# The International Symposium on Regulatory Testing and Animal Welfare: Recommendations on Best Scientific Practices for Subchronic/Chronic Toxicity and Carcinogenicity Testing

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## Introduction

Breakout Group 3 addressed the current best practices and future possibilities for incorporation of the principles of the 3Rs (in particular, refinement) in the areas of subchronic/chronic toxicity and carcinogenicity testing.[1] Participants in the group (listed at the end of this report) had previously reviewed a number of key background references (also listed at the end of the report) the group leaders had selected before the meeting. Participants were asked to consider the following questions as part of their general discussion.

## Current Considerations

1. What are the current best practices for minimizing pain and distress for subchronic/chronic toxicity and carcinogenicity testing? Are these practices adequate for preventing animals from experiencing more than minimal pain and distress? Does the Organisation for Economic Co-operation and Development (OECD[2]) guidance document (OECD 2000) represent the current best scientific practices available for defining humane endpoints for subchronic/chronic and carcinogenicity testing?
2. Where current best scientific practices are considered to be inadequate, what might be done to improve the situation? Are there animal testing situations for which best practices are needed, but are not available?
3. What steps can be taken to help ensure that current best practices are implemented uniformly and universally?
4. What processes can be used to encourage regulatory agencies to endorse current best scientific practices regarding the establishment of humane endpoints for subchronic/chronic and carcinogenicity testing and to inform the regulated community of their expectations?
5. What share of the responsibility to apply current best scientific practices should be attributable to regulated industry, research organizations, academia, and other laboratory environments, irrespective of the level of support expressed by the regulatory authorities?
6. Regarding future improvements, how best can the 3Rs be integrated into toxicity testing schemes?

## Report on Group Discussion

### Current Best Practices for Minimizing Pain and Distress

Participants unanimously agreed that the *OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation* (OECD 2000) should be regarded as providing key initial "guidance" for defining humane endpoints. However, they recognized that the document will need to evolve and will require regular updating if it is to incorporate both changing regulatory requirements and advances in animal welfare research. In addition to the key references considered by the Breakout Group, participants also identified other important references that provide helpful guidance on humane endpoints (NRC 1992, 1996) and organizations that provide related policy statements (e.g., US Public Health Service, Society of Toxicology, American College of Laboratory Animal Medicine, and Association for Assessment and Accreditation of Laboratory Animal Care International).

Participants noted that the OECD document is considered strictly as guidance and not as a legally binding document, as are OECD "test guidelines." However, all OECD test guidelines issued in 2001 state that animals should be humanely killed when they are suffering severe pain and distress or are moribund, in accordance with guidance in the OECD humane endpoints document (OECD 2000). The group also recommended that excessive tumor burden or evidence that the animal will not likely survive until the next scheduled observation should also be considered as humane endpoints.

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[1]This manuscript does not reflect official government agency policy.

[2]Abbreviations used in this report: HPV, high production volume; OECD, Organisation for Economic Co-operation and Development; QSAR, quantitative structure activity relationship; SOP, standard operating procedure.

The Breakout Group unanimously agreed that the current best practices are not adequate to prevent animals from experiencing more than minimal pain and distress for subchronic and chronic carcinogenicity testing. Earlier endpoints that are indicative of tumor burden, tissue damage, and impending organ failure are needed. It will likely be necessary to develop and validate earlier biomarkers of tumorigenesis and other toxic effects to make significant progress in further reducing pain and distress for these types of testing. New information from toxicogenomics, proteomics, metabonomics, and other research strategies may be helpful in identifying appropriate biomarkers. One approach that should be implemented immediately is the accurate recording of all clinical observations to facilitate the identification of clinical endpoints that are predictive of impending death or irreversible conditions.

More specific and detailed test-, species-, and strain-specific humane endpoints should be developed by each institution to meet the needs of the end-users (toxicologists and animal care staff). These humane endpoints should be agreed upon by relevant external bodies (e.g., regulatory bodies requiring the data and animal oversight agencies) and incorporated into protocols and particularly standard operating procedures (SOPs[2]).

