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CHAPTER 3

Development of an Evaluation Support System for Estimating Repeated-Dose Toxicity of Chemicals Based on Chemical Structure

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3.1 Introduction

In Japan, industrial chemicals are regulated by the Chemical Substances Control Law (CSCL) for the prevention of environmental pollution caused by chemical substances that exhibit a risk of impairing human health or interfering with the inhabitation and/or growth of flora and fauna. This law mandates prior examination of the hazardous properties of new chemical substances which are intended to be manufactured in or imported into Japan.

The safety evaluations of chemicals designated as "existing chemicals" under CSCL have been continuously conducted by the government since implementation of CSCL. As a result, various test data for existing chemicals were

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accumulated for certain endpoints such as biodegradation, bioconcentration, genotoxicity, repeated (mainly 28 days) dose toxicity and ecotoxicity. The test data for the existing chemicals under CSCL are disclosed on the websites of public institutions:[1,2]

Based on the test data for new and existing chemicals, the activities for developing evaluation methods by (quantitative) structure–activity relationship [(Q)SAR] and the category approach[3] have been conducted under governmental initiatives.[4–8] These methods are utilized in the prioritization of untested existing chemicals or the preparation of the reference information in the examination of new chemicals.

Repeated dose toxicity (RDT) testing is one of the important endpoints of CSCL. However, a reliable and satisfactory (Q)SAR or category approach method for evaluating the RDT of chemicals still remains undeveloped. Generally, the purpose of RDT is to determine values such as the no-observed-effect level (NOEL), no-observed-adverse-effect level (NOAEL), lowest observed effect level (LOEL), and lowest observed adverse effect level (LOAEL). These values are determined by experts in the field based on test data of various endpoints related to hematology, blood biochemistry, and histopathology. Therefore, it is difficult to directly correlate the NOEL and other parameters with chemical structure since all these parameters are influenced by many toxicological events. In order to make an *in silico* evaluation of RDT for target chemicals from their chemical structure, a comprehensive collaboration of experts possessing a variety of knowledge on the analogue chemicals is necessary.

The project, "Development of hazard assessment techniques using structure–activity relationship methods",[9] sponsored by New Energy and Industrial Technology Development Organization (NEDO) in Japan, aims to develop the "Hazard Evaluation Support System Integrated Platform (HESS)"[10] for providing decision support information to experts to evaluate the RDT of chemicals by the category approach (project period: 2007–2011). This chapter contains an overview of HESS and describes the methodology for evaluating RDT by the category approach developed in this project. The trial version of the system has been developed and will be available in 2012.

3.2 Overview of the Hazard Evaluation Support System Integrated Platform (HESS)

As shown in Figure 3.1, HESS has two main databases. One is the Toxicity Knowledge Information Database containing RDT test reports and toxicity mechanism information. The other is the Metabolism Knowledge Information Database containing metabolic maps and Adsorption, Distribution, Metabolism, and Excretion (ADME) information. HESS has two support tools for estimating the RDT of chemicals by using information from these databases—the Bayesian Net RDT Prediction Model and the Category Approach Support Function. HESS is designed to be compatible with the Organisation for

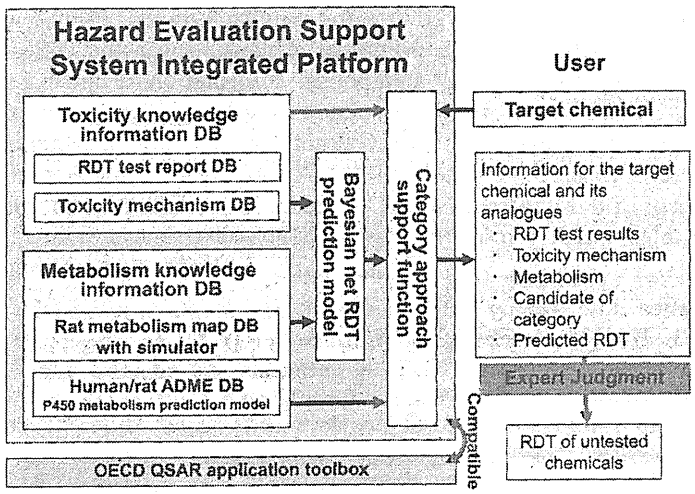


Figure 3.1 Structure of the Hazard Evaluation Support System Integrated Platform (HESS).

