Replace, Reduce, Refine – Recent Developments in Alternative Testing Methods in Pharmaceutical Toxicology and their Regulatory Acceptability in the EU

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vorgelegt von

Dr. rer. nat. Sandra Mahr

aus Berlin

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Betreuer und 1. Referent	PD Dr. Gerd Bode
2. Referent	PD Dr. Elke Röhrdanz

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List of Abbreviations

ADME(T)	Absorption, Distribution, Metabolism, Excretion, (Toxicity)
AOP	Adverse Outcome Pathway
AUC	Area Under the Curve
BPR	Biocidal Products Regulation
CaCVAM	Canadian Centre for the Validation of Alternative Methods
CAD	Carcinogenicity Assessment Document
CCAAM	Canadian Centre for Alternatives to Animal Methods
cDNA	copy Deoxyribonucleic Acid
CE	Conformité Européenne
CHMP	Committee for Medicinal Products for Human Use
CLP	Classification, Labelling and Packaging of Chemicals
c_{max}	Maximum Serum Concentration
CPMP	Committee for Proprietary Medicinal Products
CVMP	Committee for Medicinal Products for Veterinary Use
EC	European Commission
ECETOC	European Centre for the Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EDQM	European Directorate for the Quality of Medicines and Healthcare
EEC	European Economic Community
EMA/EMEA	European Medicines Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EPAR	European Public Assessment Report
ESAC	EURL-ECVAM Scientific Advisory Committee
EU	European Union
EURL	European Union Reference Laboratory
FDA	Food and Drug Administration
GCP	Good Clinical Practice
IATA	Integrated Approaches to Testing and Assessment
ICH	International Council for Harmonisation
IMI-JU	Innovative Medicines Initiative Joint Undertaking
JaCVAM	Japanese Center for the Validation of Alternative Methods

List of Abbreviations (continued)

JEG	Joint ad hoc Expert Group
J3RsWG	Joint CVMP/CHMP Working Group on the Application of the 3Rs in Regulatory Testing of medicinal Products
KE	Key Event
KoCVAM	Korean Centre for the Validation of Alternative Methods
LD_{50}	Lethal dose 50%
LOAEL	Lowest Observed Adverse Effect Level
MIE	Molecular Initiating Event
MoA	Mode of Action
MOC	Multi-Organ Chip
MPS	Microphysiological System
NIEHS	National Institute of Environmental Health Sciences
NIFDS	National Institute of Food and Drug Safety Evaluation
NIHS	National Institute of Health Sciences
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PD	Pharmacodynamics
PDMS	Polydimethylsiloxane
Ph. Eur.	Pharmacopoeia Europaea (European Pharmacopoeia)
QSA/TR	Quantitative Structure-Activity/Toxicity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic Acid
SA	Structural Alert
SME	Small-Medium-sized Enterprise
SWP	Scientific Working Party
SAWP	Scientific Advice Working Party
TG	Test Guideline
t_{max}	Time of Maximum Serum Concentration
US	United States

1 Introduction

1.1 Aim of the present thesis

In the past decades, there was significant progress in biological and medical research as well as in pharmaceutical and chemical development, also based on animal testing. To date, millions of animals are used every year in biological and medical research as well as in pharmaceutical development.

Medicinal products and chemicals need to be authorised before they can enter the market. To seek marketing authorisation for medicinal products, a comprehensive dossier needs to be compiled and submitted to the health authorities. These data are usually compiled in the Common Technical Document (CTD), recommended by the International Council for Harmonisation (ICH) in the multidisciplinary guideline called M4 [1]. This dossier includes also data on the toxicity of the respective drugs. Investigations on the potential toxicity of medicinal products and chemicals are mostly carried out using *in-vivo* experiments.

Regarding animal use, a rethinking has begun driven by both ethical and financial aspects. The principles of the 3Rs has been developed in the late 1950s and deal with the replacement, reduction and refinement of animal experiments [2]. The 3Rs have been incorporated in the European regulatory legislation [3] and therefore, pharmaceutical and chemical companies are called to implement the 3Rs into their development programs.

However, various challenges need to be met in the development of alternative testing strategies and their establishment as fully regulatory acceptable approaches.

The objective of the present work is to provide an overview about the recent developments in the field of alternative testing approaches and their status of regulatory acceptance by European Union (EU) health authorities. In addition to a general overview, the thesis will focus on highly innovative testing methods namely organoids, microphysiological systems, “omics” technologies, and *in-silico* tools.

2 Regulatory requirements

2.1 European legislation

In the EU, the requirements on the marketing authorisation of medicinal products as well as for the authorisation of clinical trials are laid down in several European Directives, Regulations as well as regulatory guidelines.

2.1.1 Directive 2001/83/EC

The regulatory requirements for marketing authorisation of medicinal products for human use are laid down in the European Directive 2001/83/EC, as amended. To gain marketing authorisation for medicinal products for human use in the EU, data on the pharmaceutical quality, efficacy as well as safety of the proposed medicinal product need to be provided in a dossier [4].

Despite the demonstration of pharmaceutical quality of the proposed medicinal product, the Directive requests the provision of non-clinical and clinical data. To that end, comprehensive information on the pharmacological properties of the medicinal product as well as its toxic potential need to be presented. According to Annex I Part IV of Directive 2001/83/EC, as amended, non-clinical data should be provided by the applicant concerning the following areas [4]:

Table 1: Non-clinical data to be provided for marketing authorisation of medicinal products

Area	Data to be presented in the dossier
Pharmacology	• Mode of action as well as dose- and time-dependent effect should be described using validated *in-vitro* and *in-vivo* assays • Undesirable pharmacodynamic effects should be described • Pharmacodynamic interaction studies should be performed • Mechanism should be investigated • Safety pharmacology studies are needed especially for life-sustaining functions
Pharmacokinetics	• Data on the absorption, distribution, metabolism and excretion (ADME) of the active substance should be provided • Especially at early stage, data on AUC and c_{max}/t_{max} should be available • *In-vitro* studies might be used to address protein-binding, drug-drug interaction or metabolism
Single-dose toxicity	• Requirement for single-dose studies withdrawn by the European Medicines Agency in 2010; Exceptions are reported in ICH M3 guideline
Repeated-dose toxicity	• Tests for short-term and long-term exposure to the drug should be performed depending on the proposed clinical use • Immunotoxic potential is often assessed in repeat dose studies

Area	Data to be presented in the dossier
Genotoxicity	• Studies investigating the mutagenic and clastogenic potential need to be performed for any new substance
Carcinogenicity	• Studies on carcinogenicity required if the medicinal product is intended for prolonged use • Not required for biologicals
Reproductive and developmental toxicity	• Studies investigating potential effects on male and female reproductive function • Studies on teratogenic effects, embryo-foetal toxicity
Local tolerance	• Potential reactions at the site of administration shall be investigated (*in-vitro* testing possible)

Additionally, Directive 2001/83/EC, as amended, refers to the Community Guidelines published by the European Commission. Volume 3 of *"The rules governing medicinal products in the European Union"* comprises scientific guidelines issued by the Committee for Medicinal Products for Human Use (CHMP) [5]. These guidelines on the quality, safety and efficacy of medicinal products should be considered during drug development. Although not legally binding, guidelines should be followed or a proper justification for any deviation should be provided.

To comply with these regulatory requirements, animal testing is usually needed. However, validated *in-vitro* tests may be used instead of animal studies provided that reliable data can be obtained for safety evaluation [4].

2.1.2 Directive 2001/20/EC and Regulation (EU) 536/2014

A further European Directive requesting toxicology data is Directive 2001/20/EC, as amended, on the *"[…] implementation of Good Clinical Practice (GCP) in the conduct of clinical trials on medicinal products for human use"* [6].

To obtain clinical trial authorisation in the EU, a dossier needs to be submitted to the respective competent authorities as well as to the concerned Ethics Committees. With this dossier, the applicant is requested to provide comprehensive data on the pharmacology and toxicology of the medicinal product under development [6]. To that end, *in-vitro* and *in-vivo* studies should usually be conducted to determine, whether the proposed drug has an acceptable safety profile that allows the administration to humans [6].

In 2014, the Regulation (EU) No 536/2014 entered into force and will be applicable probably in 2019. This Regulation will then replace the clinical trials Directive 2001/20/EC [7].

2.1.3 European Pharmacopoeia (Ph. Eur.)

The European Pharmacopoeia (Ph. Eur.) is a collection of monographs specifying the quality standards for medicinal products for both human and veterinary use with legally binding character [8]. The monographs comprise standards for ingredients, dosage forms and analytical methods. Moreover, Ph. Eur. monographs frequently request animal tests. However, as other European legislations, the Ph. Eur. is committed to reduce animal use in accordance with Directive 2010/63/EU [9].

2.1.4 Further requirements

During the development phase, several EU regulatory guidelines published by the EMA should be considered for nonclinical safety testing of pharmaceuticals. These guidelines specify requirements additional to those stipulated by the ICH, e.g. for the carcinogenicity assessment of human insulin analogues [10].

2.1.5 Regulatory requirements for medical devices, chemicals and other products

2.1.5.1 Certification of medical devices

In the EU, medical devices need to be certified before they can enter the market (CE certification). To that end, preclinical and clinical studies are required according to their risk classification. Depending on the intended human exposure to the device, studies on single- and repeated-dose toxicity, genotoxicity, carcinogenicity as well as reproductive and developmental toxicity need to be performed. The requirements for investigating the toxicity of medical devices are laid down in the Council Directive 93/42/EEC, which will be repealed by Regulation (EU) 2017/745 [11, 12].

2.1.5.2 Registration, evaluation, authorisation of chemicals (REACH)

REACH is an EU-Regulation aimed at improving environmental and human health protection from the risks provoked by chemicals while increasing the competitiveness of the chemical industry in the EU [13]. Furthermore, REACH encourages alternative methods to reduce animal testing. REACH applies to all chemical substances in our daily lives such as cleaning products, paints, clothing, furniture and electrical devices. REACH therefore affects most companies throughout the EU [13].

2.1.5.3 Regulation on the classification, labelling and packaging of chemicals

Requirements for hazard classification, labelling and packaging (CLP) of chemicals are laid down in *"Regulation (EC) no 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances"* [14]. According to this Regulation, there is no obligation to generate new toxicology data for hazard classification purposes, but

all relevant data available should be taken into consideration. Animal tests should be replaced by suitable alternative testing methods, whenever possible [14].

2.1.5.4 Biocidal Products Regulation (BPR)

Requirements concerning the marketing authorisation of biocidal products are laid down in *"Regulation (EU) no 528/2012 of the European parliament and of the council of 22 May 2012 concerning the making available on the market and use of biocidal products"* [15]. As for medicinal products, biocidal products require an authorisation before they can be placed on the market. Furthermore, all active substances included in a biocidal product need to be approved previously as well, with some exceptions. Since biocides encounter the environment, their potential toxicity needs to be assessed prior to marketing authorisation [15].

2.1.5.5 Cosmetics Regulation No 1223/2009

Registration requirements for cosmetics including toxicity assessments are laid down in the *"Regulation (EC) no 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products"*. According to this Regulation, animal tests for cosmetic products are prohibited [16].

2.2 Toxicology guidelines from the International Council for Harmonisation (ICH)

Regulatory requirements for the authorisation of medicinal products have been harmonised among the European Union, the United States (US) as well as Japan via the ICH. The regulatory requirements for the conduct of clinical trials and the authorisation of medicinal products are specified in various guidelines, which are published by the ICH. Currently, the following guidelines apply to the toxicology assessment of medicinal products for human use [17]:

Table 2: Current ICH guidelines on toxicology testing for pharmaceuticals for human use

Guideline	Reference number	Effective date
Carcinogenicity		
S1: Regulatory notice on changes to core guideline on rodent carcinogenicity testing of pharmaceuticals	EMA/CHMP/ICH/752486 /2012	09/2013
S1 A: The need for carcinogenicity studies of pharmaceuticals	CPMP/ICH/140/95	07/1996
S1 B: Testing for carcinogenicity of pharmaceuticals	CPMP/ICH/299/95	03/1998

Guideline	Reference number	Effective date
S1 C (R2): Dose selection for carcinogenicity studies of pharmaceuticals	CPMP/ICH/383/95	10/2008
Genotoxicity		
S2 (R1): Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use	CHMP/ICH/126642/08	06/2012
Toxicokinetics and Pharmacokinetics		
S3 A: Toxicokinetics: A guidance for assessing systemic exposure in toxicology studies	CPMP/ICH/384/95	06/1995
S3 B: Pharmacokinetics: Guidance for repeated-dose tissue distribution studies	CPMP/ICH/385/95	06/1995
Toxicity		
S4: Duration of chronic toxicity testing in animals (rodent and non-rodent toxicity testing)	CPMP/ICH/300/95	05/1999
Reproductive toxicology		
S5 (R2): Detection of toxicity to reproduction for medicinal products and toxicity to male fertility	CPMP/ICH/386/95	03/1995
Biotech products		
S6 (R1): Preclinical safety evaluation of biotechnology-derived pharmaceuticals	CPMP/ICH/731268/1998	12/2011
Pharmacology		
S7 A: Safety pharmacology studies for human pharmaceuticals	CPMP/ICH/539/00	06/2001
S7 B: The non-clinical evaluation of the potential for delayed ventricular repolarisation (QT interval prolongation) by human pharmaceuticals	CPMP/ICH/423/02	11/2005
Immunotoxicology		
S8: Immunotoxicity studies for human pharmaceuticals	CHMP/ICH/167235/04	05/2006
Anticancer therapeutics non-clinical testing		
S9: Non-clinical evaluation for anticancer pharmaceuticals	CHMP/ICH/646107/08	05/2010

Guideline	Reference number	Effective date
Photosafety evaluation		
S10: Guidance on photosafety evaluation of pharmaceuticals	CHMP/ICH/752211/2012	06/2014
Impurities		
M7: Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk	EMA/CHMP/ICH/83812/ 2013	01/2016
M7: Application of the principles of the ICH M7 guideline to calculation of compound-specific acceptable intakes	EMA/CHMP/ICH/ 4582894/2015	02/2018
Q3 C (R5): Impurities: guideline for residual solvents	CPMP/ICH/82260/06	08/2011
Q3 C (R6): Impurities: guideline for residual solvents	CPMP/ICH/82260/06	06/2017
Q3 D: Impurities: guideline on elemental impurities	CHMP/ICH/353369/2013	06/2016
Q3 D (R1): Impurities: guideline on elemental impurities, PDE for cutaneous application	CHMP/ICH/353369/2013	Under revision
Clinical trials		
M3 (R2): Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals	CPMP/ICH/286/95	12/2009

2.3 Current data on the use of animals for scientific purposes

According to the *"Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union"* from 2013, the number of animals used for scientific purposes was below 11.5 million, which represents a reduction of over half a million animals in the EU compared to the numbers reported in 2008 [18].

In total, more than 60% of animals were used for research and development. From 2008 to 2011, the animal numbers used for pharmaceutical research and development decreased from 22.8% to 18.8% equating to 575,518 animals, whereas the percentage of animals used for fundamental biological research has increased from 38% to 46% (equating to 715,519 animals) [18].