Implementation of humane endpoints should always be accomplished using both scientific and professional judgment. Participants also agreed on the importance of ensuring that the application of humane endpoints does not compromise the safety assessment process. In this context, recognized processes for the validation of humane endpoints and other refinement strategies are needed prior to regulatory acceptance.

Participants agreed that in addition to the implementation of certain humane endpoints, consideration should be given to improving an understanding of the relevance of animal models to the human situation. For example, pharmacokinetics could be better employed to identify target tissues and levels of the administered dose reaching the tissues in different species. Data from human studies can also contribute to improved understanding of the relevance of animal models and the design and conduct of mechanistic studies.

### *Implementation of Guidance on Humane Endpoints*

Participants recognized that scientists will require assistance both in the interpretation and the implementation of humane endpoints. Relevant and effective educational material should be made available, and the concept of humane endpoints should be included in training programs. This process will require financial resources and the cooperation of scientists, animal care staff, and others involved in the use of laboratory animals. In particular, assistance is needed to (1) decrease subjectivity in recognition and classification of levels of pain and distress in different species and strains of laboratory animals, and (2) distinguish between clinical signs of transient versus irreversible conditions.

Standardization of criteria for endpoints is of particular importance to provide consistency for the termination of studies. These problems are complicated by a lack of detailed information on pain in laboratory animals. As a first step in reducing unrelieved pain and distress (Flecknell 1994; Soulsby and Morton 2001), the overall aim should be to avoid spontaneous deaths. This aim is more likely to be achieved if administration of unnecessarily high dose levels is avoided.

Scientists should be made aware of the need for proactive monitoring and evaluation of parameters that can impact on animal well-being (Hendriksen and Morton 1999). This awareness should include consideration of the microbiological and genetic status of the animals and actions necessary to minimize or avoid spontaneous disease that could compromise both the welfare of the animals and the integrity of the study. Reduction of these and other known experimental variables can also serve as a means of reducing animal numbers. The potential for reduction and refinement by taking multi-endpoint measurements on the same animal should also be assessed where applicable.

The working group considered that there are several proactive mechanisms whereby regulatory agencies should encourage and endorse the establishment and application of humane endpoints in regulatory carcinogenicity and chronic toxicity testing. For example, regulatory agencies in the United States should publish such guidance in the *Federal Register.* Regulatory agencies should encourage discussion of humane endpoints by organizing conferences to bring together toxicologists and animal welfare experts with experience in the application of humane endpoints to serve as a forum for regulators and scientists and to develop a workable system of humane endpoints.

Responsibility for the implementation of humane endpoints should be shared by all of the major stakeholders. Participants recognized that the cultural differences and backgrounds of scientists should be addressed in the development of internationally applicable humane endpoint guidelines. The participants agreed that the OECD guidance document on humane endpoints should be referenced in all new versions of OECD test guidelines.

### *Integration of the 3Rs into Toxicity Testing Schemes*

The Breakout Group agreed that it is important for laboratories to apply strategic planning before carrying out any animal experiments in an effort to ensure appropriate implementation of the 3Rs. For example, there is tremendous potential for the increased use of screening tests to assist in prioritizing chemicals for further testing. Such an approach should also be applied to the design of new testing programs, such as the US Environmental Protection Agency High Production Volume (HPV[2]) Chemical Testing Program and the European Union's existing chemicals testing program. Strategic planning should also include establishing the rationale and necessity for conducting an animal test as well as identifying realistic goals, time lines, and appro-

priate funding necessary to incorporate refinement, reduction, and replacement strategies into studies.

Test laboratories should be made aware of any relevant test data and information to ensure the correct choice of solvents, storage conditions, and application of analytical methods. Strategic planning should include not only the provision for the use of early endpoints/markers but also built-in flexibility to change working practices, techniques, and procedures (e.g., the identification of a new blood injection method). Any strategic plan should incorporate (1) stages where formal communication with regulatory authorities takes place, (2) periodic retrospective evaluation of the outcomes of different study designs, and (3) adequate personnel training.

