**ISTITUTO SUPERIORE DI SANITÀ**

International Congress

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

Istituto Superiore di Sanità
Rome, October 19-21, 2011

**ABSTRACT BOOK**

Edited by
Isabella De Angelis and Simonetta Gemma
*Department of Environment and Primary Prevention*

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*In vitro* toxicology has grown and has acquired a major importance over the last two decades, both for the scientific advancements in this area and for the increasing influence of its approaches on the regulatory bodies. Primary aim of this congress is to provide a scientific forum for presenting novel approaches and recent results in this field allowing, at the same time, a fruitful exchange of ideas and experiences among leading scientists and young researchers. Moreover, a poster session will be organized to allow a wider discussion among all participants. The congress scientific program will cover some emerging topics in this discipline, organized in seven sessions such as: predictive toxicology, integrated *in vitro*/*in silico* approaches, stem cells in alternative methods, environmental toxicology, nanotoxicology, reproductive toxicology, multilevel approaches in toxicology. Furthermore, scientific discussion will be enriched by a Round Table on the 3Rs today: from basic research to regulatory tests.

*Key words*: *In vitro* toxicology, Cell culture, Alternative methods, 3Rs principle, Animal testing

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**Convegno internazionale. CELLTOX 1991-2011. Venti anni di tossicologia *in vitro*: risultati e prospettive future. Istituto Superiore di Sanità. Roma, 19-21 ottobre 2011. Riassunti.**
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Negli ultimi 20 anni la tossicologia *in vitro* è grandemente maturata sia per gli avanzamenti scientifici e metodologici di cui ha beneficiato sia per la sempre maggiore influenza che il suo approccio metodologico ha in ambito regolatorio. Obiettivo principale del presente convegno è quello di rappresentare un forum scientifico per la presentazione di nuovi approcci e risultati, oltre che consentire un fruttuoso scambio di idee, informazioni ed esperienze tra i partecipanti. La presenza di una sessione poster amplia la possibilità di discussione ed interazione fra i ricercatori presenti. Il programma scientifico del convegno coprirà alcuni degli aspetti emergenti dell'uso di modelli *in vitro* nella tossicologia; è organizzato in sette sessioni: modelli innovativi e valutazione del rischio, approcci integrati *in vitro/in silico*, cellule staminali e metodi alternativi, modelli per la tossicologia ambientale, nanotossicologia *in vitro*, tossicologia riproduttiva, approcci integrati. La discussione scientifica sarà ulteriormente arricchita da una tavola rotonda su le 3R oggi: dalla ricerca di base alla implementazione regolatoria.

*Parole chiave*: Tossicologia *in vitro*, Colture cellulari, Metodi alternativi, Principio delle 3R, Sperimentazione animale

Responsabili scientifici: Isabella De Angelis, Simonetta Gemma

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# SCIENTIFIC AND ORGANIZING COMMITTEE

This International Congress is organized by the Istituto Superiore di Sanità in collaboration with CELLTOX, Italian Association for *in vitro* Toxicology (Associazione Italiana di Tossicologia *in vitro*)

| | |
|---|---|
| **Francesca Caloni** | *University of Milan, Milan, Italy* |
| **Isabella De Angelis** | *Istituto Superiore di Sanità, Rome, Italy* |
| **Simonetta Gemma** | *Istituto Superiore di Sanità, Rome, Italy* |
| **Lucia Golzio** | *MerckSerono, Ivrea, Italy* |
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| **Pilar Prieto** | *European Centre for the Validation of Alternative Methods, ECVAM, Institute for Health and Consumer Protection, IHCP, Joint Research Centre, Ispra, Italy* |
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| **Chiara Urani** | *University of Milano Bicocca, Milan, Italy* |
| **Anna Zaghini** | *University of Bologna, Bologna, Italy* |

This event has been supported by unrestricted grants of:

## INVITED SPEAKERS

| | |
|---|---|
| **Jos G.M. Bessems** | *RIVM, National Institute for Public Health and Environment, Utrecht, The Netherland* |
| **Jurgen Borlak** | *Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany* |
| **Lucio Costa** | *University of Parma, Parma, Italy* |
| **Mia Emgard** | *CELLARTIS, Göteborg, Sweden* |
| **Thomas Hartung** | *CAAT, Center for Alternatives to Animal Testing Europe, Kostanz, Germany* |
| **Joachin Kreysa** | *European Centre for the Validation of Alternative Methods, ECVAM, Institute for Health and Consumer Protection, IHCP, Joint Research Centre, Ispra, Italy* |
| **Giovanna Lazzari** | *AVANTEA, Cremona, Italy* |
| **Greet Schoeters** | *University of Antwerp, Antwerp, Belgium* |
| **Per Schwarze** | *Norwegian Institute of Public Health, Oslo, Norway* |
| **Vicki Stone** | *Centre for Nano Safety, Edinburgh, United Kingdom* |
| **Emanuela Testai** | *Istituto Superiore di Sanità, Rome, Italy* |
| **Carl Westmoreland** | *Unilever's Safety & Environmental Assurance Centre, Shambrook, United Kingdom* |
| **Flavia Zucco** | *Consiglio Nazionale delle Ricerche, Rome, Italy* |

# PROGRAM

## Wednesday, October 19

12.00 Participant registration

13.30 Welcome address
**L. Musmeci**
Director of the Department of Environment and Primary Prevention
Istituto Superiore di Sanita
**I. De Angelis**
CELLTOX
Past President

### Opening lecture

*Chairs:* **L. Golzio, Y. Sambuy**

14.00 *Progress and future challenges with alternative methods to allow safety assessment without animal testing*
**C. Westmoreland**

### Session 1
### PREDICTIVE TOXICOLOGY

14.30 *A framework for risk assessment without animal testing: the role of kinetics*
**E. Testai**

### Selected oral presentations

15.00 *Assessment of acute, long-term and chronic respiratory toxicity using a long shelf-life 3D model of the human airway epithelium (MucilAir™)*
**S. Constant, S. Huang, L. Wiszniewski**

15.15 In vitro *blood-brain barrier models adapted to toxicological screening*
**M. Culot, A. da Costa, J. Hachani, C. Landry, R. Cecchelli**

15.30 *Importance of the tissue-specific 3D microenvironment in developing* in vitro *models for chondro-toxicity assessment*
**N. Steimberg, J. Boniotti, G. Zarattini, P. Barone, U.E. Pazzaglia, G. Mazzoleni**

15.45 Coffee break and poster viewing

### Session 2
### INTEGRATED *IN VITRO/IN SILICO* APPROACHES

16.15 *Integration of* in silico *and* in vitro *approaches in tiered testing strategies*
**J.G.M. Bessems**

### Selected oral presentations

16.45 *Towards a conversion of morphological features into statistical descriptors in Balb/c 3T3 cell transformation assay*
**C. Urani, R. Corvi, C. Procaccianti, F.M. Stefanini**

17.00 *Effects of time culture and prototypical CYP3A inducers on CYP2B22, CYP2C, CYP3A28 and nuclear receptors mRNAs in cryopreserved pig hepatocytes*
**V. Zancanella, M. Giantin, R.M. Lopparelli, A. Granato, C. Baratto, M.T. Vilei, M. Muraca, M. Dacasto**

17.15 *Pandora's Tox: multiple mechanisms of toxicity in one assay for early de-risking of drug discovery programs*
**H. Luithardt, S. Thomas, C. Strock, J. Gilbert, P. Metcalfe, H. Gill, K. Tsaioun**

## Thursday, October 20
*Chairs:* **S. Gemma, C. Urani**

### Session 3
### STEM CELLS IN ALTERNATIVE METHODS

9.00 *Stem cells in alternative methods*
**M. Emgard**

### Selected oral presentations

9.30 *Testing neurodevelopmental toxicity on differentiating human embryonic stem cells*
**A.C. Feutz, O. Sterthaus, C. De Geyter**

9.45 *Neurogenic-committed human pre-adipocytes: a novel* in vitro *model for evaluation the toxicity of xenobiotics/pollutants*
**C. Scanarotti, C. Guida, R. Coradeghini, M.A. Pronzato, A.M. Bassi**

10.00 *Towards the use of organotypic microtissues from primary hepatocytes and stem cells for early toxicology assessment*
**S. Stroebel, S. Messner, J. Lichtenberg, W. Moritz, J.M. Kelm**

10.15 Coffee break and poster viewing

## Session 4
## ENVIRONMENTAL TOXICOLOGY

11.00 *Ambient particles in* in vitro *toxicology: sources, components and mechanisms involved*
**P. Schwarze**

### Selected oral presentations

11.30 *Usefulness of* in vitro *bioassay in photodegradation studies of AhR-active pollutants*
**P. Macíková, J. Ospalík, J.P. Giesy, M. Bittner**

11.45 In vitro *enzymatic bioassay for the evaluation of the toxicity of aqueous matrices*
**M.G. Lionetto, E. Erroi, T. Schettino**

12.00 *Paradoxical effects of environmental concentrations of organochlorines, Lindane and Chlordecone, on cellular energy pathways in HepG2 cells*
**M.A. Benarbia, S. Faure, C. Jaques, E. Lauret, G. Simard, R. Andriantsitohaina, Y. Malthiery**

12.15 *Fumonisin B1- induced influence on functional parameters of human intestinal cell lines in relation to differentiation stage*
**F. Minervini, A. Garbetta, I. D'Antuono, A. Cardinali, L. Debellis, A. Visconti**

12.30 Lunch and poster viewing

## Session 5
## NANOTOXICOLOGY

*Chairs:* **I. De Angelis, P. Prieto**

14.00 *Nanotoxicology: relating physicochemical characteristics of nanomaterials to biological effects.*
**V. Stone**

### Selected oral presentations

14.30 *Nanotubes of the aluminosilicate Imogolite exhibit mild toxicity for macrophages and airways epithelial cells*
**O. Bussolati, B.M. Rotoli, B. Bonelli, C. Zanzottera, E. Garrone, I. Fenoglio, M. Ghiazza, E. Bergamaschi, B. Fubini**

14.45 *Characterization and investigation of cytotoxic and genotoxic effects of Zinc oxide nanoparticles in a human intestinal cell line (Caco-2)*
**A. Zijno, B. De Berardis, F. Barone, C. Andreoli, M.T. Russo, P. Degan, F. Franchini, G. Guidetti, C. Uboldi, J. Ponti, F. Rossi, I. De Angelis**

15.00 *Effects of Nickel oxide nanoparticles on human lung epithelial cells*
**M. Camatini, L. Capasso, P. Mantecca, M. Gualtieri**

15.15 *Assessment of the* in vitro *toxicological profile of amorphous silica nanoparticles and study of their intracellular fate*
**C. Uboldi, G. Giudetti, F. Broggi, J. Ponti, D. Gilliland, F. Rossi**

15.30 Coffee break and poster viewing

16. 00 **Round Table**
*The 3Rs today: from basic research to regulatory tests*
*Chair:* **A. Stammati**
*Invited participants:* **L. Costa, T. Hartung, J. Kreysa, G. Schoeters, F. Zucco**

17.30 Assemblea CELLTOX

20.00 Social dinner

## Friday, October 21

*Chairs:* **F. Caloni, M. Meloni**

### Session 6

### REPRODUCTIVE TOXICOLOGY

9.00 *Development of a battery of alternative tests for reproductive toxicity*
**G. Lazzari**

### Selected oral presentations

9.30 *The interference of pollutants with the biosynthesis of active androgens and estrogens in fish*
**D. Fernandes, C. Porte**

9.45 *The herbicide Glufosinate Ammonium is a weak androgen-like compound as emerged by a novel* in vitro *approach to identify chemicals with a prostate-mediated effect on male reproduction*
**S. Lorenzetti, I. Altieri, D. Marcoccia, L. Narciso**

10.00 *Antiestrogenic, antiandrogenic and dioxin-like activities of night and day air samples from Banja Luka Region*
**J. Novák, M. Cvešperová, J.P. Giesy, J. Klánová**

10.15 Coffee break and poster viewing

## Session 7
## MULTILEVEL APPROACHES IN TOXICOLOGY

10.45 *Toxicology Model Building and Biomarker Discovery based on –Omics datasets*
**J. Borlak**

### Selected oral presentations

11.15 *Novel multicompartmental bioreactor systems for probing multiple pathway toxicity and drug development*
**A. Ahluwalia**

11.30 *Gene expression in human cell models applied for safety assessment of chemicals*
**N. Lambrechts, S. Remy, H. Witters, R. Van Den Heuvel, G. Schoeters, I. Nelissen, J. Hooyberghs**

11.45 *Co-culture of intestinal and hepatic cells as a model to study absorption, metabolism and toxicity of dietary retinoids*
**C. Rossi, B. Guantario, D. Bellovino, S. Ferruzza, M.L. Scarino, C. Guguen-Guillouzo, Y. Sambuy**

12.00 Closing remarks
**M. Meloni**
CELLTOX President

# Opening Lecture

# PROGRESS AND FUTURE CHALLENGES WITH ALTERNATIVE METHODS TO ALLOW SAFETY ASSESSMENT WITHOUT ANIMAL TESTING

Westmoreland C.
*Safety and Environmental Assurance Centre, Unilever, Colworth Science Park, Sharnbrook, Bedford, United Kingdom*

Assuring the safety of consumers exposed to novel ingredients without the use of toxicological data generated in animals is a considerable scientific and technical challenge. In the past 20 years considerable progress has been made in the development, validation and regulatory acceptance of non-animal tests for the identification of several local endpoints (e.g. skin irritation, eye irritation). The success of these methods has resulted largely from advances in organotypic culture and tissue engineering together with robust validation studies using characterized test chemicals to compare the results of hazard identification studies in animals with those obtained in the non-animal systems.