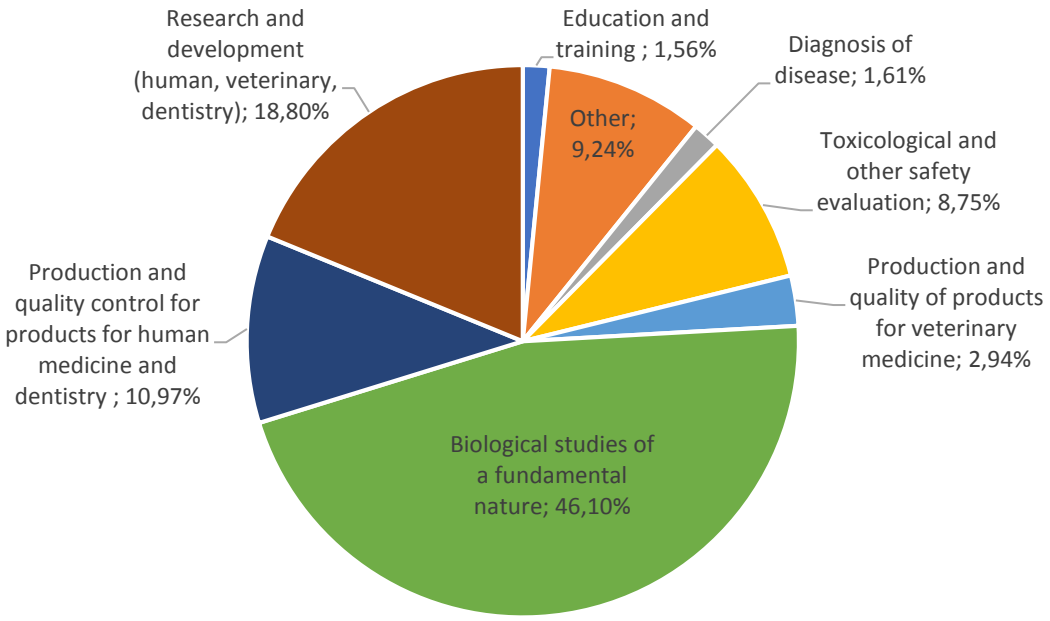

Figure 1: Purposes of animal experiments reported 2011, Source: [18]

The highest number of animals in the EU is used for pharmacological research and development as well as fundamental biological research. In 2011, 8.75% of animals were used for toxicological and other safety evaluations, representing 1,004,873 animals. Compared to 2008, 37,280 less animals were used in 2011. In general, the percentage of animals used for toxicological and safety purposes was quite stable during the last 16 years [18].

Among the animals used in the EU, the proportion of species used are specified in Table 3 below [18].

Table 3: Percentages of animals used in the EU Member States in 2011 [18]

Species	Percentages of total animals used
Rabbits and rodents (mice / rats)	80 (61 / 14)
Cold-blooded animals (reptiles, amphibians and fishes)	12.4
Birds	5.9
Artiodactyla and *Perissodactyla* (horses, donkeys, pigs, goats, sheep and cattle)	1.2
Carnivores (dogs, cats)	0.25
Non-human primates	0.05

2.4 Principle of 3Rs: Replace, Reduce, Refine

2.4.1 Background

The principle of the 3Rs was introduced by William Russell and Rex Burch in 1959 and has been broadened during the last decades [2, 3]. According to this principle, replacement of animal testing by alternative approaches, reduction of the number of animals used in the experiment and refinement of the experiments to minimise pain and distress as well as optimise animal welfare should always be considered [2, 3].

2.4.2 EU regulatory activities fostering the principle of the 3Rs

During the past 20 years, high efforts have been made to foster the principle of the 3Rs in regulatory testing of medicinal products. The European Directive 2001/83/EC, as amended, require that animal testing should be conducted according to Directive 2010/63/EC on the protection of animals used for scientific purposes [4]. In line with Directive 2010/63/EC, the principle of the 3Rs needs to be considered when selecting testing approaches intended for use in regulatory testing of human or veterinary medicinal products [9].

Table 4: EU regulatory documents facilitating the principle of the 3Rs

Regulatory document	Reference Number	Year
Replacement of animal studies by *in-vitro* models	CPMP/SWP/728/95	1997
Reflection Paper on *in-vitro* Investigation of Mitochondrial Toxicity of Anti-HIV Nucleoside Reverse Transcriptase Inhibitors	EMEA/CHMP/SWP/ 8212/2007	2007
Questions and Answers on the Withdrawal of the *"Note for Guidance in Single Dose Toxicity"*	EMA/CHMP/SWP/ 81714/2010	2010
Questions and Answers on the *"Guideline on the Limits of Genotoxic Impurities"*	EMA/CHMP/SWP/ 431994/2007 Rev. 3	2007
Guideline on non-clinical local tolerance testing of medicinal products	EMA/CHMP/SWP/ 2145/2000 Rev. 1, Corr. 1*	2015
Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes	OJ L 276/33	2010

In 2010, the *"Note for guidance on single dose toxicity"* (EMA/CHMP/SWP/ 81714/2010) has been withdrawn by the EMA due to the limited significance for the assessment of general toxicity. Instead, data on acute toxicity should be drawn from dose-escalation studies or short-duration dose-ranging studies. However, according to ICH M3, an extended single-dose toxicity study remains for certain products (e.g. for oncologics), as support for some expedited

strategies and support for clinical Phase III trials, when there is a risk for overdosage [19].

Furthermore, the 3Rs' principle has been implemented in several EU legislative acts, including Directive 2001/83/EC, REACH, CLP, BPR as well as the Cosmetics Directive 76/768/EEC [4, 13-16].

2.5 International initiatives promoting alternative testing strategies

2.5.1 EMA Joint CVMP/CHMP Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (J3RsWG)

A Joint ad hoc Expert Group (JEG 3Rs) has been established by the EMA in 2010. This group is assigned to improve and facilitate the application of the 3Rs principles to the regulatory testing of medicinal products as well as to provide advice and recommendations to both the CHMP and CVMP. Recently, the JEG 3Rs was replaced by the *"Joint CVMP/CHMP Working Group on the Application of the 3Rs in Regulatory Testing of medicinal Products"* (J3RsWG) [20]. This group is mainly composed of CHMP and CVMP working parties as follows:

- Safety Working Party (human and veterinary medicines)
- Biologics Working Party
- Immunologicals Working Party
- Efficacy Working Party
- CHMP/CVMP members
- Experts from other working parties (e.g. Vaccines Working Party, Biosimilar Medicinal Products Working Party, Joint CHMP/CVMP Quality Working Party)

Furthermore, the J3RsWG acts in close cooperation with the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) and the European Directorate for the Quality of Medicines and Healthcare (EDQM) [21].

2.5.2 European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM)

In 1991, the European Commission launched the European Centre for the Validation of Alternative Methods (ECVAM) to facilitate the activities in establishing alternative methods for regulatory testing. The tasks of the ECVAM have been assigned to the EURL-ECVAM, which has been established by the European Commission in 2011. The EURL-ECVAM is based in Italy and responsible for the validation of alternative testing approaches [22].

The activities of the EURL-ECVAM comprise research, test development as well as validation of the developed alternative testing approaches. Furthermore, the EURL-ECVAM is responsible for the coordination of the independent evaluation of the alternative tests at a European level to ensure their relevance and reliability. The EURL-ECVAM supports the European Commission and acts independently of any commercial interests or individual scientists. It provides advice in all aspects of test validation, *in-vitro* toxicology tests as well as animal welfare issues [22].

The EURL-ECVAM regularly releases strategy papers, recommendations as well as annual status reports providing an overview about the validation status of alternative testing methods [22, 23].

2.5.3 European Partnership for Alternative Approaches to Animal Testing (EPAA)

Within the European Partnership for Alternative Approaches to Animal Testing (EPAA), the European Commission, European trade associations, and companies from several industry sectors cooperate on a voluntary basis [24].

This cooperation is aimed to gather both knowledge and resources to accelerate the development, validation and regulatory acceptance of alternative testing methods to meet the overall goal to facilitate the 3Rs based on Directive 2010/63/EU [24].

According to the 2017 annual report, the following projects were ongoing [25]:

- Optimised strategies for assessing skin sensitisation: 3D skin models to assess the potential for skin sensitisation
- Clostridial vaccines for veterinary use: Novel *in-vitro* methods to replace animal-based in-process control tests
- Human Rabies vaccines: Replacement of animal-based potency tests
- Acute toxicity: Identification of clinical signs predictive of mortality
- Harmonisation of 3Rs in biologicals: Deleting international regulatory requirements for *in-vivo* safety tests
- Carcinogenicity of agrochemicals: Waiving of two-year carcinogenicity studies

2.5.4 EU-ToxRisk

EU-ToxRisk is a large-scale project that has been started in 2016. EU-ToxRisk involves 39 institutions, including academia and companies, and is publicly funded by the EU as part of the research program Horizon 2020 [26].

The overall goal is to implement the new insights in alternative testing methods in the future safety assessment of chemicals. Furthermore, new assessment approaches should be established, including animal-free *in-vitro* methods as well as “omics” and *in-silico* technologies. EU-ToxRisk is mainly focused on developments in the areas of repeated-dose toxicity as well as developmental and reproductive toxicity [26].

2.5.5 International programs outside the EU

Despite the efforts to foster the principle of the 3Rs in the EU, there are various international agencies facilitating the development and validation of alternative testing approaches.

In the US, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 2000 as part of the National Institute of Environmental Health Sciences (NIEHS). The committee comprises representatives from 16 federal regulatory and research agencies in the USA. Its key tasks are to sharpen the cooperation among US Federal regulatory agencies, to ascertain that alternative testing approaches are validated to meet US regulatory requirements as well as to reduce, refine, or replace animal use in regulatory testing [27].

In Canada, the Canadian Centre for the Validation of Alternative Methods (CaCVAM), as part of the Canadian Centre for Alternatives to Animal Methods (CCAAM), is responsible for the development and validation of alternative testing methods [28].

In Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) was established in 2005 as part of the National Institute of Health Sciences (NIHS) in Tokyo. Its key responsibilities are to facilitate the 3Rs prioritising reduction and replacement, to ensure the validation, review and the regulatory and international acceptance of alternative testing methods, which have been developed in Japan [29].

In South Korea, the Korean Centre for the Validation of Alternative Methods (KoCVAM) was established in 2009 and belongs to the National Institute of Food and Drug Safety Evaluation (NIFDS). According to the institutions in the EU, US and Japan, the KoCVAM is responsible for the validation and peer-review of alternative test methods [30].

All these international committees act in close cooperation to facilitate the development of alternative testing strategies with international regulatory acceptance.

2.5.6 Implementation of the 3Rs by the ICH

On an international level, the ICH is fostering the principle of the 3Rs aiming at an implementation of the 3Rs into the ICH guidelines. To date, several ICH documents already consider the principle of the 3Rs, among them the guidelines S1, S2, S7B, and S10 as summarised in Table 5 below [31-34]. The ICH guideline S5(R2) *"Detection of toxicity to reproduction for medicinal products & toxicity to male fertility"* is currently under revision to reduce animal use [35].

Table 5: ICH documents considering the principle of the 3Rs

Regulatory document	Reference Number	Year
Note for Guidance on Photosafety testing	CPMP/SWP/398/01	2002
Note for Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use	EMEA/CHMP/ICH/126642 /2008	2008
Note for Guidance on the Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarisation (QT Interval Prolongation) by Human Pharmaceuticals	CPMP/ICH/423/02	2005
Note for Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals	CPMP/ICH/286/95	2009
ICH M3 (R2) Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals	CPMP/ICH/286/95	2013
ICH S1 Regulatory notice on changes to core guideline on rodent carcinogenicity testing of pharmaceuticals S1 Carcinogenicity Studies	EMA/CHMP/ICH/536328/ 2013 Rev. 1	2016
ICH S2 (R1) Genotoxicity testing and data interpretation for pharmaceuticals intended for human use	CHMP/ICH/126642/08	2011
ICH S5 (R2) reproductive toxicology: detection of toxicity to reproduction for human pharmaceuticals	CPMP/ICH/386/95	under revision
ICH S7B Non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharma-ceuticals	CPMP/ICH/423/02	2005
ICH S9 Non-clinical evaluation for anticancer pharmaceuticals	CHMP/ICH/646107/08	2010
ICH S10 Photosafety evaluation of pharma-ceuticals	CHMP/ICH/752211/2012	2014

3 Regulatory acceptance of alternative testing approaches

3.1 Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches

In 2016, the CHMP adopted the *"Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches"* (EMA/CHMP/CVMP/JEG-3Rs/450091/2012) [3]. A detailed description of the guideline content is given below.

3.1.1 Regulatory acceptance criteria according to the EMA

In the *"Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches"*, the EMA defines criteria for regulatory acceptance of alternative testing methods as follows [3]:

- A defined testing approach based on standard protocols as well as clearly defined and scientifically proven endpoints should be accessible.
- The test method should be of relevance for the regulatory testing approach. The method should allow the measurement of the target effect with acceptable accuracy.
- The alternative testing method should be at least of comparable value as an already existing method or even better or it should be able to provide new data, which could not be delivered by an existing method.
- The alternative testing method should fulfil general testing requirements such as reliability and robustness.

3.1.2 Proof of scientific validity

Furthermore, the guideline requires the proof that the alternative testing method is scientifically valid. The extent of information and the application of the criteria to the alternative testing method depends on several factors such as the regulatory and scientific rationale, the kind of method, the proposed use, how this alternative test is related to the effect of interest as well as the history of this testing method [3].

3.1.3 Method validation

A further important aspect for the regulatory acceptance of an alternative testing method is its formal validation. Generally, methods used for regulatory testing should be validated to ensure sufficient specificity, linearity, accuracy, and precision. Ideally, an alternative method intended to be used in regulatory testing should be formally validated as described by the EURL-ECVAM and the EDQM. However, scientifically valid alternative methods may also be acceptable in a regulatory submission and/or can be included in regulatory guidelines even in the

absence of a formal validation. The data will then be evaluated by the competent authorities on a case-by-case basis [3].

3.1.4 Data submission

Data generated by using alternative testing approaches can be submitted in parallel to data obtained by using accepted testing methods on a voluntary basis. The data achieved by 3Rs testing methods will not be considered for the regulatory decision but will be evaluated independently regarding the regulatory acceptance of this testing method in the future [3].

3.1.5 Scientific advice and qualification of alternative testing approaches by the Scientific Advice Working Party (SAWP)

The competent authorities support the development of alternative testing approaches by providing scientific advice, which can be sought by sponsors during the drug development phase [3].

New testing approaches will be assessed by the SAWP for qualification as regulatory testing method. Applicants can submit their data to the EMA via the qualification inbox qualification@ema.europa.eu, which will subsequently be assessed by a qualification team. This qualification process will end with a CHMP opinion or a CHMP qualification advice regarding the use of the proposed alternative testing method. The scientific community will be involved in the opinion-making process via a public consultation. The new method will be available for the community after the final opinion is published by the CHMP [3].

3.2 Validation process by the EURL-ECVAM

According to Directive 2010/63/EC, the validation of alternative testing approaches should be coordinated by the EURL-ECVAM. The EURL-ECVAM validation process involves stakeholders, international partners as well as the submitters of the alternative testing methods. The EURL-ECVAM validation process is divided into four steps as shown in Figure 2 below [36].

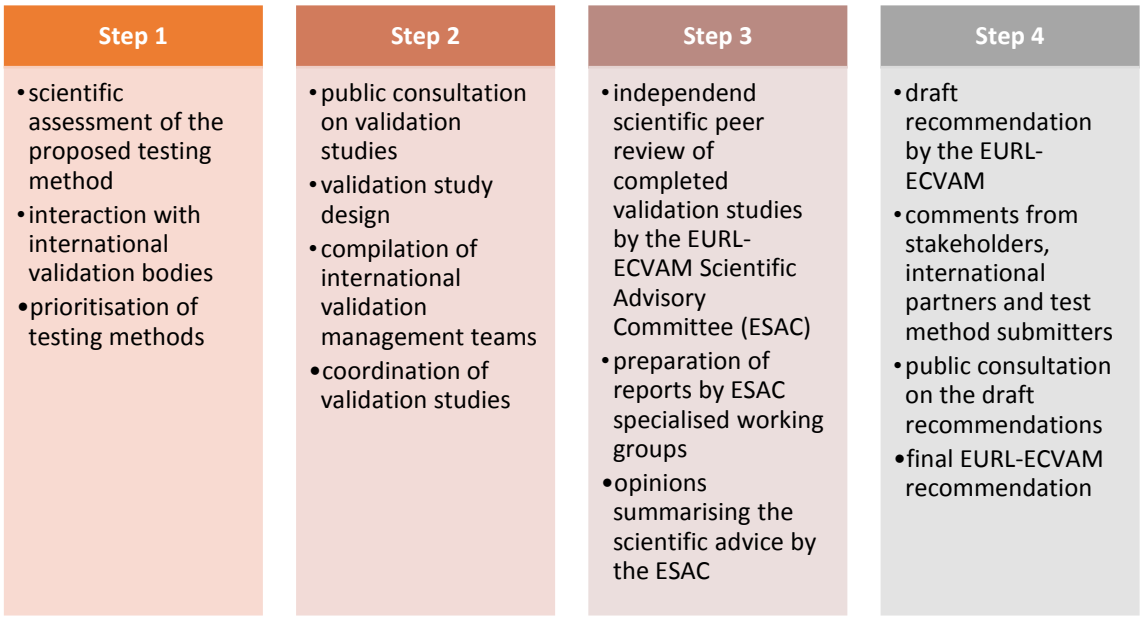

Figure 2: Illustration of the stepwise EURL-ECVAM validation process

3.3 Validation status of alternative testing methods at EURL-ECVAM

According to the EURL-ECVAM, an alternative test method is regulatory accepted in case it has been formally accepted by regulatory authorities indicating that the test method can be applied to meet a specific regulatory requirement. Regulatory acceptance includes that the alternative testing method has been formally adopted by the EU and/or OECD as an EU test method. This method will then be included in the EU Test Methods Regulation (EC) No 440/2008 (2008) and/or will be included in an OECD Test Guideline (TG), respectively.

