Molecular Life

REVIEW ARTICLES

Advances in science: next generation of lab tools, models and testing platforms used in predictive toxicology

Gertrude-Emilia Costin*

Institute for In Vitro Sciences, Inc. (IIVS), 30 W Watkins Mill Road #100, Gaithersburg, MD 20878, USA

(Received 23 February, 2017; accepted 18 March, 2017)

Abstract

Toxicology testing platforms represent the basis of the human health risk assessment process that determines whether a material or product may induce harm to humans upon exposure. Historically, safety assessment of raw ingredients or finished formulations has been performed using animal-based test methods (*in vivo*) that provide whole organism responses to toxicants. Due to the large number of products launched by industry continuously, modern toxicology shifted in recent years towards the use of novel, fast and reliable alternative methods, ranging from *in silico* to *in chemico* or *in vitro*, of which some are validated for regulatory purposes. The manuscript also addresses emerging technologies in the form of "organ/body-on-a-chip" platforms which announce to be instrumental in allowing alternative systems to *in vivo* models to assess systemic toxic effects induced by chemicals.

Keywords: predictive toxicology; *in vitro* methods; *in silico* methods; organ-on-a-chip

THE RISE OF PREDICTIVE TOXICOLOGY METHODOLOGIES

Toxicology testing platforms are an integral part of the human health risk assessment process that determines whether an ingredient or final formulation may induce harm to humans commensurate with accidental or intentional exposure. The process uses quantitative and qualitative methodologies that address hazard identification, dose-response assessment, exposure assessment, and risk characterization. It is the paramount responsibility of manufacturers within their respective industry sector (pharmaceutical, personal care, etc.) to ensure the safety of their products within a framework with various levels of regulatory requirements.

Historically, safety assessment has been performed using animal-based test methods (*in vivo*) that provide whole organism responses to toxicants. The toxicologist relied upon the animal tests to closely predict human response to hazards despite the imperfections introduced by inter-species extrapolations that are used to address the natural differences in anatomy and physiology. Due to the large number of products put on the market continuously, the requirement to protect public welfare by extensive evaluation of potential hazardous effects they may induce upon human exposure becomes a very challenging task. Therefore, novel and more efficient safety assessment methodologies need to be designed, evaluated and added to the predictive toxicology toolbox. We enter nowadays a modern scientific era that introduces Predictive Toxicology testing strategies encompassing a wide array of cutting edge methodologies that aim to reliably predict the human response to toxicants rather than observe the effects in animal-based test systems as in the classic toxicology approaches.

The lab tools, models and testing platforms currently integrated in the field of predictive toxicology can highlight areas where candidate molecules or prototypes may pose as hazardous at very early stages. Thus, they facilitate a quick and reliable selection process before further pre-clinical development and ultimately clinical trials are performed if and where applicable. Predictive toxicology reflects a paradigm shift from *in vivo* testing methods and initially promoted a wide array of alternative methods ranging from simple monoculture test systems to more complex explants or reconstructed tissue models. These available *in vitro* systems became instrumental for the investigation of cellular and molecular mechanisms underlying biological effects induced by exposure to toxicants. Furthermore, the combination between modern

*Corresponding author (Phone: 01-301-947-6524; E-mail: ecostin@iivs.org)

in silico approaches designed based on experimental data generated by *in vivo* and *in vitro* assays and cutting edge data mining algorithms contributes to the development of powerful predictive models (1). In recent years, the complexity of the testing platforms increased by the introduction of sophisticated bio-printed tissues and microphysiological systems (organs-on-a-chip/plate). They announce a yet another global paradigm shift in toxicology by enabling collection of valuable insights into acute systemic response that was not possible before. Based on its available technology platforms, the field of predictive toxicology supports ongoing 3Rs initiatives and efforts to Replace, Refine and Reduce animal usage for safety assessment of chemicals, drugs, etc.

