results also are more often affected secondarily to other processes more relevant for dose selection (e.g., inflammation). However, when haematological tissues are determined to be a target organ in pre-chronic studies, haematology results may be an appropriate basis for dose selection.

- **Organ Weights**

  Organ weights are not often the critical factor in selection of doses for chronic rodent bioassays. Chemically induced changes in organ weights, however, should be considered in conjunction with other data in the dose-selection process.

  Ideally, data from the factors and endpoints listed above would be collected from pre-chronic studies and used to select doses for chronic studies; however, not all parameters may be useful or necessary for every compound. Even when based on information concerning the points described above, dose selection for a chronic bioassay will remain an inexact process; thus, reconsideration of these same points must be made when interpreting and assessing the significance of the effects obtained in the bioassay.

Principle 5

Physicochemical factors (e.g., solubility, vapour pressure), the bioavailability of the compound, the palatability of the compound in food or drinking water, and other factors such as the potential for the substance to cause adverse effects at the site of administration (e.g., irritation, erosion, and ulceration) will influence the selection of the highest dose for chronic rodent bioassays. It is recommended that doses for chronic rodent bioassays be selected to minimise or avoid adverse nutritional, physical, organoleptic, and irritant effects.