Development of Alternative Carcinogenicity Test Methods in the Pharmaceutical Industry: Evaluation of the Current Validation/Application Status

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Abbreviations

3R	Replacement, Reduction, Refinement
ACT	Alternatives to Carcinogenicity Testing ACT
ADME	Absorption, Distribution, Metabolism and Excretion
ALTEX	Alternatives to Animal Experimentation
CAC	Carcinogenicity Assessment Committee
CAESAR	Computer assisted Evaluation of industrial chemical Substances according to Regulations
CAMM	Carcinogenicity Alternative Mouse Models
CHMP	Committee for Human Medicinal Products
CPMP	Committee for Proprietary Medicinal Products
CTA	Cell Transformation Assays
DCP	Decentralised Procedure
EC	European Commission
ECEAE	European Coalition to End Animal Experiments
ECOPA	European Consensus Platform on Alternatives
ECVAM	European Centre for the Validation of Alternative Methods
EMA	European Medicines Agency
ERGATT	European Research Group for Alternatives in Toxicity Testing
EU	European Union
FDA	Food and Drug Administration
HESI	Health and Environmental Sciences Institute
H-ras	harvey-ras
IARC	International Agency for Research on Cancer
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordination Committee on the Validation of Alternative Methods

ICH	International Conference on Harmonisation
IHCP	Institute for Health and Consumer Protection
ILSI	International Life Sciences Institute
ITS	Integrated Testing Strategies
IVMU	In Vitro Methods Unit
MIT	Massachusetts Institute of Technology
MRP	Mutual Recognition Procedure
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIHS	National Institute of Health Sciences
NRC	National Research Council
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
QSAR	Quantitative Structure-Activity Relationship
REACh	Registration, Evaluation and Authorisation of Chemicals
START-UP	Scientific and Technological Issues in 3Rs alternatives Research in the process of drug development and union politics
STP	Society of Toxicologic Pathology
Tox-21c	Toxicology in the 21st Century
TOXNET	Toxicolgy Data Network
TSCATS	Toxic Substances Control Act Test Submissions
ZEBET	Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch

1 Introduction

1.1 General Aspects of Carcinogenicity Testing

Carcinogenicity studies should be performed for any pharmaceutical whose expected clinical use is continuous for at least 6 months in the three ICH regions [1].

The most widely accepted test method of carcinogenicity testing for all kinds of chemical compounds used not only for pharmaceuticals, but also cosmetics and industrial chemicals used to be the two-year carcinogenicity test according to OECD Test No. 451: Carcinogenicity Studies [2]. The basic objective of this study 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. To assess whether a pharmaceutical compound bears carcinogenic potential, two rodent species, commonly rats and mice, are treated for two years daily. Both sexes are used and each dose group and concurrent control group contains commonly 50 animals of each sex. Parameters of these studies include measurement of body weight, food consumption, daily and detailed observations, clinical chemistry, as well as gross necropsy and histopathology. At least three dose levels and a concurrent control should be used [2]. In summary, as three different doses are tested and a control group must be included, at least 400 animals are needed for each test.

The limitations and disadvantages of the traditional paradigm of testing in two long-term rodent bioassays to evaluate the carcinogenic potential of pharmaceuticals are well known and include the large number of animals required (≥ 1,000), the significant amount of time (3+ years) and expense (>$2,000,000) involved[1], preponderance of species-specific responses that result, and the difficulty in extrapolating for effects seen at maximum tolerated doses to lower levels of human exposure. From data given in the 3rd Commission Report on the statistics on the number of animals used for experimental and other scientific purposes in the EU, it can be seen that most animals (61%) for toxicological or other safety evaluations are used in medicine [3].

When animal consumption is considered per type of regulatory toxicological test, among those test methods with the highest animal use are chronic toxicity tests (17%) [3]. As a good example the report on the ECOPA project states that, the testing of biokinetic endpoints (ADME), target organ toxicity, systemic toxicity, repeat-dose toxicity, non-genotoxic carcinogenicity,

[1] Costs and use of animals as mentioned in the scientific literature vary considerably, other sources mention up to 5 years to design, conduct and analyse, and consume as many as 400 [4] or 800 [5] mice and rats at a total cost of 1 [4] to $1.5–3 million per chemical tested [4].

reproductive toxicity, acute (dermal, inhalation) lethal toxicity, all of strategic importance for the regulatory testing of chemicals, are those that consume the highest numbers of animals [6].

The development of alternative methods is thus of high priority if one wants to reduce the number of animals that are considered to be necessary for safety evaluation of human health and environment. A number of alternative methods already exist [7], but still a lot of gaps need to be closed [3].

Coming back to the subject of carcinogenicity testing the issue exists that the results of standard carcinogenicity testing often do not contribute to a mechanistic understanding of effects, and extensive follow-up studies may be required to determine mode of action and to better understand the potential for human tumourigenic effects. [8]. Although determined to be the most practical and logical approach to determining carcinogenicity of pharmaceuticals, the time and resource intensive studies often generate results that are difficult to interpret and extrapolate to human carcinogenic risk [9]. For example, as with other surveys accessible in the literature, the data for pharmaceuticals were dominated by the high incidence of rodent liver tumours. The high susceptibility of mouse liver to non-genotoxic chemicals has been subject of numerous symposia and workshops. I was concluded that these tumours may not always have relevance to carcinogenic risk in humans and can potentially be misleading [10]. An additional point to consider when using rodent-based test systems for carcinogenicity and attempting extrapolating the results to humans is the rather consistent observation that, in general, rodents may be more susceptible to cancer than humans [11]. The predictive value of standard carcinogenicity long-term studies in rodents is therefore questioned by many authors in the field.

Finally, in the field of drug development about 92% of substances fail during clinical trials, about 20% of which are due to toxic effects in humans not identified in pre-clinical animal testing [12]. The financial loss in pharmaceutical drug development for a failed drug due to carcinogenicity findings can be enormous due to the many years lost in the development effort [13] and because carcinogenicity testing is commonly done at the end of the preclinical phase and data have to be readily available only before phase III drug trials. This is the reason why the search for more predictive alternatives has been intensified during the last two decades.

This search for alternatives goes together with another aspect, not of scientific relevance, which is the fact that there is also increasing societal interest in reducing the use of animals in scientific experimentation and safety testing [8].

In summary, the assessment of toxicological risk relies primarily on in vivo animal experiments that were designed decades ago and cost about $ 3 billion/year worldwide [14]. Their low

throughput has led to a backlog of substances whose potential toxicity remains to be adequately assessed [15], [16] and hinders front loading of toxicity testing in the drug development process [14].

Taken together, these facts underline that the classical approach of carcinogenicity testing in two rodent species for two years has to be reassessed for various reasons, incl. animal welfare.

With regard to the question when regulatory testing for carcinogenicity has to be done, the situation in the three ICH regions has been widely harmonized due to the guidance given by ICH S1A and S1B. In Japan, according to the 1990 "Guidelines for Toxicity Studies of Drugs Manual", carcinogenicity studies were needed if the clinical use was expected to be continuing for six months or longer. If there was cause for concern, pharmaceuticals generally used continuously for less than six months may have needed carcinogenicity studies. In the United States, most pharmaceuticals were tested in animals for their carcinogenic potential before their widespread use in humans. According to the US Food and Drug Administration, pharmaceuticals generally used for three months or more required carcinogenicity studies. In Europe, the Rules Governing Medicinal Products in the European Community defined circumstances when carcinogenicity studies were required. These circumstances included administration over a substantial period of life, i.e., continuously during a minimum period of 6 months or frequently in an intermittent manner so that the total exposure was similar [1].