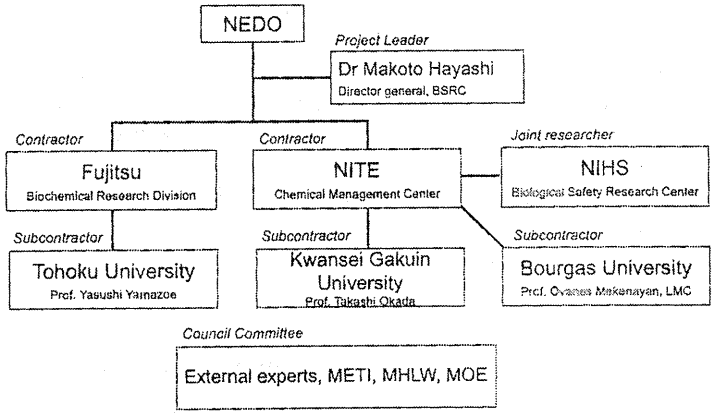


Figure 3.2 Organization of the "Development of hazard assessment techniques using structure–activity relationship methods" project.

Economic Co-operation and Development (OECD) (Q)SAR Application Toolbox.[11]

Figure 3.2 shows the organizational structure of the project. The project leader is Dr Makoto Hayashi who is the Director General of Biosafety Research Center, Foods, Drugs and Pesticides (BSRC) and former Head of the Division of Genetics and Mutagenesis, National Institute of Health Sciences (NIHS). The main contractors of the project are National Institute of

Technology and Evaluation (NITE) and Fujitsu Limited. NITE, in a joint research effort with NIHS, is primarily responsible for the collection and systemization of toxicity and metabolism information. Fujitsu Limited is primarily responsible for building the database systems. A group of experts led by Prof. Takashi Okada of Kwansei Gakuin University and a group of experts led by Prof. Yasushi Yamazoe of Tohoku University are responsible for development of the Bayesian Net RDT Prediction system and development of the metabolism prediction method, respectively.

HESS is not designed to be an "automatic prediction system". Rather, the aim is to provide support for experts such as toxicologists, pathologists and risk assessors in making toxicological decisions. Therefore, it is very important for the system to be easy to use by such experts. As such, the system is being developed primarily by toxicologists, pathologists and risk assessors. This is the most important feature of our project and is largely different from other projects in which systems are developed primarily by IT experts.

The other important feature of this project is its international focus. Since this system is designed to be compatible with the OECD (Q)SAR Application Toolbox and is intended to be used internationally, Professor Menkenyan's team from the Laboratory of Mathematical Chemistry (LMC) of Bourgas Prof. Assen Zlatarov University, responsible for building the OECD (Q)SAR Application Toolbox system, was invited to join the project. Accordingly, the project is coordinating closely with OECD activities.

In addition, recommendations from regulatory authorities are also being taken into consideration in the development of HESS. External experts in relevant fields and members of three ministries [The Ministry of Economy, Trade and Industry (METI); The Ministry of Health, Labour and Welfare (MHLW); and The Ministry of Environment (MOE)] are participating in the supporting committee of the project.

The following is an overview of each part of HESS:

3.2.1 Repeated-Dose Toxicity Test Report Database

This database contains RDT test reports (mainly 28-day RDT using rats with oral administration) for about 500 chemicals. The test data for the existing chemicals under CSCL comprise the main part of the database (about 300 chemicals). All these tests were conducted in compliance with GLP principles under the auspices of the Japanese government (MHLW, METI and NITE). In addition, other RTD test reports with detailed data and high reliability are included in the system (*e.g.*, US National Toxicology Program (NTP) data,[12] data from journal papers, *etc.*).

In this database, test report information is organized and stored in a uniform format in order to facilitate comparison of test data between chemicals. The data for hematological examinations, blood chemical examinations and histopathological examinations are grouped into individual data tables and incorporated into the RDT Test Report Database. In these group tables, the

data showing statistically significant differences from the control group are noted with a mark. However, it is difficult to compare the data among test reports using different statistical analyses methods because the statistically significant differences depend on the analysis method used.[13] Therefore, in this database, data determined to be a toxicological effect by experts are marked differently.

Histopathological terms used in the test reports generally differed between the various laboratories. In order to make searches of histopathological findings more complete and effective, we developed a thesaurus as part of the database search engine.[14]

3.2.2 Toxicity Mechanism Database

The purpose of the database is to provide the rationale for the categorization of toxicity at repeated dose levels. This database contains information from about 220 original papers that suggests the mechanism of the toxicological effects observed in the collected RDT test reports of about 70 chemicals. The toxicity mechanism information covers molecular level mechanisms, cellular level mechanisms and biological level mechanisms and contains *in vitro*, *in vivo* and *ex vivo* test results, signal transfer pathways, *etc.* We expect the database to provide appropriate information for use with the adverse outcome pathways (AOP).