The principles predictive toxicology is based upon and the various levels of complexity its methodologies can offer in multiple combinations towards the goal of ensuring human safety steadily gained popularity amongst multiple players with vested and overlapping interests in advancing the field further. Industry (manufacturers, biotech companies, contract research organizations, trade associations), academia, regulatory agencies, animal welfare groups and the public are all drivers of advanced, modern predictive toxicology methodologies. By offering the potential for reliable, reproducible, fast and more cost effective results, these new approaches can be employed in a sequential or tiered manner and have a significant impact on the product development process and on the regulatory testing framework.

THE REDUCTIONIST CONCEPT AND EMERGING TECHNOLOGIES

Toxicity testing in the 21st century reflects the process of transition from the traditional toxicant-related observations in whole animal models to mechanism-based outcomes from cell/tissue/chips-based assays and computational methods. As already demonstrated in the regulatory framework, it is however unrealistic to believe that a single assay or model will provide sufficient information on the risk or hazard posed by a chemical despite their multitude, diversity and relatively high prediction accuracy. Therefore, data need to be generated by a variety of well controlled protocols, thoroughly analyzed and integrated in a framework capable to maximize their utility for accurate predictions.

The reductionist concept of *in vitro* models and the recent orientation towards methods derived from or based on human biological components best reflects the evolution of the field and is explained in Fig. 1 using the ocular safety assessment as one key endpoint in the evaluation of raw ingredients and formulations. The safety of products was primarily performed starting in the '40s using the Draize test conducted in albino rabbits as the only available method at the time (2). Science advanced the technology towards the use of the isolated eyeball as the target organ presumed to provide similar results when exposed to toxicants without the need to use whole animal test systems. Isolated eyes (rabbit, chicken) (Fig. 1) usually obtained by testing laboratories as byproducts from abattoirs are used to assess the safety of products based on assays validated for regulatory purposes (3, 4). Following the reductionist concept, the narrowing of the test system to the primary target of a toxicant when coming in contact with the eye (the cornea) continued when pharmaceutical industry (Merck) (5) designed a now validated *in vitro* assay (6) based on excised corneas from bovine eyes obtained as byproduct from meat production. While initially designed to address severe irritants, the Bovine Corneal Opacity and Permeability (BCOP) test method was later approved for labeling of products as non-irritants and gained much popularity within numerous industry sectors. Some products however, when formulated purposely to be extremely mild, require a higher resolution of the testing methods to sense subtle changes in composition. This challenge prompted industry (manufacturers, testing labs and others in joint efforts) to design and use other test systems, albeit simpler, but more targeted and sensitive to ingredients and finished products with low toxicity profiles. Thus, the maximum simplicity of the *in vitro* test systems was reached with the monoculture (2D) systems. Continuing the reductionist concept example (Fig. 1), Statens Seruminstitut Rabbit Cornea (SIRC) cells have been used to identify minimal to moderate eye irritation potential of chemicals with a good predictive capacity (7).

It is to be pointed out that all *in vitro* methods discussed thus far use cell lineages of various animal species origins. Even though standard 2D systems are commonly used in current *in vitro* toxicology testing strategies because they allow for acquiring large data sets upon exposure to a broad variety of toxicants, their utility is cut short by extrapolation concerns regarding their ability to reliably replicate the responses of their *in vivo* counterparts (8). Furthermore, the lack of physiological cell-cell and cell-matrix interactions represents another shortcoming and accounts for the need to add exogenous metabolic activation often times not representative of natural *in vivo* environment. Due to the inherent limitations of the cell-based assays (and particularly when using lineages of animal origin), three dimensional (3D) reconstructed tissue models based on human cells gained a lot of support in the 2000s. They demonstrate acceptable replication of the *in vivo* structural and physiological characteristics, including cell viability, proliferation, differentiation, morphology, gene and protein expression battery, as well as metabolic function. As outlined in Fig. 1, human corneal epithelial cells (MatTek Corporation, Ashland, MA, USA) can be used for screening studies due to their unique human features