Certain classes of compounds may not be used continuously over a minimum of six months, but may be expected to be used repeatedly in an intermittent manner. It is difficult to determine and to justify scientifically what time represents a clinically relevant treatment periods for frequent use with regard to carcinogenic potential, especially for discontinuous treatment periods. For pharmaceuticals used frequently in an intermittent manner in the treatment of chronic or recurrent conditions, carcinogenicity studies are generally needed. Examples of such conditions include allergic rhinitis, depression, and anxiety. Carcinogenicity studies may also need to be considered for certain delivery systems which may result in prolonged exposures. Pharmaceuticals administered infrequently or for short duration of exposure (e.g., anaesthetics and radiolabelled imaging agents) do not need carcinogenicity studies unless there is cause for concern [1].

1.2 General History of alternative Method Development

Almost 25 years ago, on November 24, 1986, the animal experimentation directive 86/609/EEC came into force [17]. One of the aims of directive 86/609/EEC was to protect animals used for

experimental and other scientific purposes to ensure that any possible pain, suffering, distress or lasting harm inflicted as a consequence of procedures being conducted upon them shall be kept at a minimum. Furthermore, it was stated that EU Member States should encourage the development of new alternatives [18]. The directive also aimed to reduce the numbers of animals used for experiments by requiring that an animal experiment may not be performed when an alternative method exists, and by encouraging development and validation of alternative methods to replace animal methods [19].

Over the last years there has been an increasing interest in developing and validating alternative methods based on the concept of the 3Rs: Replacement, Reduction, and Refinement [20]. A significant early stimulus was the campaign against testing cosmetics on animals and, subsequently, European legislation outlining a timetable for the prohibition of such testing was adopted. More recently, the new European chemicals policy REACh (Registration, Evaluation Authorisation and Restriction of Chemicals) [21] has highlighted the urgent need for further progress in the development of alternatives in order to reduce the large projected animal use that will be its consequence [18].

On April 27, 2009, representatives from international agencies of validation organisations from the United States, Japan, the European Union and Canada signed a Memorandum of Cooperation establishing the International Cooperation on Alternative Test Methods (ICATM). The agreement promotes enhanced international cooperation and coordination on scientific validation of non- and reduced-animal toxicity testing methods [22].

Following the respective readings, positions and a tripartite compromise of April 2010, the revised Directive 2010/63/EU was adopted at its second reading in the European Parliament on September 8, 2010 [23]. It entered into force 20 days after its publication in the Official Journal on October 20, 2010. The new Directive will take effect on January 1, 2013. While its ultimate goal is to replace the use of animals, the Directive acknowledges that animals, including non-human primates, are still needed today [19], but no strategy to reduce and ultimately replace animal experiments is stated [24].

1.3 Development of alternative Carcinogenicity Testing Methods

The first step towards change/improvement of the paradigm of testing for carcinogenicity in two rodent species was the regulatory decision that only one of the two carcinogenicity tests has to be performed and the second one can be replaced by an alternative method [10].

The possibility of using alternative short-term carcinogenicity testing models employing transgenic mice as a substitute for a second two-year rodent bioassay in pharmaceutical testing was introduced in 1996 with the draft of a new International Conference on Harmonization (ICH) guidance (S1B) on testing for carcinogenicity of pharmaceuticals [10], [25], [26], [27], [28]. The S1B working group found, after an extensive review of available data on the carcinogenicity testing of pharmaceuticals and the regulatory decisions made that there was little if any evidence that the additional data provided by a second long-term bioassay in the mouse had influenced regulatory decision making for new drug applications [8].

Historically, the regulatory requirements for the assessment of the carcinogenic potential of pharmaceuticals in the three regions (EU, Japan, US) provided for the conduct of long-term carcinogenicity studies in two rodent species, usually the rat and the mouse. Given the cost of these studies and their extensive use of animals, it is in keeping with the mission of ICH to examine whether this practice requiring long-term carcinogenicity studies in two species could be reduced without compromising human safety [10].

In 1996, the International Conference on Harmonization (ICH) Expert Working Group on Safety acknowledged the limited utility of conventional two-year rodent bioassays for assessing the human carcinogenic potential of chemicals, including pharmaceuticals, based on past positive findings that are now considered to have little or no relevance for human risk assessment. The ICH further acknowledged the potential of several new testing models to produce meaningful information for human cancer risk assessment. The group proposed a new scheme for the carcinogenicity testing of pharmaceuticals (ICH S1B). This scheme calls for one long-term rodent carcinogenicity study, plus an additional test for carcinogenic activity in vivo, consisting of either another long-term carcinogenicity study in a second rodent species, or a short- or medium-term rodent test, to be chosen from several available alternative models [29].

Although consideration was given to reducing the requirement to one long-term bioassay in the most appropriate species (usually the rat), the S1B working group ultimately decided to preserve the standard of testing in two species, but allow for the use of alternative models, such as short-term assays in transgenic mice, that might improve the utility, mechanistic understanding, and/or human relevance of the results obtained in a second in vivo bioassay. The new S1B guideline

places the burden of proof on the sponsor to provide a scientific rationale for why a proposed alternative model might provide new information on the compound that would not otherwise be obtained from a second two-year bioassay [8]. Positive results in long-term carcinogenicity studies that are not relevant to the therapeutic use of a pharmaceutical present a dilemma to all parties: regulatory reviewers, companies developing medicinal products and the public at large. As reflected in ICH S1B, it is the overall opinion that the conduct of only one long-term carcinogenicity study (rather than two long-term studies) would, in part, allow resources to be diverted to other approaches to uncover potential carcinogenicity relevant to humans.

In consequence, according to ICH S1B, the basic testing approach comprises one long-term rodent carcinogenicity study, plus one other study. Additional tests may be short- or medium-term in vivo rodent test systems [10]:

- the initiation-promotion model in rodents,
- several transgenic mouse assays including the $p53^{+/-}$ deficient model, the Tg.AC model, the TgHras2 model, the XPA deficient model, etc. and
- the neonatal rodent tumourigenicity model.

Generally, the methods should be based on mechanisms of carcinogenesis that are believed relevant to humans and applicable to human risk assessment. Such studies should supplement the long-term carcinogenicity study and provide additional information that is not readily available from the long-term assay. There should also be consideration given to animal numbers, welfare and the overall economy of the carcinogenic evaluation process. Furthermore, emphasis is taken on the possibility to perform mechanistic studies contributing to elucidation of the way of action. Relevant tissues can be examined for changes at the cellular level by using morphological, histochemical, or functional criteria.

However, a long-term carcinogenicity study in a second rodent species is still considered acceptable [10].

There is, as a matter of fact, a dispute between those academic and scientific circles that consider alternatives as a complete substitution [11] or, referenced to the idea of the 3Rs, incl. reduction and refinement of animal testing.

Despite this dispute the 3Rs have created a movement in the scientific and regulatory community which has lead to an increasing acceptance of the notion that there are circumstances where we can, and should, depart from the classical two-year rodent bioassay for the assessment of the carcinogenic potential of chemicals [11].