Whilst these successes in the development of 'alternative methods' have without doubt had a significant effect on the use of laboratory animals for toxicity testing, significant challenges still remain. The two main challenges are: (1) How non-animal methods can be applied more widely for risk assessment purposes rather than solely for hazard identification / classification & labeling purposes, and (2) How non-animal approaches to risk assessment for systemic toxicity can be developed.

An evolution of the mindset used to develop alternative methods for safety assessment is required to build on the lessons learned from the last 20 years. For the complex safety endpoints under consideration it is clear that we are not looking for a way to do the animal test without the animal. Rather we need to develop new approaches to risk assessment that bring together information on human exposure data, effects of chemicals on key toxicity pathways and dose-response and extrapolation modeling to allow us to develop new ways of assuring consumer safety. This vision of a new paradigm for the use of *in vitro* data in safety assessment has been elegantly outlined in the US NRC Report 'Toxicity Testing in the 21st Century: A Vision and a Strategy'.

Two specific examples will be discussed that begin to explore how this new vision of the use of information on human toxicity pathways may be used in the context of risk assessment: (1) The use of a mechanistic understanding of the biology underlying human skin allergy to develop novel approaches to risk assessment, and (2) The use of DNA damage stress pathways as a case study for applying the TT21C approach to the integration of *in vitro* dose response information into consumer safety risk assessments (www.TT21C.org).

**Session 1**

## Predictive toxicology

*Chairs*

Lucia Golzio, Yula Sambuy

# A FRAMEWORK FOR RISK ASSESSMENT WITHOUT ANIMAL TESTING: THE ROLE OF KINETICS

Testai E.
*Department of Environment and Primary Prevention, Istituto Superiore di Sanità, Rome, Italy*

When developing testing strategies, kinetics is considered the crucial body of information for the design and performance of toxicological tests and for toxicity data interpretation. The knowledge of the bioavailability of a given compound by the relevant uptake routes should represent the starting point for any toxicological testing: *in vivo* the actual internal dose reaching the target is the more relevant parameter in evaluating exposure and in the quantitative risk assessment. This consideration applies also to alternative/non animal testing strategy: indeed, no or limited testing would be necessary in case of internal dose lower than the Threshold of Toxicological Concern (TTC) for the relevant route of exposure. In addition, in *in vitro* models the nominal applied concentrations rather than the actual level of cell exposure is quite often associated to an observed effects. And indeed, the difficulty in translating an *in vitro* concentration into an *in vivo* dose (*in vitro-in vivo* extrapolation) and *in vitro/in vivo* differences, often attributed to kinetics, are the major limitations of non-animal testing. In this respect, the role of *in vitro* biokinetics is crucial: despite this, only very few studies have addressed this issue. The actual intracellular concentration may be affected due to altered bioavailability (interactions with medium/plate, abiotic processes) or to physiological cellular processes (transport across the membranes, biotransformation, bioaccumulation) after acute and even more after repeated treatments. Inclusion of biokinetic measurements in the *in vitro* test protocols is essential also to know whether cells are exposed to the parent compound and/or its metabolites. Most kinetic information can be obtained from advanced non animal methods; then Physiologically Based ToxicoKinetic (PBTK) models are available for the integration of dynamic and kinetic data produced from *in vitro/in silico* methods into a biologically meaningful framework and for the extrapolation to *in vivo* conditions. This approach could make possible to derive a NOEC in *in vitro* experimental models, preferentially based on human cells representative of *in vivo* target organs, from which extrapolate the corresponding *in vivo* dose.

*The work has been partially supported by the FP7-EU funded Project PredictIV Grant n° 202222.*

# ASSESSMENT OF ACUTE, LONG-TERM AND CHRONIC RESPIRATORY TOXICITY USING A LONG SHELF-LIFE 3D MODEL OF THE HUMAN AIRWAY EPITHELIUM (MUCILAIR™)

Constant S., Huang S.,Wiszniewski L.
*Epithelix Sàrl, Geneva, Switzerland*

Most of the *in vitro* cell models for long term testing of chemicals suffer of at least two shortcomings: 1.The failure of reproducing the *in vivo* physiological characteristics of the corresponding tissues. 2. A limited shelf-life. Herein is reported the use of a standardized 3D Air-liquid Interface *in vitro* cell model of the human airway epithelium (MucilAir™) which is free of these limitations.

MucilAir™ is morphologically and functionally differentiated and it can be maintained at a homeostatic state for more than one year. The typical ultra-structures of the human airway epithelium, such as tight junctions, cilia, mucus, basal/goblet/ciliated cells can be observed. Classical airway transporters, ion channels and CypP450s are expressed and functional up to one year. The epithelia react to pro-inflammatory mediators in a physiological manner. The epithelia can be stimulated regularly with inflammatory substances to simulate chronic inflammatory reactions, up to several months. A large panel of cytokines/chemokines/metalloproteinases has been detected in MucilAir™.

Due to its unique long shelf-life of one year, this model is used for studying the human respiratory diseases, and for testing the long-term/chronic effects of drugs/chemicals on respiratory tract *in vitro*. Late effect of chemicals/mixtures (several weeks after exposure) can be observed. Several applications of MucilAir™ will be presented:

- Acute, Long-Term and Chronic Toxicity testing (first *in vitro* transposition of OECD TG412 will be presented - 28 days repeated dose study);
- Inflammatory effect assessment;
- Assessment of reversible *vs* irreversible toxic effects;
- Recent advances in the detection of respiratory sensitizers and irritants.

# *IN VITRO* BLOOD-BRAIN BARRIER MODELS ADAPTED TO TOXICOLOGICAL SCREENING

Culot M. (a,b), da Costa A. (a,b), Hachani J. (a,b), Landry C. (a,b), Cecchelli R. (a,b)
*(a) University of Lille Nord de France, Lille, France*
*(b) University of Artois, BBB Laboratory, Lens, France*

Toxicity to the CNS is a required by many regulatory programs. Cell lines derived from brain cells have shown promise as alternatives for assessing neurotoxicity. However, these *in vitro* cell assays would benefit from compound data that considered its distribution into the CNS compartment to make relevant neurotoxicity assessments. Located at the level of the cerebral capillaries, the Blood-Brain Barrier (BBB) regulates molecular exchange between the brain and systemic circulation. Therefore, BBB permeability is a key determinant for CNS exposure. BBB toxicity is equally important as a dysfunctional BBB may cause unwanted effect on brain cells either by affecting brain entry of an agent or by generating unwanted effects on neurons.

Recently, we have developed an *in vitro* BBB system suitable to make predictions about brain uptake of chemicals in a high throughput screening mode. This model, consisting in bovine brain capillary endothelial cells (BCECs) cultivated in 24-well insert plates, considerably reduces the use of animals and the technical demands to obtain a functional BBB model. Ready after only 4 days, the BCECs show a typical BBB phenotype (well-differentiated tight junctions, expression of efflux pumps). By culturing the BCECs in presence of glial cells conditioned medium, it is possible to maintain this phenotype for at least 2 weeks which enable to assess the effect of repeated dose treatment at the BBB level.

Finally, studies undertaken within the framework of Predict IV (European FP-7) prompted the importance of BBB permeability in acute as well as repeated dose neurotoxicity testing.

# IMPORTANCE OF THE TISSUE-SPECIFIC 3D MICROENVIRONMENT IN DEVELOPING *IN VITRO* MODELS FOR CHONDRO-TOXICITY ASSESSMENT

Steimberg N. (a,c), Boniotti J. (a,c), Zarattini G. (b) Barone P. (a), Pazzaglia U.E. (b), Mazzoleni G. (a,c)
*(a) Department of Biomedical Science and Biotechnology, University of Brescia, Brescia, Italy*
*(b) Clinica Ortopedica, University of Brescia, Brescia, Italy*
*(c) Istituto Interuniversitario di Miologia, Italy*

Chondrocytes represent the main cell type of Articular Cartilage (AC), a highly specialised tissue, whose main functions are to act as a low-friction, shock absorber, and load-bearing surface. In response to various intrinsic and extrinsic factors, chondrocytes are responsible for the synthesis, maintenance and turnover of AC's Extracellular Matrix (ECM), a complex and dynamic network of specific components, which confers to this conjunctive tissue its peculiar mechanical and physical properties. Due to the significant consequences of AC's suffering (health's impact and social costs), important efforts have been made during the last decades for a better understanding of chondrocytes' behaviour and responses. Nevertheless, due to the difficulties in performing *in vivo* studies, and to the lack of suitable *in vitro* systems able to model AC tissue, our knowledge of chondrocytes' physiopathology is still largely incomplete. Physiologically relevant *in vitro* models of AC could be particularly important also for the mechanistic investigation of the long-term negative effects of numerous drugs, such as antibiotics (quinolones) or local anaesthetics (buvicaine, lidocaine), known to affect chondrocytes' homeostasis. In order to improve the relevance of the *in vitro* studies on articular chondrocytes, we developed different 3D *in vitro* models of AC tissue, which, even based on the same attempt to recreate/preserve the original tissue-specific 3D microenvironment, present a different degree of complexity with respect to the native tissue *in vivo*. In the most simplified model (primary culture of homotypic aggregates of isolated hyaline chondrocytes), the synthesis of neo-ECM is promoted by the culture conditions, while in the most complex models (cartilage and bone/cartilage tissue explants), original ECM's composition, cell-ECM, and cell-cell interactions are preserved. The key point of our approach was the use of the Rotary Cell Culture System (RCCS™) bioreactor, that, by reproducing critical aspects of microgravity, generates a particular microenvironment, where high mass transfer is attained with low shear stress, thus providing optimal conditions for long-term cell survival and function within large-sized 3D constructs. The results obtained by comparing the various models (histo-morphological and bio-molecular analyses), indicate that, with regard to the traditional 2D static culture of isolated chondrocytes, all our culture methods allow, to various extent, the preservation of cell viability and differentiated phenotype over several weeks of culture, confirming their value also as a reliable alternative to *in vivo* testing of drugs potentially toxic for cartilage maturation and homeostasis.

**Session 2**

## Integrated *in vitro/in silico* approaches

*Chairs*

Lucia Golzio, Yula Sambuy

# INTEGRATION OF *IN SILICO* AND *IN VITRO* APPROACHES IN TIERED TESTING STRATEGIES

Bessems J.G.M.
*Centre for Substances and Integrated Risk Assessment, RIVM, National Institute for Public Health and Environment, Utrecht, The Netherland*

Animal experiments are necessary for safety testing of chemicals. However, there is societal pressure to reduce the use of laboratory animals. In this contribution, a vision is presented on integration of *in silico* and *in vitro* approaches in a data- and science-driven safety testing strategy aiming at significant reduction of animal bioassays.