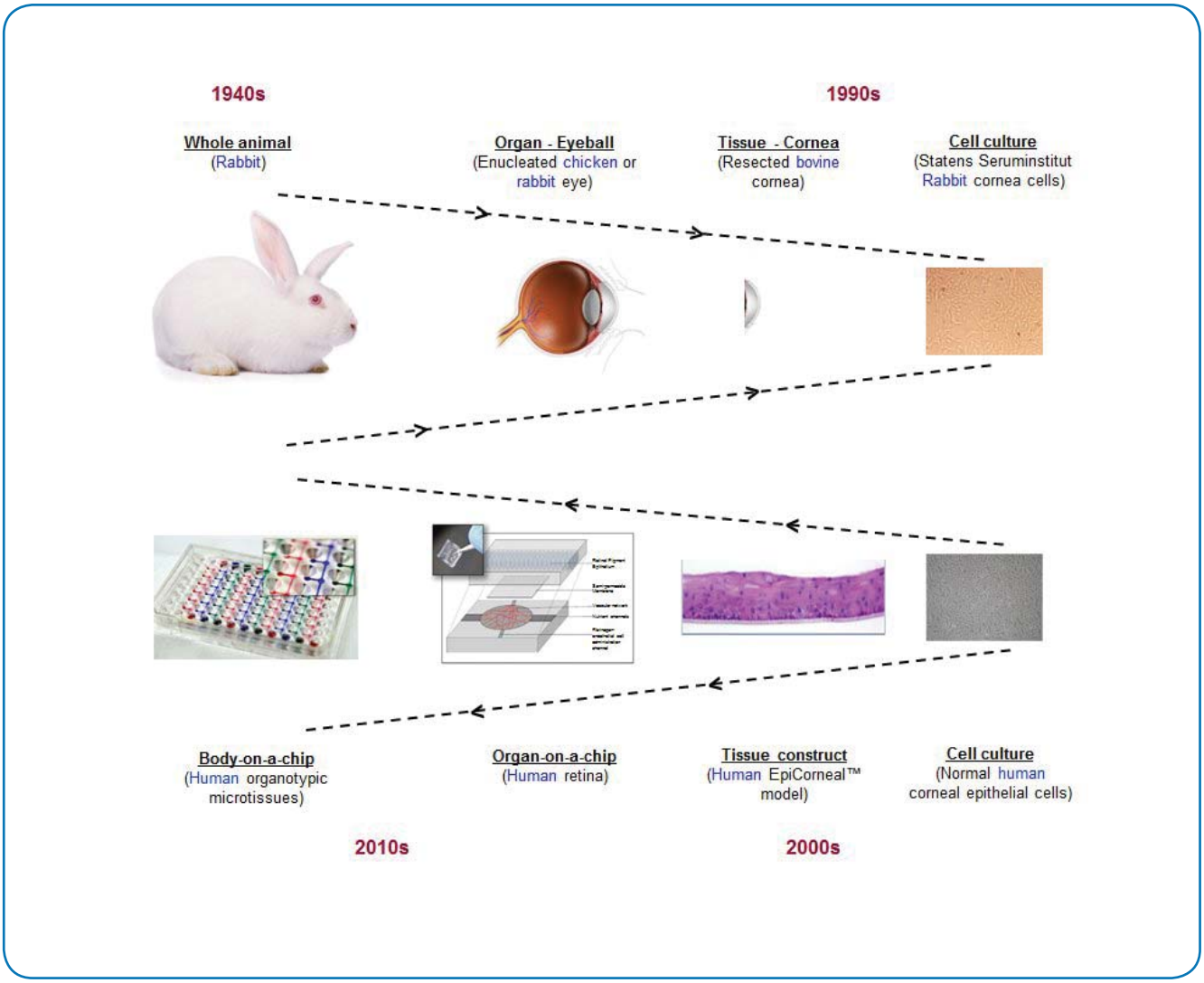

Fig. 1. – Complexity of biological test systems used in pre-clinical testing. Evolution, current status and emerging technologies. The ocular safety endpoint is used for exemplification purposes. Human corneal epithelial cells and reconstructed corneal tissue model (EpiCorneal™) – courtesy of MatTek Corporation (Ashland, MA, USA); retina-on-a-chip – reproduced with permission from Applied Stem Cell Technologies, University of Twente, Enschede, Netherlands (https://www.utwente.nl/tnw/ast/research/Retina-on-a-Chip/); body-on-a-chip – courtesy of ETH Zurich and InSphero AG, Switzerland (https://www.insphero.com/company/index.php?option=com_content&view=article&id=840); SIRC cells, courtesy of Allison Hilberer, M.S., D.A.B.T. (IIVS).

that make them a relevant testing platform compared to similar lineages from animal sources. This type of cells are also used to derive reconstructed corneal models such as the EpiCorneal™ (MatTek Corporation) (Fig. 1) that can be used to assess the irritation potential of ingredients and final formulations ranging from chemicals to drugs or cosmetics. The generation and validation of 3D models for regulatory purposes represents a huge advancement in itself because of increased similarity to native human counterparts and the accommodation of materials that otherwise could not be tested in cell-based assays due to solubility limitations and concerns. However, similar to 2D systems, the methods based on 3D tissue models generally accommodate short substance exposure times (few hours to a few days). While some tissue models can be adapted for repeated exposure regimens (*e.g.*, screening of modulators of skin pigmentation using the tissue models incorporating melanocytes), studies on the mode of action (MoA) at the organ level, and adverse outcome pathways (AOPs) (9) at the systemic organism level, cannot be implemented unless another level of complexity is to be reached by the *in vitro* lab tools.

The next generation of predictive toxicology platforms is represented by bio-printed tissues, and microphysiological systems ("organ on-a-chip"/"body on-a-chip") (reviewed in 10, 11). These platforms have the capability to incorporate the missing features of the 2D cultures (cell-cell interactions, three-dimensional architecture, etc.) and are anticipated to provide increased physiological relevance for molecular, mechanistic, and high-throughput toxicology studies prior to clinical testing. Pharmaceutical industry is once more expected to greatly benefit from these

