Meeting report

From Cells to QSAR: Alternative predictive models in toxicology

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The Italian Association for *In Vitro* Toxicology CELLTOX in collaboration with the Laboratory of Analysis and research in Physiopathology (LARF), Department of Experimental Medicine (DIMES) of the University of Genova, in April 2016 organized a three-day course on alternative predictive models in toxicology. The course was also supported by ESTIV, The European Society of Toxicology *In Vitro*.

Thomas Hartung, Professor of Evidence Based Toxicology and Director of the Center for Alternatives to Animal Testing (CAAT) at the Johns Hopkins University in Baltimore (US) opened the course with an introductory lecture on Cell "culture" in the 21st century. Playing on the double sense of culture as a way to maintain cells *in vitro* and culture in the sense of "the behaviours and beliefs characteristic of a particular group", he described the scientific and ethical advantages as well as the weaknesses and critical points of *in vitro* cell cultures, as opposed to animal models, for modern toxicology. The recent advent of differentiated cell models derived from stem cells and the use of 3D preparations for organotypic cultures, together with the application of good cell culture practices and integrated testing strategies, will improve the predictive value of cell cultures in toxicological research (Pamies et al., 2017). As an example, he described the novel mini-brains obtained from human donors' skin fibroblasts reprogrammed into induced pluripotent stem cells, then differentiated into neural stem cells and grown in 3D culture to form identical spheroid micro-physiological mini-brains that can be used to study CNS mechanisms, for developmental and neurotoxicity studies, for drug development and to study brain diseases (Pamies et al., 2016). He also introduced the read-across approaches to predict potential toxicity of new chemical substances by the use of novel screening tools built by mining the existing toxicological data (Patlewicz et al., 2014; Ball et al., 2016).

Margherita Ferro, one of the founders of CELLTOX, gave an overview of her personal experience on *in vitro* toxicological research since the mid 1980's, emphasizing the importance of the first studies on hepatic metabolism of xenobiotics using competent hepatoma cell lines, and the later development of other *in vitro* cell models for toxicological studies (Ferro et al., 1987).

Flavia Zucco, founder and first President of CELLTOX, introduced the ethical and philosophical principles underlying the choice of experimental models, alternative to the use of animals, and described the initial difficulties encountered in the pioneering years by scientists supporting the principles of the 3Rs. She also described the outcome of the recent EU project Anim.al.SEE (http://www.inemm.cnr.it/animalsee/), as an example of the recent progresses achieved in this field.

Laura Gribaldo, from JRC (Ispra, Italy), described the potential of toxicogenomic approaches for evidence-based prediction of risk. Toxicogenomic combines the information obtained by transcriptomic (genes), proteomic (proteins) and metabolomic (metabolites) approaches to understand adverse effects of chemical substances and their mechanisms of action. Toxicogenomic approaches are also useful to identify candidate biomarkers of exposure and disease and to build adverse outcome pathways (AOPs). These AOPs can be combined with cell culture data, *in silico* cheminformatics data and exposure modelling into a network of integrated testing and assessment strategies to predict safety in humans (Bell et al., 2016).

Fabiola Pizzo of the Mario Negri Pharmacological Research Institute (Milan, Italy) further expounded the concepts of read-across, the technique for predicting endpoint information for one substance by using data from the same endpoint for other substances. She explained how *in silico* approaches used to evaluate the potential toxicity of chemicals can be particularly useful when dealing with a very large number of substances. Increased computational power, new algorithms, better molecular descriptors, non-linear modelling, together with the introduction of a large number of properties and of results from complex biological endpoints have strengthened the Quantitative Structure Activity Relationship (QSAR) approaches. The computational platform VEGA (http://www.vega-qsar.eu) for QSAR analysis and ToxRead (http://www.toxgate.eu) for read-across evaluations were introduced. She also stressed that further contributions, e.g., from toxicity databases and *in vitro* data are needed to improve *in silico* methods and that results from several computational models should be compared.