Every researcher performing toxicity tests is confronted with the question: What concentration or dose should I use? The strategy to come up with a number is often driven by what numbers were used by previous researchers and which level is high enough to see at least mild toxicity. This is the hazard-driven strategy. And that is what many legislative frameworks request: investigate the hazardous properties of a substance, irrespective of the known or expected human exposure. Whereas from a risk assessment point of view in many cases the human relevance of a concentration or dose should be the leading driving force.

It is presented how an intelligent combination of exposure science, *in silico* kinetic modeling and *in vitro* toxicity testing can reduce the actual number of animal bioassays performed. A human tissue dose-based testing strategy as will be outlined could limit animal toxicity testing significantly. Animal testing could be limited much more to substance-exposure combinations where *in vitro* toxicity data at relevant human tissue dose concentrations reveal that adverse effects cannot be excluded to a reasonable extent.

# TOWARDS A CONVERSION OF MORPHOLOGICAL FEATURES INTO STATISTICAL DESCRIPTORS IN BALB/C 3T3 CELL TRANSFORMATION ASSAY

Urani C. (a), Corvi R. (b), Procaccianti C. (a), Stefanini F.M. (c)
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*(c) Department of Statistic G. Parenti, University of Florence, Florence, Italy*

*In vitro* methods for modelling the carcinogenic process and predicting the carcinogenic potential of chemicals have been proposed, and find a broad range of applications in pharmaceutical, chemical and cosmetic industry, as well as academia. The Cell Transformation Assays (CTA) are based on morphological transformation in which phenotypic changes, from normal to malignant, are observed in suitable mammalian cells (e.g., Balb/c 3T3, C3H10T1/2, SHE) exposed to test chemicals. When they undergo transformation, these cells, with typical contact inhibition properties, produce foci of aberrant cell morphology, that are scored by optical microscopy and classified into different classes. The CTA, allow for a fast and reliable assessment of the carcinogenic potential of a chemical compound in comparison with the standard two-year bioassay (OECD TG451). However, even if standard rules for foci scoring and classification have been defined, a wrong class assignment is possible due to morphologic variability in the underlying biological process, leaving room for subjective interpretation, and a possible over-/under-estimation of the carcinogenic potential of chemicals. This potential bias is a crucial issue in the standardised use of the CTA as an alternative method to animal tests.

Expert scoring of features in transformed Balb/c cells include spindle-shape cells, multilayer growth, dense and basophilic staining, random orientation at the focus edge and invasiveness of the surrounding monolayer, as described in the literature. Herewith, we propose statistical image descriptors with the aim of capturing the qualitative information contained in morphological features usually scored by experts. Quantitative descriptors are likely to successfully support the expert while classifying foci from transformed Balb/c cells.

The development and implementation of non-animal assays is strongly necessary as well as urgently required due to the enormous cost of cancer (research, substance safety assessment and therapy) to society.

*C.U. gratefully acknowledges the partial support by Fondo di Ateneo per la Ricerca Granted by Università degli Studi di Milano Bicocca.*

# EFFECTS OF TIME CULTURE AND PROTOTYPICAL CYP3A INDUCERS ON CYP2B22, CYP2C, CYP3A28 AND NUCLEAR RECEPTORS mRNAS IN CRYOPRESERVED PIG HEPATOCYTES

Zancanella V. (a)*, Giantin M. (a)*, Lopparelli R.M. (a), Granato A. (b), Baratto C. (a), Vilei M.T. (c), Muraca M. (d), Dacasto M. (a)

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The constitutive expression of major drug metabolizing enzymes like Cytochromes P450 (CYPs) and Related Nuclear Receptors (NRs) is considered of fundamental importance in drug metabolism studies made in whole-cell systems like Hepatocyte Primary Cultures (HPCs). In fresh and cryopreserved pig HPCs, time-dependent variations of CYP gene expression have been investigated by and large at the post-translational level; furthermore, few data have been published about the effect of time and known CYP3A inducers on NRs mRNAs.

In the present study, the transcriptional effects of time and prototypical CYP3A inducers upon CYP2B22, 2C, 3A28 and NR1I2, NR1I3, NR2B1 and NR3C1 were investigated by using cryopreserved pig HPCs.Materials and Methods. To measure time-dependent changes in aforementioned target genes, HPCs were stopped 24, 48, 72 and 96 hrs after plating. As regards the HPCs response to known CYP3A inducers, media containing phenobarbital (PB, 2 mM), pregnenolone 16α-carbonitrile (PCN, 10 μM), rifampicin (RIF, 10 μM), dexamethasone (DEX, 10 μM) and dimethyl sulfoxide (control) were daily added to monolayers from 24 hrs after plating and up to 72 hrs. Additionally, specific reference genes were identified by using geNormPLUS and Normfinder algorithms.

Transcriptional effects were measured by using specific qPCR assays.Results. The geometric mean of three reference genes, namely ribosomal protein large P0 (RPLP0), cyclophilin A (PPIA), glyceraldheyde 3-phosphte dehydrogenase and RPLP0, PPIA and β-actin was used to normalize time-course and induction qPCR data, respectively. CYP gene expression was strongly down-regulated as a function of time culture; at 48 hrs, CYP2B22 and CYP3A accounted (%) for 7.14±1.08 and 19.37±11.21 of the RQ value measured at 24 hrs. A low and constant constitutive expression of CYP2C, NR1I2 and NR1I3 was noticed during the whole culturing time, while NR2B1 and NR3C1 mRNA levels were increased ($p<0.001$ for NR2B1at 96 hrs). Hepatocytes were responsive to model CYP3A inducers; PB significantly increased CYP2B22 ($p<0.05$), 2C ($p<0.01$) and 3A ($p<0.001$) gene expression, while DEX induced CYP3A, NR1I2 ($p<0.001$) and NR1I3 ($p<0.05$).

Finally, RIF up-regulated only CYP3A mRNA ($p<0.05$).

Data obtained agree with previous published comparative results about time-course and induction in fresh and cryopreserved HPCs. Furthermore, present results would confirm the role played by NRs in CYP expression and regulation phenomena as well as the presence of species-differences in CYP3A drug metabolism. Confirmatory immunoblotting investigations are actually running.

**These authors contributed equally to this project and should be considered co-first authors.*

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# PANDORA'S TOX: MULTIPLE MECHANISMS OF TOXICITY IN ONE ASSAY FOR EARLY DE-RISKING OF DRUG DISCOVERY PROGRAMS

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Toxicity is a key reason for drug attrition. Identifying potential toxicity at an early stage in drug discovery can reduce the likelihood of late stage failure, saving both time and development costs. High Content Screening (HCS) is a powerful, well validated and flexible technology for quantifying and understanding the changes that occur in cells when they are exposed to potentially toxic xenobiotics. HCS allows the analysis of multi-parametric indicators of cellular toxicity, detecting cell death and mechanisms of cell death, and covering a wide spectrum of cytopathological changes. CellCiphr™ is selected by EPA ToxCast program, and combines HCS technology with a unique database comprised of marketed drugs, FDA and EPA ToxCast-submitted, and pharmaceuticals that failed in preclinical and clinical studies. CellCiphr™ HCS endpoints probe the interacting network of processes that are involved in cellular function and cellular toxic response, and have been chosen for their relevance in predicting aspects of drug-induced injury. The data generated for each compound lays the foundation for understanding the mechanisms of toxicity induced by any given compound, and the potential for different organs to undergo different responses. We present and discuss relative toxicity rankings of a selection of results for HepG2 cells and rat primary hepatocytes, and validation of this technology using FDA DILI set of compounds.

**Session 3**

## Stem cells in alternatives methods

*Chairs*

Simonetta Gemma, Chiara Urani

# STEM CELLS IN ALTERNATIVE METHODS

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Stem cells represent the most recent phase of the biotechnology revolution in medicine. Their significance is based on an understanding of how all cells develop from undifferentiated stem cells into adult tissues. Knowledge of these processes, together with the development of technologies for the manipulation of the growth, differentiation and their genetic modification, will enable these cells to be used as a potentially unlimited biological resource for cell-based human discovery tools for toxicity testing, drug discovery, and future replacement therapies.

Traditionally, safety assessments in drug discovery and evaluations of environmental stressors on human health are based on non-human material or limited sources of human tissue samples. As the use of large numbers of experimental animals and big inter-experimental variations negatively impacts the process, there is a great need to evaluate alternative options. During the last decade, human Pluripotent Stem Cells (hPSCs) have received increasing appreciation for their use in a wide range of experimental settings. Since many compound effects are species-specific, reliable toxicity assays based on human cells are of high relevance. Notably, *in vitro* differentiation of hPSCs resembles the early stages of human embryonic development, and thus, offers unique possibilities for alternative *in vitro* screening of compounds for their potential risks for adverse effects on the growing embryo.

CELLARTIS is a biotech company specialized in hPSCs technologies, and has developed several hPSC-based products for the *in vitro* and tools market space. We have developed toxicity models based on human undifferentiated stem cells, partially differentiated cells, and terminally differentiated cells. For example, at CELLARTIS, the unique features of hPSCs are taken advantage of in cellular models of developmental toxicity. Furthermore, hepatocytes and cardiomyocytes derived from hPSCs are provided from CELLARTIS in multi-well plate formats, at an unlimited supply, suitable for a wide range of applications. The cells exhibit specific markers and functional properties similar to their adult counterparts, and can be cultured for extended times *in vitro* without loss of basic functionality.

In summary, it is anticipated that new improved *in vitro* models based on physiologically relevant human cells will result in more cost-effective assays, ultimately leading to safer new drugs and better evaluations of the effects of chemicals on human health. In this presentation, the differentiation of hPSCs to specialized cells and results from safety assessment analyses utilizing hPSCs will be discussed. Moreover, the possibilities to use hESCs for artificial liver support and replacement therapies will be briefed upon.

# TESTING NEURODEVELOPMENTAL TOXICITY ON DIFFERENTIATING HUMAN EMBRYONIC STEM CELLS

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Ambiguous prognostic value of developmental toxicity studies in animals have emphasized the need for a complementary human assay. Since *in vitro* directed differentiation of human embryonic stem cells precisely reproduces the successive inductive events that occur *in vivo*, it represents a versatile promissing system for teratogenicity testing. Thus, we are now establishing, as a proof of concept, an hESC-based *in vitro* developmental neurotoxicity assay.

We compared four hESC lines created in our laboratory for their ability to generate reproducibly highly enriched neural cell populations using standardized culture conditions.

Our results indicate that all the tested hESC lines exhibit similar differentiation efficiency towards neural cells. However they greatly differ in their tendency to spontaneously commit during stem cell maintenance. As a consequence, cell lines starting with the higher degree of spontaneous neural pre-differentiation give rise to more neurons at a given time point after differentiation is initiated. In addition, such heterogeneous starting populations differentiate asynchronously making more difficult to distinguish the successive steps of the differentiation process therefore to identify the targets of toxicants. Finally slight pre-differentiation into the endodermal lineage also variably occurs affecting the level of purity of the differentiated cultures. Altogether these findings indicate that although similarly derived and cultured, hESC lines exhibit intrinsic differences and that characterization and choice of cell lines are important prerequisites to toxicological studies.

We are now comparing our different hESC lines for their response to known developmental toxicants. First, we are determining the readouts suitable to detect the effects of several well-known neuroteratogens, i.e. cyclopamine, valproic acid, methyl mercury and nicotine. Together they cover most of the deleterious effects observed during CNS development *in vivo*: defects in neural induction, neural tube formation, regionalization, neural cell growth, survival and differentiation, cell migration and network formation. Our first attempts have been limited to check the ratio of dorsal forebrain neural precursors generated, to evaluate their ability to form neuro-epithelial-like circular structures, to quantify their ability to self-renew or to migrate out of the germinal zone and differentiate into surviving, network-forming neurons. Once fully established, we will use our hESC-based assay to characterize the neurodevelopmental impact of compounds of interest and to analyze their mode of action.