The EURL-ECVAM regularly publishes information on the validation status and regulatory acceptance of alternative testing methods on the EURL-ECVAM website. The following information on the validation status of alternative testing methods was obtained from the EURL-ECVAM website (accessed July 2018) as well as from the EURL-ECVAM Status Report 2017 [23, 37].

Several test methods were validated by the EURL-ECVAM and adopted by the OECD, ICH or Ph. Eur. during the past years as summarised in Table 6 below. Several validation studies are ongoing, or assay development has been initiated.

Table 6: Validation status and regulatory acceptance of alternative testing approaches

Area	Regulatory framework	Methods validated by EURL-ECVAM / Regulatory acceptance	Methods under validation / development
Toxicokinetics	OECD TG 417: TK to be tested *in vivo* in rats [38]	• OECD TG 428: Skin absorption (2004) [39]	• Cytochrome p450 (CYP) induction test using the human cryopreserved HepaRG® cell line and cryopreserved human hepatocytes [37] • Multi-study validation trial ongoing and coordinated by EURL-ECVAM
Eye irritation*	OECD TG 405: Eye irritation/corrosion to be tested preferably in rabbits [40]	• OECD TG 437: Bovine Corneal Opacity and Permeability test (BCOP), 2009 [41] • OECD TG 438: Isolated Chicken Eye (ICE) test method, 2012 [42] • OECD TG 460: Fluorescein Leakage (FL), 2012 [43] • Cytosensor Microphysiometer (CM): OECD draft guideline not adopted [44]	• SkinEthic Human Corneal Epithelium test: immortalised human corneal epithelial cells [37] • EpiOcular Eye Irritation Test: non-transformed, human-derived epidermal keratinocytes [37] • Low Volume Eye Test (LVET): *in-vivo* refinement test method, but not recommended for prospective use [37]
Phototoxicity	OECD TG 432: 3T3-NRU *in-vitro* phototoxicity test (3T3-NRU-PT) [45]	• OECD TG 432 (2004) [45] • ICH S10 (2014) [46]	• Currently no validation studies ongoing [37]
Skin corrosion/ Irritation*	• OECD TG 404: *In-vivo* Draize rabbit test for skin corrosion/ irritation [47]	• OECD TG 430: Transcutaneous electrical resistance test (TER) [48] • OECD TG 431: Human skin models Episkin™, EpiDerm™, SkinEthic™, EpiCS® (formerly EST-1000) [49]	• No validation studies ongoing [37]

Area	Regulatory framework	Methods validated by EURL-ECVAM / Regulatory acceptance	Methods under validation / development
		• OECD TG 435: CORROSITEX® (*In-vitro* membrane barrier test) [50]	
Skin sensitisation	• OECD TG 406: Guinea Pig Minimisation test (GPMT), Buehler Test [51] • OECD TG 429: Mouse Local Lymph Node Assay (LLNA) [52] • OECD TG 442A: Mouse Local Lymph Node Assay DA (LLNA-DA) [53] • OECD TG 442B: Mouse Local Lymph Node Assay BrdU-ELISA (LLNA-BrdU) [54]	• No formally validated alternative tests for skin sensitisation available so far [37] • Reduced LLNA (r-LLNA) using 20% less animals available and included in OECD TG 429, but not suitable for hazard classification [37] • human Cell Line Activation Test (h-CLAT) included in OECD TG 442E (not applicable for pharmaceuticals so far) [55]	• Studies and/or peer-review for several methods ongoing (e.g. LuSens) [37]
Acute toxicity	• OECD TG 401: Acute Oral Toxicity deleted in 2002 [56]	• *In-vitro* cytotoxicity validation study ongoing (prediction of rodent *in-vivo* LD_{50}) [37]	• No validation studies ongoing [37]
Repeated-dose toxicity	• OECD TG 407: 28-day oral toxicity in rodents [57] • OECD TG 408: 90-day oral toxicity in rodents [58] • OECD TG 409: 90-day oral toxicity in non-rodents [59] • OECD TG 410: 21/28-day dermal toxicity in rat, rabbit or guinea pig [60]	• No validated alternative methods available so far [37]	• No validation studies ongoing [37] • PULMO-NET: EU-project aimed to improve the methodology to investigate lung function *in vitro* [37] • Predict-IV: EU-project aimed to develop integrated *in-vitro* test systems using specific biomarkers to predict repeated dose toxicity prior to *in-vivo* tests [37]

Area	Regulatory framework	Methods validated by EURL-ECVAM / Regulatory acceptance	Methods under validation / development
	• OECD TG 411: 90-day dermal toxicity in rat, rabbit or guinea pig [61] • OECD TG 412: 28-day inhalation toxicity [62] • OECD TG 413: 90-day inhalation toxicity [63] • OECD TG 452: Chronic toxicity studies in rodents [64]		
Carcinogenicity	• OECD TG 451: Carcinogenicity studies [65] • OECD TG 453: Combining chronic toxicity/carcinogenicity studies [66]	• OECD GD 214: *In-vitro* Syrian Hamster Embryo (SHE) cell transformation assay (CTA), 2015 [67] • OECD GD 231: *In-vitro* Bhas 42 cell transformation assay (CTA), 2016 [68]	• CarcinoGENOMICS FP6 project: development of toxicogenomics- and metabolomics-based *in-vitro* tests for detection of genotoxic and carcinogenic agents [37]
Genotoxicity	• OECD TG 471: Bacterial reverse mutation test (Ames test) [69] • OECD TG 476: Mammalian call gene mutation test [70] • OECD TG 474: *In-vivo* mammalian erythrocyte micronucleus test [71] • OECD TG 475: *In-vivo* mammalian bone marrow chromosome aberration test [72]	• OECD TG 473: *In-vitro* mammalian chromosome aberration test, 2014 [75] • OECD TG 478: *In-vitro* mammalian cell micronucleus test, 2014 [76] • OECD TG 476 divided into two TGs: o updated TG 476 using hprt and xprt genes (2015) [70] o OECD TG 490 using thymidine kinase gene (2015) [77]	• 3D genotoxicity assays [37] • Comet assay *in vitro* and *in vivo* [37]

Area	Regulatory framework	Methods validated by EURL-ECVAM / Regulatory acceptance	Methods under validation / development
	• OECD TG 488: *In-vivo* transgenic rodent somatic and germ cell gene mutation assays [73] • OECD TG 489: *In-vivo* mammalian alkaline comet assay [74]		
Biologicals	• Ph. Eur. monographs [8] • EU guidelines on production and quality control of biologicals [78]	• Ph. Eur. general method 2.6.30: Monocyte-activation test for *in-vitro* pyrogenicity testing adopted by Ph. Eur in 2009 and revised in 2016 [79] • Ph. Eur. general text 2.7.8: ToBI and ELISA for batch potency testing of human tetanus vaccines (2003) [37]	• No validation studies ongoing [37] • EPAA project aimed at the development/standardisation of tests for quality control of several vaccines [37]

* mainly relevant for hazard classification of chemicals / plant protection products / cosmetics

4 Recent developments in alternative testing strategies and their status of regulatory acceptance

4.1 Challenges in the development of alternative testing approaches

The development of alternative testing methods in general requires an in-depth knowledge of the provoked adverse effects and their underlying mechanism [80]. This holds particularly true for complex endpoints such as reproductive and developmental toxicity.

Furthermore, the development of *in-vitro* assays requires the downscaling of a whole organism to an *in-vitro* system based on single cells or tissue explants. Features such as blood circulation and respiration should be taken into consideration, when setting-up *in-vitro* assays involving several organ systems. In addition, cell culture test systems require reliable cell sources and standardised protocols.

Moreover, from the regulatory point of view, a major challenge for the development of reliable alternative testing methods is the need for complete validation. Tests used in registration dossiers need to be fit for the intended regulatory purpose. For alternative testing methods, the EMA issued a validation by the EURL-ECVAM as specified in the *"Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches"* (EMA/CHMP/CVMP/JEG-3Rs/450091/2012) [3]. However, scientifically valid alternative methods may also be acceptable in a regulatory submission even in the absence of a formal validation [3].

In addition to *in-vitro* assays, computerised systems such as databases and modelling methods may be useful for toxicity predictions either supplemental to *in-vivo* experiments or as full replacement. The challenge in developing computer-based approaches is the collection of high-quality data providing a reliable data basis for prediction models. Mostly, data are derived from several sources and therefore, it needs to be ensured that the underlying *in-vivo* experiments have been performed under comparable conditions [81].

4.2 Organoid systems – Organs in a dish

4.2.1 What are organoids?

Organoids are *in-vitro* tissue models consisting of several organ-specific cell types derived from pluripotent stem cells or organ progenitors. The cells differentiate and self-organise to a three-dimensional structure, which act as a miniature organ with a micro-anatomy comparable to the "real" organ [82].

The *in-vitro* technique of organoids has rapidly improved during the past years. Organoids can be generated for several tissues and organs. For human *in-vitro*

testing, organoids can be generated for liver, kidney, brain, fallopian tubes, pancreas, prostate, small intestine, lung, endometrium, salivary glands, retina and cornea [82]. Due to their organ-like properties, they can function as *in-vitro* models in several areas such as disease modelling, drug discovery or toxicology. Moreover, organoids can be used in tissue and organ replacement [82]. By now, organoids can be purchased from several companies.

To generate organoids, stem cells or progenitors need to be cultured in a 3D medium, e.g. an extracellular matrix hydrogel (e.g. Matrigel™) [82].

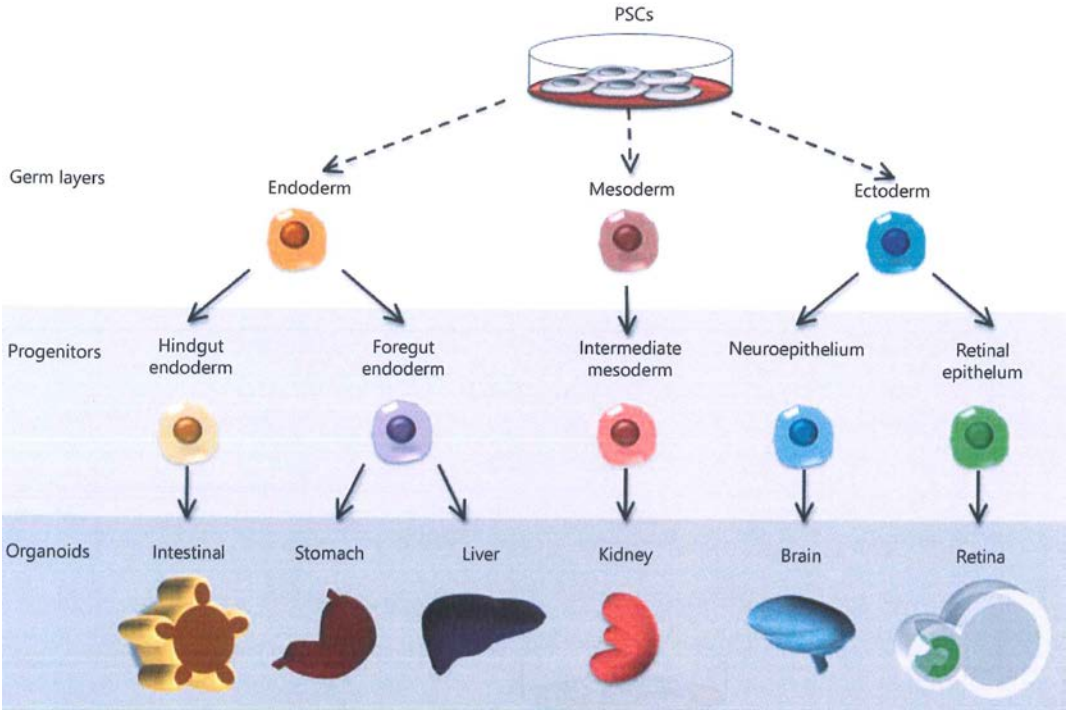

Figure 3: Schematic overview of organoid generation from pluripotent stem cells, Source: [83]

The generation of organoids involves some challenges. In the past years, efforts have been made to optimise organoid generation, e.g. on the time scheme for incorporating the different progenitors into the organoid structure as shown in Figure 4 below [84]. General challenges are the replication of individual organoids, the visualisation of living organoids, their limited perfusability and the requirement of hydrogel matrices [82].

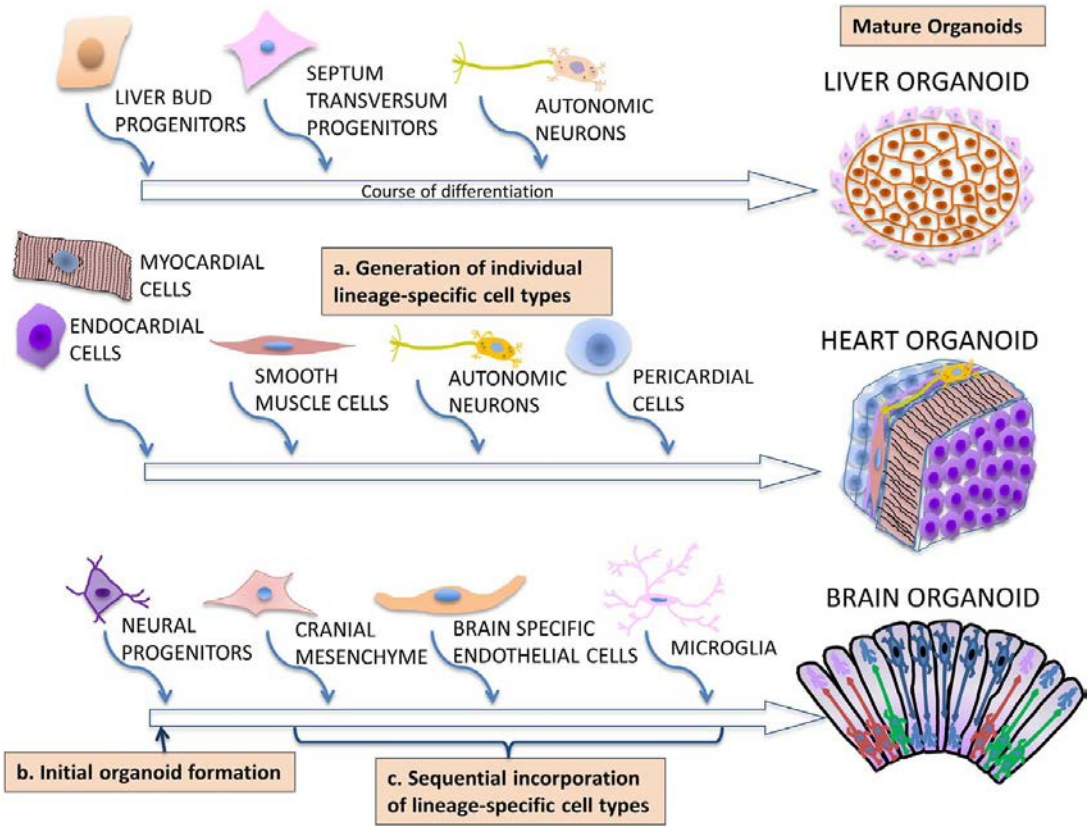

Figure 4: Generation of different organoids, Source: [84]

4.2.2 Use of organoids in regulatory toxicity testing

The general suitability of organoids for toxicity testing was demonstrated by Takasato *et al.* in 2015 [85]. Takasato and co-workers generated human kidney organoids containing nephrons together with a duct network surrounded by renal interstitium and endothelial cells. Transcription profiles showed similarity with human first trimester kidney. Regarding size and number of nephrons, the kidney organoid is quite comparable to a mouse kidney at 14.5 days post-coitum. Using these organoids, the authors were able to demonstrate nephrotoxicity of cisplatin, a well-known drug substance with nephrotoxic properties [85].