1.4 The Validation Process in the European Union

Validation is an independent assessment of a method for a defined purpose as to its reproducibility, scientific basis, and reliability/relevance [30], [31], [32]. A culture of prospective ring trials has developed, which is capable of reassuring the one-to-one replacement of a method by a better one, e.g., one limiting or refining animal use [14]. Tremendous problems arise [33], [34] where no reference method exists, the reference method is flawed or the purpose or applicability of both methods is overlapping but not identical. Unfortunately, most areas of toxicology are a mixture of those problems. This is why very few replacement methods have been accepted, and when they are, they often outperform the reference method and demonstrate its flaws [14].

In the European Union, the European Centre for the Validation of Alternative Methods (ECVAM) is an institution that supervises the official validation process of alternative methods. ECVAM was created in October 1991 to a requirement in Directive 86/609/EEC which stated that the European Commission and the member states should actively support the development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals. ECVAM was established at the Joint Research Centre in 1992, and is now part of the In Vitro Methods Unit (IVMU) of the Institute for Health and Consumer Protection (IHCP) located in Ispra, Italy. ICCVAM, the parallel institution in the U.S. was founded in 1997 and comprises representatives from 15 federal agencies conducts evaluations of new, revised and alternative test methods and promotes the scientific validation and regulatory acceptance of test methods that replace, reduce, or refine the use of animals.

Duties of ECVAM are [22]:

- Coordinating and promoting development and use of alternatives to procedures including basic and applied research and regulatory testing
- Coordinating the validation of alternative approaches at Union level
- Acting as a focal point for the exchange of information on the development of alternative approaches

- Setting up, maintaining and managing public databases and information systems on alternative approaches and their state of development
- Promoting dialogue between legislators, regulators, and all relevant stakeholders, in particular industry, biomedical scientists, consumer organisations and animal-welfare groups, with a view to the development, validation, regulatory acceptance, international recognition, and application of alternative approaches.

Validation is the process by which the reliability and relevance of a procedure are established for a specific purpose [22].

In 1995, based upon experience gained during several recent large-scale validation studies, and in consultation with various international experts (including members of ERGATT), ECVAM published recommendations focusing on practical and logistical aspects of validating alternative test methods. Five main stages in the evolution of new test methods were identified: test development; pre-validation; validation (involving a formal inter-laboratory study with the testing of coded chemicals); independent assessment and progression toward regulatory acceptance. ECVAM has implemented a pre-validation scheme, which includes three main phases: protocol refinement, protocol transfer, and protocol performance a pre-validation scheme, which includes three main phases: protocol refinement, protocol transfer, and protocol performance. The objective of the pre-validation process is to ensure that any method included in a formal validation study adequately fulfils the criteria defined for inclusion in such a study, so that financial and human resources are used more efficiently and so that there is a greater likelihood that the expectations of those in the scientific, regulatory and animal welfare communities, who seek the replacement of current animal tests by relevant and reliable alternative methods, will be met. In 2004, ECVAM published the "Modular Approach to the ECVAM Principles on Test Validity" (select from the top-menu bar the sector "Publications" followed by "ECVAM Selected Articles") that makes the validation process more flexible, by breaking down the various steps in validation into independent modules and defining for each module the information needed for assessing test validity.

More recently, ECVAM has established a formal procedure for the evaluation of the readiness of a Test Method to enter the ECVAM (pre)validation process which follows now 2 mandatory steps: Pre-submission and Complete Submission.

For the evaluation of the readiness of a test method to enter the ECVAM (pre)validation process, this test method needs to be officially submitted to ECVAM by compiling as a first step the electronic version of the Test Pre-submission Form. The entire ECVAM test submission process follows 2 mandatory steps.

Step 1: Pre-submission – it is based on the ECVAM Test Pre-submission Form (TPF) and is a mandatory requirement for a test method to be eventually considered for the ECVAM validation process. The completed TPF filled in all its parts will allow ECVAM to perform a preliminary assessment of the status of development, optimisation and/or validation of the test method and its potential relevance with regard to the 3Rs (replacement, reduction, refinement of animal testing).

Step 2: Complete Submission – it requires the compilation of a detailed Test Submission Template (TST) to be provided by ECVAM to allow a comprehensive evaluation of the submitted method.

However, further processing and duration of the validation process is not described on the ECVAM homepage. Indeed, idealised time estimates are three years for validation plus another two years for peer review and costs of $ 500,000 involving three or more laboratories [14].

2 Results

Of course there are numerous reasons for developing alternative toxicological tests and a lot has so far been done to develop alternatives to current toxicological test procedures. However, methods for research topics have to be distinguished from those for regulatory testing due to safety reasons. Only very limited methods have already been replaced, e. g skin corrosion or irritation tests (OECD 431/ Draft OECD TG) and these procedures are no longer based on animal testing. In general, the primary goal of method development mostly remains open: is research in this context performed for reasons of animal welfare or do researchers both in industry and academic circles together with regulatory staff want to refine methods in a way that mechanisms of action become clearer and prediction more reliable?

Two basic aspects should be highlighted before talking about development of alternative methods not only for testing of the carcinogenic potential, but for all kind of toxicological tests of future drug candidates: is it animal welfare and do we want to go off the general approach of using laboratory animals or, and this is according to the author's position an entirely different aspect, is it refinement and the need to develop much more precise and more profound methods?

If one reads side effects or disadvantages of the traditional modus operandi to use two different rodent species for the elucidation of the carcinogenic potential of a compound mentioned in the introduction, namely:

- enormous costs
- time- and resource consuming procedure
- high possibility of false negative and positive results
- need of further time and money consuming follow-up tests

it becomes obvious that there is not only an extreme socio-cultural need to develop alternatives to replace the animal-consuming 2-year carcinogenicity approach of testing in two rodent species but also profound scientific reasons.

As already mentioned in the introduction, the basic current approach seems to be the maintenance of performing one traditional rodent bioassay in one species and to replace the second one with an alternative method, whereas the possibility to abide by the classical way of testing in two species still remains open [10]. There are various alternatives that rarely seem to have the potential for a complete substitution of the traditional carcinogenicity test, and among those only a few are validated so far and find acceptance within the authorities. Validation,

indeed, has an integral impact on the adoption and entry of alternatives to industrial carcinogenicity screening, and the approaches and opinions in the ICH regions do not seem to be harmonised.

ECVAM initially proposed an optimistic strategy plan for the future development and validation of alternative methods [35]. Already in 2002 researchers doubted that within the next years, alternative methods would be available for most if not all endpoints. From experience of the past, Rogiers and co-workers [6] concluded that good pre-validation and validation studies are time-consuming exercises, but could be speeded up importantly because of experience gained in the previous pre-validation and validation studies. They would take up to 6–8 years before a method is validated and officially incorporated into the EU legislation [3], [6]. For most of the toxicological tests (chronic toxicity, systemic toxicity, reproductive toxicity), appropriate alternative methods have not yet been developed. Consequently they are not present in the pipeline of pre-validation/validation.

