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Finding synergies for the 3Rs – Repeated dose toxicity testing: Report from an EPAA partners' forum

Finding Synergies for the 3Rs – Repeated Dose Toxicity Testing: Report from an EPAA Partners' Forum

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Abstract

The European Partnership for Alternative Approaches to Animal Testing (EPAA) convened a Partners' Forum on repeated dose toxicity (RDT) testing to identify synergies between industrial sectors and stakeholders along with opportunities to progress these in existing research frameworks. Although RTD testing is not performed across all industrial sectors, the OECD accepted tests can provide a rich source of information and play a pivotal role for safety decisions relating to the use of chemicals. Currently there are no validated alternatives to repeated dose testing and a direct one-to-one replacement is not appropriate. However, there are many projects and initiatives at the international level which aim to implement various aspects of replacement, reduction and refinement (the 3Rs) in RDT testing. Improved definition of use, through better problem formulation, aligned to harmonisation of regulations is a key area, as is the more rapid implementation of alternatives into the legislative framework. Existing test designs can be optimised to reduce animal use and increase information content. Greater use of exposure-led decisions and improvements in dose selection will be beneficial. In addition, EPAA facilitates sharing of case studies demonstrating the use of Next Generation Risk Assessment applying various New Approach Methodologies to assess RDT.

Keywords: Repeated dose toxicity testing; alternatives; safety assessment; chemical legislation; *in vitro*; *in silico*; Threshold of Toxicological Concern (TTC); cross-sector; Integrated Approaches for Testing and Assessment (IATA); Weight of Evidence (WoE)

Highlights

- RDT tests are information rich and pivotal for safety assessment in many sectors
- Direct replacement of RDT tests by non-animal approaches is not currently possible or appropriate
- New Approach Methodologies can assist in safety decisions on systemic toxicity
- Refinements and improvements to RDT tests could reduce and optimise animal use
- There is a need to share data, information and methodologies across sectors

Abbreviations

3Cs, Communication, Collaboration and Commitment; 3Rs, Replacement, Reduction and Refinement of animal testing; ADI, Acceptable Daily Intake; AOP, Adverse Outcome Pathway; APCRA, Accelerating the Pace of Chemical Risk Assessment; BMD, Benchmark Dose; EC, European Commission; ECHA, European Chemicals Agency; ECPA, European Crop Protection Association; EFSA, European Food Safety Authority; EMA, European Medicines Agency; EPA, United States Environmental Protection Agency; EPAA, European Partnership for Alternative Approaches to Animal Testing; EU, European Union; FDA, United States Food and Drug Administration; GIVIMP, Good *In Vitro* Method Practices; H2020, Horizon 2020; IATA, Integrated Approaches for Testing and Assessment; ICCR, International Cooperation on Cosmetics Regulation; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; IFRA, The International Fragrance Association; J3rsWG, EMA's Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products; JRC, Joint Research Centre; LO(A)EL, Lowest Observed (Adverse) Effect Level; LRSS, Long Range Science Strategy; MoA, Mode of Action; NAMs, New Approach Methodologies; NGRA, Next Generation Risk Assessment; NO(A)EL, No Observed (Adverse) Effect Level; OECD, Organisation for Economic Co-operation and Development; PoD, Point of Departure; RAAF, Read-Across Assessment Framework; RDT, Repeated dose toxicity; REACH, Registration, Evaluation, Authorisation and restriction of CHemical substances; RIFM, Research Institute for Fragrance Materials, Inc.; SCCS, Scientific Committee on Consumer Safety; TTC, Threshold of Toxicological Concern; UVCB, Unknown or Variable Composition, complex reaction products or of Biological materials; WoE, Weight of Evidence.

1. Introduction

This report describes the main findings and conclusions of The European Partnership for Alternative Approaches to Animal Testing (EPAA) Partners' Forum on the topic of repeated dose toxicity (RDT) testing, held on 19 November 2018 in Brussels, Belgium. The EPAA Partners' Forum aimed to identify synergies between industrial sectors and stakeholders along with opportunities to progress these in existing research and testing frameworks. The EPAA Partners' Forum brought together 36 participants from industry and European Commission (EC), along with invited representatives from regulatory agencies and researchers from a large EU-funded project.

The invited participants represented the EC Directorates-General (DGs) Environment (ENV); Internal Market, Industry, Entrepreneurship and SMEs (GROW); Joint Research Centre (JRC); and Research and Innovation (RTD); the European Chemicals Agency (ECHA); the European Food Safety Authority (EFSA); the German Federal Institute for Drugs and Medical Devices (also as representative of the European Medicines Agency (EMA)); as well as companies from the chemicals, pharmaceuticals and vaccines, cosmetics, soaps and detergents, crop protection, animal health and fragrances sectors and their European trade associations and representatives from key EC funded projects relevant for this topic. Hans Bender (Germany) chaired the Partners' Forum and moderated the discussions.

It should be noted that this report is based on the presentations and actual discussions at the EPAA Partners' Forum aiming to achieve the stated objectives of the event. These focussed on the possibilities of each of the 3Rs (replacement, reduction and refinement of animal testing), to different extents, to be used in RDT testing as well as for the overall mission of ensuring human safety. It should not be considered a complete or comprehensive review of research efforts or potential synergies in the area of RDT testing.