emerging technologies and to reach maximum capacity and efficacy when screening libraries of compounds to obtain mechanistic insights from their crosstalk. While the minimum number of organs on a chip is yet to be defined by the scientific and regulatory community, a goal of ten organs was set by the Defense Advanced Research Projects Agency (DARPA)/National Institutes of Health (NIH) Microphysiological Systems (MPS) program (12) to determine the technical challenges arising from such complex combinations (10).

While commended for their advanced technological design and functionality, the organ/body on-a-chip platforms face several challenges that manufacturers are working assiduously to overcome. For example, they are yet to provide unlimited organoid homeostasis and currently lack physiological regenerative capabilities of cellular repair in the absence of adult stem cells and progenitor niches for local regeneration which are critical for long-term exposure testing. Despite the inherent challenges embedded in all new and yet to be optimized technologies, the recent developments on organ/body on-a-chip models increase the chances for obtaining the long sought ultimate systemic responses based on *in vitro* systems. Further optimization of these emerging technologies will make them a realistic alternative to systemic acute toxicity testing in animals.

INTEGRATION OF ADVANCED TESTING METHODS INTO PREDICTIVE FRAMEWORKS

With all tools discussed thus far at disposal and potential for new ones, predictive toxicology had the basis to create ingenious frameworks to reach virtually accurate evaluation of hazards and risks without the need for animal-based methods. The *in vitro*/*ex vivo* methods generally focus on individual key cellular and molecular events to increase the mechanistic understanding of upstream changes. When these events along with the assays capable to investigate them are integrated into an AOP, the overall understanding of how a pathway is affected by a toxicant increases significantly.