Although organoids may serve as proper test systems for toxicology assessment, some limitations of this technique still exist. Organoids often develop cavities or form clumps resulting in an altered *in-vivo* anatomy [82]. This may influence functional investigations that require perfusion and/or secretion processes [82]. One possibility to solve this problem is the cultivation of organoids in Transwell® culture systems. Furthermore, incorporating endothelial and mesenchymal stem cells in the organoid can improve *in-vitro* function [82]. Moreover, organoids often show a limited cellular diversity and are therefore not fully comparable to the tissue or organ structure *in vivo* [82]. For example, resident immune cells are missing in most organoids. Thus, inflammatory processes, which could also be

induced by toxic substances, cannot be studied using those organoids [82]. A further limitation of organoids is that they often constitute an early developmental stage compared to the mature organ *in vivo*. Therefore, such organoids can be used to assess the toxicity during early development rather than studying toxic effects on adult organ function such as intestinal peristalsis [82].

Due to their organ-like complexity, organoids may serve as a suitable alternative in toxicology testing, also for regulatory purposes. To fulfil regulatory requirements, robust protocols for the generation of organoids need to be developed in accordance with the EMA *"Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches"* [3]. Moreover, testing approaches involving organoids need to be validated, preferably by the EURL-ECVAM [79]. To date, there is no toxicity test involving organoids available that has been validated by the EURL-ECVAM and therefore, organoid-based tests will not be accepted automatically by regulatory authorities. However, regulatory acceptance may be afforded in exceptional cases, e.g. after scientific advice.

4.3 Microphysiological systems (MPS) – Organs on a chip

4.3.1 What are MPSs?

Microphysiological systems or so-called "organs-on-a-chip" are *in-vitro* models, which represents a small functional unit of organs or tissues. This functional unit has a multicellular composition comparable to the real organ or tissue and reflects the normal physiological system. The functional units are integrated in a polydimethylsiloxane (PDMS) layer and connected to each other via small channels building a microfluidic system. Integrated small pumps ensure a physiological fluid circulation. MPSs can be used for primary cells, 3D tissue culture as well as cell lines. Ideally, an MPS delivers reproducible data over a period of several weeks [82].

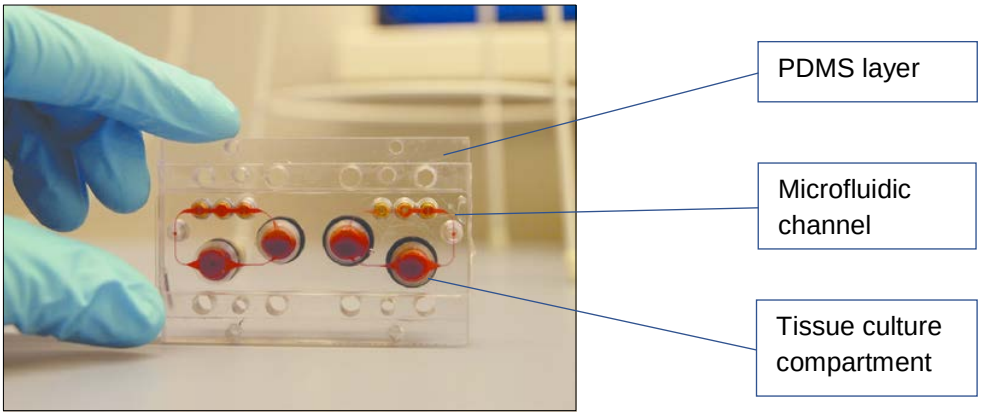

Figure 5: Multi-organ-chip with PDMS layer, Source: [86]

During the past years, high efforts have been made to further improve this technique. Multi-organ-chips (MOCs) have been developed by companies and academic institutions in close cooperation, also supported by public funding and programs such as EU-ToxRisk. To date, MPSs have been developed for the kidney glomerulus, kidney proximal tubule, female reproductive tract, placental barrier, liver, heart, skeletal muscle, microvasculature, blood vessel, blood-brain-barrier, and white adipose tissue [82]. MPSs can be used for efficacy and safety testing as well as for ADMET (absorption, distribution, metabolism, excretion, toxicity) studies [82].

4.3.2 Current MPS developments and application in regulatory toxicity testing

4.3.2.1 Liver-islet-chip

An MPS intended for use as Type II diabetes model has been established for drug testing [87, 88]. As shown in Figure 6 below, this MOC comprises two different organ cultures: liver spheroids and pancreatic islets. The tissue chambers contain 40 liver spheroids, composed of HepaRG cells and primary human stellate cells, and 10 pancreatic islet microtissues equivalent to a factor of 100,000 compared to human liver and pancreas, respectively. Both tissue chambers and the interconnecting microfluidic channel contains 610 microliters media volume. A continuous pulsatile flow is ensured by a micropump maintaining a flow of 4.94 microliters per minute [87].

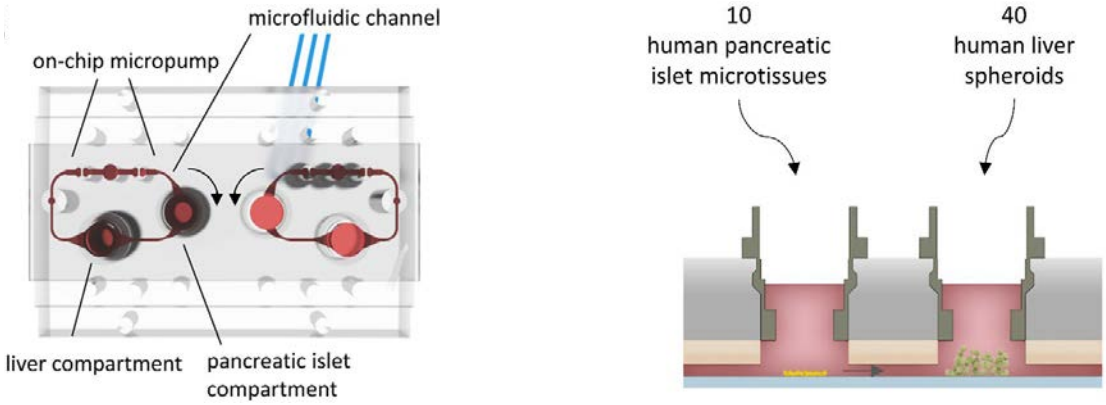

Figure 6: Two-organ-MPS (liver-islet-chip). Structure of liver-islet-chip device (left), tissue-loading scheme of organ co-culture (right), Source: [87]

Liver spheroids and pancreatic islets are co-cultured for 15 days and showed stable and reproducible circulating insulin levels over the entire period [87]. In this tissue model, insulin secreted by the islet microtissues stimulates glucose

utilisation by the liver spheroids demonstrating a cross-talk between both tissues in co-culture [87]. This co-culture system of the liver-islet-chip has been validated in different laboratories in terms of robustness and reproducibility [87]. Indeed, the pharmaceutical company AstraZeneca has adopted the liver-islet-chip for Type II diabetes drug testing [89]. This MOC will be further developed to allow the induction of insulin resistance *on-chip* and may constitute a reliable technique for Type II diabetes drug development in the future [87].

4.3.2.2 Skin-tumour model

A skin-tumour model aimed at the provision of a combined safety-efficacy assay for cancer drug candidates is currently under development at Bayer Healthcare. The drug candidate Bay1a, which is effective against lung tumour cells, causes severe skin toxicity in primates and therefore, a test system should be established to investigate potential skin toxicity of lung cancer drug candidates *in vitro* prior to initiate *in-vivo* experiments. To that end, a co-culture system comprising lung cancer spheroids (H292 cell line) and healthy skin was developed to allow simultaneous analysis of efficacy and safety, so-called “safficacy” [89].

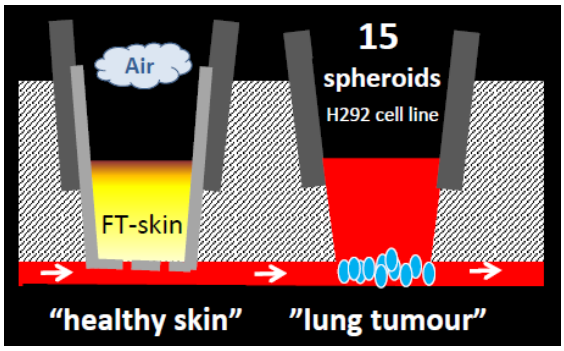

Figure 7: Skin-tumour model for cancer drug candidate “safficacy” testing, Source: [89]

The lung cancer drug candidate Bay1a was used for assay qualification. In this co-culture assay, toxic effects on skin tissue could be observed. However, efficacy on lung cancer cells could not be detected so far. Thus, further efforts will be needed to successfully establish a co-culture model for analysing both tumour efficacy and toxicity of lung cancer drug candidates [89].

4.3.2.3 Lung-on-a-chip

In 2010, Huh and co-workers first described the development of an MPS reflecting human lung function [90]. As shown in Figure 8 below, this device is composed of PDMS microchannels forming an alveolar-capillary barrier on a thin, porous, flexible PDMS membrane. This membrane is flexible and will be stretched by breathing movements, which are generated by a vacuum applied to the side chambers [90].

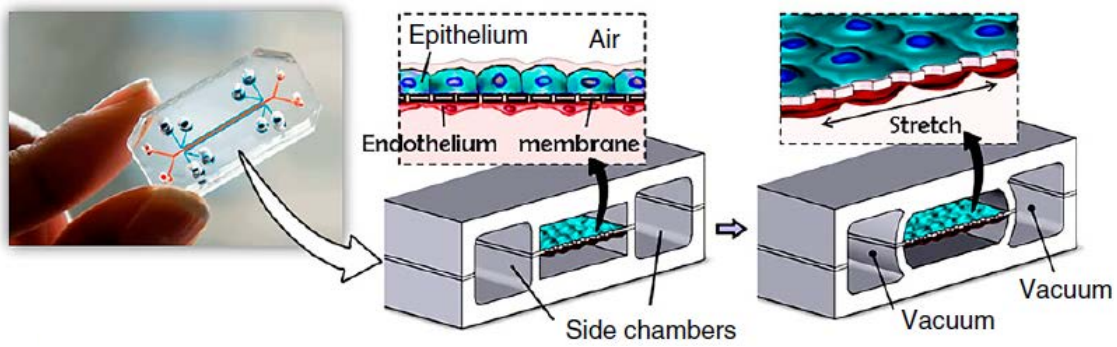

Figure 8: Lung-on-a-chip, Source: [91]

Huh and co-workers tested this device for the development of a pulmonary oedema model [91]. Pulmonary oedema constitute a common complication in cancer patients undergoing chemotherapy. In this *in-vitro* model, application of interleukin-2 into the microchannel led to a leakage of medium from the microchannel compartment resulting in fluid accumulation in the upper alveolar channel [91]. Furthermore, enzymatic reactions between plasma proteins led to the generation of fibrin clots, which are deposited in the alveolar compartment and can also be found in human pulmonary oedema. Thus, the lung-on-a-chip may be a useful *in-vitro* model to study toxic effects on human lung function [91].

4.3.2.4 Female-reproductive-tract-on-a-chip

Researchers from the North-western University (Chicago, USA) has developed an MPS replicating the female reproductive tract. The device, named “EVATAR”, comprises up to five different organs: ovary, fallopian tube, uterus, cervix and liver as shown in Figure 9 below. [92].

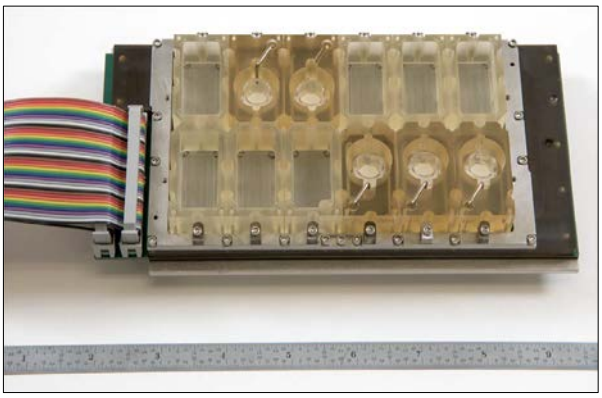

Figure 9: EVATAR, female-reproductive-tract-on-a-chip, Source: [92]

Xiao and co-workers were able to generate a 28-day-menstrual cycle by culturing murine ovarian follicles. By co-culturing ovarian follicles and peripheral organs, this MPS can mimic the female reproductive tract and the corresponding endocrine regulation [92].

This MPS enables the modelling of the menstrual cycle as well as pregnancy-like conditions and may therefore constitute a suitable *in-vitro* technology for drug development and/or toxicity assessment [92].

4.3.2.5 Four-organ-ADMET-chip

A four-organ MPS was established to enable the analysis of drug absorption, distribution, metabolism and excretion as well as toxicity assessment (ADMET). The chip is composed of two independent microfluidic circuits, which are arranged on two levels. Small intestine tissue functions as a barrier from the apical site of the intestine and is cultured in a cell culture compartment placed on top of the PDMS layer harbouring the blood flow circuit. At this site, drug absorption can take place. Drug distribution to a liver equivalent is enabled by a blood flow supported by a micropump. Renal proximal tubule cells function as kidney equivalent. These three organ equivalents can be combined with a fourth organ, e.g. skin biopsies, to analyse either a different route of absorption or analyse potential drug toxicity [93, 94].

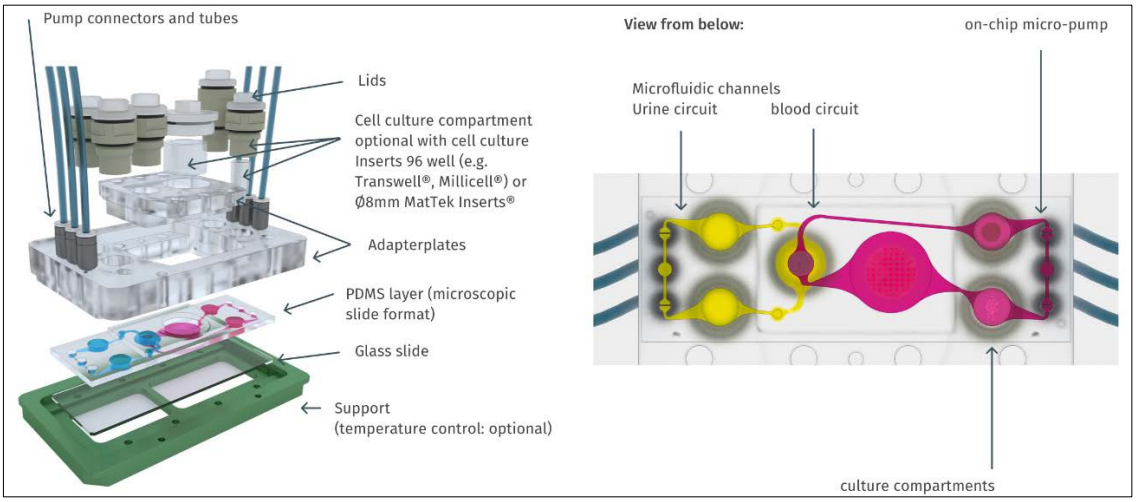

Figure 10: Four-Organ-Chip by TissUse GmbH, Berlin, Germany, Source: [93]

4.3.2.6 Human-on-a-chip

Currently, a “human-on-a-chip” is under development, which represents a systemic model covering all important human organs in one MPS. This MPS is intended for use in early clinical drug development and may allow the generation of efficacy and safety data, which are usually obtained from clinical phase I and phase II trials as well as from systemic toxicity studies in animals [95].