Chantra Eskes (SECAM, Switzerland) talked about challenges to the implementation of alternative methods in regulatory toxicology. She described the recent updates in the Organisation for Economic Cooperation and Development (OECD) guidelines for the validation of new methods for hazard assessment and for integrated approaches for testing and assessment (IATA) in the field of skin corrosion and irritation and eye damage/irritation. These guidelines were strongly driven by the EU Cosmetics Directive (EU 2003/15/EC) placing a ban on animal testing in cosmetics production, and the REACH Reg.1907/2006 imposing the revision of toxicity data for all chemicals produced in amounts greater that 1 ton/year, but also

recommending *in vitro* testing for skin and eye irritation and general rules for adopting *in vitro* tests for other effects. International acceptance of alternative methods for the characterization of chemicals toxicity is growing, as documented by the increasing number of OECD guidelines that are being developed for different toxic endpoints and for AOPs. An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment.

Anna Maria Bassi (DIMES University of Genova) discussed the biological relevance of AOPs for the prediction of risk to human health. Great concern arises from the fact that only 10 to 20% of chemicals on the market have been fully assessed for their toxic properties in humans. OECD is devoting great efforts to issue guidelines for the development of AOPs that can contribute to more reliable predictive models of human toxicity. The principal variables that control any toxic event are the Molecular Initiating Event (MIE) and the Response Matrix (RM). MIE is the initial interaction between a molecule and a biomolecule (e.g., receptor) or biosystem that can be causally linked to an outcome via a pathway, always remembering that it is the fastest reaction that typically drives *in vivo* toxicity. At the other end, the RM comprises all biological responses in all subsystems (e.g., cells, tissues, organs) and, ultimately, the *in vivo* endpoint of interest (i.e., skin sensitization). A single toxic chemical can initiate a network of AOPs, and the assessment of AOP networks can be particularly helpful when evaluating chemical mixtures. Several informatics tools are already available or are being developed to assist in the identification of relevant AOPs: AOP Wiki (https://aopwiki.org/wiki/index.php/Main_Page) represents the initial phase of a larger effort to build a complete AOP knowledge base (AOPkb) containing additional modules: Effectopedia (http://www.effectopedia.org), the intermediate effect database that will host chemical-related data derived from non-traditional methods, and AOP Xplorer (http://aopxplorer.org).

In the following section of the Course, different *in vitro* models for specific tissues were illustrated. Gastrointestinal and blood brain barrier models and their toxicological applications were introduced by **Yula Sambuy** (CREA Food and Nutrition, Rome, Italy). The human Caco-2 cell line model of the intestinal epithelium can be used to monitor toxicity of drugs, xenobiotics and food contaminants as well as alterations to mucosal permeability (Sambuy et al., 2005). Controlled culture conditions and different applications were discussed, including systems of co-culture with hepatocytes, blood cells, or endothelium. Modelling the blood brain barrier requires co-culture of endothelial cells of the microcirculation with mesenchymal cells (pericytes) and neural cells (astrocytes). Better differentiation may be achieved in microfluidic systems under shear stress conditions.

The importance of barrier models in veterinary toxicology was discussed by **Francesca Caloni** of the Department of Veterinary Medicine (Milan, Italy). Species-specific models are required to monitor exposure and toxicity for target species as well as for indirect toxicity to humans via exposure to animal products. Examples include 3D dog skin equivalent (Serra et al., 2007), bovine mammary epithelial cells (Duo et al., 2006), porcine small intestinal epithelial cells IPEC-J2 (Zakrzewski et al., 2013) and porcine alveolar epithelial cells pAEpC (Steimer et al., 2007) as well as human barrier models used to assess human exposure to animal toxicants. In the field of reproductive toxicology, bovine and pig granulosa cell models can be used to evaluate the complex ovary activity and interference from endocrine disruptors (Petro et al., 2012).

Susanna Alloisio from ETT (Genova) presented a high throughput electrophysiological assay to predict functional neurotoxicity. Microelectrode arrays (MEAs) recording electrophysiological activity of primary neuronal networks established from embryonic rat/mouse cortex can be used to rapidly assess the effects of different potentially neurotoxic substances (Alloisio et al., 2015, 2016). This model finds applications in neurotoxicity of agrochemicals, chemicals, drugs, biotoxins and "smart" drugs, and can be adapted to specific uses by introducing neural cells of different type and origin. It may be used as part of a preliminary integrated *in vitro* evidence-based testing strategy before *in vivo* tests on animals. The model is also in continuous evolution from 2D to 3D arrangements (Frega et al., 2014).