### *Reduction*

The Breakout Group participants considered several ways whereby reduction can be achieved in regulatory testing (Table 1; see also Festing et al. 1998). However, they pointed to the fact that the application of humane endpoints has the potential to increase error levels in experiments, which could necessitate the use of larger group sizes. The key to avoiding the necessity of repeating studies is to use the correct number of animals according to the experimental design and the nature of the endpoint being investigated.

### *Replacement*

Participants developed a list of potential nonanimal methods for subchronic/chronic and carcinogenicity testing, which should be further investigated for their potential to partially or fully replace animal-based tests (Table 2).

It was recognized that progress has been slow in developing replacement tests for reproductive and developmental toxicity testing, although several tissue culture screens for embryotoxicity and teratogenicity have been developed (Genshow et al. 2000, 2002), including the use of stem cells.

Participants discussed a potential general scheme for integrated toxicity testing (Figure 1). This scheme involves the application of the quantitative structure activity relationship ($QSAR^2$) as a crucial first stage, followed by the use of information from biomarkers of exposure and effect, barrier models, and basal cell cytotoxicity in conjunction with cytotoxicity to target organs and biokinetic modeling to estimate target organ doses of administered chemicals.

A major factor that inhibits the use of tissue culture methods is a concern that cell lines and other in vitro systems do not adequately mimic the response of cells in vivo at the target site within whole animals. This concern can be addressed by (1) an increased use of human primary cells, (2) the development of complex organotypic culture systems, and (3) the addition of cofactors and metabolic supplements to cell culture medium and cell immortalization to increase the longevity and decrease cell dedifferentiation.

### *Current Situation Regarding Carcinogenicity Testing*

The Breakout Group discussed the current uncertainty regarding the need for a two-species rodent bioassay for car-

**Table 1 Main ways to achieve reduction**

- Minimize experimental variation (e.g., diet, strain/stock, housing, age, gender, microbiological status).
- Increase emphasis on strategic planning (i.e., consider carefully whether an experiment is really necessary or whether it can be partially or completely undertaken without animals).
- Apply optimized experimental designs together with correct and appropriate statistical methods.
- Harmonize international guidelines (e.g., as has been achieved for pharmaceuticals through the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH]).

**Table 2 Potential nonanimal replacement methods for subchronic/chronic and carcinogenicity testing[a]**

1. Review and use of available data
2. (Q)SAR/expert prediction systems
   Predict toxicity by agents acting via genotoxic mechanisms (less appropriate for nongenotoxic mechanism)
3. Use of lower organisms (e.g., protozoa, bacteria, viruses, bacteria and nematode worms, and the fruit fly) and the early developmental stages of vertebrates
   The Ames *Salmonella typhymurium* reverse mutation test and chromosomal aberration assays have been accepted by regulatory agencies for some time.
4. Toxicogenomics (differential gene expression) and proteomics in cells
5. Tissue culture (using a variety of mammalian and human cell systems)
   Mammalian cell genotoxicity tests (e.g., mutation and chromosomal aberration assays) have been accepted by regulatory agencies for some time. Only a few human cell-based transformation systems have been developed, and no well-defined assays are as yet available.
   The SHE cell assay has been shown to be sensitive to nongenotoxic carcinogens and is currently accepted by some regulatory agencies as part of a weight of evidence for hazard identification.
6. Human studies: Volunteers/patients and epidemiology (retrospective studies)

[a]With any of these methods, more information is required on the relevance of nonanimal methods to human hazard assessment and on the scientific justification for eventually replacing the rodent with a combination of non-animal tests and subchronic and chronic studies.