In the course of this master thesis a literature survey was performed in the most prominent databases for toxicology (Toxnet, Pubmed, TSCATS) and on the homepages of international governmental and non-governmental institutions (ALTEX, ECOPA, ECVAM, ICCVAM, ICH, ILSI, NICEATM – NTP, OECD, ZEBET). It is impossible to present every single alternative for carcinogenicity testing that was mentioned in the scientific literature and to comment on its validation and/or acceptance status. However, those tests that are most promising and/or are under validation by institutions like ECVAM will be discussed hereafter. It is not the aim of this thesis to analyse a certain test method for its validity, but to summarise what has been done so far in the light of the intention to replace the current testing approach for carcinogenic potential in two rodent species.

Basic classification of so far developed alternative test methods can be divided in

- medium-term in vivo rodent test systems that abide by the identical principles of the standard carcinogenicity test but for a shorter time
- assays using transgenic rodent strains with enhanced susceptibility to carcinogens
- cell-based assays
- “omics”
- QSAR
- others

In Table 1 potential candidates for alternative methods taken from those scientific and/or regulatory publications are described. Basic principles and validation/employment status will be summarised in detail.

Table 1: Summary of Alternatives/Substitutes for the Standard Carcinogenicity Test in the Mouse/Rat as mentioned in scientific Literature and/or regulatory Framework

Assay/Test	Test Principle	References
Medium-term in vivo rodent test systems	Procedure and endpoints identical to standard carcinogenicity bioassay but for shorter time	[8], [36], [37], [38], [39]
$p53^{+/-}$ deficient model	Knockout mouse model (allele(s) of the p53 tumour suppressor gene deleted)*	[10], [11], [29], [40], [41]
TgHras2 model	Transgenic mouse model (multiple copies of the human c-Ha-ras gene inserted)*	
$XPA^{-/-}$ deficient model	Knockout mouse model (allele(s) of a nucleotide excision repair gene deleted*	
Tg.AC model	Transgenic mouse model (multiple copies of a zeta-globulin promoter/v-Ha-ras oncogene reporter construct inserted)*	
$XPA^{-/-}/p53^{+/-}$ double knockout mouse model	Double knockout mouse model*	[29], [41]
C57BL/6 (N5) – TRP53 knockout mouse model	Knockout mouse model*	[41]
Cell transformations assays	Syrian Hamster Embryo, BALB/c3T3, C3H10T1/2 cells to measure the induction of malignant features	[11], [29], [42], [43], [44]
"omics"	Methods to identify the functions of genes of a given organism	[14], [45], [46], [47]

Assay/Test	Test Principle	References
Quantitative structure-activity relationship (QSAR) based methods	Modelling tool for prediction of toxicology based on chemical structures	[13], [47], [48]
Neonatal rodent tumourigenicity model	Assay system for identification of transspecies carcinogens*	[10]
Neonatal mouse model	Model for the detection of carcinogens that operate via a genotoxic mode of action*	[29], [49]
Initiation-promotion model in rodents	Model for classification of carcinogenic compounds*	[10]
Primary monolayer and three-dimensional cell cultures of mammalian/non-mammalian cells	Primary cell models, stem cells for high throughput screening of various endpoints	[47]

*Endpoints determined in genetically modified rodent strains, neonatal animal models and the initiation-promotion model are identical to those in the standard two-year rodent bioassay, e.g. food consumption, weight gain/loss, necropsy, histopathology etc.

2.1 Medium-term in vivo rodent Test Systems

Only since 1996 (Federal Register 1996) have pharmaceutical developers been explicitly allowed to incorporate genetically modified mouse models to replace one of the two species in 2-year carcinogenicity bioassays generally recommended for regulatory product approvals internationally [50].

With an emerging consensus that the transgenic models have added value for human cancer hazard identification in carcinogenicity testing of pharmaceuticals, several new initiatives to improve the accuracy and efficiency of carcinogenicity testing strategies have focused on the question of whether a lifetime bioassay in the rat is always necessary or whether the outcome of a two-year rat carcinogenicity study can be predicted with confidence based on data from thirteen-week [36] or the six- and/or twelve-month chronic toxicity studies in rats [8], [37], [38].

No short or medium term study using non-transgenic animal models has yet been finally (pre-) validated or accepted for regulatory testing of pharmaceuticals [51].

However, there are some promising approaches that could be taken into account for supplementation and/or substitution of the long-term carcinogenicity study as stated in ICH S1B [10]. For example, the approach of Reddy and co-workers was to assess whether histopathologic lesions indicative of risk for neoplasia in a chronic study predict a two-year carcinogenic response and especially whether there was a correlation between whole animal histopathologic evidence at six and/or twelve months and carcinogenesis after two years [38]. The approach involved evaluation of results for seventy-nine compounds with closely matching doses (+25%) in six- and/or twelve-month chronic toxicity studies and two-year rat carcinogenicity studies. For the initial 79-compound Leadscope/Merck database based on the histologic evidence as defined above, or lack thereof, there was 89% (thirty-four of thirty-eight) negative predictivity and 87% (twenty-six of thirty) test sensitivity (four false negatives). There were fifteen false positives, so positive predictivity was 63% (twenty-six of forty-one).

Also van Der Laan and co-workers raised the question whether it is possible to reduce the duration of the life-span carcinogenicity studies [52]. IARC carcinogens (most of them are genotoxic) were positive within 18 months [53] and there are indications that the carcinogenic potential of a compound could also be determined earlier. However, overall accuracy of the standard 2 year rodent bioassay is only 69% [54]. The weight-of-evidence approach based on the complete package of pharmacology and toxicology and on rat carcinogenicity studies if necessary including mechanistic studies might give sufficient data to explain preneoplastic lesions seen after 12 months [55].

Tamano and co-workers recently presented medium-term liver and multi-organ bioassay systems for detection of not only genotoxic but also non-genotoxic carcinogens [39]. Positive results obtained in a relatively short period closely correlated with long-term carcinogenicity. A combination of liver and multi-organ bioassay systems is indicated for detection of potential hazard of chemicals to humans. Rodent systems like those could be regarded as appropriate alternatives for assessment of carcinogenic risk.

Numerous further publications using rats or mice for studying the carcinogenic potential of drug candidates over the amount of less than the compulsory 24 months can be found in the scientific literature [39], [56], [57]. Unfortunately, all of them bear a lack of predictivity and/or sensitivity as described already in the context of the newly developed assay by Reddy and co-workers [38]. The overall accuracy of assays using transgenic animals as $p53^{+/-}$, $p53^{+/-}$ (only genotoxic componds) or $XPA^{-/-}$ and/or $XPA^{-/-}/p53^{-/-}$ knockout mice seems to be much higher (81%/75%/83%) [8], and therefore even higher than the corresponding mouse or rat bioassay (69% overall accuracy) [54] respectively.

2.2 Assays using transgenic rodent Strains

Six of the models described in Table 1 are based on genetically modified mouse models that have been tested under the auspices of the ILSI HESI Alternatives to Carcinogenicity Testing (ACT) Committee [29]. When screening scientific literature for potential alternatives to the standard carcinogenicity test, it seems that it is indeed those procedures that use genetically modified animals that gain most attention and that bear highest potential to substitute the 2-year study completely.