3.2.3 Rat Metabolism Map Database with Metabolic Simulator

This database contains metabolic maps for about 680 chemicals with repeated dose toxicity test reports. All parental chemicals and metabolites in the database can be searched by chemical structure, and the database can be used for investigating metabolites that cause toxicological effects. In addition, a simulator for estimating metabolites in rat liver has been built based on the metabolic maps.

3.2.4 Human/Rat ADME Database (P450 Metabolism Prediction Model)

This database contains information from about 280 original papers related to ADME information on humans and rats for about 80 chemicals with RDT test reports. A model for predicting metabolites of human P450 (CYP2E1, *etc.*) based on the substrate structure was developed. The prediction results for about 100 chemicals with repeated dose toxicity tests are also included in the database. This database can be used for comparing species differences in toxicity between humans and rats based on their different metabolisms.

3.2.5 Bayesian Net RDT Prediction Model

In this model, causality of toxicity is expressed as a network of conditional probabilities (Bayesian network) to predict the probability for a chemical to induce a specified toxicity.[15] Detailed repeated dose toxicity test data (hematological examinations, blood chemical examinations and histopathological examinations) were analyzed by the cascade model,[16] a data mining method to extract structure alerts used in the Bayesian Net RDT Prediction Model.[17] The model was built using the expertise of toxicologists and pathologists.

3.2.6 Category Approach Support Function

Chemicals considered to have the same AOP in RDT tests were categorized based on information in the Toxicity Knowledge Information Database and the Metabolism Knowledge Information Database. The definitions of the categories (structure, parameter, mechanism of action and metabolism boundaries) were entered in the system as a category library of 36 categories.

The system operates in the following way: When a user inputs a target chemical for evaluation, the system searches the category library for possible categories to which the target chemical belongs. Information about the target chemical and its analogues, extracted from the Toxicity Knowledge Information Database and the Metabolism Knowledge Information Database, are displayed in a format that can be easily analyzed by the system user. The user can also investigate the category to which the target chemical belongs and fill in data gaps using data from the analogue chemicals.

3.3 Example of the Category Approach for Evaluating Repeated-Dose Toxicity Tests

It is important to establish a methodology for applying the category approach to complex endpoints in the field of chemical management. OECD is proposing using AOP principles in applying the category approach to complex endpoints.[18] The AOP indicates the mechanistic pathway to the outcome in the test from the molecular initiating event. We also included AOP principles in applying the category approach to repeated dose toxicity tests based on the OECD proposal.[19]

The outcomes observed in repeated dose toxicity tests largely depend on the dose levels tested. They are a complex combination of the findings recognized as the effects of the test chemical. Accordingly, we described an adverse effect as a combination of findings. We also described the mechanism by which these adverse effects are induced from molecular level to *in vivo* level as the AOP for repeated dose toxicity.

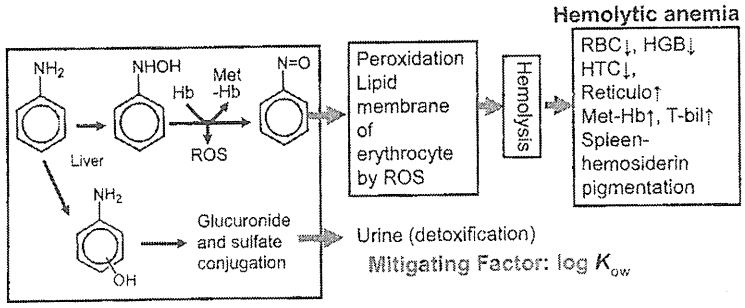


Figure 3.3 AOP for describing the hemolytic anemia induced by anilines.

Figure 3.3 shows the AOP for describing the hemolytic anemia induced by anilines in repeated dose toxicity tests in rats. *N*-hydroxyanilines, metabolites of anilines in the liver, react with hemoglobin to produce methemoglobin (Met-Hb) and reactive oxygen species (ROS). ROS then causes peroxidation of lipids in the membranes of erythrocytes, resulting in hemolytic anemia. When hemolytic anemia is induced, hematological examinations yield the following observations: a decrease in the number of erythrocytes (RBC↓), a decrease in hemoglobin (HGB↓), a decrease in hematocrit (HTC↓) and an increase in reticulocytes (Reticulo↑). In addition, an increase in total bilirubin (T-bil↑) can be observed in blood biochemical examinations, and pigmentation of hemosiderin in the spleen is typically observed in histopathological examinations.