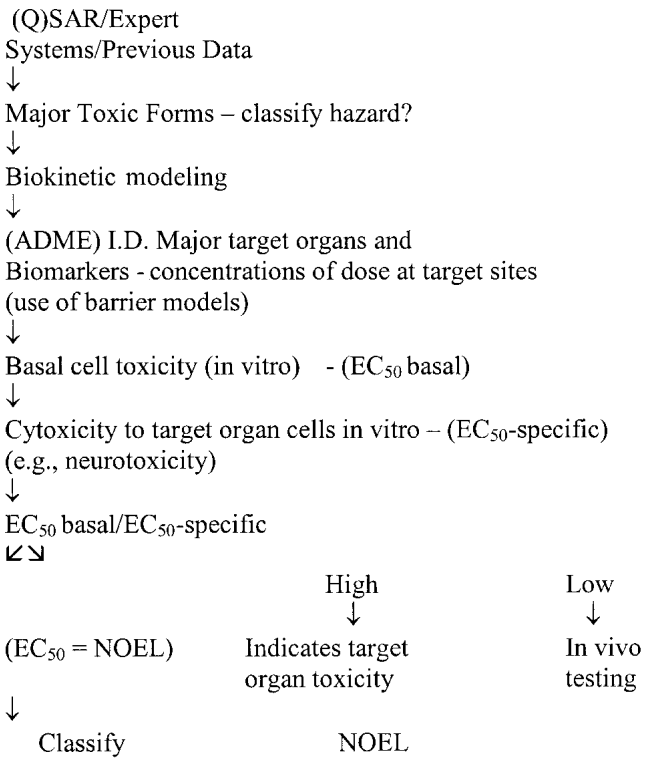


**Figure 1** Integrated toxicity testing, a possible scheme. $EC_{50}$, the test substance concentration that results in a 50% reduction in the effect measured; NOEL, no observed effect level; QSAR, quantitative structure activity relationship.

cinogens, rather than the potential use of one bioassay in the rat, accompanied by a transgenic mouse assay. Although transgenic animal models have been developed for both mutagenicity and carcinogenicity testing in rodents, these test methods are still in the process of being validated. However, at least one regulatory agency (US Food and Drug Administration) is accepting data from transgenic mouse models as part of the safety assessment of selected pharmaceuticals. There is a need for assessment of the International Life Sciences Institute coordinated studies on these models, and for regulatory agencies to determine the usefulness, limitations, applicability, and acceptability of these models for their regulatory testing needs. In addition, appropriate harmonization should be pursued among regulatory agencies with respect to regulatory uses of transgenic models, as has been reached under the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Hierarchical and integrated testing schemes are currently being implemented in many testing programs. Apart from genotoxicity testing, examples where hierarchical schemes have either been proposed and/or implemented and where their use needs to be encouraged include (1) the 2-yr mouse carcinogenicity bioassay, (2) endocrine disruptor testing, and (3) HPV chemicals testing.

Currently, two biomarkers are being used as early signs of tumorigenesis and none are used for predicting other chronic effects. It is expected that biomarker use will gradually increase with the growing use of genomic and proteomic analysis.

## Summary of Breakout Group Recommendations

### Current Best Practices for Minimizing Pain and Distress

- The OECD document on humane endpoints and other relevant references should be used and incorporated into regulatory testing guidelines as appropriate.
- Humane endpoint guidance should be regularly updated to incorporate advances in animal welfare research and changing regulatory requirements.
- Scientific and professional judgment should always be used when interpreting guidelines and procedures for the application of humane endpoints.
- Humane endpoints should be applied in a manner that allows achievement of safety assessment testing objectives.
- Each institution should develop more specific and detailed protocols and SOPs for humane endpoints, incorporating the principles of the OECD guidance document and other relevant references. Details for monitoring should be specific to each endpoint, species, and strain/stock.
- Understanding of the relevance of animal models and studies to human hazard identification should continually be improved.

### Implementation of Current Best Practices

- Information on humane endpoints should be provided on intranet and internet sites.
- Results and experiences from the implementation of earlier, more humane endpoints should be published.
- Useful criteria for endpoints should be standardized, especially for subjective endpoints (e.g., moribund condition).
- Extreme endpoints (e.g., signs of severe pain and distress, excessive tumor burden, and moribund condition) should be avoided whenever possible.
- The concept of humane endpoints should be introduced into training programs with relevant and effective educational material, including courses and refresher courses for certification (e.g., American College of Laboratory Animal Medicine and the UK Module 5 training courses).
- Scientists should be made aware of the need for proactive monitoring and evaluation of animal well-being.
- Recognized processes for validating humane endpoints should be established.