The discovery and cloning of oncogenes and tumour suppressor genes in the 1980s and early 1990s and rapid development of technologies to create transgenic mice overexpressing or underexpressing these critical target genes in cancer development provided the tools to toxicologists to begin to leverage the growing knowledge base of molecular mechanisms underlying human cancer in the development of new carcinogenicity test models. The expectations were that these models would (1) provide opportunities to understand mechanisms of carcinogenesis, (2) respond more selectively to known human carcinogens, and (3) significantly reduce the number of animals, time, and costs involved in carcinogenicity testing [8].

In 1998, at the International Conference of Harmonization (ICH), an agreement titled ‘‘Testing for Carcinogenic Potential of Pharmaceuticals’’ was reached between regulatory agencies and pharmaceutical manufacturers in the United States, Europe, and Japan acknowledging the need to improve methods to identify carcinogens [58], [59], [60]. Included in this agreement was a regulatory approval to substitute an alternative model of carcinogenicity for one of the two-year conventional rodent species assays. As a result, several transgenic rodent strains with enhanced susceptibility to carcinogens were developed as potentially useful models in assessing chemical carcinogenesis. Potential advantages of transgenic mice over the non-transgenic counterparts included a decreased assay time from two years to six months, decreased incidence of spontaneous tumours, decreased animal usage, improved understanding of cancer mechanisms, and resource savings [9].

Variants of transgenic mouse strains, such as the $p53^{+/-}$ knockout mouse, TgrasH2 micro-injected mouse, $XPA^{-/-}$ knockout mouse and the Tg.AC microinjected mouse [10], [11], [29], [40], [41], were identified as ‘‘promising’’ by a consortium of laboratories organised by the International Life Sciences Institute (ILSI), and were recommended for further evaluation [58] and are those that were mentioned mostly in the scientific literature.

In the US, the Carcinogenicity Alternative Mouse Models (CAMM) Working Group of the Society of Toxicologic Pathology (STP) surveyed its members to define current practices and opinions in industry regarding the use of alternative mouse models for carcinogenicity testing [40]. The results of the survey indicated that CAMM are used most often to fulfil a regulatory requirement (e.g., to replace the two-year mouse bioassay) and are being accepted by regulatory agencies. Alternative models are also sometimes used for internal decision making or to address a mechanistic question. The CAMM most commonly used are the $p53^{+/-}$ and rasH2. The rasH2 appears to be the currently accepted model for general carcinogenicity testing. Problems with study interpretation included lack of historic background data, unexpected tumour finding, and tumour identification/characterisation of early lesions. Problems with implementation or conduct of the study included extent of the pathology evaluation, numbers of animals, survival and study duration. Recommendations were developed for frequency and type of positive control testing, extent of histopathologic examination of test article–treated and positive control animals, current use and future development of diagnostic criteria, increased availability and use of historic data, and use of other genetically modified mice in carcinogenicity testing.

When asked how they would use a CAMM assuming equal regulatory acceptance, a slightly greater number of respondents (eight vs. six out of sixteen responses) indicated that they would replace the standard two-year mouse model with the rasH2 model. Five respondents would use the $p53^{+/-}$ model to address a potential genotoxicity question. The rasH2 model was selected most often as the model to be used to answer mechanistic questions (three responses), but all models listed had at least one response for this use [40].

The $p53^{+/-}$ model is of particular value for compounds with residual concern that genotoxic activity may contribute to tumourigenesis. The rasH2 model is an appropriate alternative without regard to evidence of genotoxic potential [8].

MacDonald and co-workers summarised conclusions from a workshop of ILSI HESI held up on the utility of transgenic assays for risk assessment [29]. The purpose of this workshop was to reach an understanding of how data from genetically modified mouse models are viewed by different regulatory bodies in the pharmaceutical sector and, based on this understanding, to identify areas in which more experimental work may be needed to increase the utility of data derived from these assays. Conclusions of this workshop are summarised in Table 2.

Table 2: Summary of Positions of CPMP, FDA and NIHS with regard to the Utility of genetically modified Mouse Models for pharmaceutical Risk Assessment

Model	CPMP*	FDA	NIHS
$p53^{+/-}$	- acceptable for regulatory purposes - not limited to genotoxic compounds	- appropriate alternative model when limited to compounds that are clearly or equivocally genotoxic	
Tg.rasH2	- appropriate alternative model for regulatory purposes for both genotoxic and non-genotoxic compounds		
Tg.AC	- useful for screening the carcinogenic potential of dermally administered pharmaceuticals	- useful for dermally applied products (review of data from products intended for systemic administration but assayed using dermal route)	- concerns regarding the stability of this model's phenotype
$XPA^{-/-}$ and $XPA^{-/-}/p53^{+/-/-}$ double knockout	- promising models but need for further development	- no experience with these models	
Areas of general agreement	- the alternative assays currently under consideration have value in carcinogen identification - these assays can serve as an alternative to the standard mouse 2-year bioassay in a testing program - the testing paradigm is, in general, accepted - results of these assays should not be considered on their own, but rather integrated with other available data and considered as part of a weight of evidence approach for risk assessment purposes		

According to MacDonald et al, modified [29]

* Today: Committee for Medicinal Products for Human Use (CHMP)

Regarding the submission status of alternative carcinogenicity test methods, MacDonald and co-workers provided a summary in the three ICH regions [29]: In the US, 25% of the proposed mouse carcinogenicity study protocols received by the FDA's Center for Drug Evaluation and Research's Executive Carcinogenicity Assessment Committee (probably from 2001-2003) had been for an alternative model. Data from approximately 90 protocols and 24 completed genetically modified mouse or other alternative assays had been received and evaluated. Most of these protocols and completed studies had used the $p53^{+/-}$ assay.

The regulatory experience gathered in the European system until 2003 had consisted mainly of requests for advice regarding study design and discussions with companies about the potential acceptability of such a test in the non-clinical package for a marketing authorisation. Five product applications had been received for marketing authorisation of new active substances including studies with $p53^{+/-}$ mice in their dossiers; all five studies showed negative results for carcinogenicity. These studies were accepted as contributing to the weight of evidence, in combination with results from the long-term rat study (available for four of five products) and data from the genotoxicity tests. A Tg.rasH2 study was also included in a dossier for which a $p53^{+/-}$ study was already present. The company carried out this additional study because of lack of experience with the $p53^{+/-}$ model with regard to compounds that may be specifically carcinogenic in the gastrointestinal tract. Finally, in a dossier of an orally administered compound, a dermal Tg.AC study was included. This study was not accepted as contributing to the weight of evidence, as the route of administration was considered to be inappropriate, and the study did not add to the body of information regarding the mechanism of tumour formation in the rat.

Regarding the submission status at this time in Japan, the $p53^{-/-}$ model was used in studies conducted at the NIHS aimed at understanding mechanisms of carcinogenesis, with emphasis on dose-response relationships and discerning possible thresholds. Additionally, researchers at NIHS were working toward developing a $p53^{-/-}$ model on a C3H/He background that might be capable of responding to genotoxic carcinogens within ten weeks of treatment [29].

2.3 Cell Transformation Assays

Cell transformation is defined as the induction of certain phenotypic alterations in cultured cells that are characteristic of tumourigenic cells [61]. These phenotypic alterations can be induced by exposing mammalian cells to carcinogens. Assays measure induction of malignant features in mammalian cells after treatment with tested chemicals and entail morphological, biochemical

and molecular changes such as alteration of cell morphology and growth control, acquisition of anchorage-independent proliferation and tumourigenicity in susceptible animals [62], [63], [64].