1.1 Definitions and Context

For the purposes of this report, the term "RDT testing" is assumed in its broadest context and across as wide a group of industries and use scenarios as possible. The EPAA Partners' Forum acknowledged that there are a variety of "standard" Organisation for Economic Co-operation and Development (OECD) RDT tests which range from 28 to 90 days and longer (up to 2 years duration in rodent and non-rodent species). The main tests for regulatory use are summarised in Table 1. RDT tests are considered to be studies that are designed to evaluate a wide range of effects *in vivo* upon prolonged exposure. As such, RDT testing provides information on the potential profile of toxicity in animals that can be used in the context of defining safety in humans. In addition, information from RDT testing may trigger additional investigations for reproductive toxicity, immunotoxicity, neurotoxicity or

carcinogenicity. There is an historical assumption that current RDT tests in animals are predictive of effects on human health, although interspecies variability (which may reveal lack of relevance) is acknowledged when using such data for safety assessments in humans. As such, in many sectors, despite the potential limitations, the results from RDT tests are one of the cornerstones of ensuring safety of consumers, patients and for occupational exposure.

Table 1. Summary of the key standard tests and OECD Test Guideline studies for repeated dose toxicity

Short-term repeated dose toxicity study (28-day)

- Repeated Dose 28-day Oral Toxicity Study in Rodents (OECD 407)
- Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test (OECD 422)
- Subacute Inhalation Toxicity: 28-Day Study (OECD 412)
- Repeated Dose Dermal Toxicity: 21/28-day Study (OECD 410)

Sub-chronic toxicity study (90-day)

- Repeated Dose 90-Day Oral Toxicity Study in Rodents (OECD 408)
- Subchronic Inhalation Toxicity: 90-day Study (OECD 413)
- Subchronic Dermal Toxicity: 90-day Study (OECD 411)

Long-term repeated dose toxicity studies

- Chronic toxicity studies (OECD 452) primarily in rodents
- Combined chronic toxicity/carcinogenicity studies (OECD 453), typically tested in rats

Whilst the use of many standard tests of varying exposure time was acknowledged, the EPAA Partners' Forum focussed much of its attention on the 90-day assays – at the same time appreciating that these tests are not performed in the cosmetics industry. Typically the 90-day RDT test requires two species and an appropriate route of exposure, most commonly oral, but dermal and inhalation may also be required. Dosing at a range of concentrations up to the maximum tolerated dose is performed regularly, e.g. daily, and observations are compared to a control. The observations should include clinical, histopathological, behavioural and many other measurements. Testing may also include

range-finding and palatability studies, usually of short duration. Observations of endpoints in RDT test may trigger further testing for specific effects. Details of experimental design and procedures are provided in the Test Guidelines referred to in Table 1 although there are many variations and additional requirements as summarised below.

The EPAA Partners' Forum heard that, with the exception of the cosmetics industry, RDT testing is commonly performed across all industrial sectors. It is considered to provide a rich source of data and information on the effects of a chemical on an organism. Industrial sectors such as pharmaceutical, crop protection and biocides have considerable expertise in RDT testing with a relatively comprehensive inventory of historical data. In the case of pharmaceuticals, the safety assessment of a new drug may also be supported by human data. As such, the results of RDT testing, especially the 90-day test, are currently pivotal to many industries to ensure safety of products to humans.

1.2 Regulatory Importance, Status and Challenges of RDT Testing

The EPAA Partners' Forum heard that the use of RDT testing is governed by a multitude of regulations, directives and guidelines. The regulations cover different industrial sectors and global regions and it is inevitable that there are different requirements within individual sectors and geographies, those presented at the Forum are summarised briefly below. However, at the core of all regulations is the recognition of the use of OECD Test Guideline studies, mostly due to Mutual Acceptance of Data within and outside of OECD countries. The 90-day RDT test is frequently required due to the depth of information it provides and the understanding of the results. As well as being a regulatory information requirement, the results of RDT testing for the most sensitive species and endpoint can be used to identify points of departure (PoD), notably the No Observed (Adverse) Effect Level (NO(A)EL), Lowest Observed (Adverse) Effect Level (LO(A)EL) and, where possible, benchmark dose (BMD). The PoD can then be used in a safety context e.g. to set the reference doses for non-dietary safety evaluation or Acceptable Daily Intake (ADI) for dietary exposure assessment. In addition, the information from the 90-day RDT test can inform regulatory decisions such as classification and labelling and identification of specific hazards that may require further investigation.

Even within a single geographical area, there are a large number of regulations covering the various types and uses of chemicals. For instance, the European Union (EU) has various regulations covering different topics including industrial chemicals, cosmetics products, plant protection active ingredients biocidal active ingredients and related products. In addition, other regulations such as for pharmaceuticals and activities such as Community Strategies on Endocrine Disruptors and Combined

Exposures to Mixtures (European Commission, 1999) need to be taken into account. The result is a variety of requirements, some of which may even be considered contradictory with each other.

Further information was provided to the EPAA Partners' Forum relating to the role of individual European Agencies in using information from RDT tests. Under the Registration, Evaluation, Authorisation and restriction of CHemical substances (REACH) regulation, the European Chemicals Agency (ECHA) has minimum requirements for data dependent on tonnage and other conditions. However, ECHA's database which is available through ECHA's dissemination portal (cf. https://echa.europa.eu/information-on-chemicals) has many data gaps for RDT studies and, with the aim of avoiding as much animal testing as possible, the REACH regulation allows for adaptation of standard information requirements e.g. by using alternatives such as read-across. The European Food Safety Authority (EFSA) recognises the critical role of the 90-day study as a data requirement in six types of regulated products (i. food packaging and contact materials, ii. food ingredients, iii. feed additives, iv. genetically modified organisms, v. dietetic products, nutrition and food allergies, novel foods, and vi. pesticides). Within the data requirements, the 90-day study may be used differently, e.g. it is required by default for pesticides and as part of a tiered approach for food contact materials.