From specialized cell/tissue models to the mechanisms of toxicity, **Chiara Urani** from the Department of Environmental and Earth Sciences of the Università degli Studi Milano Bicocca (Milan, Italy), gave an overview of *in vitro* models to predict carcinogenesis. A carcinogen induces the formation of tumors, increases their incidence or reduces their time to appearance. The rodent carcinogenicity assay is still performed (OECD TG 451 and TG 453) despite its limited transferability to humans, long duration and high costs. Cell transformation assays (CTAs) are various and widely used by academic, industrial, governmental and regulatory agents. However, their use is limited to the characterization of carcinogenetic risk without a quantitative output. Detailed *in vitro* carcinogenicity test guidelines have been issued by OECD (Vasseur et al., 2012) and EURL/ECVAM recommendations include the need to develop human cell assays, encourage studies on tumor promotion mechanisms and develop automation systems for assay scoring (EURL ECVAM 2013; Urani et al., 2009).

Emma Di Consiglio from the Italian Institute of Health (Rome, Italy) discussed approaches to improve the predictive value of *in vitro* studies for the toxic evaluation of chemical substances. The aim is to apply an integrated testing strategy for quantitative *in vitro/in vivo* extrapolation (QIVIVE). Biokinetic processes are often responsible for *in vitro/in vivo* differences (e.g., differences between nominal concentrations and real levels of cell exposure can significantly shift the dose response curve; single or repeated exposures can affect the response). The effects of factors influencing the bioavailability were described and discussed with respect to the challenge of prediction and QIVIVE in different *in vitro* models and human *in vivo* re-

sponses. An example for ibuprofen, tested as single or repeated treatments in three different hepatic *in vitro* experimental models, was presented (Truisi et al., 2015).

Stefano Lorenzetti (ISS, Rome) explained that exposure to endocrine active substances (EAS, able to directly interact with components of the endocrine system), or endocrine disruptors (ED, able to alter functions of the endocrine system causing adverse health effects) (Rovida et al., 2015) may cause male infertility and associated-diseases/malformations or obesity. The search for alternative molecules to these substances considered "of very high concern" has become a public health priority and a regulatory problem. Endocrine activity must be considered as a collection of modes of action, potentially leading to adverse outcomes, rather than an (eco)toxicological hazard in itself (EFSA, 2013). Thus, although much emphasis is placed on *in vitro* methods based on human cell lines representative of endocrine-targeted tissues (e.g., prostate), looking at subcellular mechanisms (e.g., nuclear receptor interaction) and on functional biomarkers of clinical relevance (e.g., PSA secretion in human prostate epithelial cells), it is necessary to define the predictive value of molecular or biochemical changes within an AOP (Lorenzetti et al., 2015). These approaches are being developed within the LifeEDESIA EU project (http://www.iss.it/life/). Alternative methods can successfully be used for the screening, identification and characterization of endocrine interfering substances, and of alternative molecules to the substances of very high concern. *In vitro* toxicology allows the identification of endocrine-mediated adverse effects by using clinical biomarkers that are indicative of a cell-specific and endocrine-dependent functional effect.

Silvia Letasiova from MatTek (Bratislava, Slovakia) described tests to characterize skin topical toxicity. Different OECD guidelines exist for *in vitro* or *ex vivo* tests for skin corrosion, skin irritation, phototoxicity and percutaneous absorption. *In vivo* tests on rabbits are rapidly being substituted by *in vitro* tests also as consequence of their variability and poor predictive value, as well as for ethical reasons. Corrositex® (OECD TG 435) measures the penetration capacity of substances across protein membranes. Reconstructed human epidermis (RhE) both in 2D and 3D arrangements, are used for skin corrosion testing *in vitro*. Among these, EpiSkin™, EpiDerm™, SkinEthic™, and EpiCS™ skin corrosion tests, commercialized by different companies, are all valid in the context of OECD TG 431. Some of the RhE models are also used for skin irritation testing *in vitro*, and different protocols are applied (OECD TG 439). Phototoxicity (photoirritation) is a chemically induced non-immunologic acute skin irritation requiring light. Phototoxicity *in vitro* tests utilize either the 3T3 fibroblasts neutral red uptake (NRU) test or the EU pre-validated EpiDerm™ phototoxicity test. Finally, percutaneous absorption tests utilize diffusion of chemicals into and across excised skin (human or pig) and reconstructed skin models (OECS TG 428).