# NEUROGENIC-COMMITTED HUMAN PRE-DIPOCYTES: A NOVEL *IN VITRO* MODEL FOR EVALUATION THE TOXICITY OF XENOBIOTICS/POLLUTANTS

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Stem cell models offer an opportunity both for therapeutic use and for the assessment of alternative *in vitro* models. Human lipoaspirate is a source of adult stem cells (pre-adipocytes), which are able to differentiate into various phenotypes, such as neurogenic lineage. Several reports suggest that neural disorders may begin early in life, and that air pollutants, drugs and other xenobiotics play a crucial role in this process. Here we assess the suitability of human multipotent adult stem cells, derived from fat tissue, and their neurogenic derivates in screening exogenous compounds, such as environmental pollutants (i.e. Polycyclic Hydrocarbons, PAH), foodstuffs, drugs, etc. that may affect adipose cells and neurogenic development. Neurogenic differentiation was confirm by analysis of cholinergic system and acetylcholinesterase immunoreactivity. PAH heterocyclic derivatives contain inducers of CYP1A1, so we explored the activity of CYP1A1-related enzymes, i.e. 7-ethoxycoumarin- and 7-ethoxyresorufin O- deethylase (ECOD and EROD, respectively) in both cell systems in basal conditions and after exposure to non-cytotoxic doses of BNF, a well-known PAH-type inducer. This study is the first to demonstrate that pre-adipocytes and their neurogenic derivates display basal ECOD and EROD activity, and that this latter is expressed at lower levels in neurogenic derivate cells than pre-adipocytes. Both activities are induced in BNF-treated cells and neurogenic derivates showed higher levels of ECOD than pre-adipocytes. EROD and immunoblotting confirmed the presence and inducibility of the specific CYP1A1 protein. The salient side of our data is that these performed analysis confirm the usefulness of our models for verify increase of CYP1A protein and related enzymatic activities in neurogenic differentiating cells and in pre-adipocytes. Other relevant findings can be summarize in these points: 1) pre-adipocytes are available in large number from liposuction procedures and represent a source of human adult mesenchymal stem cells, by getting over ethical issues; 2) since pre-adipocytes can be differentiated to several different cell types, our innovative models may be useful for analyzing, on the same donor, the potential of xenobiotics on several cell-specific biological functions; 3) pre-adipocytes represent the reservoir of lipophylic compounds, i.e. PAH, and so we propose this *in vitro* model for analyzing effects related of unintended exposure to contaminants; 4) neurogenic derivatives, from pre-adipocytes, can be used for studying the first steps of the morphological and functional fetal-embryonic development.

# TOWARDS THE USE OF ORGANOTYPIC MICROTISSUES FROM PRIMARY HEPATOCYTES AND STEM CELLS FOR EARLY TOXICOLOGY ASSESSMENT

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Cell-based assays represent powerful and indispensible tool to assess substance efficacy and safety. Primary and stem cells are used more frequently to assess substance toxicology. However, current standard cultivation technologies negatively impacts cell functionality. We present a new production format which allows the formation of microtissues using either mouse embryonic stem cells or primary rat and human hepatocytes.

The Embryonic-Stem-Cell Test (EST) is a cell based assay to evaluate embryotoxicity of substances and belongs to the portfolio of ECVAM validated *in vitro* assays. However, the current protocol is time consuming and labor-intensive mainly due to manual transfer of EB's into an adhesive 96-well plate. In this study the HD-method for EB production was adapted to a novel top-loadable HD-plates in a 96-well format for a reliable and more efficient EST-process. The process eliminates the requirement of plate inversion enabling the implementation into an automated process. EBs were grown for 5 days in the HD-plate to achieve complete ESC aggregation and sufficient size to induce cardiomyocyte differentiation of embryonic stem cells. Differentiation of EB culture in flat bottom plates displayed cardiomyocyte contraction efficiency of 88%±13% at day 10. We adapted the traditional manual HD method to develop a more efficient process using a top-loadable hanging drop culture format leading to approximately 80% time saving per 96-well plate. So far, hepatoxicity *in vitro* is assessed mainly on monolayer cultures of primary hepatocytes to study drug metabolism, enzyme induction and compound toxicity. In order to preserve liver-specific functions of hepatocytes over extended time periods and to provide a versatile platform for toxicology testing, we developed scaffold-free, organotypic rat and human liver microtissue models. The architecture mimics closely native liver tissue when co-cultured with non-parenchymal liver cells. Morphological characterization indicate tight cell-cell contacts, extensive glycogen storage and formation of bile canaliculi between hepatocytes. The rat liver microtissues show stable cell survival and CYP3A induction over 5 weeks in culture. Moreover, in the heterotypic cultures comprising Kupffer cells, indiosyncratic toxicology could be displayed the first time in an *in vitro* rat and human liver model.

**Session 4**

## Environmental toxicology

*Chairs*

Simonetta Gemma, Chiara Urani

# AMBIENT PARTICLES IN *IN VITRO* TOXICOLOGY: SOURCES, COMPONENTS AND MECHANISMS INVOLVED

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Ambient particles consist of a wide variety of components including minerals, organic and inorganic compounds, microbial and other biological components and carbon. All of these can contribute to health effects of particles. For an effective abatement strategy the knowledge of which sources and components contribute the most to the observed effects, is of great importance. In the interest of susceptible subpopulations the knowledge of how these effects may be exerted could help to mitigate pathological processes. Inflammation seems to play a very important role in these processes. Though inflammation is difficult to study *in vitro*, the production and release of cytokines that are involved in inflammation, are important biomarkers of a response. Also, mechanisms for how all these effects are elicited, provide useful information, not the least with respect to susceptibility issues. However, these responses need to be verified in *in vivo* studies. Particles from diesel exhaust and wood burning are able to elicit inflammatory responses. Our studies different types of cell cultures (lung cell lines, co-cultures, primary lung cells) indicate that the organic fraction of particle extracts is most important for the cytokine release. Residual organic compounds on the washed particles seem to elicit the induction of CYP 1A1, although at much lower concentrations than the induction of cytokine release needs. Preliminary data indicate that the cytokines are primarily induced by a subfraction of the organic extract that contains organic compounds. The presence of small amounts of microbial components may strongly enhance the responses through the dual pathway trigger of the inflammasome response. An important question is whether the different components trigger widely different pro-inflammatory responses, qualitatively and quantitatively. The selected components commonly found in PM were ultrafine carbon black (ufCB), ZnCl2, FeSO4, 1-nitropyrene (1-NP), lipopolysaccharide (LPS), and crystalline silica. Screening of the responses and important signalling pathways involved indicates surprisingly similar responses, qualitatively and quantitatively, though differences are observed. The predominant response appeared to be increased gene expression of neutrophil-recruiting CXC-chemokines, expecially CXCL8. An important mechanism in the IL-8 response seems to be the TACE-, TGFα- and EGFR-linked pathway. In addition to the conventional cytokines investigated more complex *in vitro* systems may be used to study other markers of inflammation. In triple and two-cell cocultures VEGF and TGFβ release is observed. In addition, the cultures show increased levels of the long penthraxin PTX-3, which may have a function as a local, anti-inflammatory protein.

# USEFULNESS OF *IN VITRO* BIOASSAY IN PHOTODEGRADATION STUDIES OF AHR-ACTIVE POLLUTANTS

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Hydrophobic Organic Compounds (HOCs) were shown to cause many adverse effects in organisms; often via activation of Arylhydrocarbon Receptor (AhR). At various levels, they are present in all environmental matrices, thus, it is almost impossible to avoid the exposure of the organisms to these chemicals. HOCs can be degraded and removed from the environment by many processes, such as volatilization, adsorption to solid-phase particles, leaching, bioaccumulation, biodegradation, chemical oxidation or photooxidation.

The aim of this study was to assess changes of both AhR-mediated activity and concentration of benzo[a]pyrene (B[a]P), dibenz[a,h]anthracene (DB[a,h]A), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and a mixture of these three pollutants in the water solution after UV-VIS irradiation by a medium pressure mercury lamp. Samples were irradiated in Pyrex tubes (eliminating wavelengths below 300 nm) at concentrations 37 µg/L B[a]P, 0.5 µg/L DB[a,h]A, and 4.8 ng/L TCDD; in HOCs mixture, concentration of each pollutant was one third. AhR-mediated toxicity was assessed using *in vitro* bioassay based on the H4IIE-luc transgenic cell line.

The H4IIE-luc cells are stably transfected with a DRE-driven firefly luciferase reporter gene construct; the transcriptional activation of which occurs in an AhR-dependent manner. The most potent known activator of AhR is TCDD, but a number of structurally diverse compounds have also been found to activate AhR. Simultaneously, chemical analysis of concentration of all pollutants was performed using GC/MS (GC/MS/MS for TCDD). As we expected, decrease of both AhR-mediated activity and concentration of DB[a,h]A and TCDD as sole compounds caused by irradiation was observed. Nevertheless, in case of B[a]P, *in vitro* analysis revealed formation of highly AhR-active photoproducts which was observed as an enhancement of AhR-mediated activity of aqueous B[a]P sample during first 12 hours of irradiation.

In the same time, concentration of B[a]P assessed by GC/MS decreased exponentially. In the case of HOCs mixture or mixture of HOCs with humic substances (as a natural compartment of aquatic environment), no significant enhancement of AhR-mediated

activity was observed; both AhR-mediated activities (*in vitro* bioassay) and concentrations (GC/MS) of all the pollutants decreased exponentially during the irradiation.

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# *IN VITRO* ENZYMATIC BIOASSAY FOR THE EVALUATION OF THE TOXICITY OF AQUEOUS MATRICES

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The aim of the present research is the application of a new *in vitro* bioassay (recently patented by the University of Salento, MI2008A008813, PCT/EP2008/064703) based on the measurement of the inhibition of carbonic anhydrase activity as screening tool for the general toxicity measurement of aqueous environmental samples (water, interstitial water, elutriates, percolates, waste waters, etc.). Carbonic anhydrase is a metalloenzyme catalyzing the reversible hydration of $CO_2$ in H+ and $HCO_3$-. It is a ubiquitous enzyme in bacteria, plant and animal kingdoms, playing a fundamental role in a number of physiological processes.

The proposed method consists in the measurement of the *in vitro* inhibition of the enzymatic activity when the enzyme is placed in contact with aqueous environmental matrices containing toxic substances. The extent of enzyme inhibition is proportional to the degree of toxicity of the sample.

The bioassay is sensitive to the main classes of environmental pollutants such as heavy metals (Cd, Hg, Cu, As), pesticides, PCBs, IPA. When the carbonic anhydrase test was applied to real samples, a significant correlation was observed between the results obtained with the *in vitro* test and data obtained with standardized tests based on the use of living organisms.

The proposed method represents a quick, simple, and low cost tool for rapid screening of the toxicity of aqueous environmental samples avoiding the use of living organisms.

# PARADOXICAL EFFECTS OF ENVIRONMENTAL CONCENTRATIONSOF ORGANOCHLORINES, LINDANE AND CHLORDECONE, ONCELLULAR ENERGY PATHWAYS IN HEPG2 CELLS

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Persistent environmental pollutants (pesticides, heavy metals...) and food contaminants are aserious public health problem. In fact many studies have demonstrated a link between theexposure to these contaminants and the prevalence of several diseases in regions that showhigh levels of contamination. Chlordecone and Lindane are organochlorine pesticides whichhave been used worldwide. Residues of these compounds are still found in rivers and foodstuffs even though their use is currently banned in most countries.The mitochondrion plays an important role in cell regulation and survival, mainly byproducing ATP which is the main source of cellular energy. This makes it a likely target oforganochlorine toxicity. There are several studies that assessed Chlordecone and Lindane toxicity. In most of thesestudies, the concentrations used are relatively higher than those found in the environment andplasma blood however effects of low concentration remain unknown.

In the present study, we investigated the effect of environmentally and clinically relevant lowconcentrations of Lindane and Chlordecone on mitochondrial function using the HepG2 cellline. Cells were exposed for 24 and 48 hours. The cell viability after these different exposure timeswas assessed by the MTT assay was. Parameters that are in a link with mitochondrial functionwere assessed: cellular respiration was analyzed by polarography and the intra cellularATP/ADP was extracted from cells and quantified by HPLC. Nitric oxide and superoxideanion release were evaluated by electronic paramagnetic resonance using the spin-trappingmethod.

Our results show that even if low concentration of both toxics had no effect on cell viabilityand oxidative stress; however, they both increased nitric oxide production. Interestingly, toxics had paradoxical effects on cell respiration. Indeed, while Lindane decreased uncoupledrespiration and the enhanced maximal respiration capacity of cells, Chlordecone had no effecton uncoupled respiration and impaired the maximal respiration capacity. Moreover, bothtoxics increased ATP production to the same extent. Our study highlights an important feature: cell function and regulation can be altered byxenobiotics without affecting cell viability. Chlordecone and Lindane, at low concentrations, induced changes in the metabolic pathways of HepG2 cells suggesting a link between thedevelopment of metabolic diseases and the exposure to pesticides in a manner that involves mitochondrion OXPHOS alterations. Further studies to investigate this process *in vivo* maylead to preventive and curative strategies to xenobiotics contamination.