In 2007, the OECD published a detailed review paper on cell transformation assays for detection of chemical carcinogens [43] with the objective to provide an overview of the three main CTAs, (the Syrian hamster embryo cell (SHE), the BALB/c 3T3 and the C3H10T1/2 assays) and to correlate them with in vivo rodent assays and assess their performances in predicting chemical carcinogenicity and the main goal to judge whether any of these assays is ready to be suggested for further development into OECD Test Guidelines.

It was concluded that although there is insufficient information on mechanism of action and usage for pharmaceuticals, the SHE assay had the potential of being used as a screen for pharmaceutical testing as a part of a tiered testing strategy. In addition to its ability to identify potential rodent carcinogens, it showed promise for identifying carcinogens that are not genotoxic. Because of the ability to identify potential rodent carcinogens, it had been proposed for use as a second level in vitro screening test for carcinogenic potential. Another proposed use of the assay was for the identification of potential carcinogens that have no evidence of genetic toxicity in the currently used assays. The assay could also be a replacement for the in vitro mammalian cell mutagenicity assays with similar or lower predictive capacity. The overall conclusion at this stage in 2007 was that the performances of the SHE and the BALB/c 3T3 CTAs were adequate for recommending that they are integrated into official OECD Test Guidelines. The C3H/10T1/2 was not considered to fulfil the need at this time. On the basis of these conclusions and on recommendations of two expert meetings on cell transformation held at the European Centre for the Validation of Alternative Methods [65] a formal pre-validation study on the Syrian hamster embryo (SHE) and Balb/c 3T3 CTAs was set up to address issues of standardisation of protocols, within-laboratory reproducibility, test method transferability and between-laboratory reproducibility [42].

Hence, the conclusion and the actual status as published in May 2010 [51] and January 2011 was that the SHE pH 6.7, and the SHE pH 7.0 protocols and the assays system are transferable between laboratories, and are reproducible within and between laboratories, that for the Balb/c 3T3 method an improved protocol has been developed, which allowed to obtain reproducible results. These results in combination with the extensive database summarised in the OECD DRP31 [43] support the utility of in vitro CTAs for the assessment of carcinogenicity potential.

This development is very pleasant as e.g. van der Laan and co-workers concluded in 2002 that the SHE assay can only support the outcome of carcinogenicity data of conventional long-term

rodent studies, without adding further knowledge and that for final risk assessment this assay was not useful [52]. The authors assumed that it might be used as a rapid screening tool to select uncomplicated compounds and not to contribute to regulatory weight of evidence.

However, it has to be mentioned that the SHE test requires animal sacrifice and preparation of new pools of target cells, is labor intensive, and requires the addition of an S-9 fraction [66].

2.4 "omics"

In general, "omics" techniques comprise various different methodologies such as genomics, toxicogenomics, proteomics and metabolomics [47]. A milestone in toxicology was the emergence of toxicogenomics, resulting from the application of knowledge gained from genomics science of conventional toxicology [67]. This growing research field specifically tackles the complex interactions between toxic effects, elicited by exogenous stimuli (e.g. chemical compounds), and the structure and/or activity of the genome [68], [69]. The basic tool in toxicogenomics, the DNA microarray, allows simultaneous analysis of thousands of individual genes and thus permits assessment of characteristic modifications in gene expression profiles induced by toxic compounds. In recent years a number of European initiatives have been launched to speed up the search for alternative methods, especially suitable for studying chronic toxicity induced by xenobiotics. Among those, the carcinoGENOMICS project was raised to develop omics-based in vitro screens for testing the carcinogenic potential of chemical compounds [67].

Vinken and co-workers published detailed information regarding this project which is considered to be a basic step of development [67]. Its innovative character lies in the combination of optimised organotypical cell culture systems, including stem cell-derived models, with both transcriptomics and metabonomics as well as with phenotypic anchoring. A key step in the first phase of carcinoGENOMICS implies the establishment of a collection of data that can serve as a platform to predict the carcinogenic potential of chemicals in lung, liver and kidney. This is achieved by including a set of chemicals that display well-characterised carcinogenic properties during the optimisation of the in vitro assays. In the second phase of carcinoGENOMICS, the designed in vitro toxicogenomic assays will enter the process of pre-validation, according to ECVAM's guidelines [31]. Multi-laboratory validation is foreseen upon adding high-throughput features to the developed assays. Special attention is paid to the robustness of the assays as well as to the further evaluation of their predictive power. An additional and trickier set of chemicals will be used for this fine-tuning of the in vitro tests.

The major advantage of using technologies that screen across many different cellular metabolic parameters is their ability to produce metabolic fingerprints of toxicants. Because changes in these metabolites are thought to precede toxic outcomes, appropriate changes may serve as early, sensitive indicators of potential toxicity and can then be used to help guide decision making with regard to compound classification. An important issue that remains unsolved is how to integrate metabonomics into testing strategies. Compared with transcriptomics, metabonomic approaches have been less frequently used in predictive toxicology [45].

Ellinger-Ziegelbauer and co-workers also do research in the field of toxicogenomics and underlined the possibility of using data of short-term bioassays and application in the identification of early biomarkers for carcinogenicity [46]. Using gene expression profiles from the livers of rats treated up to 14 days with genotoxic and non-genotoxic carcinogens used in short-term repeated dose studies, they identified characteristic gene expression profiles for these two groups of carcinogens, applied these profiles to extract biomarkers discriminating genotoxic from non-genotoxic carcinogens and calculated classifiers based on the support vector machine (SVM) algorithm. These classifiers then predicted a set of independent validation compound profiles with up to 88% accuracy depending on the marker gene set. The study could be taken as proof of the concept that a classification of carcinogens based on short-term studies may be feasible.

Van Vliet admits “omics” technologies to play a key role in establishing the proposed toxicity pathway-based risks assessment and to provide excellent opportunities to identify perturbations leading to toxicity [47].

No information can be taken from the ECVAM website that “omics”-based methodology has already been finally (pre-) validated or accepted for regulatory testing of pharmaceuticals [51] or even taken into account of such. A basic problem of all “omics” approaches is their specificity. In other words it is not clear whether a pattern seen in one cell type has any predictive value for other cell types incl. the problem that “omics” results are usually very different between in vitro and in vivo conditions.

2.5 Quantitative Structure-Activity Relationship (QSAR) based Methods

Quantitative structure-activity relationship or QSAR analysis can predict toxicological properties of a compound based on its chemical structure and therefore be valuable for testing strategies [70], [71]. Validated predictive QSAR software for carcinogenicity can offset the need for costly and resource intensive 2- year rat or mouse cancer bioassays for some applications [48]. These

considerations and improvements in informatics and computer technology are attracting greater attention to the development of valid alternative methodologies worldwide [72], [73], [74], REACH, http://ecb.jrc.it/REACH/).

Although QSAR analysis alone is generally not enough for risk assessment, various authors state that it can be used to categorise compounds into different toxicity classes and thereby identify the most appropriate tests to continue the testing strategy [75], [76].

Valerio and co-workers demonstrated successful QSAR predictive modelling of naturally occurring carcinogens found in the human diet by using an external validation test [48]. The QSAR predictive modelling approach employed in this study was a high-throughput method employing discriminant analysis. According to the authors, the high-throughput approach could be very valuable in risk assessment and priority setting for the vast number of untested natural products, certain food additives and dietary constituents when used in combination with experimental evidence of rodent carcinogenic potential, structural alert classification schemes and other aforementioned approaches in predictive toxicology.

