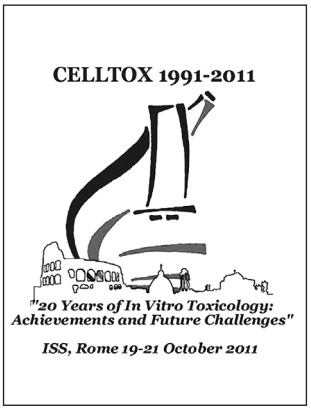
CELLTOX 1991-2011: Twenty years of *in vitro* toxicology: achievements and future challenges

Rome, Italy, October 19-21, 2011



The CELLTOX meeting, held in Rome in October 2011, was organized to celebrate the 20th anniversary of the Italian Association for *In Vitro* Toxicology, CELLTOX. The Association was created by a group of Italian scientists who pioneered alternative methods in toxicology since the early 1980s (Zucco, 1980; Stammati et al., 1981). In 1984 in Urbino, Italy, they organized the Third International Workshop on Tissue Culture Applications in Toxicology (Anon., 1985) that focused on cytotoxicity and teratogenicity and discussed the most recent developments of *in vitro* screening tests. Since the establishment of CELLTOX in 1991, the field of *in vitro* toxicology has greatly expanded and has acquired increasing recognition in the scientific community (De Angelis et al., 2010). In parallel, the pressure to comply with incoming regulation and animal testing bans in the life sciences, together with a growing awareness and public concern about animal welfare, have contributed to the move towards reduction, refinement and replacement in the use of animals in research and regulatory toxicology. The 20th anniversary meeting brought together researchers of international standing as well as many young scientists to present and discuss recent advances in different fields of *in vitro* toxicology. One hundred participants from 16 countries convened at the Istituto Superiore di Sanità (ISS – Italian Institute of Health), co-organizer of the meeting together with CELLTOX. The program was organized in seven scientific sessions opened by a keynote lecture on a broad topic of special interest, followed by oral communications selected from submitted abstracts. A poster session exhibited during the meeting. Throughout the meeting the attendance was excellent and the discussion very fruitful.

Carl Westmoreland, senior scientist in the Safety and Environmental Assurance Centre at Unilever (UK), opened the meeting with an introductory lecture on "Progress and future challenges of alternative methods to allow safety testing without animal testing." He presented the challenges ahead to achieve a more effective reduction of the use of laboratory animals for toxicity testing. In particular, he emphasized the need to address human health risk assessment rather than simply hazard identification, taking into account potency and integration of effects on several endpoints, using new approaches and alternative methods in toxicology. The majority of existing alternative methods are in fact only suitable for hazard identification of cosmetic ingredients and do not give information on potency (Adler et al., 2011). Animal experimentation for safety decisions is still far from being replaced for most toxicology endpoints, with the only exception of skin sensitization for which animal testing is expected to be fully replaced by 2017-2019. The aim for the future should not just be to perform animal tests without animals, but to develop new methods that can provide information on toxicological mechanisms as well as accurate predictions of human exposure risk. As stated in the US National Research Council report, "Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods that evaluate changes in biological processes using cells, cell lines, or cellular components, preferably of human origin" (Krewski et al., 2010). This concept is at the basis of a new paradigm for the use of *in vitro* data in safety assessment outlined in the report "Toxicity Testing in the 21st Century: A Vision and a Strategy."[1] Critical for this new approach is the integration of data into models for toxicity pathways and systemic exposure, using computational approaches. To achieve this it is important to better understand the underlying biological pathways leading to a toxic effect or to a reactive response. To allow *in vitro-in vivo* extrapolation for human risk assessment, it is essential to consider the concentrations of toxic substances on target sites *in vivo* and other factors contributing to effective exposure doses. With this aim, more alternative tests will be needed to monitor the different aspects of the toxic response to a substance, and closer collaboration with clinical scientists would be advantageous to develop new tests and models. Validation and acceptance of the new approaches remains one of the future challenges.