# FUMONISIN B1- INDUCED INFLUENCE ON FUNCTIONAL PARAMETERS OF HUMAN INTESTINAL CELL LINES IN RELATION TO DIFFERENTIATION STAGE

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Fumonisin B1 (FB1) is a mycotoxin considered as food contaminant because often present in cereals and the human gastrointestinal tract represents its important target. This study aims to investigate the modifications induced by FB1 on two human intestinal lines (HT-29 and Caco-2) at different stage of differentiation. In relation to the modifications induced by FB1 on sphingolipid pathway, the inhibition of cell proliferation (by MTT test), the induction of oxidative stress (by determination of GSH content) and Transepithelial Electrical Resistance (TEER) were selected as functional parameters. A dose- (from 0.5 to 69 μM) and time-dependent (up to 72 hrs) experiments were carried out on undifferentiated (HT-29) and differentiated (Caco-2) intestinal cells, after assessment of differentiation stage by enzymatic activity (alkaline-phosphatase content) and TEER determinations. Toxic effects induced by FB1 were related to differentiation stage of intestinal cells. Concerning the MTT assay, the differentiated Caco-2 cells resulted more sensitive reducing the 30% of cellular proliferation after 24 hrs exposure to 4.3 μM of FB1. Instead, the reduction of 13-22% was observed with undifferentiated HT-29 cells after 72 hrs exposure to 8.6 and 17.2 μM by using high (500,000 cells/ml) and low (100,000 cells/ml) cellular density, respectively. Concerning the induction of oxidative stress, the determination of GSH content was assessed by using monochlorobimane probe. Pro-oxidant activity, observed as significant reduction of GSH amount (almost 13%), was found on HT-29 cells after 72 hrs incubation with FB1 up to 8.6 μM. Preliminary experiments on Caco-2 cells showed pro-oxidant activity trend at lower FB1 concentrations after 48 hrs. Concerning TEER assessment, the damage of cellular integrity induced by FB1 was linked to differentiation stage: in particular TEER values on Caco-2 cells were reduced (almost 15%) up to 60 min exposure only at highest FB1 concentration, albeit not significantly. Instead, on HT-29, the influence of FB1 on cellular integrity was time and dose-dependent. Studies are in progress in order to evaluate a time-course localization of FB1on two intestinal cell models by using fluorescence microscopy.

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**Session 5**

## Nanotoxicology

*Chairs*

Isabella De Angelis, Pilar Prieto

# NANOTOXICOLOGY: RELATING PHYSICOCHEMICAL CHARACTERISTICS OF NANOMATERIALS TO BIOLOGICAL EFFECTS

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There are thousands of different types of engineered nanomaterials under development, with currently over 1300 consumer products containing nanomaterials on the market (http://www.nanotechproject.org/). Such nanomaterials vary in composition, size, surface area, shape, charge, crystal structure, solubility, strength and electrical conductivity. Due to their wide-spread exploitation it is important to assess the potential hazards associated with intentional and incidental exposure of humans. This is also important because the extra-ordinary characteristics that make these materials so interesting for industrial use also influence their biological reactivity.

The ability of particles to induce cellular responses such as oxidative stress, intracellular signaling and pro-inflammatory gene expression have been related to their size, surface area and charge.

Nanomaterials such as carbon nanotubes with a fiber like shape (or high aspect ratio) have been shown to induce inflammatory and pathological changes that are similar to pathogenic fibres such as asbestos, while relatively low toxicity materials such as $TiO_2$ have been shown to modify their toxicity according to their crystal structure.

This presentation will explore in more depth how physico-chemical characteristics can influence the biological responses to nanomaterials. In addition, the presentation will discuss how modifications in protocol can also influence the response obtained.

For example, we have demonstrated that modification of the dispersants used to add the nanomaterials to the culture system can greatly influence pro-inflammatory cytokine production by macrophages and hepatocytes exposed to Au nanoparticles.

# NANOTUBES OF THE ALUMINOSILICATE IMOGOLITE EXHIBIT MILD TOXICITY FOR MACROPHAGES AND AIRWAYS EPITHELIAL CELLS

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Among nanomaterials, Carbon Nanotubes (CNT) and, possibly, other High Aspect Ratio Nanoparticles (HARN), are a cause for concern due to their asbestos-like morphology. HARN impair airway barrier function and are toxic to macrophage lines.

Since airways represent the first barrier for inhaled particles, the effects of novel nanomaterials on the cells of the Lung-Blood Barrier (LBB) should be investigated. In view of a correct conduction of toxicological studies on the pathogenicity of HARNs, there is also an urgent need to identify appropriate reference materials acting as positive and negative controls made up by particles of similar size and shape.

Nanotubes of Imogolite (INT), a hydrated alumino-silicate with the formula $(OH)_3Al_2O_3SiOH$, could be envisaged as a possible negative control for HARN toxicity studies. Given the very little information available on INT toxicity, we investigate the effects of INT on the barrier properties and on LBB cells.

INT were synthesized via sol-gel procedure and found organized into fibers at FESEM. As *in vitro* models of LBB cells, we used two murine macrophage cell lines (Raw264.7 and MH-S) and the human airway epithelial Calu-3 cells. Cell viability was assessed with resazurin. RT-PCR was used to study the expression of *Nos2* and *Arg1*, markers of, respectively, macrophage classical or alternative activations, and concentration of nitrites in the culture medium was adopted as an indicator of NO production. Epithelial barrier integrity was evaluated from the Trans-Epithelial Electrical Resistance (TEER).

Compared to MWCNT and SWCNT, INT caused much smaller effects on macrophage viability and no significant damage was observed up to 40 $\mu g/cm^2$ of monolayer for exposure times up to 24h.

The incubation of macrophages with INT at doses as high as 120 $\mu g/cm^2$ for 72h did not alter either *Nos2* or *Arg1* expression nor increased NO production. Whereas a prolonged incubation with SWCNT or MWCNT progressively impaired the barrier properties of airway epithelial cells in a dose-dependent way, Calu-3 cells monolayers exposed to INT did not show significant change in permeability and lowered TEER. Cell viability, evaluated with resazurin method in the same monolayers, was not significantly affected by any nanotube.

In spite of their fibrous nature, INT appear not markedly toxic for *in vitro* models of LBB cells and could represent a low-toxicity reference for *in vitro* toxicological studies on HARN, should further tests confirm their inertness.

# CHARACTERIZATION AND INVESTIGATION OF CYTOTOXIC AND GENOTOXIC EFFECTS OF ZINC OXIDE NANOPARTICLES IN *IN VITRO* HUMAN INTESTINAL CELL LINE (CACO-2)

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Increasing industrial and medical use of Zinc Oxide (ZnO) has led to growing concerns about its safety, requiring a careful evaluation of its potential toxic effects. In this study ZnO cytotoxicity and genotoxicity was estimated in Caco-2 cell line that maintains the morphological and functional characteristics of small intestine lining. Initial efforts of the study were devoted to thorough physical characterization of the nanoparticles (NPs) in terms of size, surface area, number particles, agglomeration state, surface charge, chemical composition, impurities, by Dynamic Light Scattering (DLS), electron microscopy (SEM and TEM), Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

Mean sizes of ZnO measured by DLS were 340 nm for NPs suspended in ethanol and 942 nm for particles in culture medium. Primary size of particles detected by TEM was 45-170 nm, larger than those declared by the manufacturer (50-70 nm). Particles formed large aggregates of an average diameter, estimated by SEM, of 199 nm and 128 nm in ethanol and culture medium, respectively. Particle surface was negatively charged.

Cell viability was measured by Neutral Red Uptake assay. A dose-dependent decrease of cell viability was observed in presence of ZnO NPs already after 6 h of treatment. The presence of Fetal Calf Serum (FCS) strongly reduced Zinc toxic effects, probably through its interaction with NP surface. Colony forming efficiency test confirmed a toxic effects of ZnO at 6 hours of treatment and a reduced toxicity in presence of FCS in culture medium.

Level of intracellular Reactive Oxygen Species (ROS) was measured by 2,7-Dichlorofluorescein Diacetate (DCFDA) dye. A dose dependent ROS increase was detected after both 6 and 24 hours treatment. Oxidative DNA damage was evaluated by HPLC detection of 8-oxodG in Caco-2 cells treated for 2, 4, 6 and 24 hours. A significant increase of oxidated guanine levels was detected at 6 hours of treatment.

Genotoxic effects were evaluated by cytokinesis blocked micronucleus assay. Cells were treated with 1-5 μg/cm2 of ZnO for 6 or 24 hours in absence of FCS and further cultured for 24 hours in presence of FCS and 4.5 μg/ml cytochalasin B. A dose dependent reduction of cell proliferation and an increase of micronuclei were observed at both treatment time.

Our results highlighted cytotoxic and genotoxic effects of ZnO NP in this cell line, mainly mediated by oxidative damage. However, a direct interaction of ZnO with DNA or other macromolecules affecting chromosome integrity cannot be excluded.

# EFFECTS OF NICKEL OXIDE NANOPARTICLES ON HUMAN LUNG EPITHELIAL CELLS

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Human exposure to nanoparticles (<100 nm) is inevitable since nanotechnology continues to improve its widely use. Epidemiological studies suggest an association between increased risk of respiratory diseases and inhalation exposure to dust containing nickel ions. Nevertheless till now nickel oxide nanoparticles (NiO-NPs) have received little attention from scientific community and their effects on human health have been poorly investigated.

NiO NPs (Sigma Aldrich) were characterized with Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) to estimate their real dimension and behaviour in liquid media. The ζ-potential of the NPs was also evaluated. Cell viability, pro-inflammatory response and DNA damage of NiO dose (20-40-60-80-100 μg/ml) were evaluated in BEAS-2B and A549 cell lines exposed for 2, 3, 6 and 24h.

NiO NPs presented a tendency to agglomerate/aggregate in aqueous solutions and the ζ-potentialmeasurements confirmed this evidence. NiO NPs produced a significant decrease in cell viability in both cell lines and induced an increase of IL-6 and IL-8 release. The interleukin release pathway involved two MAP-kinases: the phosphorylated forms of p-38 and JNK increased after 2h of treatment. The phosphorylation of NF-kB and IkB-α were evaluated after treatment with the dose of 100 μg/ml. NiO exposure induced Rab5 (early endosomes) increase after 2h of treatment in A549 cells; Rab7 levels (late endosomes) remained augmented at 24h of treatment. BEAS-2B did not present endocytic markers. NiO NPs induced a significant increase of phospho-ATR and phospho-ATM in both cell lines. Immunohistochemistry had shown a translocation of phospho-ATR into the nuclei. Cell cycle profile, analysed with flow cytometry at 24h of treatment, evidenced a G2/M phase arrest in A549 cells and a G1 phase arrest in BEAS-2B cells.

These results demonstrate the cytotoxic, genotoxic and pro-inflammatory potential of NiO-NPs, as already reported for Ni2+ ions. Additional studies are required to investigate the effects induced by ambient or workplace human exposure, and to verify if such NPs may be really involved in pulmonary diseases.

# ASSESSMENT OF THE *IN VITRO* TOXICOLOGICAL PROFILE OF AMORPHOUS SILICA NANOPARTICLES AND STUDY OF THEIR INTRACELLULAR FATE

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Amorphous silica nanoparticles ($aSiO_2$NPs) are currently used in many industrial applications such as glass production, cosmetics, dentistry and telecommunications, and are FDA-approved animal feed additives. In addition, due to their low or absent toxicity, $aSiO_2$NPs are candidate tools in biomedical applications such as drug-delivery, gene-therapy and cancer diagnosis.