Recently the EU funded a project called “CAESAR” which was specifically dedicated to develop QSAR models for the REACH legislation. Five endpoints with high relevance for REACH have been addressed within CAESAR, among them also carcinogenicity. The CAESAR application is a JAVA™ web application that allows the access to all toxicity predictive models developed within the CAESAR project.

No information can be taken from the ECVAM website that QSAR-based methodology has already been finally (pre-) validated or accepted for regulatory testing of pharmaceuticals [51] or even taken into account of such.

2.6 Others

2.6.1 Neonatal Mouse Model

The neonatal mouse model, in various forms, has been used experimentally since 1959 and a large number of chemicals have been tested. The neonatal model is known to be very sensitive to the detection of carcinogens that operate via a genotoxic mode of action. In contrast, it is known not to respond to chemicals that act via epigenetic mechanisms commonly observed in the two-year carcinogenicity studies. As such, the model has a high sensitivity and specificity in its response [49].

CPMP has accepted proposals for the use of this model. FDA considers this to be an appropriate model in select limited circumstances for compounds that are clearly or equivocally genotoxic. NIHS has some experience with this model [29].

2.6.2 Initiation-Promotion Model in Rodents

The possibility of application of these assay types as additional in vivo tests for carcinogenicity is mentioned in ICH S1B [10]. One initiation-promotion model for detection of hepatocarcinogens (and modifiers of hepatocarcinogenicity) employs an initiator followed by several weeks of exposure to the test substance. Another multiorgan carcinogenesis model employs up to five initiators followed by several months of exposure to the test substance.

No information can be taken from the ECVAM website that assays or methods based on the initiation-promotion methodology have already been finally (pre-) validated or accepted for regulatory testing of pharmaceuticals [51] or even taken into account of such.

2.6.3 Primary Monolayer and three-dimensional Cell Cultures of mammalian/non-mammalian Cells

Advances in the life sciences have provided a variety of new technologies to investigate the adverse effects of environmental agents in a more mechanistic, less expensive and time saving manner. Van Vliet recently provided an all-embracing summary of current in vitro techniques that partly show promising potential to contribute to the toxicological mechanism of action and could serve as screening tools [47]. Some of the most promising include primary cell culture models, human stem cells, imaging technologies and systems biology. Of course, all of them are far beyond validation, application for regulatory purposes and/or substitution of the traditional carcinogenicity testing strategy, but could serve as supplementary tools for an integrated test strategy and further elucidation of the mode of action of toxicological pathways. As for the other methods, no information is available that any of these techniques are taken into account for regulatory purposes.

3 Discussion and Conclusion

During the last 30 years, efforts have been made by all stakeholders to develop 3R-alternatives, and to validate and practically apply these [77].

However, during the process of developing and validating no clear distinction was made between the development and use of alternatives in either research or regulatory testing. As far as the availability of alternatives for research purposes is concerned, the actual situation is often described as good and a lot of alternative methodologies (e.g., cell and tissue culture) and new technologies (e.g., ‘‘-omics’’) are used to elucidate mechanisms of action or toxicity, for example, in preclinical drug development [3].

The situation, however, becomes quite different when alternative methods are considered for regulatory purposes, namely, to be used as replacement methods for the in vivo methods used in regulatory testing for the safety of drugs. For this purpose scientists tend to be more critical and less enthusiastic and also quality assurance systems like GLP (Good Laboratory Practice) ask for documented validation and acceptance by formal guidances (ICH or OECD).

A summary of the overall results as mentioned above is rather short and can be formulated as stated in ICH S1B [10]: one long-term animal experiment can be substituted by another short-term animal experiment.

With regard to the regulatory acceptance of alternative carcinogenicity testing, a milestone was set in 1996 with the new ICH guidance S1B [10] on testing for carcinogenicity of pharmaceuticals that is still the gatekeeper in the three ICH regions and enables usage of alternative short-term carcinogenicity testing models employing transgenic mice as a substitute for a second 2-year rodent bioassay in pharmaceutical testing. Applicants were allowed to substitute one of the 2-year standard carcinogenicity tests with one of the following transgenic mouse assays: the $p53^{+/-}$ deficient model, the Tg.AC model, the TgHras2 model, the XPA deficient model, etc. The “etc” was not specified in detail, but as depicted in Table 2, assays using different models were not accepted for drug approval or probably considered supplementary and not pivotal data.

13 years after entry of ICH S1B alternative carcinogenicity assays based on transgenic mice do not play a significant role and very few are included to the application package sent to EMA for evaluation during the centralised procedure in the EU as reported by Friedrich [78], to be discussed further down. In general, only transgenic mouse assays seem to play a significant role when alternatives to the 2-year rodent assay are taken into account from part of the

pharmaceutical industry as no hints on application regarding other possibilities depicted in S1B can hardly be found in the scientific literature, e.g. the initiation-promotion model in rodents, short- or medium-term in vivo rodent test systems or the neonatal rodent tumourigenicity model.

It is, as already mentioned in the introduction, not always obvious whether a strict limitation in animal use or method refinement is the key issue in this context. From the position of animal welfare it makes no sense to replace one of the 2-year rodent bioassays under usage of a maximum of 1,000 animals including those for dose range finding studies by several of so called "alternative methods" that might contribute to the identification of the mode of action of the substance, but consume the double amount of animals.

However, pharmaceutical companies have two main interests: to be assured by their toxicologists (or clinicians at a later stage) upon the safety of their compound and to gain market access as early as possible. Both goals could, from the logistic point of view, be achieved by an intelligent application of state-of-the art alternatives to traditional toxicological methods. However, as the development of a new drug is a very risky and expensive exercise the companies tend to keep the risk of unexpected and unexplainable data/results as low as possible. This results in a rather conservative attitude. Methods are only adopted when validation has clearly shown what can be expected.

One of the lessons learned so far from the long-lasting development process is the fact that from the perspective of regulators alternatives could contribute to faster market access due to decreased investigation periods [8]. As a matter of fact, they believe that the pharmaceutical industry should be more than open towards the application of alternatives to standard assays, not only in the field of carcinogenicity testing. Regulators regret that reality is contrary as the following example shows: an evaluation of carcinogenicity studies of medicinal products for human use authorised via the centralised procedure from 1995-2009 [78] revealed that from a total of 144 new drug applications containing carcinogenicity data, 116 of 144 in total (80.5%) were submitted with the standard package, namely two rodent carcinogenicity studies. One long-term carcinogenicity study in rats and one transgenic mouse study were part of 8 applications (5.5%). 13 applications (9%) contained only one long-term carcinogenicity study in mice or rats and one application contained only one transgenic mouse model (1%). No carcinogenicity studies were performed in case of six applications (4%).

In conclusion, the majority of applicants that had to include information on potential carcinogenicity of their compound to the application dossier decided to abide to the traditional way and performed two rodent bioassays. Long and co-workers gave hints to come across the

reasons for this discrepancy [40]: problems with the alternative model or with study interpretation were reported more frequently than problems with implementation or conduct. The problems reported, with relative frequency, were lack of historic background data (one to five responses for each model), unexpected tumour finding (one to three responses for each model), and tumour identification/characterisation of early lesions (one or two responses for each model) [40].