Emanuela Testai, from the *Istituto Superiore di Sanità* in Rome, introduced the session "Predictive Toxicology". She presented "The role of kinetics in the framework for risk assessment without animal testing." In particular, the importance of kinetics in the design and interpretation of toxicological tests was stressed, since the bioavailability of a substance by the relevant uptake routes influences the actual "internal" dose

[1] www.TT21C.org

to which a tissue is exposed. In alternative non-animal testing strategies, no or limited testing would be required in case of actual internal doses being lower than the threshold of toxicological concern. However, in *in vitro* test methods, the observed effect should be correlated with the actual concentration of exposure, rather than with the nominal concentration, taking into account factors such as non-specific binding to culture vessels, cells, proteins in the medium, as well as other factors that may alter bioavailability such as transport, metabolism, and bioaccumulation. This uncertainty in the level of exposure is further amplified when chronic toxicity is investigated by repeated treatment experiments. She illustrated the importance of kinetics *in vitro* with some results obtained in the context of the EU FP7 project Predict-IV, which has among its aims to identify *in vitro* relevant kinetic parameters and to measure the real exposure of cells to xenobiotics, including their metabolites[2]. The biokinetic information from *in vitro* tests should then be integrated into physiologically-based toxicokinetic models for extrapolation to *in vivo* conditions.

This aspect linked well to the following session on "Integrated *in vitro*/*in silico* approaches" introduced by **Jos G. M. Bessems** of the National Institute for Public Health and Environment of The Netherlands, who showed the advantages of integrated testing strategies in chemical risk assessment. The potentially hazardous effects of a substance should be investigated taking into account its human relevance in terms of the levels of known or expected exposure. Efficient risk assessment should therefore start by combining realistic exposure assessment with expected effects derived from analysis of Quantitative Structure-Activity Relationships (QSARs), and with results of batteries of *in vitro* tests, including human cell models, *in silico* and toxicogenomic approaches, leading to prioritization of further *in vivo* testing. These integrated testing strategies offer an opportunity to significantly reduce the number of chemicals/substances that would need to be tested *in vivo*. In addition, animal experiments could further be limited to doses relevant to human exposure and to those combinations of substances and exposures where *in vitro* toxicity testing cannot exclude to a reasonable extent the occurrence of adverse effects.

The third session on "Stem cells in alternative methods" was introduced by **Mia Emgard** from Cellartis[3], Sweden. She showed how recent advances in the technologies that allow *in vitro* reproduction and maintenance of the developmental process, leading from multipotent undifferentiated stem cells to adult differentiated cell types, opened a potentially unlimited source of cell-based human models for toxicity testing, drug discovery and future replacement therapies. *In vitro* differentiation of human Pluripotent Stem Cells (hPSCs) resembles the early stages of human embryonic development and thus offers unique possibilities for alternative *in vitro* screening of compounds that are potentially toxic to the growing embryo. Dr Emgard presented two cellular models developed at Cellartis, i.e., cardiomyocytes and hepatocytes, which can be used as toxicity tests in different configurations (2Ds or 3Ds) and at different stages of development. These cells exhibit markers and functional properties specific for their level of tissue-specific development, can be cultured for extended periods of time and are provided in different multi-well formats that allow several testing applications, including -omic technologies and medium/high-throughput screening. Among the recent applications of these models is the prediction of hepatic chemical carcinogenicity (Yildirimman et al., 2011) and studies of cardiotoxicity endpoints, such as drug-induced myocardial injuries, ventricular arrhythmias and contractility (Jonsson et al., 2011).

The session on "Environmental Toxicity" was opened by **Per Schwarze** from the Norwegian Institute of Public Health, talking about "Ambient particles in *in vitro* toxicology: sources, components and mechanisms involved". Ambient particles are very heterogeneous, consisting of a wide variety of components including minerals, organic and inorganic compounds, carbon compounds and other biological components, and their study presents practical limitations linked to the physico-chemical properties. Human exposure to these particles almost invariably leads to inflammatory processes that are difficult to study *in vitro*, although production and release of inflammatory cytokines are important biomarkers of this response. Studies using different types of cell cultures (lung cell lines, co-cultures, primary lung cells) indicated that the organic fraction of particle extracts is principally responsible for cytokine release. However, screening of microparticles of different composition for their effects on the major inflammatory signaling pathways and genes resulted in very similar patterns of response. Some interesting co-culture models of epithelial pneumocytes, monocytes and endothelial cells exposed to silica particles showed that conditions of culture of the three cell types induced different patterns of pro-inflammatory cytokine release depending on whether there was contact or not between the cells. Monocytes and pneumocytes mutually reduced the other cell type's release of mediators in response to silica particles, and the presence of endothelial cells in the same culture dish, but not in direct contact with pneumocytes and monocytes, further modified the response of the other cells in co-culture (Herseth et al., 2008).