In order to create a category of anilines that induce hemolytic anemia, it is necessary to specify anilines that induce hemolytic anemia by the same pathway as shown in Figure 3.3. Table 3.1 shows some anilines with information related to hemolytic anemia. This information is contained in the Toxicity Knowledge Information Database and the Metabolism Knowledge Information Database. The findings related to hemolytic anemia shown in the table is obtained from RDT tests in male rats. The test for chemicals No. 4 and No. 8 is the Combined Repeat-Dose and Reproductive/Developmental Toxicity Screening test. The tests for the other chemicals are 28-day repeated dose toxicity tests.

This table shows that, as a rule, anilines with the potential to induce hemolytic anemia have a log $K_{ow} > 1$ (Nos. 1–8). On the other hand, two chemicals (amino phenols Nos. 9 and 10) with low log K_{ow} values of 0.24 showed only a weak potential to induce hemolytic anemia at high-dose levels. Three chemicals, amino benzene sulfonic acids (Nos. 11–13) that had even lower log K_{ow} values (negative values) lack the potential to induce hemolytic anemia even at high-dose levels. The reason aminophenols and amino benzene sulfonic acids exhibit weak or no potential to induce hemolytic anemia can be explained by their high excretion rate in urine due to their high water solubility.[20] These results indicate that a low log K_{ow} value could be used as a mitigating factor in the formation of category boundaries.

Table 3.1 Evidence related to the adverse outcome pathway in Figure 3.3 for anilines.

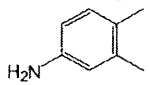
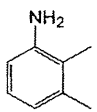
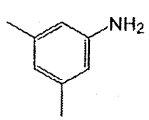
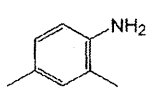
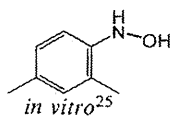
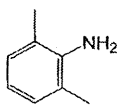
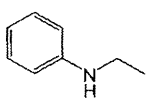
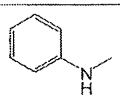
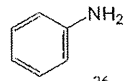
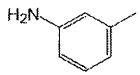
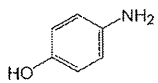
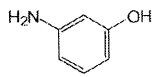
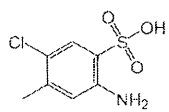
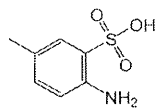
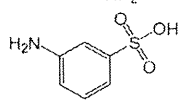
No.	*Structure*	*Observed findings related to hemolytic anemia in the repeated dose toxicity tests for male rats (dose mg^{-1} kg^{-1} day^{-1})*	*log K_{ow}**	*Mechanism information*		*Metabolism information*
				Met-Hb	*HBI*[23–24]	
1		RBC ↓ • Hgb ↓ · Hct ↓ · Ret ↑ Spleen pigmentation (250)	2.17	*in vitro*[21] *in vivo*[21]	0.7	—
2		Hgb ↓ (60) RBC ↓ · Hct ↓ · Ret ↑ · MetHb ↑ T-Bil ↑ (300)	2.17	*in vitro*[21] *in vivo*[21]	—	—
3		Hgb ↓ · Hct ↓ · Ret ↑ Spleen pigmentation (60) RBC ↓ (360)	2.17	*in vitro*[21] *in vivo*[21]	14	—
4		Bil ↑ (2) Hgb ↓ (10)	2.17	*in vitro*[21] *in vivo*[21]	2.3	 *in vitro*[25]
5		RBC ↓ · Hgb ↓ · Ret ↑ · Met-Hb ↑ Spleen pigmentation (250)	2.17	*in vitro*[21] *in vivo*[21]	1.1	—
6		RBC ↓ (5) Hgb ↓ · Hct ↓ · Ret ↑ · Met-Hgb ↑ Spleen pigmentation (25)	2.11	—	—	—

Table 3.1 *(Continued)*

No.	*Structure*	*Observed findings related to hemolytic anemia in the repeated dose toxicity tests for male rats (dose mg^{-1} kg^{-1} day^{-1})*	*log K_{ow}**	*Mechanism information*		*Metabolism information*
				Met-Hb	*HBI*[23-24]	
7		RBC ↓ · Hgb ↓ · Hct ↓ · Ret ↑ Spleen pigmentation (25) Bil ↑ (125)	1.62	—	—	 *in vitro*[26]
8		Spleen pigmentation (30) RBC ↓ · Hgb ↓ · Hct ↓ · Bil ↑ (100)	1.62	*in vitro*[22] *in vivo*[22]	4.9	—
9		RBC ↓ (500)	0.24	—	—	—
10		Bil ↑ · Spleen pigmentation (720)	0.24	—	—	—
11		—	−0.89	—	—	—
12		—	−1.53	—	—	—
13		—	−2.08	—	—	—

*Calculated by KOWWIN 1.67 (US EPA).