As above mentioned information regarding the conduct of carcinogenicity studies concern the centralised procedure [78], mechanisms of scientific advice and/or protocol assistance and data interpretation are much better than in case of other procedures as MRP or DCP, where national authorities in single member states are to be consulted. One could speculate that regulatory assistance from part of the authorities is not driven by strong will to substitute traditional assays. European authorities prefer to raise questions rather than express strong positions [52] commented van der Laan in this context in 2002.

These findings together with problems reported by Long and co-workers [40] could be an explanation to the fact that we are still at a very preliminary step facing entire substitution of animal testing. It should be taken in mind that the idea to replace animal-based safety assessment is not a new idea: Russell and Burch published already in 1959 “The Principles of Humane Experimental Technique” in which they stated that all animal experiments should incorporate, as far as possible, the 3Rs: replacement (of animals with alternative methods), reduction (in the numbers of animals used to achieve scientific objectives) and refinement (of methods to minimise animal suffering) [20].

It is not always realised by all stakeholders that for regulatory testing the lack of alternative methods, developed for that particular purpose is rather dramatic. The actual lack of 3R-alternative methods for regulatory testing purposes has been clearly identified by European regulatory institutions [3], but not much has been done to improve this condition. This became also clear from the low number of research proposals on 3R alternatives that was sent in during the first two calls of the Sixth Framework Research Programme of the EU. One of the reasons for this low interest could be that the EU procedure is very difficult and time-consuming, scaring off scientists from both industry and the academic world [5]. However, in case of the cosmetic regulation, from March 2013 onwards all animal testing is banned and products tested on animals, even outside Europe, are completely banned from the EU market [79]. No such pressure is put on the pharmaceutical industry and will probably never been applied, because in case of doubts it is of course human and not animal welfare that has to be assured. Therefore, the need to

develop, validate, implement and routinely apply animal-free methodology in regulatory safety testing of pharmaceuticals within a limited period is practically not given.

However, as recently summarised by Bottini and Hartung, the backlog of overdue method renewal in regulatory toxicology is most remarkable, since hardly any scientific field continues to use experimental set-ups developed more than 40 years ago [80]. Looking for ways out of this condition, an improved and much more intense cross-linking of stakeholders, e. g. academic circles, pharmaceutical industry and regulatory bodies would be beneficial. It is obvious that pharmaceutical companies fear delayed market access of their products and in most of cases they probably do not dare to select alternative testing strategies or run them in parallel, resulting in a lack of historical data. This, as a matter of fact, even worsens the situation as no further data will be generated on behalf of the pharmaceutical industry.

The MIT-economist Michael E. Porter has most prominently put forward the hypothesis that regulations stimulate innovation [81]. Interestingly, life science is considered to be one of the fastest growing disciplines: its knowledge base is said to double every 5 to 7 years [80], but the regulatory framework in the field of alternative method acceptance virtually remains on the same level: ICH S1B came into force in March 1998, more than 13 years ago and is far behind the scientific knowledge. Hartung recently delivered a fierce explanation in this context: scientific truth was established on the basis of irrefutable evidence; majority of opinion governed regulatory toxicology. The process would be driven by historic requirements, contemporary scientific knowledge, individuals who happen to be in charge, political decisions, etc. and would thus be circumstantial rather than strategic [14].

Therefore, a much more flexible and courageous approach from part of the international regulatory institutions would be desirable. Fixed deadlines should be implemented on a regular basis for revision not only of guidelines for carcinogenicity testing. The holistic concept of integrated test strategies [82] (combination of existing data, chemical categories/grouping, in vitro test battery and in silico, computer based assessment) is of course reasonable in the context of combination of refinement, reduction and replacement methodologies and strategies, but bears the potential of regulatory diversion and therefore further delay.

One promising, although authority-driven project regarding the bottlenecks in reduction, refinement and replacement of animal testing in pharmaceutical discovery and development is START-UP (Scientific and Technological issues in 3Rs Alternatives Research in The process of drug development and Union Politics) [83]. Global harmonisation was considered as one of the highest priorities for further success in the implementation of the 3Rs.

Finally, CHMP published a concept paper on the need for revision of the position on the replacement of animal studies by in vitro models in February 2011 expecting that a draft of the revised guideline will be released for consultation in 2011 [84]. The CHMP recommends revising the position on the replacement of animal studies by in vitro models in order to propose a clear process for regulatory acceptance of 3R alternatives. Whether this will be a breakthrough in the development and particularly the acceptance of alternative methods or not cannot be answered but it is of course an important step and underlines the awareness of regulatory bodies to promote alternatives.

Overall, the regulatory framework for alternative carcinogenicity testing is developing, but needs further discussion. Development of alternative methods and their validation is of high interest. Due to the complexity of cancer genesis, a unique testing strategy is highly improbable and will presumably be done on a case by case basis. Replacement and/or substitution of the standard approach and application of animal-free methodology is a long-term goal, but could be speeded up if the validation process could be accelerated.

4 Summary

The assessment of the carcinogenic potential of pharmaceuticals for many decades has relied upon the results of chronic, two-year rodent bioassays. According to the standard protocol, the test substance is administered daily in graduated doses to several groups of test animals for the majority of their life span, normally by the oral route. The limitations and disadvantages of this approach are well known and include the large number of animals required, the significant amount of time, money, personal resources and the difficulty in extrapolating for effects not only seen at maximum tolerated doses to lower levels of human exposure.

Over the last 20 years there has been an increasing interest in developing and validating alternative methods based on the concept of the 3Rs: Replacement, Reduction, and Refinement. The possibility of using alternative short-term carcinogenicity testing models employing transgenic mice as a substitute for a second two-year rodent bioassay in pharmaceutical testing was introduced in 1996 with the drafting of a new International Conference on Harmonization (ICH) guidance (S1B) on testing for carcinogenicity of pharmaceuticals.

In the course of this master thesis a literature survey was performed in the most prominent databases for toxicology and on the homepages of international governmental and non-governmental institutions with the aim to identify those tests that were mentioned in the scientific literature and to comment on its validation and/or acceptance status. Those tests that are most promising and/or are under validation by institutions like ECVAM are discussed in detail.

Several knockout mouse models, cell transformations assays, “omics” technology, quantitative structure-activity relationship (QSAR) based methods, neonatal rodent models and models for high throughput screening of various endpoints were mentioned mostly.

CPMP, FDA and NIHS came across that genetically modified mouse models for pharmaceutical risk assessment currently under consideration have value in carcinogen identification, that these assays can serve as an alternative to the standard mouse 2-year bioassay in a testing program, but that results of these assays should not be considered on their own, but rather integrated with other available data and considered as part of a weight of evidence approach for risk assessment purposes. Other test systems mentioned above are not considered as substitution of the rodent bioassay for regulatory carcinogenicity testing.

Overall, the regulatory framework for alternative carcinogenicity testing is developing, but needs further discussion. Development of alternative methods and their validation is of high interest,

but due to the complexity of cancer genesis, a unique testing strategy is highly improbable and will presumably be done on a case by case basis. Replacement and/or substitution of the standard approach and application of animal-free methodology is a long-term goal, but could be speeded up if the validation process could be accelerated.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

Unterschrift