One of the endpoints assessed as part of the safety evaluation of potential toxicants is skin sensitization that is induced when susceptible individuals are exposed to an allergen and could subsequently develop allergic contact dermatitis upon additional exposure(s). The current legislation mandates that investigation of skin sensitization potential and potency of toxicants be assessed using alternative methods to classic animal-based systems (12). Extensive knowledge of the cellular and molecular components and factors of the skin sensitization pathway has prompted the development of *in silico*, *in chemico* and *in vitro* methods addressing specific key events (KEs) and pioneered the development of the AOP for this particular endpoint (Fig. 2a). The AOP for skin sensitization identifies four KEs: 1) the covalent binding to skin proteins (haptenation) of the parent toxicant/its derivatives following abiotic/metabolic activation (postulated to be the molecular initiating event); 2) activation of epidermal keratinocytes; 3) activation (maturation) and mobilization of Langerhans cells and dermal dendritic cells (DC); and 4) DC-mediated antigen presentation to naïve T-cells and proliferation/activation of allergen specific T-cells. The clinical presentation is an inflammatory response (*e.g.*, erythema, edema, blisters, itching) that occurs in the skin upon re-challenge with an allergen (elicitation phase, Fig. 2a).

At the beginning of the decision process based on the AOP for skin sensitization are various computational approaches [*e.g.*, Organisation for Economic Co-operation and Development (OECD) Quantitative Structure-Activity Relationship (QSAR) Tool box] that have now reached a stage of maturity and acceptance that allows their participation in the screening cascade to detect sensitizers. Their performance is directly related to the accuracy of the data imported and upon which the algorithm is constructed. The ideal computational model should be capable to correctly identify true negatives with few false positives (high specificity) as well as the true positives with few false negatives (high sensitivity). One shortcoming of these models is the incomplete understanding of the effect chemical substitutions may have on the toxicants' reactivity and/or their susceptibility for metabolic activation. One immediate use of the computational models is in the initial screening designed to eliminate toxicants with obvious sensitization potential before further investigation. This approach aims to minimize false positives and accept false negatives (toxicants that may be later predicted positive when subsequent, more sophisticated and sensitive testing occurs). The end goal of this strategy is to detect skin sensitizers before human subjects are exposed to them.

The KE#1 (covalent binding to proteins) can be addressed by the Direct Peptide Reactivity Test (DPRA) (14) which is an *in chemico* method that measures the depletion of synthetic heptapeptides containing either cysteine or lysine following the incubation with the toxicant. Depletion of the peptide in the reaction mixture is measured by High Performance Liquid Chromatography (HPLC) using ultraviolet (UV) detection and separates sensitizers and non-sensitizers based on the level of reactivity with the peptides. The KE#2 of the skin sensitization AOP is addressed by the KeratinoSens™ (15), a luciferase reporter gene assay which quantifies the luciferase gene as an indicator of the activity of the Nrf2 transcription factor in keratinocytes following exposure to the toxicant. Finally, the human cell line activation test (h-CLAT) addresses KE#3 of the skin sensitization AOP by quantifying changes in the expression of cell surface