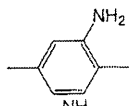
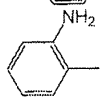
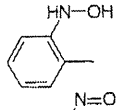
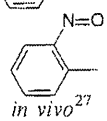
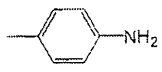
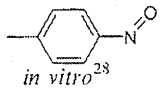
Mechanism and metabolism information can be used as supporting evidence for the pathway shown in Figure 3.3. For example, Met-Hb is not measured in many repeated dose toxicity tests, but the production of Met-Hb can be strong evidence for the pathway shown in Figure 3.3. However, such an information gap can be complemented by other studies.[21,22] For example, hemoglobin binding index (HBI)[23,24] values measured in other studies and the presence of metabolites such as *N*-hydroxy-, or nitroso-amines observed in other studies can be evidence for the pathway.

In comparison to the other anilines in the Table 3.1, 2,4-dimethylaniline (No. 4 in Table 3.1) did not show the same apparent potential to induce hemolytic anemia. This can be explained by the fact that the chemical was tested at rather low dose levels. However, the possible potential for this chemical to induce hemolytic anemia can be predicted by the pathway shown in Figure 3.3 by the supporting evidence that the production of Met-Hb was observed[21] as a result of the production of *N*-hydroxy-2,4-dimethylaniline as a metabolite[25] of the target chemical.

In the case of *N*-methylaniline (No. 7), the existence of aniline as a metabolite[26] in the *in vitro* test supported the fact that *N*-methylaniline induces hemolytic anemia. Therefore, it is suggested that the boundary of the category can be extended from primary aniline to *N*-alkyl aniline.

Based on the above discussions, chemical Nos. 1–8 in Table 3.1 can be specified as the category members inducing hemolytic anemia by the pathway shown in Figure 3.3. The boundary of the category can be defined not only by

Table 3.2 Evidence related to the adverse outcome pathway in Figure 3.3 for untested anilines.

		Observed findings related to hemolytic anemia in the repeated dose toxicity tests for male rats (dose $mg^{-1}\ kg^{-1}\ day^{-1}$)		*Mechanism information*		
No.	*Structure*		*log K_{ow}**	*Met-Hb*	*HBI*[23,24]	*Metabolism information*
14		Untested	2.17	*in vitro*[21] *in vivo*[21]	7.3	—
15		Untested	1.62	—	4.0	  *in vivo*[27]
16		Untested	1.62	—	4.3	 *in vitro*[28]

*Calculated by KOWWIN 1.67 (US EPA).

the chemical structure but also by the activity of the chemicals. In the case of the members mentioned above, the boundary of the category can be defined as monocyclic anilines and alkylated anilines with log $K_{ow} > 1$. In HESS, the boundaries of RDT categories defined by such an approach are registered in the category library.

It is also necessary to consider AOPs using evidence other than actual RDT data in order to find an appropriate category for chemicals lacking experimental data. For example, from the structure of an untested chemical, we can predict the reactivity in the molecular initiating event in an AOP based on, for example, the nature and position of a substitution group. Table 3.2 shows examples of chemicals in a category defined by the method described above and the supporting evidence. Based on the RDT test data of the category members (Nos. 1–8 in Table 3.1), these chemicals (Nos. 14–16 in Table 3.2) can be estimated to induce hemolytic anemia at dose levels less than 250 mg kg^{-1} day^{-1}.

3.4 Conclusions

In this chapter, we have given an overview of HESS and the category approach for evaluating RDT tests, as well as an example of categorization by this system. Because this system is designed to provide detailed RDT test data in conjunction with mechanism and metabolism information, the system can provide reliable information based on scientific evidence to the experts who evaluate the safety of chemicals. Completion of the final version of the system is planned for 2012.

Acknowledgements

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Reduction of use of animals in regulatory genotoxicity testing: Identification and implementation opportunities—Report from an ECVAM workshop☆

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ABSTRACT

In vivo genetic toxicology tests measure direct DNA damage or the formation of gene or chromosomal mutations, and are used to predict the mutagenic and carcinogenic potential of compounds for regulatory purposes and/or to follow-up positive results from *in vitro* testing. These tests are widely used and consume large numbers of animals, with a foreseeable marked increase as a result of the EU chemicals legislation (REACH), which may require follow-up of any positive outcome in the *in vitro* standard battery with appropriate *in vivo* tests, regardless of the tonnage level of the chemical.