RDT studies are particularly valuable to the pharmaceutical industry to support clinical drug development in both Phase 1 and Phase 2. For pharmaceuticals, under the ICH M3(R2) regulations in the EU, there are generally differences in RDT studies for small molecules and biologicals. There is strong evidence of international collaboration e.g. between the EU, USA and elsewhere through the acceptance of a number of pieces of legislation from the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

For cosmetics ingredients, since March 2013 there has been a full ban on animal testing in the EU with several other countries also imposing a ban – raising a strong possibility that this may become a global ban. Despite the ban, it is emphasised by Cosmetics Europe that there is a need for information regarding systemic toxicity. However, with regard to regulatory submissions to e.g. the EC's Scientific Committee on Consumer Safety (SCCS), it is recognised that several test methods and guidelines for endpoints relating to RDT exist but acceptance of non-animal tests for systemic toxicity to assure safety is not guaranteed.

The EPAA Partner's Forum identified a number of challenges relating to the regulatory use and acceptance of RDT testing and specifically the implementation of alternatives and the 3Rs:

- There is a very slow pace of change in regulatory acceptance of updates to RDT testing, specifically relating to the replacement (and to a lesser extent refinement) of *in vivo* tests and understanding and implementing the best new technology and innovation.

- There is a lack of harmonisation and consistency in the data requirements in regulations between sectors and also between regions.
- There is varied, but often limited, implementation of alternatives to RDT testing in regulatory toxicology of which none are validated as a full replacement. Some sectors, however, are creating an environment to implement alternatives e.g. the International Cooperation on Cosmetics Regulation (ICCR) Principles for new methodologies in the risk assessment of cosmetic ingredients (Dent et al., 2018).
- There is a lack of coherent and transferable data resources for e.g. the *in vivo* tests and also the alternatives. Such resources could ensure that testing is not repeated unnecessarily and could assist with the validation of alternatives. In addition, retrospective studies of data can assist in the refinement of existing tests.

The challenges for the use of 3Rs in RDT for regulatory purposes were considered by the EPAA Partners' Forum and the initiatives attempting to address them are discussed in Section 2 along with opportunities in Section 3 below.

1.3 Impact of the 3Rs and other Alternatives on RDT

The EPAA Partners' Forum concluded that whilst there are currently no valid or validated non-animal alternatives that replace RDT tests directly, there is increasing use of alternatives in decision making e.g. for exposure-driven risk assessment in the cosmetics industry. Further, despite it being highly unlikely that a direct and complete one-to-one replacement of RDT testing will be possible, dependent on context, (non-validated) alternatives and different approaches are being increasingly applied to assist in safety decision making e.g. in the cosmetics industry. The lack of validated alternatives is due to the complexity of the RDT endpoint and the wealth of information that it provides on organ level and many other effects as well as the nature of the current validation paradigm. The information provided from the current RDT tests is, at present, essential in many industry sectors to assuring human safety.

Whilst the EPAA Partners' Forum acknowledged the lack of any suitable direct alternatives to RDT testing, there was unanimous support for greater effort in their development, implementation and acceptance. There are many drivers for these alternatives including ethical concerns, but also to provide better and more human-relevant safety information and to fill gaps in toxicological knowledge. In the context of the 3Rs, all aspects of alternatives were considered by the EPAA Partners' Forum including knowledge of exposure as well as knowledge from New Approach Methodologies

(NAMs) encompassing *in chemico* and *in vitro* assays, omics technologies (e.g. metabolomics and transcriptomics) and *in silico* approaches. In addition to the methodologies, strategies for their implementation and acceptance were discussed, as well as potential improvements (e.g. refinements) to existing RDT tests that could enhance the knowledge gained. Details on current projects and initiatives to develop and implement the 3Rs and alternatives to RDT testing are provided in Section 2.

2. Initiatives, Projects and Current Use of Alternatives for RDT

Many initiatives and funded projects in the area of RDT that have attempted to develop alternatives were described in the EPAA Joint Partners' Forum, these are summarised in Table 2 with a broader discussion of their relevance given below. It is however recognised in this report that others exist and may not be mentioned herein.

Table 2. Summary of main initiatives and projects relating to the development and increased acceptance of non-animal approaches for repeated dose toxicity testing (RDT) discussed at the EPAA Partners' Forum. Further details are available from the reference provided.

Initiative or Project	Funding agency, organiser etc	More information
Funded Projects		
Historical European Commission funding (pre-SEURAT-1)	European Commission (FP5 – H2020)	https://cordis.europa.eu/
SEURAT-1	European Commission (FR7) / Cosmetics Europe	Gocht et al. (2015); http://www.seurat-1.eu/
EU-ToxRisk	European Commission H2020	Daneshian et al. (2016); http://www.eu-toxrisk.eu/
Accelerating the Pace of Chemical Risk Assessments (APCRA)	ECHA, EPA, Health Canada and others	Kavlock et al. (2018)
Long-Range Science Strategy (LRSS)	Cosmetics Europe	Desprez et al. (2018); https://www.lrsscosmeticseurope.eu

CE-ToxGPS (example of RDT project included in LRSS)	Cosmetics Europe	https://www.lrsscosmeticseurope.eu
RDT Ontology (example of RDT project included in LRSS)	Cosmetics Europe	Desprez et al. (2019); https://www.lrsscosmeticseurope.eu
Various initiatives e.g. QSAR	EFSA	https://www.efsa.europa.eu/en/data/chemical-hazards-data
Feasibility study on data sharing	European Parliament	European Parliament (2018)
Use of omics to derive PoDs	EPA, Health Canada	Farmahin et al. (2017)
Microphysiological Systems Program	FDA, NIH	Wikswo et al. (2013)
Roadmaps / Strategies		
FDA Roadmap	US FDA	US FDA (2017)
EMA identified alternatives	EMA	EMA (2019a, 2019b)
Map of RDT Mechanisms	JRC	Prieto et al. (2014, 2019)
Project proposal for a Blue-sky workshop: Soliciting input for new ideas to address repeated dose toxicity	EPAA	https://ec.europa.eu/growth/sectors/chemicals/epaa_en
Workflows		
SEURAT-1 Workflow	EC / Cosmetics Europe	Berggren et al. (2017); OECD (2017)
LRSS Workflow	Cosmetics Europe	Desprez et al. (2018)
Fragrance Material Safety Evaluation Process	RIFM	Api et al. (2015)
ICCR Principles	ICCR	Dent et al. (2018)