The toxicological profile of 15-35-85 nm-sized $aSiO_2$NPs was assessed *in vitro* on Balb/3T3 immortalized mouse fibroblast studying cytotoxicity, genotoxicity and carcinogenic potential by Colony Forming Efficiency assay, Micronucleus test and Cell Transformation Assay, respectively. Neither cytotoxicity nor genotoxicity and carcinogenic potential were observed under our experimental conditions. We have then investigated the uptake of 85 nm-sized $aSiO_2$NPs fluorescently-labelled with Tris (2,2′-bipyridyl) dichlororuthenium (II) hexahydrate ($aSiO_2$-$Ru(bipy)_3$NPs). The internalization has been studied by fluorescence microscopy and at different exposure times (6-72h). We have observed that $SiO_2$-$Ru(bipy)_3$NPs are easily internalized by Balb/3T3 mouse fibroblasts, thus suggesting that although NPs are internalized, they are not able to exert any effect, and therefore these particles might represent an useful tool for future biomedical applications. In addition, our current results are the starting point for future experiments to specifically address the pathway that is implicated in the internalization of these specific nanoparticles.

**Session 6**

## Reproductive toxicology

*Chairs*

Francesca Caloni, Marisa Meloni

# DEVELOPMENT OF A BATTERY OF ALTERNATIVE TEST FOR REPRODUCTIVE TOXICITY

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The reproductive cycle is an integrated physiological process that encompasses a number of complex steps involving the development of female and male gametes, with their supportive somatic cells, and the long process of embryo development bringing to the establishment of pregnancy and ending up with the birth of the offspring.

Due to this complexity and to the many cell types and physiological mechanisms involved, it is clear that any alternative testing approach for reproductive toxicity must rely on a large number of different assay systems.

The development of such alternative strategy has been pursued within the European integrated project REPROTECT in which more than 20 different *in vitro* assays have been developed aiming to predict adverse chemical effects on male and female fertility, on implantation, or on embryonic development. The variety of targeted toxicological endpoints included estrogen and androgen receptor activity, oocyte maturation, oocyte fertilisation and formation of female and male pronuclei, blastocyst formation and hatching, growth and morphology of rat embryos, embryonic stem cell differentiation.

Within the course of the project over 150 testing chemicals have been selected amongst well known reproductive toxicants. Given the different toxicological endpoints of each assay a limited overlap of common chemicals were used by the test developer laboratories. In the final year of the project the 14 most promising tests were challenged with a battery of 10 chemicals, selected by a panel of independent experts, upon blind conditions for potential adverse effects on the reproductive system in order to obtain an indication on the complementarity of the tests used in the battery. The biological effects of the active chemicals were revealed by the testing battery with relatively high accuracy.

This study provides the first proof of principle that complementary *in vitro* assays can correctly predict chemical effects relevant for reproductive toxicity testing.

# THE INTERFERENCE OF POLLUTANTS WITH THE BIOSYNTHESIS OF ACTIVE ANDROGENS AND ESTROGENS IN FISH

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There is now clear evidence that numerous xenobiotic compounds act as Endocrine Disrupters (EDs) in fish by affecting reproductive functions. Some of these compounds exert their action by binding to steroid receptors, and several well established *in vitro* methods can assess this mode of action. However, few *in vitro* methods are available to detect non-genomic mechanisms of ED action. This study aims at the application of alternative *in vitro* tools to assess the interference of synthetic chemicals with key enzymatic activities involved in the synthesis and metabolism of active steroids in fish. The enzymatic pathways selected for the study were (a) testicular synthesis of oxy-androgens, (b) ovarian synthesis of estradiol, and (c) sulfation and glucuronidation of active hormones. The method is based on the use of gonad and liver subcellular fractions from different fish species, viz. carp –*Cyprinus carpio*– and sea bass –*Dicentrarchus labrax*– and allows a first screening of those compounds with a high potential to alter fish reproduction by inhibiting the synthesis and metabolism of active androgens and estrogens. A wide range of environmental pollutants, including pharmaceuticals, synthetic musks, organotin compounds, alkylphenols and Polycyclic Aromatic Hydrocarbons (PAHs) were investigated. Regarding the synthesis of oxy-androgens, *in vitro* assays were performed by incubating testes mitochondrial fractions with labelled precursors (17α-hydroxyprogesterone and androstenedione) in order to assess the activity of the CYP17 and CYP11β enzymes, involved in the synthesis of androstenedione and 11β-hydroxy-androstenedione, respectively. Among the tested xenobiotics, hydroxylated PAHs had a significant inhibitory effect on both enzymes, being 9-hydroxy-phenanthrenethe strongest inhibitor of CYP17 (IC50: 10.8±4.2 μM) and CYP11β (IC50: 31.3±5.2 μM), followed by nonylphenol (IC50: 100 μM for CYP17), the polycyclic musks, galaxolide and tonalide (IC50: 213-225 μM for CYP17), and the pharmaceuticals, fluvoxamine and fluoxetine (IC50s: 321-335 μM for CYP17; 244-550 μM for CYP11β). Interestingly, 9-hydroxy-phenanthrene also inhibited ovarian P450 aromatase (CYP19) activiy (IC50: 4.3 μM). Triphenyltin, tributyltin and nonylphenol inhibited the sulfation of estradiol (IC50s: 17, 18 and 41 μM) and the glucuronidation of testosterone and estradiol. Overall, these *in vitro* methods allow the detection of selected chemicals that by interfering with these key enzymatic pathways might finally affect physiological processes, such as gamete growth and maturation in fish.

# THE HERBICIDE GLUFOSINATE AMMONIUM IS A WEAK ANDROGEN-LIKE COMPOUND AS EMERGED BY A NOVEL *IN VITRO* APPROACH TO IDENTIFY CHEMICALS WITH A PROSTATE-MEDIATED EFFECT ON MALE REPRODUCTION

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Although prostate function is critical for male fertility, so far prostate was an overlooked target in reproductive toxicity assays. Within the EU integrated project ReProTect, the LNCaP human prostate cell line was used as a model system to investigate chemicals affecting prostate epithelium functionality by means of a tiered approach integrating two different toxicological endpoints: cell viability and PSA secretion. Both toxicological endpoints have been measured using well established and widely used commercial assays with the aim to implement them in *in vitro* reproductive toxicology. A training set of five well-known (anti)androgenic chemicals and a ReProTect feasibility set of ten blinded chemicals were used. Obtained results showed that several compounds reduced PSA secretion only at cytotoxic concentrations whereas the androgens DHT and MT markedly increased PSA secretion as did, unexpectedly, the herbicide glufosinate ammonium/GA, not yet known as an androgen agonist. On the other side, the anti-androgens 2OH-flutamide, linuron, vinclozolin, di-n-butyl phthalate also increased PSA secretion, but the magnitude of their effect was much lower than for androgens. The ER-binder bisphenol A/BPA also reduced PSA secretion, while increasing cell viability. Overall, our approach properly discriminated androgenic compounds as well as yielded no false positives, as based on available toxicological evidences. The unexpected results obtained with GA deserves further attention since it was known only to affect embryo implant via impaired glutamine biosynthesis. Indeed, GA does not bind the androgen receptor/AR and, as we shown here, recognize only the mutated ART877A present in the LNCaP cell line, resembling the already known selective interaction of BPA toward the LNCaP-mutated ART877A.

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# ANTIESTROGENIC, ANTIANDROGENIC AND DIOXIN-LIKE ACTIVITIES OF NIGHT AND DAY AIR SAMPLES FROM BANJA LUKA REGION

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Air pollution has been connected mainly with induction lung and heart diseases. However, recent studies indicate that the air pollutants could act also as Endocrine Disruptors (ED). The research of endocrine disruption caused by air-pollution focused mainly on the compounds associated with air Particulate Matter (PM). Recently, it has been shown that considerable part of endocrine disruptors is present also in the Gaseous Phase of the air (GP).

Thus, we assessed the potential of both particulate and gas phase fraction of air pollutants from Banja Luka Region (Bosnia-Herzegovina) to produce specific biological effects *in vitro*. The samples have been collected at residential part of Banja Luka, a former pulp mill areal at Banja Luka and a background locality nearby National Park Kozara. Different sets of samples have been taken during day and night-time. The sample extracts were assessed for priority chemical pollutants and specific effects using *in vitro* bioassays. Dioxin-like activity, antiandrogenicity and estrogenicity with antiestrogenicity were assessed using H4IIE-*luc* (rat hepatocarcinoma), MDA-kb2 (human breast carcinoma) and MVLN (human breast carcinoma) reporter gene cell models, respectively.

The results have confirmed the endocrine disruptive potential of the air samples. The potential was connected both with PM and GP fraction of the air. Interestingly, night-time samples from residential site showed higher dioxin-like effects than their corresponding day-time samples. On the other hand, at background site and industrial locality, dioxin-like toxicity displayed opposite trend in GP fraction but PM fraction effects did not differed between night and day. Estrogenic effects of the samples were not significant but PM fractions were antiestrogenic. The lowest antiestrogenic effects were elicited by both day and night samples from background site and day sample from residential site. The greatest effects were produced by day and night samples from industrial site. Antiandrogenicity was observed after exposure of both GP and PM samples from all localities. The results show utility of usage of biotests in assessment of specific effects of complex environmental samples.

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**Session 7**

## Multilevel approaches in toxicology

*Chairs*

Francesca Caloni, Marisa Meloni

# TOXICOLOGY MODEL BUILDING AND BIOMARKER DISCOVERY BASED ON "OMICS" DATASETS

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Information on the carcinogenic potential of chemicals is primarily available for primary High Production Volume (HPV) products. Because of the limited knowledge gain from routine cancer bioassays and the fact that HPV chemicals are tested only there is the need for more cost effective and informative testing strategies. Here the application of advanced genomics to a cellular transformation assay to identify toxicity pathways and gene signatures predictive for carcinogenicity is reported.

Genome wide gene expression analysis and qRT-PCR were applied to untransformed and transformed mouse fibroblast Balb/c 3T3 cells that were exposed to either 2, 4-diaminotoluene (DAT), Benzo(a)Pyrene (BaP), 2-cetylaminoflourene (AAF) and 3-Methycholanthrene (MCA) at IC20 and different time points, respectively. Bioinformatics was applied to define toxicity pathways and gene signatures predictive of the carcinogenic risk of these chemicals.

Based on published data and the MTS assay IC20 conditions for the *in vitro* carcinogenicity assay were defined. While bioinformatics revealed distinct differences for individual chemicals at the gene level pathway analysis identified common perturbation that resulted in an identification of 13 genes whose regulation in cancer tissue had already been established. Strikingly, this gene signature was identified in short term untransformed and transformed cells therefore validating the predictive power of this gene signature.

The testing strategy identified commonly regulated carcinogenic pathways in an *in vitro* carcinogenicity assay. A gene signature was defined that predicted the risk for carcinogenicity. The results warrant validation of the assay.

# NOVEL MULTICOMPARTMENTAL BIOREACTOR SYSTEMS FOR PROBING MULTIPLE PATHWAY TOXICITY AND DRUG DEVELOPMENT

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Cell culture is the work horse of biologists, toxicologists, tissue engineers and a whole host of research fields in both academia and industry. Having explored individual molecular mechanisms inside cells for decades using traditional cell culture techniques, researchers have only just begun to appreciate that the intricate interconnectivity between cells and cellular networks as well as with the external environment is far more important to cellular orchestration than are single molecular events inside the cell. For example many questions regarding cell, tissue, organ and system response to drugs, environmental toxins, stress and nutrients cannot possibly be answered by concentrating on the minutiae of what goes on in the deepest recesses of single cells. To address this gap, we have developed new models based on modular or connected culture bioreactors to investigate cellular cross talk between different cell types and to construct complex *in vitro* models. I will present data on a new *in vitro* model of glucose and lipid metabolism as well a model of hepatotoxicity and discuss how we can design and use new bioreactor modules to construct more complex models of absorption, metabolism and exchange for probing multiple pathway toxicity and drug development.

# GENE EXPRESSION IN HUMAN CELL MODELS APPLIED FOR SAFETY ASSESSMENT OF CHEMICALS

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In modern society, one cannot imagine life without personal care and life style products that mostly contain chemicals. However, chemical consumption may be associated with certain risks for human health, such as the potential to induce sensitization which affects over 20% of the adult population. The European Union, with the rest of the world in its footsteps, aims to avoid these risks by enforcing its legislation on safety assessment of all existing and newly produced chemicals. To date however, the only available validated assays to screen the sensitizing potential of substances are directed to skin as a target organ and are animal-based. Consequently, the sacrifice of more than 1.8 million animals is expected. For respiratory sensitization, no validated assays are even available at the moment.