A 2-day workshop with genotoxicity experts from academia, regulatory agencies and industry was hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in Ranco, Italy from 24 to 25 June 2008. The objectives of the workshop were to discuss how to reduce the number of animals in standard genotoxicity tests, whether the application of smarter test strategies can lead to lower animal numbers, and how the possibilities for reduction can be promoted and implemented.

The workshop agreed that there are many reduction options available that are scientifically credible and therefore ready for use. Most of these are compliant with regulatory guidelines, i.e. the use of one sex only, one administration and two sampling times *versus* two or three administrations and one sampling time for micronucleus (MN), chromosomal aberration (CA) and Comet assays; and the integration of the MN endpoint into repeat-dose toxicity studies. The omission of a concurrent positive control in routine CA and MN tests has been proven to be scientifically acceptable, although the OECD guidelines still require this; also the combination of acute MN and Comet assay studies are compliant with guidelines, except for sampling times.

☆ This document represents the consensus view of the participants as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

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Based on the data presented at the workshop, the participants concluded that these options have not been sufficiently utilized to date. Key factors for this seem to be the uncertainty regarding regulatory compliance/acceptance, lack of awareness, and an in many cases unjustified uncertainty regarding the scientific acceptance of reduction options. The workshop therefore encourages the use and promotion of these options as well as the dissemination of data related to reduction opportunities by the scientific community in order to boost the acceptance level of these approaches. Furthermore, experimental proof is needed and under way to demonstrate the credibility of additional options for reduction of the number of animals, such as the integration of the Comet assay into repeat-dose toxicity studies.

1. Introduction

In vivo genetic toxicology tests are used to predict the mutagenic and carcinogenic potential of compounds for regulatory purposes and/or to follow-up positive results from *in vitro* testing. They measure direct DNA damage, its repair or the formation of gene or chromosomal mutations following induction of DNA damage by test compounds. These tests are widely used for pharmaceuticals, industrial chemicals, pesticides, biocides, food additives and cosmetic ingredients. The results form the scientific basis for risk assessment and are used for classification and labelling (C&L) of chemical substances in the EU (the Dangerous Substances Directive 67/548/EEC [1] and Regulation (EC) No. 1272/2008 on the classification, labelling and packaging of substances and mixtures [2]), and across the world (UN Globally Harmonised System (GHS) [3]).

In the scientific community efforts are increasing to replace *in vivo* tests by appropriate *in vitro* tests, especially driven by regulations enforced within the European Union (EU) such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) [4] and the 7th Amendment to the Cosmetics Directive [5]. The 7th Amendment prohibits any acute *in vivo* genotoxicity tests for cosmetic ingredients after March 2009, thereby triggering search for innovative hazard and risk assessment concepts. Tens of thousands of substances being tested for genotoxic potential for the purposes of REACH registration, using a classical testing scheme, could potentially lead to a very high number of additional tests in animals. The requirements as described in the REACH Integrated Testing Strategy (ITS) (Guidance on information requirements and chemical safety assessment) [6] signal the follow-up of any positive outcome in the *in vitro* standard battery with appropriate *in vivo* tests, regardless of the tonnage level of the chemical.

Taking into account the problem of low specificity of the current standard *in vitro* tests to discriminate rodent non-carcinogens from carcinogens [7,8], it becomes clear that the improvement of current *in vitro* tests (i.e. reduction of "false positives"), and the search for new approaches to *in vivo* testing that could lead to a reduction in the number of animals, present key challenges for genetic toxicologists. In a workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2006, strategies to reduce false positive results from *in vitro* tests were discussed [9] and research is under way to address this. While this will likely reduce the number of *in vivo* studies required to follow-up positive outcomes from *in vitro* tests, additional efforts will be needed to ensure a reduction – rather than further increases – of the total number of animals. This is because of the very large number of chemical substances that will be subject to the REACH information requirements and the mandatory *in vivo* testing requirements that still apply to some categories of substances (e.g. pharmaceuticals, pesticides).