2.1 European Union (EU) Funded Projects

The EU has provided considerable support through various funding schemes for research into animal-free toxicology. Since 1998 (the Fifth Framework Programme, FP5) until the current time (Horizon

2020, H2020) the EU has funded over 200 international projects with over 700€ million, with funding increasing with each cycle of Framework Programmes. In addition, over 150€ million has been provided in support by industry for 3Rs-relveant safety testing (25€ million from Cosmetics Europe for SEURAT-1; 85€ million and 40€ million from the European pharmaceuticals industry for IMI and IMI2 projects respectively). Over three quarters of the funding has been directed towards mammalian toxicology, of which a substantial part was devoted to RDT. The contribution of past and on-going EU projects to the 3Rs was recognised by the EPAA Partners' Forum and more details were provided on two of the larger initiatives and projects, as described below.

One of the most significant EU funding initiatives for RDT was "SEURAT-1". This was a cluster of six research projects (2011-2015) which ranged from the development of assays from stem cells, to *in vitro* biomarkers and a microfluidic bioreactor, coupled to computational models and databases (Gocht et al., 2015). The SEURAT-1 Workflow, constructed on existing data, *in silico* modelling and biokinetic considerations, was one of the most important outputs which aimed to assess chemical safety without relying on animal testing (Berggren et al., 2015, 2017; OECD, 2017). Whilst the Workflow was designed with cosmetic ingredients in mind, it is relevant to RDT and applicable to other chemicals, e.g. pharmaceuticals, plant protection products or biocides, etc. The current EU funded "flagship" project relating to RDT is EU-ToxRisk (Daneshian et al., 2016). This is a six year (2016-2021), multidisciplinary project with approximately 30€ million of funding. The aims of EU-ToxRisk are to develop pragmatic, robust read-across procedures incorporating mechanistic and toxicokinetic knowledge through the use of case studies. Implementation of alternatives is a key aspect of EU-ToxRisk and it works closely with stakeholders including regulatory authorities (through a Regulatory Advisory Board) to make the alternatives fit-for-purpose.

2.2 Industry Funded Projects and Initiatives

The cosmetics industry has a long history of supporting the development of non-animal approaches to RDT. This has gained increased importance due to the full implementation of a ban on animal testing for cosmetics ingredients which came into force in the EU in March 2013. Through Cosmetics Europe, the European cosmetics industry co-funded the SEURAT-1 initiative, as noted above. The SEURAT-1 Workflow proposed by Berggren et al., (2017), became the starting point for Cosmetics Europe's "Long Range Science Strategy" (LRSS) programme which included RDT as part of its 2016-2020 framework. The LRSS has three main goals, namely, to develop relevant non-animal NAMs; to apply and implement the NAMs in Next Generation Risk Assessments (NGRAs); and to ensure NAMs and NGRAs fit to the regulatory framework. These concepts were expanded upon by Desprez et al., (2018) who

implemented and extended the SEURAT-1 Workflow into the LRSS. The updated Workflow has incorporated three tiers to understand risk assessment for systemic toxicity which were extended by the ICCR who proposed nine principles for using NAMs in (human-relevant) risk assessment (Dent et al., 2018).

Amongst a significant number of projects funded through the LRSS to develop NAMs and demonstrate their use for NGRA, two were shown during the EPAA Partners' Forum as examples of activities ongoing in the field of RDT. The first example relates to defining an ontology that includes Mode of Action (MoA) elements for RDT and in which links are made with (internal) exposure and chemistry (Desprez et al., 2019). The second example introduced at the EPAA Partners' Forum was the development of a chemoinformatics platform (CE-ToxGPS). The CE-ToxGPS platform develops further the COSMOS database (https://cosmosdb.eu/cosmosdb.v2/) and is intended to extend the role of the system from data storage to data integration with active workflows and inclusion of predictive capabilities to help risk assessors.

Related to cosmetic products, the safe use of fragrance materials is ensured by the fragrance industry's self-regulatory programme through its members and affiliates IFRA and the Research Institute for Fragrance Materials (RIFM) Inc, in which scientific data are generated, evaluated and distributed for the safety of fragrance raw materials found in personal and household care products. In order to determine safety, a four step procedure with evaluation from an Expert Panel is applied (Api et al., 2015) and the findings are made available through the Food and Chemical Toxicology Fragrance Material Safety Assessment Center (RIFM, 2019).

The fragrance industry's safety evaluation procedure is updated on a regular basis through specific projects. For instance, to assess aggregate consumer exposure RIFM continues to improve exposure information through the use and refinement of the Creme RIFM Aggregate Exposure Model (https://www.cremeglobal.com/products/creme-rifm/; Safford et al., 2017). Computational and chemistry-based approaches, including read-across, have been used to evaluate the safety of fragrance materials where there are data gaps, although there is an on-going challenge with the justification of chemical similarity (which goes beyond the fragrance industry). In addition, the use of the Threshold of Toxicological Concern (TTC) has had a significant impact on decreasing the need for *in vivo* testing, since many fragrance ingredients are only used in very small concentrations (Bhatia et al., 2015).