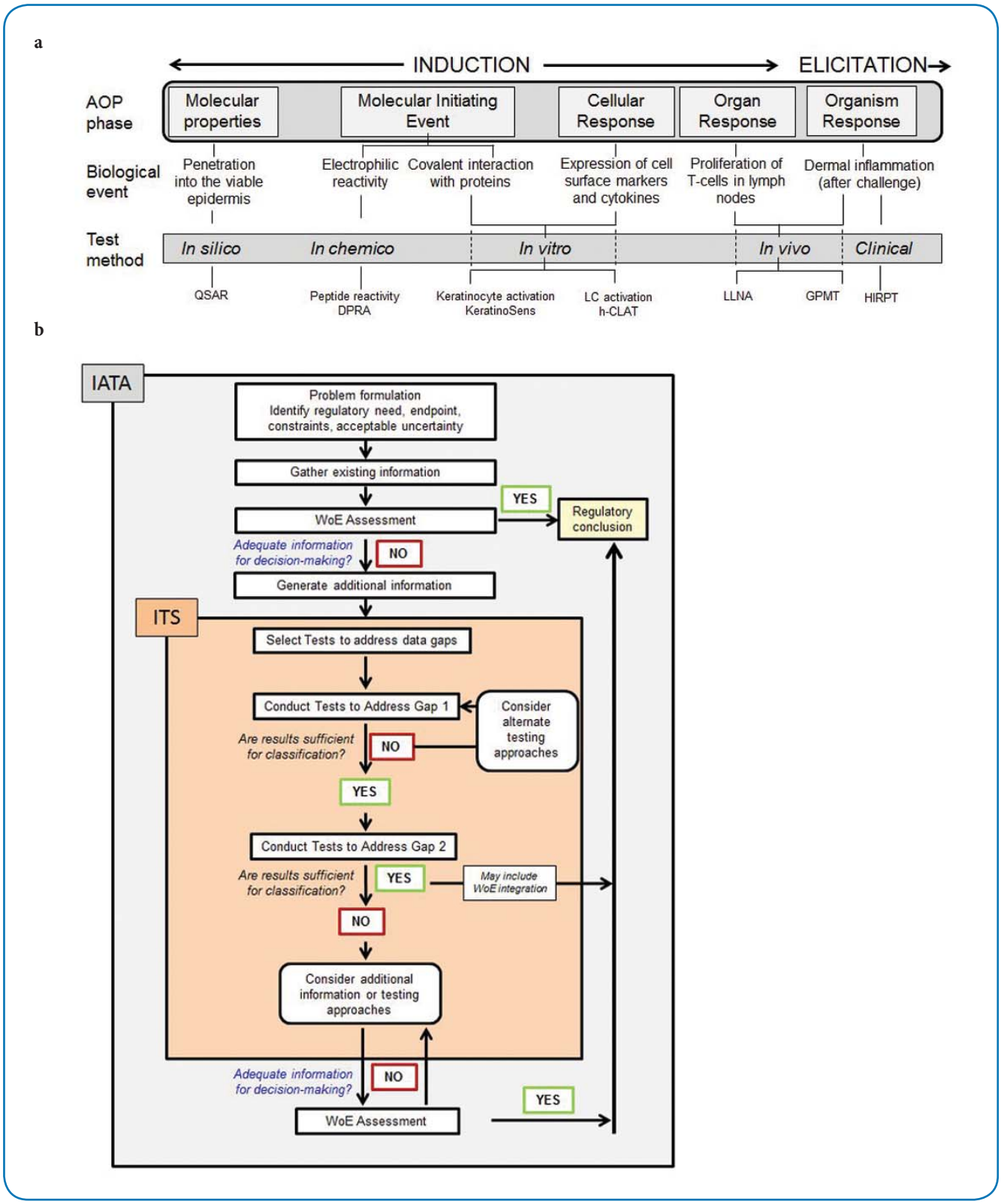

Fig. 2. – *a)* The modern concept of AOP. Cascade of events participating in the skin sensitization pathway is used as example. *Abbreviations:* AOP, Adverse Outcome Pathway; DPRA, Direct Peptide Reactivity Assay; GPMT, Guinea Pig Maximization Test; h-CLAT, human-Cell Line Activation Test; HRIPT, Human Repeated Insult Patch Test; LC, Langerhans Cells; LLNA; Local Lymph Node Assay; OECD, Organisation for Economic Co-operation and Development; QSAR, Quantitative Structure-Activity Relationship; TG, Test Guideline. Only *in vitro* assays validated for regulatory purposes (*i.e.,* associated with OECD TGs) are listed. *b)* Evolving toxicology concepts of ITS and IATA. Integration of *in vitro* methods, platforms and technologies into progressive approaches to predictive toxicology. Abbreviations: IATA, Integrated Approach on Testing and Assessment; ITS, Integrated Testing Strategy; OECD, Organisation for Economic Co-operation and Development; WoE, Weight of Evidence. Modified from: http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm.