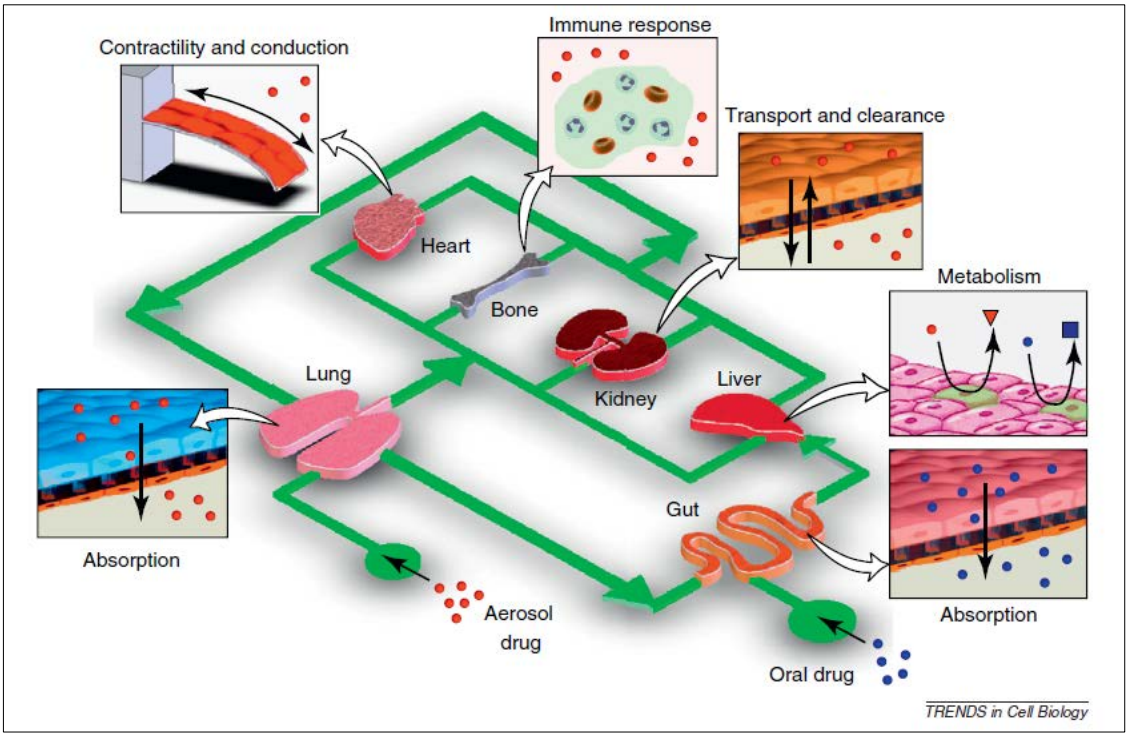

Figure 11: Illustration of the human-on-a-chip concept, Source: [96]

The “human-on-a-chip” concept illustrated in Figure 11 is intended to provide information on drug/substance effects regarding the most-important organ systems of the human body. An MPS including cultured tissue from lung, heart, gut, liver, kidney and bone can be applied to analyse the absorption of inhaled drugs (indicated by red dots in Figure 11) from the lung to the microcirculation. Furthermore, investigations on cardiotoxicity, transport metabolism in the liver, clearance (kidney), and influence of potential immune responses (bone) can be performed. Drug substances administered orally (indicated by blue dots in Figure 11) enter the gut compartment and will subsequently being transported via the microcirculation to the liver [96].

4.3.3 Use of MPSs in regulatory toxicity testing

Several microphysiological systems were developed during the past years including multi-organ-chips designed for ADMET investigations. Multi-organ-chips and associated control units are commercially available. MPSs can be used during the whole drug development phase, from drug discovery to drug safety assessment. In the past years, microphysiological systems attracted more attention and indeed, a liver-islet-chip intended for diabetes Type II drug testing was already adopted by the industry [87, 89].

One of the big challenges in cell-based technologies is the availability of reliable cell sources [89]. However, several reliable cell sources, e.g. immortalised human cell lines and stem cells, could already be assured making cell-based methodologies more secure [89].

A further hurdle is the qualification and validation of assays based on this technology. All devices and associated instrumentation should be qualified for the intended application. To ensure reproducibility, robustness and reliability of a cell-based assay, both the rules of "Good Cell Culture Practice" and the recommendations for improving the quality and utility of organotypic models are to be considered mandatory [97, 98]. In fact, there are only a few studies published dealing with the qualification of data achieved by an MPS-based approach against those revealed by conventional *in-vivo* studies [88]. As validation of complex multi-organ-chips is considered challenging, Marx and co-workers suggested a two-tiered approach [88]. First, single-organ-chips should be used to qualify the MPS-based tool "fit-for-purpose" [88]. Validation data obtained in this first step can then be used to further qualify and validate multi-organ-chips in a second step [88]. To establish MPS-based tools in regulatory testing, general guidance from OECD, EMA, and ICH should be considered [88].

To date, no MPS was formally validated, especially by the EURL-ECVAM. Therefore, general requirements for the regulatory acceptability of alternative testing methods are not met so far.

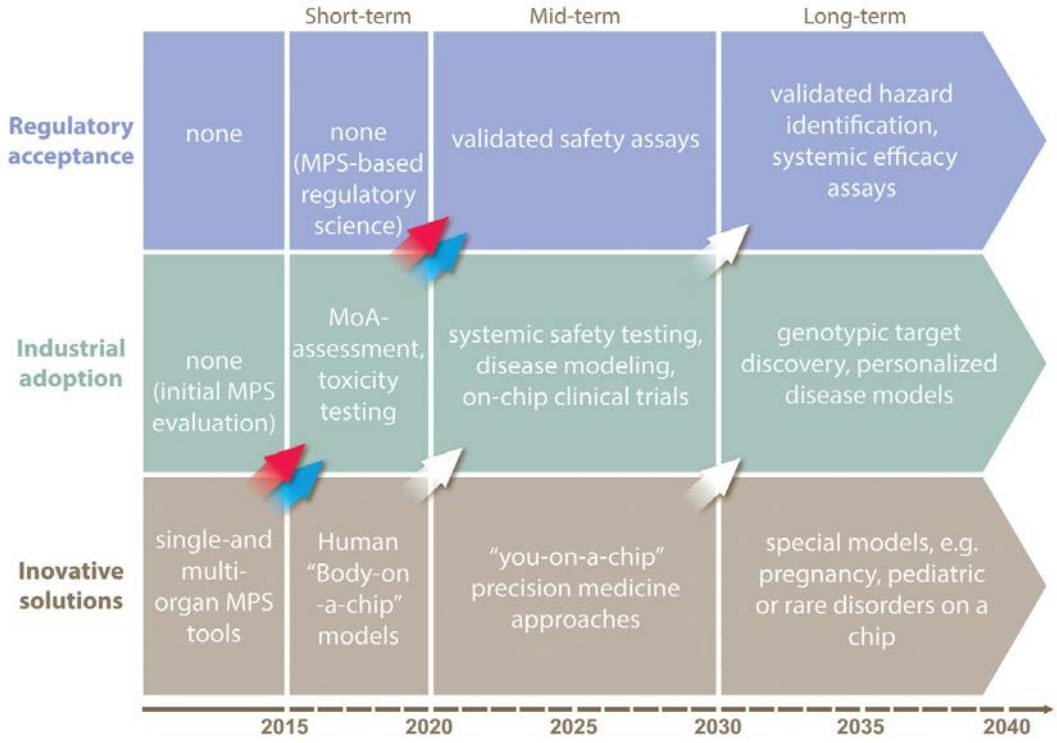

Figure 12: Time schedule towards the reduction and replacement of in-vivo studies by MPS-based assays, Source: [88]

In 2016, Marx and co-workers published a time schedule providing an estimation for the development of MPS-technology towards a full regulatory acceptance (see Figure 12). They assume that MPS-based tools may be validated and reach regulatory acceptance as an alternative testing approach between 2020 and

2030 [88]. As a future perspective, MPS-tools may be personalised ("you-on-a-chip") and could serve as an appropriate patient-specific disease model [88].

4.4 "Omics"

4.4.1 What is "omics" technology?

The term "omics" describes the analytics of systemic genome responses using microarrays, commonly referred to as "gene chips". Microarrays have been developed in the late 1990s. A usual gene chip is composed of a solid surface with immobilised copy DNA (cDNA) probes representing the whole genome of the target species (see Figure 13). Current microarrays for human genome analysis harbour up to 54,000 probe sets [99].

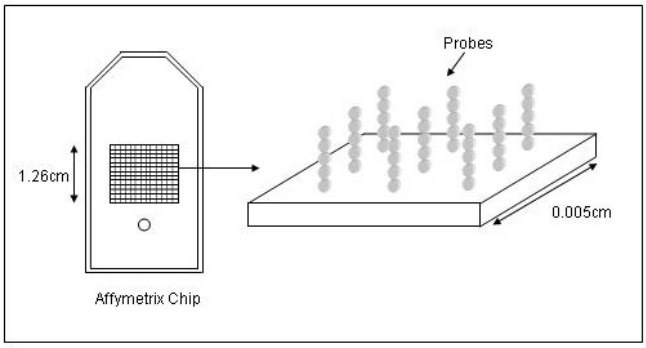

Figure 13: Illustration of an Affymetrix Gene Chip, Source: [99]

To analyse gene expression, messenger RNA needs to be extracted from cells or tissues and labelled with fluorophores or chemiluminescent reagents. After incubation with labelled cellular RNA extracts, the target RNA molecules bind to the corresponding cDNA probes resulting in a hybridisation signal, which is subsequently detected and quantified using a specialised scanner system.

Microarrays offer a way to analyse genome-wide cellular responses and to gain insights into gene regulation during different disease states. For evaluation of these huge data sets, bioinformatics needs to be applied.

In the past decades, microarrays have widely been used in research and development resulting in more and more applications. Currently, microarrays can be applied in the following areas [100]:

- Genomics — Analysis of genomic structure and function
- Transcriptomics — Analysis of alterations of RNA expression
- Proteomics — Analysis of protein expression
- Metabolomics — Analysis of metabolite profiling

- Epigenomics — Analysis of reversible heritable alterations in gene function
- Regulomics — Analysis of transcription factors and other molecules involved in gene regulation

4.4.2 Application of “omics” technology in regulatory toxicity testing: toxicogenomics

In toxicity assessments, the “omics” technology can be applied to evaluate potential whole genome alterations induced by chemical or biological substances. By analysing the toxic cellular response on the whole-genome level (toxicogenomics), insights into the mode-of-action (MoA) of an adverse reaction can be gained provided that the test substance causes alterations at the RNA, protein or metabolite level. In 1998, Marton and co-workers analysed the effect of the immunosuppressant FK506 on gene expression in the yeast *S. cerevisiae* [101]. Indeed, the authors identified both changes in the calcineurin pathway as direct result of drug-binding and “off-target” effects, which were independent of the binding to the target, proving that gene chips may be a suitable tool for identifying drug effects [101].

An “omics”-based tool developed for toxicogenomics is the ToxExpress™ program. This system supports the evaluation of potential drug toxicity markers. ToxExpress™ is composed of a proprietary reference database derived from *in-vivo* toxicity studies in rats as well as in primary rat and human cells, techniques for gene expression analysis, and powerful software tools. Gene expression profiles are compared with toxin-associated gene expression patterns derived from a reference database [102].

Moreover, in 1999, the microarray ToxChip has been developed by NIEHS researchers in the US. This microarray comprises 2900 human genes including, among others, genes responsible for the regulation of DNA replication, DNA repair, apoptosis and oxidant stress [103]. As current information on this microarray is lacking, it is not clear whether this platform is still available or further developed.

A further gene chip developed for customised toxicogenomic analyses is *ToxBlot*. *ToxBlot* arrays have been constructed for both human and mouse comprising around 2400 cDNA sequences corresponding to about 600 genes [104].

However, despite the benefits gained from “omics” technology, changes on the molecular level do not necessarily result in phenotypic alterations, i.e. a measurable adverse reaction [100]. Thus, this technique does not deliver direct information on adverse drug reactions or toxicity of chemicals [80]. Therefore, microarray results should be carefully interpreted, and understanding of complex physiological processes is required. Nevertheless, microarray data may provide

important information on potential biomarkers and pathways of toxic reactions [80].

One of the challenges of “omics” technology and its use in toxicology testing is the high amount of data generated requiring bioinformatics evaluation and proper data interpretation. To date, a standardisation of microarray evaluation, which would be a prerequisite for regulatory testing, is not yet established [80]. Moreover, no formal validation of microarrays for regulatory purposes have been conducted [37].

In 2016, a survey among 11 members of the “*European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC)”* revealed that “omics” data have been used in some regulatory submissions. In one case, a 2-year carcinogenicity study could be waived based on “omics” data [100].

In 2006, members of the EURL-ECVAM published a procedure for the validation of “omics”-based technologies for regulatory purposes. Researchers in the EU are requested to submit their “omics”-based test methods to the EURL-ECVAM for initial appraisal. A formal validation process will then be started by the EURL-ECVAM if the method is considered suitable [105].

Interestingly, the EURL-ECVAM took part in the CARCINOGENOMICS FP6 project [37]. The goal of this project was to develop *in-vitro* tests based on toxicogenomics and metabolomics for the identification of potential genotoxic and carcinogenic agents. Two toxicogenomics-based assays were chosen for further optimisation [37]:

- Assay in HepaRG cells for detection of liver carcinogenicity
- Assay in RPTEC/TERT1 cells for detection of kidney carcinogenicity

The EURL-ECVAM coordinated the optimisation and pre-validation of these assays including the testing of 15 chemicals, assessment of transferability and reproducibility of this technology as well as the development of bioinformatics solutions [37].

Altogether, “omics” technology holds great promise as alternative testing strategy, particularly if used as a pre-screening tool and if combined with cell culture methods. However, consistent methods for the analysis of “omics” data are lacking so far. Currently, from a regulatory point of view, “omics” technologies are considered not acceptable as an alternative testing method to *in-vivo* data so far, at least as a stand-alone approach. Standardisation and validation of “omics”-based approaches are still needed to achieve regulatory acceptance [100].

4.5 *In-silico* tools

4.5.1 What are *in-silico* methods?

In addition to the *in-vitro* tools described above, *in-silico* models have been developed to predict toxicity of drugs or chemicals based on computerised tools, which should complement existing toxicity tests to minimise animal use and costs. *In-silico* tools are computational methods based on algorithms, databases or specialised software solutions. *In-silico* tools can be applied in high-throughput screening making them favourable for the toxicity assessment of chemicals [81].

In the past years, several *in-silico* tools were developed for toxicity prediction including

- Databases containing toxicity information
- Modelling approaches
- Data mining
- Visualisation methods
- Specialised software solutions [81, 106, 107]

Important *in-silico* approaches are modelling methods, which can predict the toxicity of substances based on their molecular properties. For example, so called structural alerts (SA) or toxicophores are known chemical structures indicating toxicity of the substance of interest. *In-silico* tools using that information may help to predict toxicity of a potential drug candidate before initiating *in-vitro* or animal experiments [81]. However, limitations of SAs are that they offer only a “black-or-white" result (presence or absence of SAs) and no information on the underlying pathway is provided. Moreover, SAs contained in the prediction tool used may not be complete leading to false-negative results [81].

SAs are included in many toxicity prediction tools based on Quantitative Structure-Activity/Toxicity Relationships (QSAR/QSTR) such as OECD QSAR, Toxtree, OCES, Derek™ Nexus, HazardExpert, Meteor, CASE, PASS or cat-SAR [81]. QSAR/QSTRs are one of the most important developments in the field of *in-silico* modelling. QSAR/QSTR modelling builds a quantitative relationship between a pharmacological, chemical, physical or biological effect of the substance of interest and its chemical structure. Thus, these computer-based mathematical technologies allow predictions of physical properties or biological effects, e.g. adverse drug reactions, based solely on the molecular structure of a chemical [107].

One of the QSAR-based commercially available software solutions is DS TOPKAT®, which uses cross-validated QSTR models. Among others, DS TOPKAT® enables the prediction of the Lowest Observed Adverse Effect Level (LOAEL) for chronic toxicity in the rat [108].

A further QSAR-based *in-silico* tool is Derek™ Nexus, a software based on expert knowledge. Derek™ Nexus provides toxicity predictions regarding several toxicological endpoints. Importantly, Derek™ Nexus can be used in the context of the ICH M7 guideline *"Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk"*. Furthermore, the software can be used for prediction of skin sensitisation [109].