Different approaches to ocular irritation testing were illustrated by **Laura Ceriotti** (VItroscreen, Milan, Italy). Following recent EU regulations (Cosmetics Regulation, REACH and Directive 2010/63/EU) as well as ethical considerations, alternatives to the Draize rabbit eye test have been developed. In 2012, the update of OECD TG 405 recommended the use of a sequential testing strategy, limiting the *in vivo* test to the last step. Organotypic models employ corneas of animal origin (bovine, rabbit and chicken) while other methods are based on primary human keratinocytes (Epiocular™ eye irritation test) or immortalized human corneal epithelium (Skin-Ethic™). *In chemico* methods that rely on macromolecular matrices have also been proposed (e.g., Ocular Irritection™). Alternative methods allow the identification of severe irritants/corrosives and non-irritants, while irritants (Class 2) requires the application of a multiple test strategy. A multiparametric approach may increase predictive power for non-classified substances and mixtures.

Human exposure to nanoparticles has become a serious toxicological problem, since chemicals in nano-size acquire distinct properties. **Isabella De Angelis** of the Italian National Institute of Health (Rome, Italy) indicated that the respiratory, cardiovascular and central nervous system appear to be the major targets of nanoparticle toxicity caused by the formation of reactive oxygen species. A large information gap still exists on the characterization, mechanisms of toxicity, risk evaluation and regulation on limits of exposure for nanomaterials on the European market. The EU project NANoREG (http://www.nanoreg.eu) aims to provide tools for risk assessment and decision making and to develop new testing strategies adapted to different nanomaterials. Different analytical tools need to be combined for correct analysis of nanoparticles, particularly since nanoparticles tend to form aggregates in aqueous medium. Critical aspects of cytotoxic assays of nanoparticles include their tendency to bind to reagents, contributing non-specifically to the output signal. The micronuclei test and Comet assay are generally used for genotoxicity testing of nanoparticles. Some critical aspects of biological assays of nanoparticles also impact on *in silico* methods, as the latter strongly rely on the quality of existing experimental data.

The reproducibility of *in vitro* cell models largely depends on the standardization of cell culture conditions. Animal-derived supplements such as foetal bovine serum exhibit large inter-batch variability in composition. **Anita Muraglia** from Biorigen Srl (Genova) introduced platelet lysates from platelet-rich plasma (PLRP) as valuable alternatives to foetal calf serum for the culture of different cell types. An allogeneic platelet lysate was characterized and optimized into a "ready to use" standardized two-component cell culture supplement for research use, that demonstrated high performance in a broad spectrum of cell types, supporting cell growth and viability (www.lyset.it).

Milena Mastrogiacomo from DIMES (University of Genova, Italy) spoke on the use of PLRP for mesenchymal stem cell culture in regenerative medicine. Adult human bone marrow stromal cells (BMSC) can be induced to proliferate *in vitro* using PLRP, maintaining their differentiation potential for longer when properly stimulated with appropriate factors (Muraglia et al., 2015). Differentiated BMSC may be seeded on scaffolds of

different chemical composition, resorption rate and architecture to reconstitute damaged bone.

Arti Ahluwalia from Centro Piaggio (Pisa, Italy) described the development of multi-chamber bioreactors to reproduce the tissue/cell interactions occurring in the human metabolic system. Combining liver, adipose and endothelial cells in different connected compartments connected by medium allows metabolic and toxicity studies. Recent developments in bioreactor design involve moving membranes that reproduce peristalsis and systems applying cyclic squeeze contactless pressure (Mazzei et al., 2010; De Maria et al., 2011; Giusti et al., 2014).

Giovanna Mazzoleni of the Department of Clinical and Experimental Sciences, of the University of Brescia (Italy) described the principal techniques of and models of 3D culture, illustrating advantages and limits of their use. Tissue engineering approaches, biofluidic studies, micro- and nano-technologies are helping to generate controlled tissue-specific microenvironments.

Practical session

The third day of the course offered a practical training session, considering the very positive feedback received at the end of previous Basic and Advanced Theoretical and Practical courses, organized by the LARF team since 2008. The practical session was opened to 20 participants, and included two training blocks, focused on a human skin model for prediction of the irritation potential of chemicals, and on 3D dynamic *in vitro* models. A panel discussion among experts and trainees, during hands-on sessions and in the final roundtable, contributed to create a scientific network on alternative methods. The evaluation questionnaires, completed at the end of the Theoretical and Training Modules, resulted in a high level of satisfaction by all participants, who requested that other courses be scheduled for acquisition of basic techniques or for improvement of existing expertise on alternative methods.

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