markers associated with the process of activation of monocytes and DC (*i.e.*, CD86 and CD54) in the human monocytic leukaemia cell line THP-1 following exposure to the toxicant (16). Beyond these cellular events, the organ and organism responses (including sensitization potency) are addressed using *in vivo* or clinical studies (Fig. 2a).

Given the complexity of the skin sensitization pathway and the multitude of cellular and molecular events involved, it is unlikely that a single *in vitro* method is able to accurately assess this critical safety endpoint. Complex analysis of datasets generated in multiple *in vitro* assays addressing skin sensitization achieved a higher accuracy in predicting a human response when combined (17, 18). The AOP for skin sensitization offers the perfect example of how Integrated Testing Strategies (ITS) work in presenting a structured, prescriptive, rule-based conceptual framework allowing for the cumulative synthesis of information and maximization of its analysis (Fig. 2b). Furthermore, another evolving concept is represented by the Integrated Approach on Testing and Assessment (IATA) which combine, analyze and integrate results from one or multiple methodological approaches [(Q)SAR, read-across, Weight of evidence (WoE), *in chemico*, *in vitro*, *ex vivo*, *in vivo*] or omic technologies (*e.g.*, toxicogenomics) and follow an iterative strategy to inform regulatory and labeling decisions (19).

CONCLUSIONS

The cross-disciplinary nature of toxicology provided the scaffold for novel, state-of-the art technologies to converge and methodically generate and integrate multiple datasets into frameworks used to ultimately predict human responses to toxicants and to support regulatory and labeling decisions. Initiatives from the U.S. Environmental Protection Agency (EPA) (ToxCast program) or National Research Council (NRC) (Toxicity Testing in the 21st Century: A Vision and a Strategy) (20) guided the direction of toxicity testing away from animal models and towards a wide range of alternative tools discussed herein. This cohesive movement came to meet the need to evaluate the safety of a continuously increasing number of ingredients and mixtures and led to an unavoidable paradigm shift in toxicology testing that soon became a widely accepted vision by leading scientific and regulatory groups for future toxicology testing. The overarching goal of predictive toxicology is to rely on high-throughput, specific, sensitive, validated, reliable, reproducible, relevant, easy to perform, transferable, affordable, fast alternative methods to animal-based test systems for the prioritization of compounds for further investigations, identification of MoAs and ultimately the development of prediction models for adverse health effects in humans.

Taking into consideration the complexity of human responses to the toxicants, it is anticipated that combinatorial analysis of multiple datasets generated in a battery of *in vitro*, *in silico*, *in chemico*, ex vivo, etc. methods is needed to inform a regulatory decision. The integration of alternative methods in the AOPs, ITS and IATAs represents a practical reflection of contemporary concepts in toxicology converging towards reliable frameworks for the prediction of human responses to toxicants. The various computational approaches, high-throughput screening assays and increasingly complex model systems described herein are now capable to provide a comprehensive and clinically relevant toxicological profile and will continue to be refined, improved and advanced to ensure safety of products for human use.

Acknowledgments: The author thanks Hans Raabe, M.S. and Allison Hilberer, M.S., D.A.B.T. (IIVS) for reviewing the artwork included in the manuscript.