4.5.2 EU development projects on *in-silico* tools

One EU-wide project is eTOX (Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the *in-silico* prediction of toxicities), which was initiated in 2010 and funded by the Innovative Medicines Initiative Joint Undertaking (IMI-JU). The goal was to compile toxicity data archived at pharmaceutical companies in order to generate a high-quality database aimed at data mining and toxicity prediction [110]. eTOX comprised 13 pharmaceutical companies, seven academic institutions as well as six small-medium-sized enterprises (SMEs). During seven years of project lifespan, the integrated modular package "eTOXsys" has been developed for toxicity prediction [110].

eTOXsys provides a database including over 1,800 substances and 7,000 studies. Additionally, predictive models for several endpoints comprising transporter inhibition, cardiotoxicity, phospholipidosis, hepatotoxicity as well as safety pharmacology are available [111].

4.5.3 Application of *in-silico* methods in regulatory toxicity testing

In-silico methods are aimed to complement the assessment of drug toxicity, which is mainly based on *in-vitro* and *in-vivo* studies [81]. One of the benefits is their applicability prior to synthesising the substance of interest as toxicity estimations are based on molecular properties and data, which have been obtained from substances of similar structure. In general, *in-silico*-based toxicity assessment may help to potentially reduce and refine animal toxicity studies resulting in lower costs and shorter development time [81].

As all methods used for toxicity assessment, also *in-silico* tools need to be validated to achieve regulatory acceptance. It should be noted that the EURL-ECVAM has no mandate to validate *in-silico* methods [107]. Therefore, the validation of those computational approaches lies in the responsibility of the user, i.e. the pharmaceutical/chemical industry. For validation of QSAR models, the OECD QSAR validation principles should be followed [112].

The European Chemicals Agency (ECHA) regularly publishes reports on the regulatory applicability of non-animal approaches under the REACH, CLP and BPR. Table 7 summarises the information on the applicability of *in-silico* methods in toxicity assessment specified in the ECHA report from 2017 [80].

Table 7: Availability of QSAR models for different toxicity endpoints

Endpoint	Available QSAR tools for prediction of toxicity
Dermal absorption	Flynn's algorithm, Magnuson's rule, OECD QSAR toolbox, Danish QSAR Database, DERMWIN (EPISUITE)
Distribution	Waterbeem's rules
Metabolism and Excretion	OECD QSAR Toolbox, Danish QSAR Database, Meta-print 2D (metabolism), SMARTcyp (metabolism)
Acute toxicity	DS TOPKAT® (rat LD_{50})
Skin and respiratory sensitisation	OECD QSAR Toolbox (with limitations), Derek™ Nexus
Repeated-dose and chronic toxicity	QSAR models for human liver, kidney, heart toxicity
Mutagenicity	Toxtree, OECD QSAR Toolbox, Danish QSAR Database,VEGA, Derek™ Nexus
Carcinogenicity	OECD QSAR toolbox, Toxmatch, Oncologic (US), T.E.S.T., VEGA, Danish QSAR Database
Reproductive toxicity	Danish QSAR Database, VEGA, T.E.S.T., ADMET predictor, CASE Ultra, DS TOPKAT®, Leadscope models, TIMES
Neurotoxicity	OECD QSAR Toolbox

Among the QSAR tools mentioned in the ECHA report, some are free of charge such as the OECD QSAR Toolbox, which is outlined below.

In order to facilitate the development and applicability of QSAR methods in the toxicity assessment of chemicals, the OECD has developed the OECD QSAR Toolbox. The project is supported by international institutions and chemical and pharmaceutical companies [113].

This *in-silico* platform should enable the user to

- identify structural features of relevance and the potential MoA,
- identify other chemicals with identical structural features and/or MoA,
- use experimental data, which are at hand to complete the data set, and
- predict skin sensitisation [113].

The development of the OECD QSAR Toolbox has run in several phases. Version 1.0 has been released already in 2008 followed by the launch of version 2.0 two years later. Version 3.0 has been released in 2012 followed by the fourth version in 2017. OECD QSAR Toolbox v.4.2 was launched in February 2018 [113].

Altogether, the use of either commercial or freely available *in-silico* tools is regulatory accepted, at least to reduce or refine animal experiments. However, current *in-silico* tools do not cover all toxicity endpoints needed such as the No Observed Adverse Effect Level (NOAEL) [80]. Furthermore, QSAR models are currently not reliable for being used for the prediction of complex endpoints such as reproductive toxicity [80].

Results from QSAR models can be used for regulatory submissions if

- the respective tool is scientifically valid,
- the substance of interest falls within the scope of the model applied,
- the prediction model is capable of being used in regulatory testing and
- the model used is properly documented [80].

4.5.4 Further promising developments in alternative testing strategies

Additional to the methods described in the sections above, some further developments in the area of alternative testing strategies should not go unmentioned. One of these methods is the concept of Adverse Outcome Pathways (AOPs) dealing with the combination of biological processes and adverse effects, including mode of action (MoA), upstream exposure and downstream effects at the single organism level. Frequently, adverse outcomes are reported endpoints of *in-vivo* toxicity studies performed in accordance with OECD guidelines [114].

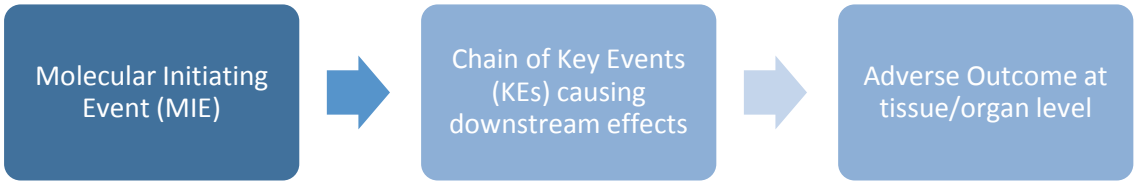

Figure 14: Concept of Adverse Outcome Pathways

An AOP for skin sensitisation has been published by the OECD first in 2012 (revised in 2014) [115]. Furthermore, an AOP was recently approved within the framework of OECD guideline 442E, which is, however, not yet applicable to pharmaceuticals [55].

AOPs can also be part of integrated approaches to testing and assessment (IATA). IATAs are based on existing scientific information together with newly generated data achieved by toxicity tests [114].

4.6 Information on regulatory acceptability of alternative testing methods from EMA assessment reports

According to published OECD guidelines and ECVAM reports, none of the new *in-vitro* technologies such as “omics” or MOCs is already validated and therefore, their regulatory acceptance is questionable. However, scientifically valid alternative methods may also be acceptable in a regulatory submission even in the absence of a validation by the EURL-ECVAM [3]. The use of those testing approaches may be possible upon scientific advice by regulatory authorities.

To figure out, whether “omics” or organ-chip-technologies have already been used in registration dossiers, European Public Assessment Reports (EPARs) of medicinal products for human use have been studied. In total, 186 EPARs of medicinal products for human use published by the EMA after approval or refusal via the centralised procedure between July 2016 and July 2018 were evaluated.

None of the EPARs contained indications on the use of organoids, microphysiological systems or “omics” technologies in toxicity testing. In most cases, the toxicology has been assessed based on a comprehensive set of *in-vivo* studies. In the EPAR of the medicinal product Aplidin (EMEA/H/C/004354), gene expression profiling has been described for pharmacodynamic purposes without further specification on the method used [116]. For biosimilars, the comparability exercise for demonstration of biosimilarity with the reference product should be based on *in-vitro* testings as requested by current guidelines. *In-vivo* studies in animals should be considered dependent on the *in-vitro* results [117]. In the EPARs evaluated, the reported comparability exercise has been performed mainly *in vitro*. However, animal studies have been performed as well (see Annex).

In-silico modelling for the estimation of toxicity has been used in the registration dossier of 12 medicinal products [118-129]. For pharmacodynamics (PD) purposes, *in-silico* modelling has been used once [130]. In one dossier, *in-silico* tools have been used for ecotoxicity estimations [131].

Altogether, non-validated alternative testing approaches such as organoids, MPS or “omics” technologies have not been used in registration dossiers of medicinal products, for which marketing authorisation has been sought via the centralised procedure during the last two years. However, it should be noted that alternative test methods used in the early drug development phase, e.g. in high-throughput screening assays, are not necessarily included in the CTD and therefore not mentioned in the EPARs. *In-silico* models have been applied in some cases (see above). The low amount of EPARs containing *in-silico* approaches may arise from the fact that drug candidates are often screened very early in the development phase and pre-selected according to their potential toxicity. This pre-selection process might therefore not be included in the registration dossier.

5 Conclusion and Outlook

During the last 10 years, the development of alternative testing approaches to replace, reduce or refine animal experiments for toxicity testing has been accelerated significantly. Moreover, several alternatives have already been validated by the EURL-ECVAM and may therefore being used in registration dossiers.

Highly innovative *in-vitro* techniques such as organoids, microphysiological systems and “omics” technologies have been introduced into the area of toxicity testing but have mostly been used in academic research in the past. During the last years, these methods became more attractive for the industry supported by EU projects such as EU-ToxRisk. However, a broad industrial adoption of these innovative technologies is still missing. Moreover, none of these methods has been validated by the EURL-ECVAM for regulatory purposes so far and therefore their regulatory acceptability is still questionable.

Among the recent advances, *in-silico* tools have been developed, which offer the possibility to predict the potential toxicity of chemicals based on their molecular properties. An advantage of this approach is the capability to preselect potential candidates even prior to synthesize them and its applicability for high-throughput screening [81]. Substances with potential toxicity could then be either modified or rejected for further development offering an optimised development process by spending time and costs as well as reduces animal use [81].

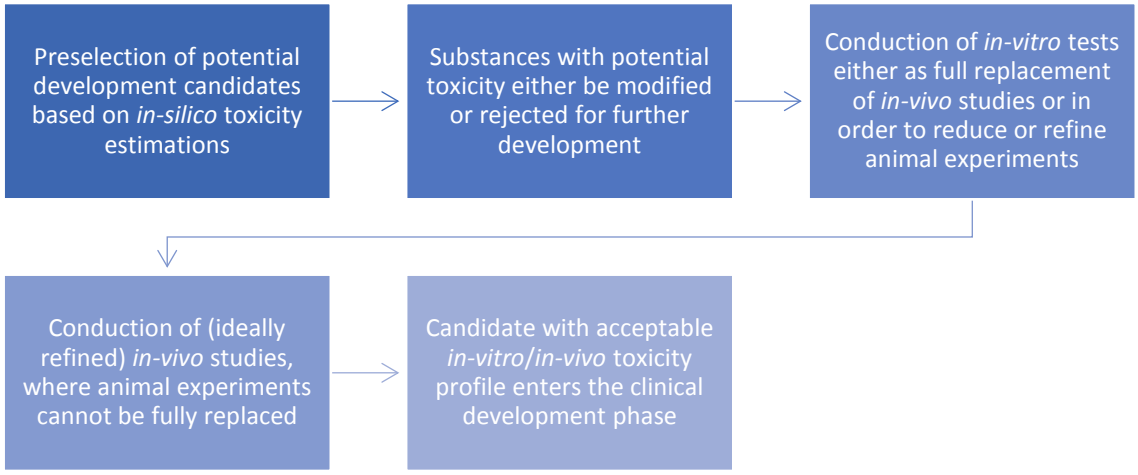

Figure 15: Optimised development process starting with in-silico preselection of potential candidates

The great advantage of *in-silico* tools is their growing database, which is shared by the members of these tool groups. Furthermore, these tools may provide early signals for any adverse effect such as genotoxicity or teratogenicity. Accordingly, the complex toxicology program can be refined and improved early on. This refinement helps to avoid repetition of studies and certainly supports the protection of patients against undesirable effects. Among several *in-silico* tools

that are commercially available, the OECD offers the free QSAR Toolbox for *in-silico* toxicity estimations. Due to their great advantages, *in-silico* methods are already accepted for toxicity estimations by regulatory authorities, if OECD requirements on the validation of *in-silico* methods are met.

Although there was important progress during the last years, the development of reliable and robust alternative testing methods is still challenging, particularly for addressing complex endpoints. To date, no alternatives to the *in-vivo* testing of reproductive and developmental toxicity exist. However, current advances such as the “female-reproductive-tract-on-a-chip” making confident that the process is on a promising path.

Regarding the implementation of innovative technologies such as “omics” in regulatory testing, both health authorities and the industry need to learn how to optimise the interpretation of the assay results and how can these data be used for decision making, e.g. decisions important for the support of first-in-man clinical trials. This learning process has already started in the industry and regulatory authorities are keen to participate in this process as well. As such, the US Food and Drug Administration (FDA) is offering what is called “safe harbour”, which means that the industry can submit their data to the FDA for review but without using them for any decisive conclusions to the drug development or market authorisation [132]. There is hope that, on a neutral basis, further discussions clarify the value of such results.

The fact that several alternative testing methods have already been validated or are currently under validation by the EURL-ECVAM should encourage the further development of alternative testing strategies. International programs such as EU-ToxRisk are important in fostering the development of alternative testing methods as it is an expensive and time-consuming process. Moreover, sharing experiences across companies and institutions may help to further accelerate the development of alternative testing strategies. Hereby, publication of supportive data as well as failures would accelerate the process of useful data mining for a final characterisation of new compounds. When the development of drug candidates has been decided to be stopped, then a publication may help other researchers to improve their development strategies. Data being archived without access are of no help at all.

A promising example for a cooperation between stakeholders to reduce animal testing is the revision process of ICH guideline S1A on rodent carcinogenicity testing [133-135]. This guideline specifies the need for 2-year rodent carcinogenicity studies. Usually, 2-year rodent carcinogenicity studies in mice and rats are required prior to authorisation of a small molecule medicinal product intended for long-term treatment. However, data obtained from rodent carcinogenicity studies comprising 182 compounds allow the conclusion that the outcome of these studies may be predictable from available chronic toxicity data [136]. Therefore, there are considerations about waiving the 2-year rodent

carcinogenicity studies if there is no indication for carcinogenicity on a whole-animal basis from chronic toxicology studies in rats [136]. In 2013, the ICH-S1 Expert Working Group published the *Regulatory notice on changes to core guideline on rodent carcinogenicity testing of pharmaceuticals*, adopted in 2016 by CHMP [137]. Sponsors can submit their Carcinogenicity Assessment Documents (CADs) to the participating regulatory authorities (US FDA, EMA, Japanese Pharmaceuticals and Medical Devices Agency, Health Canada, and SwissMedic) for review. The evaluation will lastly be reviewed by the ICH-S1 Expert Working Group for guideline revision [133-136]. A process like this involving all stakeholders at an international level paves the way for future implementation of the 3Rs in regulatory toxicity testing.

Altogether, although recent advances in the development of alternative testing methods have been made, the complete replacement of animal experiments by alternative testing methods will still be a long way off. Nevertheless, repeat thinking about how to improve evaluations of new drug candidates regarding their efficacy and toxicity is continuing. The present thesis has outlined a number of new options, with deeper insight into their values and collection of greater experience improvements may be faster in future.

6 Summary

In the past decades, there was significant progress in biological and medical research as well as in pharmaceutical and chemical development, also based on animal testing. To date, millions of animals are used every year in biological and medical research as well as in pharmaceutical development. Medicinal products and chemicals need to undergo toxicological tests before they can seek marketing authorisation, and their potential toxicity is mainly investigated *in vivo* as requested by the regulatory legislation and guidelines.

However, a rethinking has begun driven by both ethical and financial aspects. The principle of the 3Rs aiming at the replacement, reduction and refinement of animal tests has been introduced in the late 1950s and has already been incorporated in the European regulatory legislation. Therefore, the industry is requested to implement the principle of the 3Rs into their development programs. During the last 20 years, some exciting advances have been made such as organoids (“organ-in-a-dish”), microphysiological systems (“organs-on-a-chip”), “omics” technologies (e.g. “gene chips”), and *in-silico* tools. However, most of these technologies have not reached full regulatory acceptance so far, except for *in-silico* models that have already been used for toxicity estimations in registration dossiers of pharmaceuticals.