The agrochemicals industry, (in part through the European Crop Protection Association (ECPA)) are investigating multiple approaches to use omics data e.g. from the study of responses such as RNA molecules at the transcriptome level or chemical processes involving metabolites at the metabolomic

level to provide more efficient means of defining PoDs. In this regard industry is working alongside regulatory agencies e.g. recommendations from a joint United States Environmental Protection Agency (EPA) and Health Canada study (Farmahin et al., 2017) are being investigated. The agrochemicals industry has also demonstrated the use of methods such as metabolomics for read-across (van Ravenzwaay et al., 2016) as well as other efforts demonstrating the utility of epigenetics in safety assessment (LaRocca et al., 2017) and omics technologies in chemical risk assessment (Buesen et al., 2017).

2.3 Initiatives from Governmental and Regulatory Agencies

Within Europe a number of agencies have recognised the potential use of alternatives to RDT and are involved in initiatives to support their implementation. ECHA reported that adaptations to REACH requirements for RDT commonly include read-across, whilst acknowledging the difficulty in this approach due to the lack of scientifically sound approaches and justification occurring frequently in the dossiers. ECHA is also involved in the Accelerating the Pace of Chemical Risk Assessment (APCRA) project (Kavlock et al., 2018). APCRA was initiated by the United States Environmental Protection Agency (EPA~) with the aim of bringing together international governmental regulators and researchers to discuss progress and barriers in applying NAMs to prioritisation, screening, and quantitative risk assessment of differing levels of complexity. There are a number of (mainly regulatory) organisations within Europe, USA, Canada and South Korea. Within APCRA, ECHA leads a case study which aims to provide a qualitative and quantitative comparison of NAMs and traditional RDT animal toxicity testing for data-poor chemicals.

EFSA also has a number of initiatives to provide information for data-poor substances. These initiatives cross a number of endpoints but are also relevant to RDT. They include, but are not limited to, the assessment of, and models for, dermal absorption; the use of QSARs and read-across to make predictions of effects; the promotion of the use of NAMs for the parent compound and metabolites; the use of AOPs; and assays for *in vitro* hepatic metabolism.

The EC's Joint Research Centre (JRC) has been at the forefront of evaluating the use of alternatives to *in vivo* toxicity testing for several decades. A part of applying these techniques has been the use of Integrated Approaches to Testing and Assessment (IATA) that attempt to integrate and weight all existing evidence and guide the targeted generation of new data, for the purpose of making regulatory decisions (OECD, 2016). Previous work from the JRC focussed on the assessment of mammalian acute toxicity and demonstrated the possibility of identifying and defining the mechanisms and hence pathways associated with acute oral toxicity (Prieto et al., 2014; 2019). The JRC is proposing to

undertake an analysis of RDT studies to gather, organise and analyse mechanistic knowledge, alongside data, related to toxicological effects on target organs in animal models after repeated exposure to chemicals, i.e. to map out the mechanisms related to RDT. The outcome of this analysis will be the description of a set of characteristics of chemicals inducing repeated dose systemic toxicity which will inform the development of alternative approaches and help to enhance standard *in vivo* studies to maximise the information they provide.

The EMA supports the use of the 3Rs and alternatives to evaluate the safety of medicinal products (EMA, 2019a). Through the EMA's Joint Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (J3RsWG) it is providing reflection papers and guidelines on the development of the 3Rs to identify toxicity, including RDT, in addition to recommendations on the 3Rs for the European Pharmacopoeia (EMA, 2019b). The series of reflection papers (EMA, 2016, 2017, 2018a,b) have provided the context for the use of alternatives for medicinal products. The reflection document (EMA, 2018b; page 8) provides information on 3Rs opportunities in RDT that are already implemented and accepted by the regulators.

The US Food and Drug Administration (FDA) aims to integrate emerging predictive technologies in safety assessment and identify priority challenges. However, it recognises the challenges faced by regulatory toxicologists in keeping pace with scientific and technological developments, specifically, the balance of ensuring safety whilst supporting innovation and the need to carefully define the context of the use of the alternative. The FDA has formed a Senior Toxicologist Working Group comprising senior toxicologists from all six FDA program Offices in addition to the National Center for Toxicological Research and the Office of the Commissioner. The purpose of the Working Group is to share information on new methods in toxicology as well to allow FDA regulatory and research scientists to become familiar with emerging toxicology tests and their potential usefulness in risk assessment. The FDA Predictive Toxicology Roadmap (US FDA, 2017) sets out the vision to identify critical priority activities for the integration of emerging predictive toxicology methods and new technologies into regulatory risk assessments. The Roadmap is intended to emphasise the context of use and the "qualification" of a model or assay i.e. whether it can be relied upon to have a specific interpretation and application in product development and regulatory decision-making for a particular use. Partnerships are an essential part of the Roadmap – as such the "3Cs" themes run through all the roadmaps and initiatives, these are Communication, Collaboration and Commitment.

3. Opportunities for the 3Rs in RDT Testing

A key objective of the EPAA Partners' Forum was to identify opportunities to progress the synergies between industrial sectors to rationalise and improve RDT testing. The key opportunities are summarised in Table 3. In this section these opportunities are organised into various themes whereby needs or on-going research in one (or more) sectors could be more broadly applied. The purpose here is to foster an on-going dialogue and a move towards more synergy and understanding across sectors.

Table 3. Key opportunities and needs to implement the 3Rs for RDT testing. Full details are provided in Section 3.