The ECVAM Task Force Genotoxicity and Carcinogenicity has recently conducted a survey on opportunities to reduce the number of animals in genotoxicity testing. A questionnaire was sent to industry and contract research organisations (CROs) with the goal to investigate current practice regarding animal use in the *in vivo* micronucleus and chromosomal aberration tests (e.g. number of animals, gender, use of negative and positive controls, etc.). The results of this survey revealed that the opportunities for reduction provided by the OECD guidelines and IWGT recommendations [10,11], although apparent for many years, have not been implemented generally [12]. For example, although there is the possibility to use one sex of test animal only and an option to omit some controls, the majority of laboratories still use animals of both sexes and a concurrent positive control with every assay. When asked whether a reduction of the size of the positive control group would be acceptable, all participants of the survey were prepared to follow this – provided that this would be accepted by the regulatory authorities. A further trigger for a subsequent workshop was the ongoing revision of the ICH guidelines for genotoxicity testing, which strongly promotes the integration of genotoxicity tests into repeat-dose toxicity (RDT) studies, an approach also mentioned as an option in the REACH ITS.

The 2-day workshop was hosted and sponsored by ECVAM in Ranco, Italy from 24 to 25 June 2008. Seventeen genotoxicity experts from academia, regulatory authorities and industry were invited to contribute their experiences. The objectives of the workshop were:

- To discuss how to reduce the number of animals in standard genotoxicity tests.
- To discuss whether the number of animals can be reduced by application of smarter test strategies.
- To find a way forward how these possibilities for reduction can be best promoted and implemented.

The overriding premise agreed by all participants was that a reduction of the number of animals in genotoxicity testing should not compromise the safety standards, and that a poorly conducted and poorly designed *in vivo* study (i.e. with too few animals) is a waste of animals.

2. Summaries of the presentations given at the workshop

Information from the presentations given by various participants relevant to decision-making is summarised below.

2.1. Regulatory background

Raffaella Corvi from ECVAM, Italy informed the group that the Joint Research Centre of the European Commission estimated that, under REACH, genotoxicity is among the endpoints for which the highest number of *in vivo* tests will be needed [13]. Stefan Pfuhler from Procter & Gamble, Switzerland, summarized the opportunities that the REACH ITS [6] offers to the toxicologist to reduce the number of animals used in this area. These include the integration of genotoxicity endpoints into repeat-dose toxicity studies "if scientifically justified", and the need for a second *in vivo* study only if it is required to conclude on the relevance of the positive results *in vitro* (as opposed to the performance of a mandatory

second *in vivo* study triggered by tonnage only [4]). Furthermore, the *in vivo* Comet assay is listed as a suitable follow-up for positive results from both *in vitro* gene mutation and chromosomal aberration (CA) endpoints. This may enable registrants to omit a second *in vivo* assay irrespective of the *in vitro* profile. It was recognised that the Comet assay does not detect possible aneugenic chemicals; these, however, can be picked up at the *in vitro* testing stage.

The impact of the revision of the ICH S2 guideline on reduction in animal usage was presented by Peter Kasper from the German Federal Institute for Drugs and Medical Devices (BfArM). The revised guideline, which at the time of the workshop was one step away from finalization, touches several areas that are expected to affect (lower) the number of animals to be used for genotoxicity testing of pharmaceuticals. The main changes compared to the guideline currently in place are:

- The highest concentration used in *in vitro* mammalian cell assays is decreased from 10 to 1 mM, which should reduce the number of irrelevant positive results and lead to a decrease of *in vivo* follow-up studies.
- One sex only approach as a default option for classical genotoxicity studies such as the bone marrow micronucleus (MN) test or the assay to detect unscheduled DNA synthesis (UDS) measured in hepatocytes *ex vivo*. The use of both sexes will only need to be considered if any existing data indicate a toxicologically meaningful sex difference in the species used.
- It is sufficient to include a positive control animal group (either concurrently or separately) only periodically, after a laboratory has established competence in the use of the assay.
- Integration of *in vivo* genotoxicity endpoints into repeat-dose toxicity (RDT) studies is preferable. In cases where this does not meet the criteria for a sufficient exposure, acute studies can be performed but testing of different endpoints and/or tissues should be combined into one study, where possible.

Two different scenarios for *in vivo* testing will be available for the pharmaceutical industry, see Fig. 1.

2.2. Use of one sex versus two sexes

David Kirkland from Covance, UK, presented data from 23 rat and 7 mouse MN studies where the positive control, cyclophosphamide (CPA), was compared in animals of both sexes. For CPA in rats, the MN response was greater in males than females in almost every study (Fig. 2A and B). The MN responses in female rats were similar or greater than in males only at 40 mg/kg but the responses in males were still positive. This dose is probably too high for routine use. In mice, the responses to CPA in males were never less than the responses in females (Fig. 2C).