To date, although promising technologies have been developed, a broad adoption of alternative testing methods by the pharmaceutical industry is still missing. Pharmaceutical companies mainly prefer to rely on long-established animal models and shy to introduce alternative testing methods, which are not yet fit for regulatory purposes. International programs fostering the principle of the 3Rs such as EU-ToxRisk involving all stakeholders need to be expanded and continued to accelerate the implementation process of alternative testing strategies into drug development. A promising example is the process for revision of the ICH guidance S1A involving both the industry and regulatory authorities. The results of this project may lead to the conclusion that 2-year carcinogenicity studies in rodents could be waived in most cases and that the carcinogenic potential of pharmaceuticals could be predicted based on long-term toxicity data. Projects like this make confident that the implementation process of the 3Rs in regulatory testing is in progress.

However, sharing and publication of scientific data by academia and industry as well as a close cooperation with regulatory authorities are needed to further accelerate the implementation process of the 3Rs in regulatory toxicity testing. If all stakeholders pull together, a broad implementation of alternative testing methods in regulatory toxicity testing is within reach.

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234. European Medicines Agency, European Public Assessment Report: Rixathon, EMA/303207/2017, 21 April 2017.

235. European Medicines Agency, European Public Assessment Report: Riximyo, EMA/440905/2017, 21 April 2017.

236. European Medicines Agency, European Public Assessment Report: Refixia, EMA/346525/2017, 23 Marchl 2017.

237. European Medicines Agency, European Public Assessment Report: Brineura, EMA/31226/2017, 21 April 2017.

238. European Medicines Agency, European Public Assessment Report: Trumenba, EMA/CHMP/232746/2017, 23 March 2017.

239. European Medicines Agency, European Public Assessment Report: Axumin, EMA/237809/2017, 23 Marchl 2017.

240. European Medicines Agency, European Public Assessment Report: Ivabradine Accord, EMA/263015/2017, 23 March 2017.

241. European Medicines Agency, European Public Assessment Report: Dinutuximab beta Apeiron, EMA/263814/2017, 23 March 2017.

242. European Medicines Agency, European Public Assessment Report: Emtricitabine/tenofovir disoproxil Krka d.d., EMA/CHMP/236203/2017, 23 February 2017.

243. European Medicines Agency, European Public Assessment Report: Natpar, EMA/180882/2017, 23 February 2017.

244. European Medicines Agency, European Public Assessment Report: Roteas, EMA/158410/2017, 23 February 2017.

245. European Medicines Agency, European Public Assessment Report: Varuby, EMA/239011/2017, 23 February 2017.

246. European Medicines Agency, European Public Assessment Report: Chenodeoxycholic acid sigma-tau, EMA/650359/2016, 15 September 2016.

247. European Medicines Agency, European Public Assessment Report: Jylamvo, EMA/78284/2017, 26 January 2017.

248. European Medicines Agency, European Public Assessment Report: AMGEVITA, EMA/106922/2017, 26 January 2017.

249. European Medicines Agency, European Public Assessment Report: Daptomycin Hospira, EMA/109959/2017, 26 January 2017.

250. European Medicines Agency, European Public Assessment Report: SOLYMBIC, EMA/106921/2017, 26 January 2017.

251. European Medicines Agency, European Public Assessment Report: Tadalafil Lilly, EMA/CHMP/23344/2017, 26 January 2017.

252. European Medicines Agency, European Public Assessment Report: Yargesa, EMA/103540/2017, 26 January 2017.

253. European Medicines Agency, European Public Assessment Report: Rolufta, EMA/CHMP/148961/2017, 26 January 2017.

254. European Medicines Agency, European Public Assessment Report: LEDAGA, EMA/CHMP/13156/2017, 15 December 2016.

255. European Medicines Agency, European Public Assessment Report: Pregabalin Zentiva k.s., EMA/14344/2017, 15 December 2016.

256. European Medicines Agency, European Public Assessment Report: Truxima, EMA/CHMP/75695/2017, 15 December 2016.

257. European Medicines Agency, European Public Assessment Report: Alecensa, EMA/197343/2017, 15 December 2016.

258. European Medicines Agency, European Public Assessment Report: LIFMIOR, EMA/114647/2016 Corr.1, 17 July 2017.

259. European Medicines Agency, European Public Assessment Report: Vihuma, EMA/CHMP/814221/2016, 15 December 2016.

260. European Medicines Agency, European Public Assessment Report: Cystadrops, EMA/738656/2016, 13 October 2016.

261. European Medicines Agency, European Public Assessment Report: Zinplava, EMA/853812/2016, 22 November 2016.

262. European Medicines Agency, European Public Assessment Report: Movymia, EMA/88527/2017, 10 November 2016.

263. European Medicines Agency, European Public Assessment Report: Suliqua, EMA/800280/2016, 10 November 2016.

264. European Medicines Agency, European Public Assessment Report: Fiasp, EMA/CHMP/50360/2017, 10 November 2016.

265. European Medicines Agency, European Public Assessment Report: Tadalafil Generics, EMA/803097/2016, 10 November 2016.

266. European Medicines Agency, European Public Assessment Report: Vemlidy, EMA/793580/2016, 10 November 2016.

267. European Medicines Agency, European Public Assessment Report: AFSTYLA, EMA/CHMP/699390/2016, 10 November 2016.

268. European Medicines Agency, European Public Assessment Report: Darunavir Mylan, EMA/851324/2016, 10 November 2016.

269. European Medicines Agency, European Public Assessment Report: Terrosa, EMA/84371/2017, 10 November 2016.

270. European Medicines Agency, European Public Assessment Report: Emtricitabine/Tenofovir disoproxil Mylan, EMA/CHMP/636453/2016, 13 October 2016.

271. European Medicines Agency, European Public Assessment Report: OCALIVA, EMA/725757/2016, 13 October 2016.

272. European Medicines Agency, European Public Assessment Report: Rekovelle, EMA/11072/2017, 13 October 2016.

273. European Medicines Agency, European Public Assessment Report: Emtricitabine/Tenofovir disoproxil Krka, EMA/CHMP/522913/2016, 13 October 2016.

274. European Medicines Agency, European Public Assessment Report: SomaKit TOC, EMA/734748/2016, 13 October 2016.

275. European Medicines Agency, European Public Assessment Report: Tenofovir disoproxil Mylan, EMA/798405/2016, 13 October 2016.

276. European Medicines Agency, European Public Assessment Report: Venclyxto, EMA/725631/2016, 13 October 2016.

277. European Medicines Agency, European Public Assessment Report: NINLARO, EMA/CHMP/594718/2016, 15 September 2016.

278. European Medicines Agency, European Public Assessment Report: Granpidam, EMA/645375/2016, 15 September 2016.

279. European Medicines Agency, European Public Assessment Report: Glyxambi, EMA/749639/2016, 15 September 2016.

280. European Medicines Agency, European Public Assessment Report: Ivabradine JensonR, EMA/648588/2016, 15 September 2016.

281. European Medicines Agency, European Public Assessment Report: Ivabradine Zentiva, EMA/73167/2017, 15 September 2016.

282. European Medicines Agency, European Public Assessment Report: Parsabiv, EMA/664198/2016, 15 September 2016.

283. European Medicines Agency, European Public Assessment Report: Emtricitabine/Tenofovir disoproxil, EMA/650230/2016, 15 September 2016.

284. European Medicines Agency, European Public Assessment Report: IBRANCE, EMA/652627/2016, 15 September 2016.

285. European Medicines Agency, European Public Assessment Report: Lartruvo, EMA/CHMP/742133/2016, 15 September 2016.

286. European Medicines Agency, European Public Assessment Report: Onivyde, EMA/CHMP/589179/2016, 21 July 2016.

287. European Medicines Agency, European Public Assessment Report: Truberzi, EMA/549473/2016, 21 July 2016.

288. European Medicines Agency, European Public Assessment Report: Inhixa, EMA/536977/2016, 21 July 2016.

289. European Medicines Agency, European Public Assessment Report: Mysildecard, EMA/533666/2016, 28 July 2016.

290. European Medicines Agency, European Public Assessment Report: Sialanar, EMA/555265/2016, 21 July 2016.

291. European Medicines Agency, European Public Assessment Report: Tenofovir disoproxil Zentiva, EMA/532453/2016, 21 July 2016.

292. European Medicines Agency, European Public Assessment Report: Thorinane, EMA/536972/2016, 21 July 2016.

293. European Medicines Agency, European Public Assessment Report: CABOMETYX, EMA/664123/2016, 21 July 2016.

294. European Medicines Agency, European Public Assessment Report: Kisplyx, EMA/578759/2016, 21 July 2016.

295. European Medicines Agency, European Public Assessment Report: Atazanavir Mylan, EMA/503216/2016, 23 June 2016.

296. European Medicines Agency, European Public Assessment Report: Aerivio Spiromax, EMA/486136/2016, 23 June 2016.

297. European Medicines Agency, European Public Assessment Report: Airexar Spiromax, EMA/486131/2016, 23 June 2016.

298. European Medicines Agency, European Public Assessment Report: Nordimet, EMA/527385/2016, 23 June 2016.

299. European Medicines Agency, European Public Assessment Report: Zalmoxis, EMA/CHMP/589978/2016, 23 June 2016.

300. European Medicines Agency, European Public Assessment Report: CINQAERO, EMA/CHMP/481610/2016, 23 June 2016.

301. European Medicines Agency, European Public Assessment Report: Bortezomib Hospira, EMA/CHMP/421198/2016, 23 May 2016.

302. European Medicines Agency, European Public Assessment Report: Bortezomib Sun, EMA/CHMP/449636/2016, 26 May 2016.

303. European Medicines Agency, European Public Assessment Report: Pemetrexed Fresenius Kabi, EMA/CHMP/407425/2016, 26 May 2016.

304. European Medicines Agency, European Public Assessment Report: Zepatier, EMA/419807/2016, 26 May 2016.

305. European Medicines Agency, European Public Assessment Report: Qtern, EMA/428168/2016, 26 May 2016.

306. European Medicines Agency, European Public Assessment Report: EndolucinBeta, EMA/CHMP/404078/2016, 28 April 2016.

307. European Medicines Agency, European Public Assessment Report: Epclusa, EMA/399285/2016, 26 May 2016.

308. European Medicines Agency, European Public Assessment Report: Zinbryta, EMA/458317/2016, 28 April 2016.

8 Annex

Table 8: Information on alternative testing methods in EPARs published between July 2016 and July 2018

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of "omics", MPS, organoids and/or *in-silico* approaches
Myalepta [138]	EMEA/H/C/004218	metreleptin	Biological	Toxicity assessment based mainly on animal studies; no information on "omics", MPS, organoids or *in-silico* approaches
Aimovig [139]	EMEA/H/C/004447	erenumab	Biological	Toxicity assessment based mainly on animal studies; no information on "omics", MPS, organoids or *in-silico* approaches
Alsitek [140]	EMEA/H/C/004398	masitinib	Small molecule	Toxicity assessment based mainly on animal studies; no information on "omics", MPS, organoids or *in-silico* approaches
Halimatoz [141]	EMEA/H/C/004866	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on "omics", MPS, organoids or *in-silico* approaches
Hefiya [142]	EMEA/H/C/004865	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on "omics", MPS, organoids or *in-silico* approaches
Hyrimoz [143]	EMEA/H/C/004320	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on "omics", MPS, organoids or *in-silico* approaches
Nityr [118]	EMEA/H/C/004582	nitisinone	Small molecule	Mainly literature-based assessment, *in-silico* genotoxicity assessment

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Trazimera [144]	EMEA/H/C/004463	trastuzumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on “omics”, MPS, organoids or *in-silico* approaches
Carmustine Obvius [145]	EMEA/H/C/004326	carmustine	Small molecule	Literature-based assessment
Aplidin [116]	EMEA/H/C/004354	plitidepsin	Small molecule	PD studies: Gene expression profiling, cultured cell suspensions from tumour specimens; Toxicity assessment based mainly on animal studies
Tegsedi [130]	EMEA/H/C/004782	inotersen	Small molecule	PD: specificity and selectivity confirmed *in silico*; Toxicity assessment based mainly on animal studies
Verkazia [146]	EMEA/H/C/004411	ciclosporin	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature; no information on “omics”, MPS, organoids or *in-silico* approaches
Dzuveo [147]	EMEA/H/C/004335	sufentanil	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature; no information on “omics”, MPS, organoids or *in-silico* approaches
Biktarvy [148]	EMEA/H/C/004449	bictegravir / emtricitabine / tenofovir alafenamide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Amglidia [149]	EMEA/H/C/004379	glibenclamide	Small molecule	Literature-based assessment

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Rubraca [119]	EMEA/H/C/004272	rucaparib	Small molecule	Toxicity assessment based mainly on animal studies; *in-silico* genotoxicity assessment (DEREK) and PK drug-drug interaction modelling
Pemetrexed Krka [150]	EMEA/H/C/003958	pemetrexed	Small molecule	Literature-based assessment
Zessly [151]	EMEA/H/C/004647	infliximab	Biological	Similarity to reference product demonstrated *in vitro*; no information on “omics”, MPS, organoids or *in-silico* approaches
Juluca [152]	EMEA/H/C/004427	dolutegravir / rilpivirine	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
KANJINTI [153]	EMEA/H/C/004361	trastuzumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on “omics”, MPS, organoids or *in-silico* approaches
Prasugrel Mylan [154]	EMEA/H/C/004644	prasugrel	Small molecule	Literature-based assessment
Trydonis [155]	EMEA/H/C/004702	beclometasone / formoterol / glycopyrronium bromide	Small molecule	Informed consent application with reference to the medicinal product Trimbow
Riarify (previously CHF 5993 Chiesi Farmaceutici S.p.A.) [156]	EMEA/H/C/004836	beclometasone / formoterol / glycopyrronium bromide	Small molecule	Informed consent application with reference to the medicinal product Trimbow

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Mylotarg [157]	EMEA/H/C/004204	gemtuzumab ozogamicin	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Alpivab [158]	EMEA/H/C/004299	peramivir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Alofisel [159]	EMEA/H/C/004258	darvadstrocel	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Lamzede [160]	EMEA/H/C/003922	velmanase alfa	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Segluromet [161]	EMEA/H/C/004314	ertugliflozin / metformin hydrochloride	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Semglee [162]	EMEA/H/C/004280	insulin glargine	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Steglujan [163]	EMEA/H/C/004313	ertugliflozin / sitagliptin	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Lokelma [164]	EMEA/H/C/004029	sodium zirconium cyclosilicate	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Shingrix [165]	EMEA/H/C/004336	herpes zoster vaccine (recombinant, adjuvanted)	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Steglatro [166]	EMEA/H/C/004315	ertugliflozin	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Hemlibra [167]	EMEA/H/C/004406	emicizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Crysvita [168]	EMEA/H/C/004275	burosumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Anagrelide Mylan [120]	EMEA/H/C/004585	anagrelide	Small molecule	Mainly literature-based assessment, *in-silico* genotoxicity assessment (ICH M7)
Alkindi [169]	EMEA/H/C/004416	hydrocortisone	Small molecule	Literature-based assessment
Herzuma [170]	EMEA/H/C/002575	trastuzumab	Biological	Similarity to reference product demonstrated *in vitro*; no information on “omics”, MPS, organoids or *in-silico* approaches
Efavirenz/Emtricitabine/Tenofovir disoproxil Krka [171]	EMEA/H/C/004274	efavirenz / emtricitabine / tenofovir disoproxil	Small molecule	Literature based assessment
Ozempic [172]	EMEA/H/C/004174	semaglutide	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Darunavir Krka [173]	EMEA/H/C/004273	darunavir	Small molecule	Literature-based assessment
Darunavir Krka d.d. [174]	EMEA/H/C/004891	darunavir	Small molecule	Literature-based assessment
Fanaptum [175]	EMEA/H/C/004149	iloperidone	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
MVASI [176]	EMEA/H/C/004728	bevacizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Adynovi [177]	EMEA/H/C/004195	rurioctocog alfa pegol	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Fasenra [178]	EMEA/H/C/004433	benralizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Fulvestrant Mylan [179]	EMEA/H/C/004649	fulvestrant	Small molecule	Literature-based assessment
Intrarosa [180]	EMEA/H/C/004138	prasterone	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Jorveza [181]	EMEA/H/C/004655	budesonide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Ocrevus [182]	EMEA/H/C/004043	ocrelizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Onzeald [183]	EMEA/H/C/003874	etirinotecan pegol	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Prevymis [184]	EMEA/H/C/004536	letermovir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Tacforius [185]	EMEA/H/C/004435	tacrolimus	Small molecule	Literature-based assessment
Adlumiz [121]	EMEA/H/C/003847	anamorelin	Small molecule	Toxicity assessment based mainly on animal studies; *in-silico* model used for safety pharmacology (cardiovascular)
Zejula [186]	EMEA/H/C/004249	niraparib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Elebrato Ellipta [187]	EMEA/H/C/004781	fluticasone furoate / umeclidinium / vilanterol	Small molecule	Literature-based assessment
Imatinib Teva B.V. [188]	EMEA/H/C/004748	imatinib	Small molecule	Literature-based assessment
Ontruzant [189]	EMEA/H/C/004323	trastuzumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Trelegy Ellipta [190]	EMEA/H/C/004363	fluticasone furoate / umeclidinium / vilanterol	Small molecule	Literature-based assessment
Cyltezo [191]	EMEA/H/C/004319	adalimumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Miglustat Gen.Orph [192]	EMEA/H/C/004366	miglustat	Biological	Literature-based assessment
Nyxoid [193]	EMEA/H/C/004325	naloxone	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Ritonavir Mylan [194]	EMEA/H/C/004549	ritonavir	Small molecule	Literature-based assessment
TOOKAD [122]	EMEA/H/C/004182	padeliporfin	Small molecule	Toxicity assessment based mainly on animal studies; impurities assessed *in silico* for potential carcinogenicity, chromosomes damages, genotoxicity and mutagenicity
Tremfya [195]	EMEA/H/C/004271	guselkumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
VeraSeal [196]	EMEA/H/C/004446	human fibrinogen / human thrombin	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Zubsolv [197]	EMEA/H/C/004407	buprenorphine / naloxone	Small molecule	Literature-based assessment