- Development of common data resources
- Improvement of mechanistic understanding
- Creation of common ontologies to link exposure, kinetics, chemistry, MoA and effects
- Better use of IATA or Weight of Evidence (WoE) strategies
- Incorporation of NAMs or other data to supplement lacking data
- Improvement in validation of NAMs to facilitate acceptance
- Optimisation of RDT *in vivo* test guidelines
- Harmonisation as far as possible of regulations across sectors and geographies
- Increasing dialogue between stakeholders to increase awareness of new technologies
- Direct projects and case studies to solve specific problems
- Definition and agreement on the information needs that data from RDT tests currently fill in different industry sectors/different regulatory settings i.e. decision-making context

3.1 Raising Cross-Sector Awareness and Collaboration to Define Cross-Sector Opportunities to Improve, and Ultimately Replace, RDT

The EPAA Partners' Forum appreciated a key opportunity that underpins much potential progress in embedding the 3Rs and alternatives in RDT testing is to ensure collaboration between all stakeholders. Collaboration will speed progress in the refinement of tests and as well as the development of alternatives. Collaboration across sectors and geographical areas will assist with harmonisation of

tests and the acceptance of alternatives. Overall, the need for dissemination (see Section 3.7) and collaboration is seen as being pivotal to identifying the needs, maintaining momentum, and establishing a community to support delivery of new predictive toxicology methods.

In order to improve, and provide the possibility for the ultimate replacement of, RDT, there is a need to understand the needs for individual safety decisions which may vary between industry sectors. Progress will be made, in part at least, by breaking RDT down into component pillars e.g. route of exposure, target organs, effect etc. Once the components of RDT have been established, suitable technologies can be identified to replace them. In this context the use of NAMs is ideal to provide information to assist in the improvement, and ultimate replacement, of RDT. However, the use of NAMs needs to be properly mapped out onto the needs of RDT in a holistic manner, rather than being a piecemeal approach.

The use of the information from RDT should also be considered in the development and application of alternatives. The concept of NGRA, which was initiated by the EPA to develop a new paradigm for the next generation of risk science (US EPA, 2014; Krewski et al., 2014), is an opportunity to remove the barrier to acceptance of the tests and to ensure their development is relevant to safety assessments.

3.2 Needs Drive the Opportunities – Reasons for Tests Redefined Through Proper Problem Formulation

There are different, but clearly defined, reasons across the sectors for undertaking a RDT test; some reasons are common across sectors whilst others may be specific to a regulation. For instance, most, if not all, sectors require knowledge of PoDs for safety assessment (predominantly from NO(A)ELs) and it should be considered whether NAMs would (in some instances) provide more relevant PoDs for the question in hand than a PoD derived from animal testing. The understanding of the information required depends on a number of factors especially relating to the protection goals to be achieved, the decisions to be made, the legal requirements and safety assessment to be met. More emphasis has to be put on the appropriate level of information that is needed to make the decisions and, more specifically, the confidence to enable acceptance of the decision and an appreciation of when the information is incorrect or insufficient. The definition of the issues to be addressed needs to be considered through better problem formulation. This will assist in the use and understanding information from alternatives for specific purposes. The information will be context dependent, despite this there is an opportunity to develop this knowledge across the needs of all sectors. Indeed,

within the process of problem formulation, there is the possibility to (re-)define the roles of alternatives and strategies in their use for RDT more thoroughly.

3.3 Methodological Development

The cross-sector EPAA Partners' Forum was in agreement that there are various opportunities for the development of all areas of RDT methodology from test design to the reporting of outcomes. The clear opportunity here is to align new research (and hence funding may be required) for better problem formulation to support the overall goal of safety to humans. The main areas to be considered were summarised as being with regard to the information and data derived from RDT and related studies, the integration of the data to provide a solution and use of appropriate benchmarks to provide assurance of the outcome.

In terms of the design of RDT various adaptions could be foreseen aligned to the better design of the test. These could be to take account of preliminary information from e.g. *in vitro* tests to identify target organs and effects to investigate. In addition, redesign of the 90-day RDT could allow for the integration of further measurements into the existing studies to improve the information that was obtained to support better and more far-sighted analysis. The EMA's reflection document (EMA, 2018a,b) has identified various opportunities for the implementation of the 3Rs including the expansion of the concept of integration of additional endpoints in RDT studies.

The EPAA Partners' Forum heard further positive proposals for refinements that could be made to RDT tests through integrated and intelligent study design. The aim of such refinements is to combine multiple endpoints traditionally assessed in separate studies into a single test to provide more information of high quality and greater relevance, however with the use of fewer animals. Various opportunities were noted to obtain better information on toxicokinetics, neurotoxicity, immunotoxicity, *in vivo* genotoxicity (i.e. integrated micronucleus test) and on MoA.

There are further opportunities to refine the design of RDT studies. One opportunity is to set up tests to support the derivation of BMD as opposed to NO(A)ELs to obtain the reference dose or PoD. The design of dosing is currently, in part at least, performed in accordance to regulations i.e. the desire for hazard characterisation at high doses.

3.4 Implementation of New Methodologies

The EPAA Partners' Forum recognised the need for implementation of new technologies, methodologies and strategies, as well as refinements to existing study types, as a key need and

opportunity for the 3Rs in RDT. Implementation in this context implies that the new approaches are suitable and acceptable to make safety decisions relating to RDT. In turn, the EPAA Partners' Forum concluded that the new technologies must give the same level of information to support safety assurance and current RDT studies.

The acceptance of a new approach (in the broadest context) requires some assessment of the alternative and elements of validation. There is an opportunity and need to move away from the "standard" methods of validation to a process that is more rapid, responsive and fit for purpose, bearing in mind that it should also be transferable across sectors and geographies. One clear method where the usefulness of 3Rs alternatives can be demonstrated (if not formally validated) in RDT is through the use of well-designed case studies.