Vicki Stone from the Center for Nano Safety in Edinburgh (UK) introduced the next session with a presentation on "Nanotoxicology: relating physicochemical characteristics of nanomaterials to biological effects". She gave a wide overview of the extension of engineered nanomaterials under development, to which we are or will be exposed in the future, with a growing market that currently counts over 1300 consumer products containing nanomaterials[4]. Assessment of the potential hazards associated with intentional and incidental human exposure is further complicated by the differing behavior of each material when in the nanoscale, the very same character-

2 http://www.predict-iv.toxi.uniwuerzburg.de/

3 www.cellartis.com

4 www.nanotechproject.org

istic that confers their extraordinary properties also influences their biological reactivity. The ability of nanoparticles to induce cellular responses, such as oxidative stress, intracellular signaling, and pro-inflammatory gene expression, is related to their size, surface area and charge. Relatively low toxicity materials, such as titanium oxide, have been shown to modify their toxicity according to their crystal structure. As another example, the inflammatory effects and pathological changes induced by multiwalled carbon nanotubes (MWCNTs) with a long and narrow fiber-like shape have been shown to resemble those produced by other pathogenic fibers such as asbestos, known to cause mesotheliomas. Cytotoxicity of MWCNTs in human mesothelial cells *in vitro* was shown to depend on their long shape and small diameter, with thick or tangled nanotubes being less toxic, inflammogenic and carcinogenic (Nagai et al., 2011). Enhanced production of reactive oxygen by macrophages was also observed as a consequence of "frustrated" phagocytosis attempts of the long straight nanotubes. Good experimental models are therefore required to predict biological effects from physicochemical characteristics of nanomaterials, and to assess their potential toxicity (Stone et al., 2009).

The "Reproductive Toxicology" session was opened by **Giovanna Lazzari** from Avantea with a lecture on "Development of a battery of alternative methods for reproductive toxicity". Due to the complexity of the reproductive cycle and the lack of validated alternative tests for most of the steps included in the cycle, *in vivo* testing is presently the only tool available for hazard assessment of reproductive toxicants. In recent years, however, EU projects were funded to develop/optimize *in vitro* models able to detect adverse effects and mechanisms associated with reproductive toxicity, such as the FP6 ReProTect project[5] and the FP7 ESNATS project[6]. ESNATS was more specifically aimed at the development of an "all-in-one" toxicity test platform based on embryonic stem cells (ESC), in particular human ESC (hESCs), to accelerate drug development, reduce related R&D costs and propose a powerful alternative to animal tests. Within ReProTect, fifteen promising tests reflecting various toxicological mechanisms that can impair mammalian female and male fertility (i.e., effects on Leydig and Sertoli cells, folliculogenesis, germ cell maturation, motility of sperm cells, steroidogenesis, endocrine system, and on the preimplantation embryo) were developed and challenged with 10 blinded chemicals with toxicologically well-documented profiles. Comparative analysis together with a weight-of-evidence approach allowed a robust prediction of adverse effects on fertility and embryonic development of the test chemicals *in vivo*, although substances that required metabolic activation were intentionally excluded from the study (Schenk et al., 2010). Among these *in vitro* tests, the ReProGlo assay that uses a mouse embryonic stem cell line stably expressing a fluorescent reporter system capable of detecting drug-induced alterations in the canonical Wnt/β-catenin signaling pathway, is involved in the regulation of early embryonic development (Uibel et al., 2010). A major drawback of most of these and other *in vitro* test systems is their lack of metabolic competence required to metabolize chemicals to their biologically active forms, and it may therefore be necessary to refine these systems including (i.e., in co-culture models) metabolically active hepatocytes. A different approach in the field of alternative methods in reproductive toxicology is the one pursued by scientists of the US Environmental Protection Agency in a research program, the Virtual Embryo Project[7], aimed at developing new methods that use high-tech computer modeling and vast collections of data and biological knowledge bases in place of traditional laboratory tests. The complexity of the questions asked will certainly require more time and effort to achieve good predictions from non-animal tests in the field of human reproductive toxicity, although the work that is already under way appears very promising and will certainly benefit from more multidisciplinary approaches.