Our research aims at ensuring chemical safety through development, validation, and implementation of alternative, *in vitro* tests for assessment of potentially sensitizing chemicals using an animal-sparing and mechanistically relevant approach.

In the past decade we developed a generic approach to construct predictive *in vitro* models. Firstly, as a model we usually choose a relevant and preferably human cell type: e.g. primary human $CD34^+$ progenitor derived dendritic cells (skin sensitization) and the human bronchial epithelial cell line BEAS-2B (respiratory sensitization). Further, with respect to the measurement technology we prefer gene expression. In a first screening stage, we use a set of reference compounds and we apply the microarray technology from which we extract without prior bias candidate gene markers. These markers are challenged in a next stage with new compounds in which we also switch to the RT-qPCR technology. Finally, the surviving gene markers are combined in a predictive mathematical model by performing e.g. a discriminant analysis. Through this approach one ends up with a limited number of markers for which the mechanistic relevance can be assessed experimentally. Being human in origin, these models might help to explore the underlying cellular and molecular mechanism of sensitization and consequently may be employed in human risk assessment or efficacy studies.

Currently we are also investigating the possibility to employ these predictive gene markers in more challenging types of exposures (e.g. gaseous substances, nanoparticles or chemical mixtures).

By applying *in vitro* cell-based models to assess the skin and/or respiratory sensitizing potential of substances, we contribute to chemical hygiene without animal suffering.

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# CO-CULTURE OF INTESTINAL AND HEPATIC CELLS AS A MODEL TO STUDY ABSORPTION, METABOLISM AND TOXICITY OF DIETARY RETINOIDS

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*In vitro* cell culture models have been proved extremely useful to study molecular mechanisms of transport, metabolism and toxicity of nutritionally or pharmacologically relevant molecules. Among their limitations, however, is that single cell types are cultivated isolated from other cells that *in vivo* are in constant close physiological interplay. To reproduce *in vitro* a co-culture model of transport and metabolism in small intestinal enterocytes and hepatocytes, we used the human adenocarcinoma Caco-2 cell line as intestinal cell model and two different hepatic cell models: murine 3A cells and human HepaRG cells.

To test the performance of this model we have investigated dietary retinoids transport and metabolism, that *in vivo* involves differentiated enterocytes of the small intestinal mucosa and liver hepatocytes for secondary metabolism and storage.

In co-culture experiments, Caco-2/TC7 were grown and differentiated on filter inserts for three weeks, then transferred to culture wells with 3A or HepaRG cells seeded on the bottom of the culture wells and maintained in co-culture for 24 hours. During the co-culture period β-carotene or retinol were added to the apical side of the intestinal cells. Functionality of the co-culture model was assayed by different parameters: Caco-2/TC7 monolayer integrity, expression of specific genes involved in retinol metabolism (BCMO1, CYP26A1), and the retinol-dependent secretion of RBP4 from the hepatocytes. We found that both retinol and β-carotene added to the apical medium of Caco2/TC7 cells induced a reduction in the intracellular levels of RBP4 in underlying hepatocytes, indicating that transport and metabolism had occurred in both cell types. We also measured the induction of CYP26A1 mRNA in human HepaRG, which is strongly regulated by retinoic acid in hepatocytes, as an alternative endpoint to test the performance of this co-culture model.

This *in vitro* model has shown to be a promising tool to analyse the absorption and metabolism of retinoids and could further be extended to investigate transport and metabolism of molecules of toxicological and pharmacological interest.

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**Poster session**

# P 1.1 DIFFERENCES BETWEEN MC-RR AND MC-LR IN GSH CONJUGATION BY RECOMBINANT HUMAN GLUTATHIONE-S-TRANSFERASES (GST)

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Microcystins (MC) are a group of ≈80 congeners of toxic cyclic heptapeptides produced by different cyanobacteria differing from each other for amino-acids substitutions in specific positions.

The accepted pathway for MC detoxication and excretion is GSH conjugation. The GSH adduct formation can occur spontaneously at alkaline pH and enzymatically, catalysed by GSTs, both *in vitro* with cell from aquatic organisms or *in vivo* in rodents, fish and mussels. Recently the adduct formation between MC–LR and GSH with human recombinant GSTs has been demonstrated *in vitro*. In the range of MC-LR concentrations tested (0.25-50 μM), GST-P1 showed a temperature-dependent sigmoidal allosteric curve, GST M1 and A3 were linear whereas GST T1 and A1 showed a typical saturation curve. The efficiency score was T1>A1≅M1≅A3>>P1 (0.075-0.0064 pmol GSMC-LR (μg prot min μM)$^{-1}$). In particular where MC-LR has a leucine residue in position 2; the substitution of leucine with arginine as in MC-RR caused a ten-fold difference in the acute toxicity of the two congeners (50 vs 500 μg/kg in mouse for i.p. $LD_{50}$ values)

To investigate possible differences in detoxication, the GSH conjugation catalyzed by human GSTs recombinant with MC-RR was investigated. The GSMC-RR adduct produced spontaneously at alkaline pH, was identified by DAD-HPLC and confirmed by LC/MS/MS and used as standard for the calibration curve to quantify the adduct formation. The experimental conditions used were selected in order to minimise the influence of the chemical reaction on the enzymatic formation of the conjugate. In the range of MC-RR concentrations tested (2.5-100 μM) the GST isoenzymes (M1, T1, P1, A1 and A3) demonstrated to produce significantly higher amounts of adduct compared with MC-LR. GST T1, A1 and A3 showed a typical saturation curve, whereas GST P1 and A3 were linear. The obtained biochemical kinetic parameters ($V_{max}$, $K_m$, $CL_i$ and kcat) indicate as efficiency score T1>>A1≅P1>M1>A3 (0.161- 0.057 pmol GSMC-RR (μg prot min μM)$^{-1}$). Kinetic differences in detoxification between MC-RR and MC-LR, exists, and if these data obtained with recombinant enzymes will be confirmed with human liver cytosol, were all GSTs (not only those tested here) are simultaneously, the information could contribute in explaining differences in toxicity among congeners.

# P 1.2 THE RELEVANCE OF BIOKINETIC DATA IN THE EVALUATION OF IBUPROFEN TOXICITY IN HEPATIC CELLULAR MODELS FOLLOWING REPEATED EXPOSURE

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In *in vitro* studies, the toxicity of a compound is usually related to the nominal rather than to the actual concentration to which cells are exposed which also changes over time, due to uptake into the cells, adsorption to the plastics, binding to medium components, evaporation, hydrolysis, and last but not least biotransformation.

In the frame-work of the EU project-PREDICT-IV, a tiered strategy was developed to identify the *in vitro* biokinetic parameters and determine the actual level of exposure to selected test compounds following repeated treatments in different hepatic cellular models (i.e. primary rat and human hepatocytes, HepaRG cell line). In the present study results obtained with Ibuprofen (IBU) will be presented.

Cells were treated for 13 days with IBU at 2 non cytotoxic concentrations. On the first (day0) and the last (day13) day of treatment cell lysate and supernatant samples were collected at five different time points and IBU content was quantified. The IBU stock solution and the time0 culture medium were also tested and set as starting point for quantification. A procedure was set up to measure adsorption to the plastics or physical sequestration by Matrigel and stability in the medium was checked: results indicate no IBU loss due to “abiotic” process.

Following the time course of IBU content in primary rat hepatocytes, on day0 a rapid (2 min) and progressive uptake into the cells was noted, corresponding to a proportional decrease in the supernatant at both concentrations. After 24 hours IBU intracellular content was dramatically decreased: the low mass balance suggests the occurrence of IBU biotranformation. At the low concentration, different kinetics were observed at day 0 and 13 suggesting a potential of IBU to accumulate into the cells. Preliminary experiments performed with HepaRG and primary human hepatocytes, evidenced in both human models a slower uptake and some differences in IBU kinetic when compared to the rat model that should be confirmed.

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# P 1.3 USE OF *IN VITRO* SKIN AND EYE IRRITATION/CORROSION DATA FOR REGULATORY AND INDUSTRIAL PURPOSES

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Considerable progress took place within the last years regarding the international regulatory acceptance of alternative test methods to animal testing. In particular, the adoption of the skin irritation test method based on reconstructed human epidermis models by the EU, now allows, in conjunction with the previously adopted *in vitro* test methods for skin corrosion, the full replacement of the animal test for assessing acute dermal irritation & corrosion. The same test guideline was later adopted at the OECD level as TG 439 but the replacement of the animal test at the international level still depends upon country-specific regulatory requirements.

In the area of eye irritation and corrosion, four OECD test guidelines have been accepted at the OECD level (TG 437 on the BCOP test; TG 438 on the ICE test; and two draft TG on the cytosensor microphysiometer and fluorescein leakage assays). All of these assays allow for the identification of ocular corrosives and represent as such partial replacements to the animal test. Furthermore, the cytosensor microphysiometer test method also allows for the identification of non-classified water-soluble surfactants and surfactant-based formulations.

Based on such progresses non-animal data shall now be used in the EU as a full replacement to determine skin irritation and corrosion hazards, and as a partial replacement to determine ocular corrosion hazard. This results into new approaches of hazard assessment with no animal data which in turn opens a number of practical questions regarding the industrial and regulatory applicability of such *in vitro* data. In particular the following issues were raised:

- how to best construct and assess suitable testing strategies;
- the applicability of adopted *in vitro* tests to assess mixtures, preparations and dilutions;
- the information requirements which are critical for the assessment of the *in vitro* data;
- the applicability of *in vitro* data to specific national regulatory requirements.

To address some of these issues, scientists in Europe coming from both industry and regulatory bodies are working together in order to find harmonized agreements and recommendations on the best way forward. An overview of these current challenges and how they are being handled with will be presented.

# P 1.4 *IN VITRO* ESR MEASUREMENTS: POWERFUL TOOL TO STUDY TOXIC EFFECTS ON CELLS

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Electron Spin Resonance (ESR) spectroscopy is a highly efficient technique to access a wide range of information on unfavourable effects caused in cell by chemicals or drugs.

ESR in spin labelling is useful to study the membrane, and in particular to detect the change in lipid bilayer organization induced by drugs. Recently, our team developed a method to quantify the lipid bilayer effective microviscosity of membrane and applied this method to investigate on the fluidity effect produced bypropofol. More recently, we described the effect of the randomly methylated beta cyclodextrin (Rameb) on both liposomes as membrane model and colon carcinoma cell line (HCT-116). In particular, rameb was proven to influence the cholesterol concentration in the inner part of membrane that is known to play a vital role for signal transduction and protein sorting.

ESR in spin trapping is used to both identify and quantify the Reactive Oxygen Species (ROS) in cells. With an ESR study on human colon carcinoma cell line we highlighted the cytotoxicity of the photosensitizer pyropheophorbide-a methyl ester. In fact, after photoexcitation of this dye, superoxide anions, hydroxyl radicals and singlet oxygen have been detected using the intracellular located spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN).

ESR is also one of the most sensitive method for measuring cellular oxygen consumption. We will show our studies on the alterations of oxygen respiratory in human renal tubular cells treated with an endotoxin (Lipopolysaccharide, LPS). The incubation of HK-2 cells with LPS elicited a lower consumption of oxygen suggesting a decreased cell metabolism.

# P 1.5 MONITORING OF CICLOSPORIN A KINETICS IN KIDNEY, LIVER AND CNS CELLS *IN VITRO*

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The major goal of the FP7 project Predict-IV is to develop strategies that will help to improve assessment of drug safety in the early stage of development and late discovery phase. After the establishment of appropriate kidney, liver and CNS *in vitro* systems, representing target organs mostly affected by toxic reactions, the dynamics and kinetics of cellular responses to toxic effects of already well described drugs will be characterized. Kinetic processes have an enormous influence on the amount of a drug actually reaching its biological target and being responsible for the pharmacological action as well as for the onset of adverse drug reactions, and therefore seem to be an explanation for the differences between results obtained *in vivo* and *in vitro*.