Other aspects of implementation include their proper and appropriate use through guidance and guidelines. Recent advances in topics such as the OECD's Guidance Document on Good *In Vitro* Method Practices (GIVIMP) (OECD, 2018) are important and the process of "good practice" could be extended to other approaches e.g. *in silico* techniques. Relating to this, appropriate reporting is required that is consistent and fit for purpose, as well as being transferable from industry to regulators and being of an appropriate depth and quality to fulfil regulatory requirements. Many examples exist of reporting templates and evaluation schemes. Using the example of read-across for regulatory submission, ECHA has developed the Read-Across Assessment Framework (RAAF) to evaluate the completeness of a read-across under certain scenarios (ECHA, 2017).

3.5 Data Sharing

The sharing of data across sectors was seen by the EPAA Partners' Forum as a very large opportunity to progress the 3Rs for RDT. There are a number of aspects to this. The first is the sharing of the results of RDT tests themselves to provide access to more data which would prevent the need for repeat and unnecessary testing. In addition, a good data source will provide the basis for models as well and the evaluation and eventual validation of alternatives. The sharing of data should also extend beyond the standard tests to include data for toxicokinetics, alternatives, omics analyses, mechanistic information and data from human clinical trials, amongst others. Such an (ambitious) data framework may allow ultimately for the assurance of no human toxicity from non-clinical data.

Whilst the broadest possible sharing of data was endorsed by the EPAA Partners' Forum it is acknowledged that in order for data to be shared there are a number of challenges to be overcome in terms of the practical aspects, legal ownership and confidential nature of the data. In terms of the practical storing and sharing of data a number of on-line databases are available including, for

regulatory purposes, ECHA's dissemination portal, and to share safety data (e.g. NO(A)ELs) COSMOS DB – there are also many other databases including commercial ones. The eTOX Project (Cases et al., 2014; Sanz et al., 2017) has demonstrated the possibilities for sharing data from the pharmaceutical industry through the development of the eTOX database in the eToxSys platform (https://www.etoxsys.com/the-database.htm). Many learnings on the extraction, curation and storage of data from legacy RDT study reports were made in the EU IMI eTOX Project (Cases et al., 2014; Sanz et al., 2017).

The sharing of data would be greatly assisted by the digitalisation of data and use of an appropriate electronic format – there is a clear opportunity to harmonise data storage to facilitate sharing at various levels e.g. between industry and the appropriate regulatory agency as well as with other scientists. As the data matrices become more complex with different types of data, so will the associated databases. The EU IMI eTransafe Project (http://etransafe.eu/) is attempting to create such a translational database to support human safety assessment. Also from the European perspective, the European Parliament is funding a feasibility project on the joint sharing of data across sectors. The EU Agencies harmonised approach for safety data access and submission will investigate the possibility of sharing data between ECHA, EFSA and EMA (European Parliament, 2018).

3.6 Regulatory Needs

The opportunities to inform regulatory science, regulations and regulators of updates in the 3Rs were highlighted in the EPAA Partners' Forum. The motivation here is to bring about and maintain acceptable change, hence the dialogue with regulators must be open and frank (see Section 3.7 on Dissemination). A number of opportunities were identified to assist in regulatory science. One of the key needs of regulatory science must be that it keeps pace with the underlying technology (see Section 1.2). The acceptance of new methods for regulatory purposes is also a fundamental need. The EPAA Partners' Forum heard that there are opportunities to facilitate and improve acceptance in a number of ways. There is a requirement for validation of new methods and there may be opportunities to streamline the current process to improve the uptake of new methods.

With regard to legislation and regulations, there is an opportunity to increase harmonisation across global regions and sectors. It is appreciated that different industries will, inevitably, have different requirements for RDT studies, however, increased harmonisation in areas such as which studies are required (and any additional testing) should decrease unnecessary repetition of testing. Global harmonisation of RDT tests and mutual acceptance of data will potentially allow for a significant reduction in testing.

584

585 A further opportunity is to improve knowledge on RDT for mixtures and natural products. ECHA noted
586 that approximately two thirds of REACH dossiers were for unknown or variable composition, complex
587 reaction products or of biological materials (UVCB) substances or mixtures. Currently there is little
588 known about many of these, other than a small proportion of their constituents. There is, therefore,
589 an opportunity, to use the existing alternative tests and approaches more efficiently to support
590 regulatory decision making.

591

592 ***3.7 Dissemination and Stakeholder Engagement***

593 The EPAA Partners' Forum recognised the on-going need for dissemination regarding the 3Rs in RDT.
594 Dissemination is a key opportunity as it will allow for a full dialogue and engagement between all
595 stakeholders from industry, to the regulatory community across the world, to academics and
596 businesses that may be developing alternatives. There are several aspects to dissemination with
597 specific tasks required to raise awareness in the developers of alternatives to RDT studies as well as
598 how they may be validated (e.g. through EURL ECVAM), implemented and accepted. Conversely
599 regulators need to be informed of the new technologies and improvements and / or refinements in
600 standard tests that may be occurring. Lastly, the users of existing and new alternatives for RDT, e.g. in
601 industry, need to be made aware of the utility and possible acceptance of such approaches.

602

603 ***3.8 Capacity Building and Training in the 3Rs***

604 The increased need for expertise in all areas of safety assessment related to RDT was confirmed by
605 the EPAA Partners' Forum. Capacity building has the opportunity of increasing the number of trained
606 toxicologists and safety assessors who can implement the 3Rs whilst assuring the same level of
607 confidence on the outcome. A key aspect of capacity building is through training of new and existing
608 scientists which will enable them to understand and utilise the new technologies as well as to refine
609 the existing tests for RDT.