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Dupixent [198]	EMEA/H/C/004390	dupilumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Entecavir Accord [199]	EMEA/H/C/004458	entecavir	Small molecule	Literature-based assessment
Lutathera [200]	EMEA/H/C/004123	lutetium (177lu) oxodotreotide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Symtuza [201]	EMEA/H/C/004391	darunavir / cobicistat / emtricitabine / tenofovir alafenamide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Tecentriq [202]	EMEA/H/C/004143	atezolizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Bavencio [203]	EMEA/H/C/004338	avelumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Entecavir Mylan [204]	EMEA/H/C/004377	entecavir	Small molecule	Literature-based assessment
Lacosamide Accord [205]	EMEA/H/C/004443	lacosamide	Small molecule	Literature-based assessment
Rydapt [206]	EMEA/H/C/004095	midostaurin	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Xermelo [207]	EMEA/H/C/003937	telotristat ethyl	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Human IgG1 monoclonal antibody specific for human interleukin-1 alpha XBiotech [208]	EMEA/H/C/004388	human IgG1 monoclonal antibody specific for human interleukin-1 alpha	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Masipro (RFD) [209]	EMEA/H/C/004159	masitinib	Small molecule	PD only demonstrated *in vitro*; toxicity assessment based mainly on animal studies no information on “omics”, MPS, organoids or *in-silico* approaches
Cuprior [123]	EMEA/H/C/004005	trientine tetrahydrochloride	Small molecule	*In-silico* evaluations of the genotoxic potential of Cuprior and three main impurities using DEREK and Leadscope assays
Efavirenz/Emtricitabine/Tenofovir disoproxil Mylan [210]	EMEA/H/C/004240	efavirenz / emtricitabine / tenofovir disoproxil	Small molecule	Literature-based assessment
Fotivda [211]	EMEA/H/C/004131	tivozanib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Imraldi [212]	EMEA/H/C/004279	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; comparative *in-vivo* study; no

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
				information on “omics”, MPS, organoids or *in-silico* approaches
Nitisinone MDK (previously Nitisinone MendeliKABS) [213]	EMEA/H/C/004281	nitisinone	Small molecule	Literature-based assessment
Kisqali [214]	EMEA/H/C/004213	ribociclib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
MAVENCLAD [215]	EMEA/H/C/004230	cladribine	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Maviret [216]	EMEA/H/C/004430	glecaprevir / pibrentasvir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Vosevi [217]	EMEA/H/C/004350	sofosbuvir / velpatasvir / voxilaprevir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Insulin lispro Sanofi [218]	EMEA/H/C/004303	insulin lispro	Biological	Similarity to reference product demonstrated *in vitro*; comparative *in-vivo* study; no information on “omics”, MPS, organoids or *in-silico* approaches
Veltassa [124]	EMEA/H/C/004180	patiromer	Small molecule	*In-silico* assessment of local tolerance (two models)

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Efavirenz/Emtricitabine/Tenofovir disoproxil Zentiva [219]	EMEA/H/C/004250	efavirenz / emtricitabine / tenofovir disoproxil	Small molecule	Literature-based assessment
Kyntheum [220]	EMEA/H/C/003959	brodalumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Trimbow [221]	EMEA/H/C/004257	beclometasone / formoterol / glycopyrronium bromide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Blitzima [222]	EMEA/H/C/004723	rituximab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Reagila [223]	EMEA/H/C/002770	cariprazine	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Ritemvia [224]	EMEA/H/C/004725	rituximab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Rituzena (previously Tuxella) [225]	EMEA/H/C/004724	rituximab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Spherox [226]	EMEA/H/C/002736	spheroids of human autologous matrix-associated chondrocytes	Biological	No conventional toxicity studies performed

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
OXERVATE [227]	EMEA/H/C/004209	cenegermin	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
BESPONSA [228]	EMEA/H/C/004119	inotuzumab ozogamicin	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Erelzi [229]	EMEA/H/C/004192	etanercept	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Kevzara [230]	EMEA/H/C/004254	sarilumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Skilarence [231]	EMEA/H/C/002157	dimethyl fumarate	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Ucedane [232]	EMEA/H/C/004019	carglumic acid	Small molecule	Literature-based assessment
Febuxostat Mylan [233]	EMEA/H/C/004374	febuxostat	Small molecule	Literature-based assessment
Rixathon [234]	EMEA/H/C/003903	rituximab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Riximyo [235]	EMEA/H/C/004729	rituximab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Elmiron [131]	EMEA/H/C/004246	pentosan polysulfate sodium	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature; Ecotoxicity: Bioaccumulation potential - log Kow assessed *in silico*
Refixia [236]	EMEA/H/C/004178	nonacog beta pegol	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Brineura [237]	EMEA/H/C/004065	cerliponase alfa	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Spinraza [125]	EMEA/H/C/004312	nusinersen	Small molecule	Toxicity assessment based mainly on animal studies; Potential interaction with “off-target” sequences assessed *in silico*
Trumenba [238]	EMEA/H/C/004051	meningococcal group b vaccine (recombinant, adsorbed)	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Axumin [239]	EMEA/H/C/004197	fluciclovine (18f)	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Ivabradine Accord [240]	EMEA/H/C/004241	ivabradine	Small molecule	Literature-based assessment
Qarziba (previously Dinutuximab beta EUSA and	EMEA/H/C/003918	dinutuximab beta	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Dinutuximab beta Apeiron) [241]				
Emtricitabine/Tenofovir disoproxil Krka d.d. [242]	EMEA/H/C/004686	emtricitabine / tenofovir disoproxil	Small molecule	Literature-based assessment
Natpar [243]	EMEA/H/C/003861	parathyroid hormone	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Pemetrexed Hospira UK Limited [126]	EMEA/H/C/004488	pemetrexed	Small molecule	Literature-based assessment; *in-silico* studies used to characterise change in formulation compared to reference product
Roteas [244]	EMEA/H/C/004339	edoxaban	Small molecule	Informed consent application referring to the medicinal product Lixiana
Varuby [245]	EMEA/H/C/004196	rolapitant	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Chenodeoxycholic acid Leadiant (previously known as Chenodeoxycholic acid sigma-tau) [246]	EMEA/H/C/004061	chenodeoxycholic acid	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature
Jylamvo [247]	EMEA/H/C/003756	methotrexate	Small molecule	Literature-based assessment

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
AMGEVITA [248]	EMEA/H/C/004212	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; toxicity assessed *in vivo*; no information on “omics”, MPS, organoids or *in-silico* approaches
Daptomycin Hospira [249]	EMEA/H/C/004310	daptomycin	Small molecule	Literature-based assessment
SOLYMBIC [250]	EMEA/H/C/004373	adalimumab	Biological	Similarity to reference product demonstrated *in vitro*; toxicity assessed *in vivo*; no information on “omics”, MPS, organoids or *in-silico* approaches
Tadalafil Lilly [251]	EMEA/H/C/004666	tadalafil	Small molecule	Informed consent application referring to the medicinal product Cialis
Xeljanz [127]	EMEA/H/C/004214	tofacitinib	Small molecule	Toxicity assessment based mainly on animal studies; *In-silico* tools DEREK and SARAH Nexus used for mutagenicity assessment of impurities
Yargesa [252]	EMEA/H/C/004016	miglustat	Small molecule	Literature-based assessment
Rolufta [253]	EMEA/H/C/004654	umeclidinium	Small molecule	Informed consent application referring to the medicinal product Incruse
LEDAGA [254]	EMEA/H/C/002826	chlormethine	Small molecule	Literature-based assessment
Pregabalin Zentiva k.s. [255]	EMEA/H/C/004277	pregabalin	Small molecule	Literature-based assessment
Truxima [256]	EMEA/H/C/004112	rituximab	Biological	Similarity to reference product demonstrated *in vitro*; toxicity assessed *in vivo*; no

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
				information on “omics”, MPS, organoids or *in-silico* approaches
Alecensa [257]	EMEA/H/C/004164	alectinib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
LIFMIOR [258]	EMEA/H/C/004167	etanercept	Biological	Application completely refers to reference product Enbrel
Olumiant [128]	EMEA/H/C/004085	baricitinib	Small molecule	Toxicity assessment based mainly on animal studies; *In-silico* toxicity assessments of impurities (ICH M7)
Vihuma [259]	EMEA/H/C/004459	simoctocog alfa	Biological	Informed consent application referring to the medicinal product Nuwiq
Cystadrops [260]	EMEA/H/C/003769	mercaptamine	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Zinplava [261]	EMEA/H/C/004136	bezlotoxumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Movymia [262]	EMEA/H/C/004368	teriparatide	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Suliqua [263]	EMEA/H/C/004243	insulin glargine / lixisenatide	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Fiasp [264]	EMEA/H/C/004046	insulin aspart	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Talmanco (previously Tadalafil Generics) [265]	EMEA/H/C/004297	tadalafil	Small molecule	Literature-based assessment
Vemlidy [266]	EMEA/H/C/004169	tenofovir alafenamide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
AFSTYLA [267]	EMEA/H/C/004075	lonoctocog alfa	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Darunavir Mylan [268]	EMEA/H/C/004068	darunavir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
LUSDUNA [129]	EMEA/H/C/004101	insulin glargine	Biological	Similarity to reference product demonstrated *in vitro*; toxicity assessed *in vivo*; *In-silico* toxicity assessments of impurities (immunogenicity)
Terrosa [269]	EMEA/H/C/003916	teriparatide	Biological	Similarity to reference product demonstrated *in vitro* and *in vivo*; toxicity assessed *in vivo*
Emtricitabine/Tenofovir disoproxil Mylan [270]	EMEA/H/C/004050	emtricitabine / tenofovir disoproxil	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
OCALIVA [271]	EMEA/H/C/004093	obeticholic acid	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Rekovelle [272]	EMEA/H/C/003994	follitropin delta	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Emtricitabine/Tenofovir disoproxil Krka [273]	EMEA/H/C/004215	emtricitabine / tenofovir disoproxil	Small molecule	Literature-based assessment
SomaKit TOC [274]	EMEA/H/C/004140	edotreotide	Small molecule	Literature-based assessment
Tenofovir disoproxil Mylan [275]	EMEA/H/C/004049	tenofovir disoproxil	Small molecule	Toxicity assessment based mainly on animal studies and scientific literature; no information on “omics”, MPS, organoids or *in-silico* approaches
Venclyxto [276]	EMEA/H/C/004106	venetoclax	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
NINLARO [277]	EMEA/H/C/003844	ixazomib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Granpidam [278]	EMEA/H/C/004289	sildenafil	Small molecule	Literature-based assessment
Glyxambi [279]	EMEA/H/C/003833	empagliflozin / linagliptin	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Ivabradine JensonR [280]	EMEA/H/C/004217	ivabradine	Small molecule	Literature-based assessment
Ivabradine Zentiva [281]	EMEA/H/C/004117	ivabradine	Small molecule	Literature-based assessment
Parsabiv [282]	EMEA/H/C/003995	etelcalcetide	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Emtricitabine/Tenofovir disoproxil Zentiva [283]	EMEA/H/C/004137	emtricitabine / tenofovir disoproxil	Small molecule	Literature-based assessment
Ibrance [284]	EMEA/H/C/003853	palbociclib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Lartruvo [285]	EMEA/H/C/004216	olaratumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Onivyde [286]	EMEA/H/C/004125	irinotecan	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Truberzi [287]	EMEA/H/C/004098	eluxadoline	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Inhixa [288]	EMEA/H/C/004264	enoxaparin sodium	Biological	Toxicity assessment based on *in-vitro* studies and scientific literature; no information on

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
				“omics”, MPS, organoids or *in-silico* approaches
Mysildecard [289]	EMEA/H/C/004186	sildenafil	Small molecule	Literature-based assessment
Sialanar [290]	EMEA/H/C/003883	glycopyrronium bromide	Small molecule	Literature-based assessment
Tenofovir disoproxil Zentiva [291]	EMEA/H/C/004120	tenofovir disoproxil	Small molecule	Literature-based assessment
Thorinane [292]	EMEA/H/C/003795	enoxaparin sodium	Biological	Toxicity assessment based on *in vitro* studies and scientific literature; no information on “omics”, MPS, organoids or *in-silico* approaches
CABOMETYX [293]	EMEA/H/C/004163	cabozantinib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Kisplyx [294]	EMEA/H/C/004224	lenvatinib	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Atazanavir Mylan [295]	EMEA/H/C/004048	atazanavir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Aerivio Spiromax [296]	EMEA/H/C/002752	salmeterol / fluticasone propionate	Small molecule	Literature-based assessment
Airexar Spiromax [297]	EMEA/H/C/004267	salmeterol / fluticasone propionate	Small molecule	Literature-based assessment

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
Nordimet [298]	EMEA/H/C/003983	methotrexate	Small molecule	Literature-based assessment
Zalmoxis [299]	EMEA/H/C/002801	allogeneic T cells genetically modified with a retroviral vector encoding for a truncated form of the human low affinity nerve growth factor receptor (ΔLNGFR) and the herpes simplex I virus thymidine kinase (HSV-TK Mut2)	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
CINQAERO [300]	EMEA/H/C/003912	reslizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Bortezomib Hospira [301]	EMEA/H/C/004207	bortezomib	Small molecule	Literature-based assessment
Bortezomib Sun [302]	EMEA/H/C/004076	bortezomib	Small molecule	Literature-based assessment
Pemetrexed Fresenius Kabi [303]	EMEA/H/C/003895	pemetrexed	Small molecule	Literature-based assessment
Zepatier [304]	EMEA/H/C/004126	elbasvir / grazoprevir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Qtern [305]	EMEA/H/C/004057	saxagliptin / dapagliflozin	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Medicinal product	Product number	International non-proprietary name	Small molecule/ Biological	Toxicity studies: Use of “omics”, MPS, organoids and/or *in-silico* approaches
EndolucinBeta [306]	EMEA/H/C/003999	lutetium (177 Lu) chloride	Small molecule	Literature-based assessment
Epclusa [307]	EMEA/H/C/004210	sofosbuvir / velpatasvir	Small molecule	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches
Zinbryta [308]	EMEA/H/C/003862	daclizumab	Biological	Toxicity assessment based mainly on animal studies; no information on “omics”, MPS, organoids or *in-silico* approaches

Eidesstattliche Erklärung:

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

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Ort, Datum	Dr. Sandra Mahr