- Regulatory agencies should publish their guidelines and/or requirements for the definition and use of humane endpoints.
- Regulatory agencies should encourage discussion of the use of humane endpoints, including data sharing of information and experiences concerning the use of humane endpoints and other refinement strategies (Langley et al. 1999).
- All institutions should have animal care committees, and should educate members on the concept and potential for applying humane endpoints.
- All institutions using animals for testing should implement a strategic planning scheme for each study to maximize the application of the 3Rs.
- Scientists should be encouraged to maximize data collection from animals without subjecting them to an increased number of procedures.
- The OECD guidance document should be referenced in all official good laboratory practice guidelines and in all new versions of existing and novel OECD test guidelines, and scientists should be required to consult the document.
- The International Council of Laboratory Animal Science should encourage OECD Member Countries to require scientists working with animals in their countries to follow a recognized guidance document on the 3Rs.
- Strategic planning should include the identification of earlier humane endpoints/markers that can further reduce pain and distress, recognizing that this inclusion may increase statistical variation and necessitate larger group sizes.
- Editors of scientific journals should insist on evidence of the use of best practices for research data submissions.
- Professional societies and accreditation bodies should recognize and promote the use of humane endpoints.
- Effective communication is needed between all those involved in animal testing and associated staff in industry and regulators.

## Future Work

- New clinical biomarkers of exposure and effect that indicate predictive signs and that differ from those found in control animals should be sought.
- The results of ongoing International Life Sciences Institute studies on transgenic mouse models for carcinogenicity should be assessed for the usefulness, limitations, applicability, and acceptability of these models for regulatory testing requirements.
- More in vitro screens for nongenotoxic carcinogens need to be developed. This research will require funding and coordination.
- The use of screening and computer prediction models to prioritize chemicals for further testing should be increased.
- Toxicity testing data should be used retrospectively to improve QSAR[1] models. These predictive approaches should be validated formally as soon as possible.

The future use of QSAR will require (1) more reliance on mechanisms of toxicity than on structural analogues, (2) research to identify new relevant receptors to incorporate into the models, (3) the incorporation of information from drug development and from the human genome mapping project, (4) better use of information derived by extrapolating from receptor structure and properties to toxicity, (5) the availability of high-quality reliable and relevant information to construct extensive databases, (6) better interdisciplinary dialogue between scientists undertaking fundamental and applied research, and (7) increased targeted funding.

- Human cells and tissues need to become more widely available to facilitate the identification of potential health effects of chemicals.
- The search for solutions to problems in data handling, interpretation, and experimental reproducibility for genomics and proteomics needs to be promoted for these approaches to become more useful for subchronic, chronic, and carcinogenicity regulatory testing.
- Genomic and proteomic techniques should continue to be investigated for their potential usefulness for screening purposes and for understanding the basic mechanisms of toxicity.
- A wide range of complementary studies that might supply diverse toxicity information and might be considered for incorporation into integrated testing schemes needs to be investigated.

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## Integrated Testing Strategies—Examples

- <http://www.epa.gov/opptintr/chemrtk/ceoltr.htm#B>.
- Original letter from EPA regarding need for HPV chemical testing <http://www.epa.gov/opptintr/chemrtk/ceoltr1.htm>.
- Letter from EPA underlining animal welfare-related concerns <http://www.epa.gov/opptintr/chemrtk/ceoltr2.htm>.
- Letter from EPA reiterating animal welfare-related concerns <http://www.epa.gov/opptintr/chemrtk/ceoltr3.htm>.
- Test Smart program for HPV Testing B Center for Alternatives to Animal Testing <http://caat.jhsph.edu/programs/workshops/testsmart/hpv-intro.htm>.
- Test Smart Program for Endocrine Disruptors <http://caat.jhsph.edu/programs/workshops/testsmart/endo-intro.htm>.
- Commission of the European Communities (2001). White Paper: Strategy for a Future Chemicals Policy

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