610

611 ***4. Current Culture of Synergy and Optimisation of 3Rs for RDT***

612 Sections 2 and 3 indicated that many current, and future, opportunities for synergies and optimisation
613 in the 3Rs for RDT were identified in the EPAA Partners' Forum. In addition, an encouraging culture of
614 many types of synergies, bringing together regulatory agencies and stakeholders, was evident with

clear motivation for on-going progress. This section details some of the main synergies that are occurring to make progress in the 3Rs for RDT that are not described above.

A key focus of synergies between stakeholders is to enable and encourage collaboration and the development of a continuous dialogue in areas such as the development of the full range of alternatives to the standard RDT tests (i.e. *in silico*, *in vitro*, omics etc.), the implementation and acceptance of alternatives, and the development of IATA, strategies and workflows for safety assessments. Synergies for the promotion and optimisation of 3Rs approaches usually start within an organisation (especially when it is large) and spread outwards. The EPAA itself is based on collaboration between different industry sectors and regulators and aims at identifying and fostering effective synergies among its members.

One area where there is scope for greater synergy, but less evidence of actual progress, is cross-sector collaboration in research projects i.e. different industrial sectors working together. Therefore, the EPAA Partners' Forum has been designed as an opportunity to facilitate this. Cross-sector synergies offer many opportunities for the 3Rs, e.g. the EMA (EMA, 2018a,b) and others have suggested the integration of further endpoints in a more intelligent design of tests and the increased use of NAMs to provide better information – all of these and other proposals could have significant positive impact on other sectors. The EPAA Partners' Forum discussed more ways of encouraging and implementing synergies in the 3Rs for RDT. One method is the use of case studies with input from all partners. Another valid approach to developing synergies for the 3Rs in RDT is to address a specific problem or issue, such as a joint EU and US project on the identification of the most sensitive organ in RDT.

5. Summary and Conclusions

The EPAA Partners' Forum on RDT testing aimed to identify synergies between sectors and opportunities to progress these in existing research frameworks. The EPAA Partners Forum heard that, with the exception of the cosmetics sector, RDT testing on animals is still a regulatory requirement across all industries. It is done to comply with legislation/regulations as well as to provide a rich source of information from which to perform safety assessments. A variety of tests are performed, with tests such as the 90-day rodent assays being viewed as valuable, and often essential, to assist in the identification of sub-chronic, organ-level adverse effects.

Immediate replacement of tests for RDT across all sectors is unlikely due to the complexity of the knowledge they provide about the test substance. The level of information obtained is often seen as extremely valuable and necessary to make safety assessment decisions following long-term, low dose exposure. It is acknowledged that a direct one-to-one replacement of the 90-day RDT test by a single

assay, even at the organ level, is not possible. However, despite the difficulty in finding non-animal approaches to allow safety decisions to be made about systemic toxicity, there has been much effort at the basic research level with significant funding from the EU's historical Framework Programmes and current H2020 Programme, in addition to efforts elsewhere on the globe. The EPAA Partners Forum was able to appreciate that real opportunities for the 3Rs in RDT testing will come from a combination of problem formulation, better study design (including dose level selection) and the use of NAMs, AOPs and other targeted MoA testing that may be needed to improve hazard identification and risk assessment.

Read-across of effects between "similar" molecules is one paradigm that was reported to provide information to support risk assessment following repeated exposure. Currently there is much debate about how, and if, read-across can provide information to allow safety decisions to be made about RDT in a regulatory context. One proposed solution is to support read-across through a body of evidence supplemented by data from NAMs. Other approaches to making safety decisions for repeated exposure relate to the development and use of various testing strategies and workflows integrating various types of data. Whilst the workflows are distinct for different applications, in practice there are commonalities between them including the use of exposure information, read-across or *in silico* predictions as well as other data from NAMs. They are generally designed to enable decision making from minimum experimental outlay. The workflows and schemes for safety assessment often include an early element relating to exposure e.g. the use of TTC or other exposure-based waiving.

Clear opportunities for synergies across stakeholders were identified at the EPAA Partners' Forum. For instance, the lack of harmonisation of regulations within and between sectors and geographical areas could be addressed. In addition, there is a recognised need to develop the 90-day RDT test further to provide more and better information, e.g. better dosing regimes and the increased use of omics or other NAMs to identify additional testing and / or analysis needed to support, for example, the assessment of neurotoxicity or endocrine disruption. Non-animal (*in silico* and *in vitro*) alternative approaches are being developed, however, it was appreciated that regulatory science need to keep pace with the rapid changes and improvements in technology to allow for their implementation.

As an outcome of the EPAA Partners' Forum on repeated dose toxicity testing, the following conclusions were made:

- Applying alternative methods when assessing systemic toxicity is a major challenge due to the complexity of interactions in the living body and for certain industries (e.g. cosmetics) this is critical due to regulatory requirements;

- Although EPAA partners are committed to the 3Rs, it was recognised that up until now animal tests on systemic toxicity are pivotal for supporting many safety decisions;
- Given the comprehensive data set provided by the traditional animal RDT testing, the full replacement with alternatives represents a major challenge. Breaking down the questions addressed by RDT (e.g. POD, identification of target organs) is required to make progress;
- Any replacement effort requires close cooperation amongst all safety assessors (in industry, regulatory agencies, academia) at a very early stage during alternative method design and development, ideally at a global scale;
- EPAA is well placed to enable cross-fertilisation, help set future research agendas and convene key players;
- EPAA facilitates sharing of case studies where novel approaches to safety decision making have been used successfully.

Conflicts of interest

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Disclaimer

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713

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