References

1. Kavlock R and Dix D, *Computational toxicology as implemented by the U.S. EPA: providing high throughput decision support tools for screening and assessing chemical exposure, hazard and risk,* ***J. Toxicol. Environ. Health B. Crit. Rev.***, **13**, 197-217 (2010).
2. Draize JH, Woodward G, Calvery HO, *Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membrane*, ***J. Pharmacol. Exp. Ther.***, **82**, 377-390 (1944).
3. NIH, *ICCVAM-recommended test method protocol isolated rabbit eye test method*, ***NIH Publication***, No. 10-7553, B51-B65 (2010).
4. OECD, Test No. 438, *Isolated chicken eye test method for identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage*, ***OECD Publishing***, Paris, 1-20 (2013).
5. Gautheron P, Dukic M, Alix D, Sina JF, *Bovine corneal opacity and permeability test: an in vitro assay of ocular irritancy*, ***Fundam. Appl. Toxicol.***, **18**, 442-449 (1992).
6. OECD, Test No. 437, *Bovine corneal opacity and permeability test method for identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage*, ***OECD Publishing***, Paris 1-27 (2013).
7. OECD, *Draft guideline for the testing of chemicals. The short time exposure in vitro test method for identifying i) Chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage*, ***OECD Publishing***, Paris 1-12 (2014).
8. Yamada KM and Cukierman E, *Modeling tissue morphogenesis and cancer in 3D*, ***Cell***, **130**, 601-610 (2007).
9. Marx U, Walles H, Hoffmann S, Linder G, Horland R, Sonntag F, *et al.*, *'Human-on-a-chip' developments: a translational cutting*

edge alternative to systemic safety assessment and efficiency evaluation of substances in laboratory animals and man?, ***ATLA***, **40**, 235-257 (2012).
10. Marx U, Andersson TB, Bahinski A, Beilmann M, BEken S, Cassee FR, *et al.*, *Workshop Report. Biology-inspired microphysiological system approaches to solve the prediction dilemma of substance testing*, ***ALTEX***, **33**, 272-321 (2016).
11. Kim JY, Fluri DA, Kelm JM, Hierlemann A, *Frey O, 96-well format-based microfluidic platform for parallel interconnection of multiple multicellular spheroids. **J. Lab Autom.***, **20**, 274-282 (2015).
12. Hartung T and Zurlo J, *Alternative approaches for medical countermeasures to biological and chemical terrorism and warfare*, ***ALTEX***, **29**, 251-260 (2012).
13. European Union, *Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products*, ***OJL***, **342**, 59-209 (2009).
14. OECD, Test No. 442C, *In chemico skin sensitisation: Direct Peptide Reactivity Assay (DPRA) OECD guidelines for the testing of chemicals*, ***OECD Publishing***, Paris, 1-19 (2015a).
15. OECD, Test No. 442D. *In vitro skin sensitisation: ARE-Nrf2 Luciferase Test Method OECD guidelines for the testing of chemicals*, ***OECD Publishing***, Paris, 1-20 (2015b).
16. OECD, Test No. 442E. *In vitro skin sensitisation: human Cell Line Reactivity Test (h-CLAT) OECD guidelines for the testing of chemicals*, ***OECD Publishing***, Paris, 1-21 (2015c).
17. Bauch C, Kolle SN, Ramirez T, Eltze T, Fabian E, Mehling A, *et al*, *Putting the parts together: combining in vitro methods to test for skin sensitizing potential*, ***Regul. Toxicol. Pharmacol.***, **63**, 489-504 (2012).
18. Natsch A, Ryan CA, Foertsch L, Emter R, Jaworska J, Gerberick F, *et al.*, *A dataset of 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation*, ***J. Appl. Toxicol.***, **33**, 1337-1352 (2013).
19. Jaworska J and Hoffmann S, *Integrated testing strategy (ITS) – opportunities to better use existing data and guide future testing in toxicology*, ***ALTEX***, **27**, 231-242 (2010).
20. National Research Council (NRC), *Toxicity Testing in the 21st Century: A Vision and a Strategy*, ***National Academy Press***, 1-4 (2007).