At the end of the second day, a Roundtable discussed "The 3Rs today: from basic research to regulatory tests" with the participation of Lucio Costa (Professor of Toxicology, Department of Environmental and Occupational Health Sciences at the University of Washington, Seattle, WA, USA), Thomas Hartung (Director of CAAT, Center for Alternatives to Animal Testing, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA), Joachim Kreysa (Head of the Validation of Alternative Methods Unit hosted by the Joint Research Centre's Institute for Health and Consumer Protection in Ispra, Italy), Greet Schoeters (Scientific Director of CARDAM, the Centre of Advanced Research and Development of Alternative Methods and President of ESTIV), Flavia Zucco (former Research Director at CNR and coordinator of several EU projects focusing on alternative methods), and Anna Laura Stammati (former President of CELLTOX) as chairperson. A few questions were addressed to the participants by the Roundtable chair on the problems and possible solutions to further implement the 3Rs principles, not only in industry and in regulatory testing, but also in basic research and in education. The recent Directive 63/2010 (on the protection of animals used for scientific purposes) represents an important step forward but is still not sufficient. There is still a great need to launch strong initiatives aimed at better informing the scientific community involved in basic research, the referees responsible for evaluating new grant proposals, and the members of the ethical committees about existing alternative methods and their power to reduce animal experimentation. In addition, it is important to inform them of the risks of overestimating the power of extrapolation from animal experimentation to the human situation. In recent years the 3Rs principles have significantly advanced, especially in terms of Refinement and Reduction, while Replacement is still a distant chimera in the widespread false belief that "if not tested

[5] www.reprotect.eu

[6] www.esnats.eu

[7] www.epa.gov/ncct/v-Embryo/

in vivo it cannot be predictive for humans". Further dissemination of the 3Rs principles will depend on the availability of research grants for the development of new tests and new models that could be applied in the regulatory field. There is an urgent need to get better ways of funding and, in this regard, it was considered crucial to pose the right questions and to find a good label for making it attractive for scientists to advance this field. The way towards a reduction of animal experiments in regulatory testing is the development of integrated approaches, involving batteries of *in vitro* tests and *in silico* modeling that will allow better prediction of human *in vivo* effects. The role of databases in this respect is extremely important, as they favor the dissemination of information on the best and more appropriate models, tests and methods to address basic research questions. The bioethical aspects were also discussed, emphasizing the importance of addressing the animal welfare issue in basic research and in education, since full implementation of the 3Rs will require more than is already written in the new Directive. The overall message of the Roundtable was that there is the need to promote information and dissemination on alternative methods, and the scientific and ethical issues that accompany them, and to expand the 3Rs beyond the field of toxicology, searching for new models and approaches from the life sciences and clinical fields in the attempt to further reduce animal experiments. It remains clear that with the new paradigm in toxicity testing new approaches and technologies are needed and that basic research and a good collaboration between different disciplines will play an important role.

The CELLTOX President-elect, Marisa Meloni (Vitroscreen, Italy), concluded with a short take-home message that summarized the most relevant concepts discussed and underlined the need to apply a quality system to *in vitro* research. She closed the meeting by thanking the speakers, the participants, the ISS and the Organizing and Scientific Committee, especially in the name of former CELLTOX President Isabella De Angelis, and wishing a bright outlook for *in vitro* toxicology and for future initiatives to promote information and education for the advancement of the 3Rs principles in research, education and regulatory testing.

The Congress abstract book can be downloaded from http://www.iss.it/publ/cong/cont.php?id=2517&lang=1&tipo=6&anno=2011

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