Covance has also examined test chemical data from three rat studies using both sexes in which the test chemical gave a positive response. For these chemicals, positive responses were observed in all male rats whereas 1 out of 3 compounds were negative in female rats. Females only showed stronger MN responses than males when bone marrow toxicity was greater.

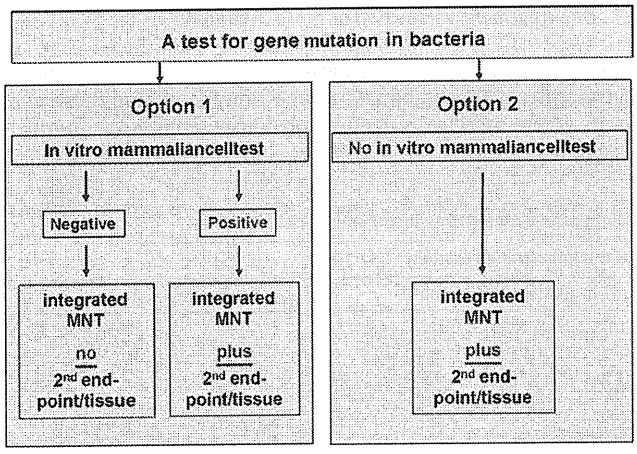

Fig. 1. Schematic of the draft revised ICH S2 (R1) guideline which provides the pharmaceutical industry with two options to perform the basic genotoxicity battery (integrated MNT = rodent micronucleus test with haematopoietic cells integrated into repeat-dose toxicity study).

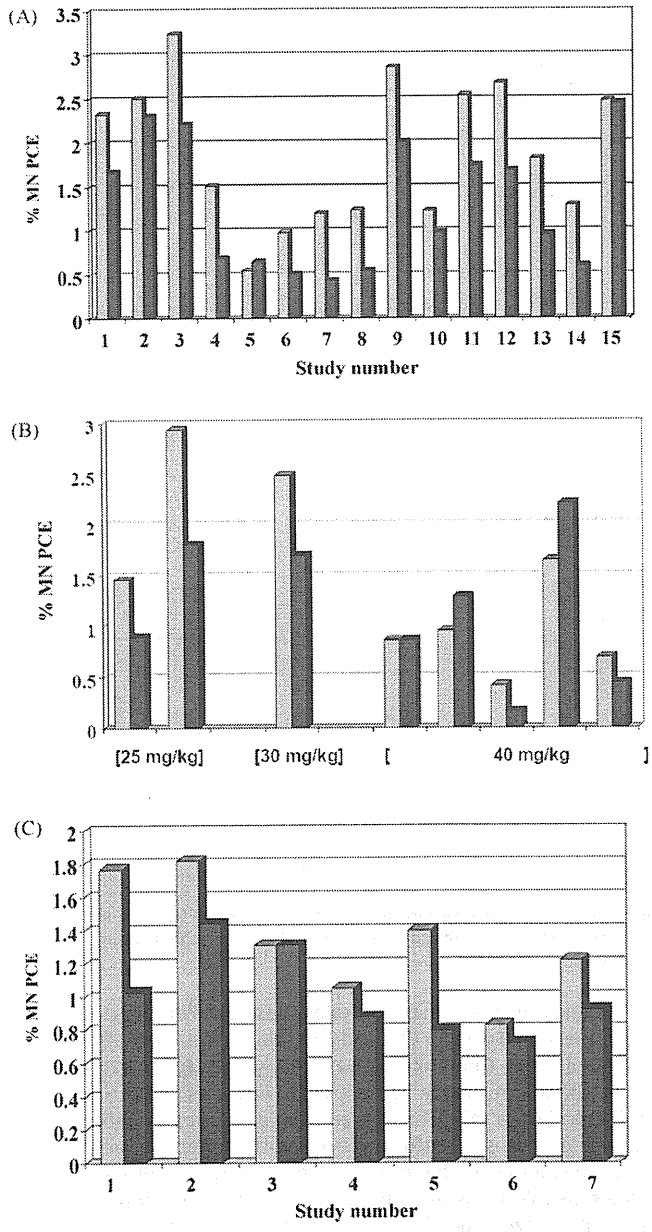

Fig. 2. Comparison of positive control group (cyclophosphamide) results between male (□) and female (■) rats from 23 rat and 7 mouse MN BM studies. All studies were carried out in the same laboratory. Studies compared were from male and female rats treated with 20 mg/kg cyclophosphamide (A), male and female rats treated with low, medium and high doses of cyclophosphamide (B) and male and female mice treated with 40 mg/kg cyclophosphamide (C).