The immunosuppressant Ciclosporin A (CsA) has been chosen as test compound due to distinct nephro-, hepato- and neurotoxic effects in transplantation patients as a result of overdosage. For evaluation of CsA *in vitro* kinetics, kidney, liver and CNS cells have been treated repeatedly with low and high doses for 14 days. Because of its high sensitivity and selectivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was the favored analytical method to determine the actual compound concentration in cells and corresponding supernatants. First results showed a broad spectrum of kinetic characteristics of CsA - from strong accumulation (more than twofold in kidney tubular epithelial cells and threefold in primary rat hepatocytes, respectively) to high metabolism (almost 100% conversion in hepatoma cells). Analysis in neuronal cells showed neither an accumulation potential nor metabolic activities.

*In vitro* kinetics data of CsA will be used in combination with advanced pharmacokinetic modelling with simultaneous consideration of possible individual metabolic variability and omics findings to develop an integrated modelling approach for prediction of *in vivo* drug effects in humans.

# P 1.6 HUMAN HEPATOCYTES IN THREE-DIMENSIONAL CULTURE ON INNOVATIVE BIOPOLYMERIC SCAFFOLDS AS AN USEFUL SYSTEM FOR *IN VITRO* TOXICOLOGY TESTS

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Many innovative biomaterials have recently be developed as scaffolds to replace physiological matrix components and their improvement has led to significant advances in culture techniques in terms of cell survival, quantitative expansion, maintenance of differentiated phenotype and specific cell functions. A key point in achieving these goals has been to maintain a three-dimensional culture and the typical cyto-architecture of the tissue by improving the extracellular matrix geometry and by promoting cell-cell contacts and reciprocal adhesions. These bio-artificial systems represent a real hope as functional substitutes for damaged organs and tissues and have provoked a great interest in the field of regenerative medicine. Concerning hepatocyte cultures, since the liver is the main organ involved in detoxification processes and in the defence of organisms against harmful molecules, in addition to their biomedical applications, these systems can be utilized as invaluable tool for toxicology tests for analyzing the effects on metabolism of new drugs, or for screening potentially toxic substances. The aim of our research was to identify the most suitable biomaterial for the technological applications with hepatocytes. Since the possibility to improve the performance of these systems depends strongly on the methods used to create the scaffolds, here we analyzed porous matrices made of gelatin or blends of gelatin and glycosaminoglycans, obtained with different methods for the culture of the C3A cell line, considered a good model of human hepatocytes. Scaffolds were obtained using either a concentrated emulsion-templating technique known as High Internal Phase Emulsion (HIPE) or a gas foaming technique; the latter method uses an inert gas instead of the internal liquid phase toluene, avoiding the use of organic solvent and allowing the creation of scaffolds with larger pores and interconnections. Cell viability was analysed using MTS and LDH assays; ultrastructural morphology and three-dimensional cell organization into the scaffold were assessed by SEM; albumin and urea secretion, as the main metabolic markers of hepatocyte functions, were monitored using, respectively, an ELISA kit and a colorimetric assay. Finally Cytochrome P450-3A4 activity was quantified by a luminescent method. Values of activity of this important enzyme of the detoxification system, obtained in the absence or in the presence of specific inducing molecules, were compared between the different culture conditions.

# P 1.7 *IN VITRO* EVALUATION OF THE SENSITIZING CAPACITY OF TEXTILE COLOURANTS

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The triphenylmethane dye malachite green is manufactured both as chloride and oxalate salts. It is used to colour synthetic fibrers, silk, leather and paper products. At the moment, malachite green is classified as non sensitiser based on the Magnusson and Kligman test. Two old German papers however, reported occupational sensitization to malachite green. Concern about possible sensitization properties must be carefully evaluated, also considering that the main use of malachite green is to color materials in direct contact of human skin.

The overall objective of this work was to implement a testing strategy for assessing the sensitizing potential (hazard) of colorants using *in vitro* methods. The testing strategy was based upon a selection of two *in vitro* sensitization tests developed within the Framework Program (FP) 6 funded project Sens-it-iv (www.sens-it-iv.eu).

The NCTC2544 IL-18 assay was used to assess the effect of malachite green salts on IL-18 production in keratinocytes, and the THP-1 activation test was used to assess the effects on dendritic cells activation (CD86 expression and IL-8 release). In both models, preliminary experiments were conducted to establish the dose range based on cell viability (CV75 for the THP-1 assay and CV80 for the NCTC2544/IL-18 assay, where CV represents the Cellular Viability at 75 or 80% relative to vehicle treated cells). A CV80 of 0.25 μg/ml (0.68 μM for the chloride; 0.55 μM for the oxalate) was calculated for both compounds, and CV75 of 0.56μg/ml (1.5 μM) was calculated for the chloride and 0.59μg/ml (1.3 μM) for the oxalate.

Both salts induced a dose-related increase in intracellular IL-18 content, in CD86 expression and IL-8 results. Based on the results obtained, both malachite green salts resulted positive in both assays. Accordingly to the results, malachite green salts should be considered as potential contact sensitizers. On a molecular weight basis, the oxalate appears to be more potent than the chloride.

# P 2.1 HEPATOCYTE PRIMARY CULTURES (HPCS) AS AN USEFUL BIOASSAY TO CHARACTERIZE METABOLISM AND BIOACTIVITY OF ILLICIT STEROIDS IN CATTLE

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Cattle HPCs have been successfully used to investigate the metabolism and bioactivity of Illicit Steroids And Prohormones (ISAPs). Nevertheless, their usefulness as a multi-parametric screening bioassay has never been investigated so far. Thus, cattle HPCs were incubated with known ISAPs and submitted to investigations aiming to characterize: (a) their effects on mRNA levels of Drug Metabolizing Enzymes (DMEs) and related Transcription Factors (TFs); (b) on CYP3A28 apoprotein and catalytic activity; (c) the ISAPs metabolite profiling. HPCs were incubated with 100 μM Boldenone (BOLD), its precursor Androsta-L,4-Diene-3,17-Dione (ADD), Dehydroepiandrosterone (DHEA) or an association of BOLD:ADD (90:10 μM). ISAPs effects on 16 DMEs and 5 TFs mRNAs were measured, after 6 hrs of incubation, by qPCR; their effect on CYP3A28 apoprotein and catalytic activity, after 24 hrs of incubation, by immunoblotting and a Testosterone (TST) HPLC assay. Finally, the time-dependent (0, 3, 6 and 24 hrs) metabolite profiling of ADD, BOLD and ADD:BOLD was characterized by using a LC-HRMS technique. Cells exposed to DHEA showed a significant up-regulation of CYP2C9, GSTA1, DHEA-ST, 17βHSDII, CAR, PXR, RXRα and PPARα mRNAs. In contrast, ISAPs did not alter mRNA levels in BOLD-, ADD- or BOLD:ADD-exposed cells, except for a significant reduction of CYP1A1 mRNA in ADD-incubated hepatocytes. No effect of ISAPs was ever noticed on CYP3A28 gene and protein expression, while a significant inhibition of 6β, 2β and 16β-OHTSTase was observed in cells exposed to ADD and BOLD. Finally, the production of ADD and α-BOLD from BOLD as well as of α-BOLD and β-BOLD from ADD as main metabolites was demonstrated. Furthermore, several OH-ADD derivatives were detected, and 16-OH-ADD epimers were more abundant than 6-OH-ADD ones. As observed with other anabolic steroids, four mono-hydroxylated BOLD derivatives were isolated and tentatively identified. Cattle HPCs represent an useful *in vitro* model to study species-specific differences in xenobiotic metabolism and molecular mechanisms involved in DMEs expression and regulation, as well as a complementary tool for the screening of ISAPs abuse in cattle. If liver tissue collected at the slaughterhouse was useful to obtain viable HPCs, this *in vitro* model might reduce the use of experimental animals, according to the 3R's philosophy.

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# P 4.1 COMPARATIVE STUDIES ON THE DRUG METABOLIZING ENZYMES ASSOCIATED MODEL ACTIVITIES INTERACTIONS IN HUMAN HEPATIC MICROSOMES BY N-IN-ONE AND SINGLE SUBSTRATE ASSAYS

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The objective of this study was to compare the n-in-one assay, developed for high-throughput measurement of CYP-xenobiotic inhibitory interactions screening, with single substrate assay. The effects of eighteen xenobiotics on CYP-selective activities were determined in pooled human liver microsomes by both assays. The $IC_{50}$ values for inhibitors (concentration causing 50% reduction of control activity) were determined and the correlation analysis of the remaining activities at the highest concentration, 100 μM, was investigated. Briefly, the same inhibition values were obtained with both assays for CYP1A2, CYP2B6, and CYP2C19. Structurally related group of compounds were the most potent and extensive inhibitors towards CYP1A1/2 and CYP2B6 model activities in both assays. Potent interference between CYP2E1 model activity, chlorzoxazone-6-hydroxylation and the incubation mixture has been observed. The correlation study indicated that the remaining activity of CYP1A2 (melatonin-6-hydroxylation and 7-ethoxyresorufin-O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C9 (tolbutamide hydroxylation), CYP2C19 (omeprazole hydroxylation) and CYP3A4 (omeprazole sulfoxidation) in single substrate assay were significantly correlated ($P<.05$) with the remaining activities in cocktail assay. The correlation was significant at ($P<.001$) for CYP2C8 (amodiaquine N-deethylation) model reaction. In contrast CYP2D6 (dextromethorphan-*O*-demethylation), CYP2E1 (chlorzoxazone-6-hydroxylation) and CYP3A4 (midazolam 1'hydroxylation) model reactions showed no correlation between the two assays.

# P 4.2 COBALT-INDUCED ATROPHY IN CULTURED C2C12 MYOTUBES: A CYTOTOXIC AND MECHANICISTIC STUDY

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Cobalt can cause toxic health effects due to over-medication with cobalt-containing compounds, consumption of cobalt enriched foods/drinks, or to occupational exposure. In orthopaedic joint implants, cobalt may be released in ionic form or, after implant replacement, in form of particulate wear debris, resulting in local cell toxicity. The particles stimulate adjacent macrophage infiltration that destroys bone and soft tissue, increases cobalt blood levels, and causes neurological impairment. Beside neural cells, cobalt may act on many cell types leading to cell death by apoptosis and necrosis. The mechanisms of cobalt action concern DNA fragmentation and oxidative stress; mitochondria appear to be the main target of its toxicity. Moreover doses ≥200 μM $Co^{2+}$ mimic hypoxic stress condition, by enhancing the levels of hypoxia-inducible factor HIF-1α, a modulator of mitochondrial oxygen consumption.

Actually, cobalt side-effects on skeletal muscle are poorly investigated.In the present study, in order to mimic the effects produced by implant-released cobalt ions on skeletal muscle, we administered for up to 72 hours increasing concentrations of $CoCl_2$ (1- 400 μM) to *in vitro* skeletal C2C12 myotubes. We performed a detailed analysis of $Co^{2+}$-induced cytotoxic effects by MTT assay and cell apoptosis or necrosis detection. We evaluated then the myotubes integrity by morphologic and morphometric analyses, and the expression of a set of stress chaperones correlating to specific sub-cellular insults.

Within 24 hours of treatment with doses ≥10 μM $CoCl_2$, C2C12 myotubes significantly diminished cell viability. Long-term cobalt exposure (10-200 μM for 72 hours) clearly induced cell death mainly by necrosis, whereas apoptosis was detectable only after 72 hours of 100-200 μM treatments. Moreover, already after 24 hours of treatment, $CoCl_2$ produced a dose-dependent morphological impairment of myotubes (also at concentrations unaffecting cell viability) consisting on a progressive reduction in size, a process commonly defined as atrophy. Atrophy was also confirmed by the expression of a subset of hallmark signs, including the up-regulation of atrophy-related genes, such as Atrogin-1. After 24 hours of treatment and already from 10 μM $CoCl_2$, immunoblot analysis revealed a dose-dependent up-regulation of the following chaperones: HSP25, marker of cytoskeleton and membrane impairments, HSP70, index of oxidative stress; and GRP75 and GRP94, whose over-expression are related to mitochondrial and endoplasmic reticulum altered functions respectively.

These results suggest that Co2+ induces dose- and time-dependent atrophy and necrosis, and activates cell stress response in skeletal C2C12 myotubes.

# P 4.3 DIOXIN-LIKE AND ANTIESTROGENIC ACTIVITIES OF AMBIENT AIR SAMPLES FROM PASSIVE SAMPLING IN CENTRAL EUROPE REGION

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Outdoor air pollution represents one of the major problems in many countries. Airborne pollutants have been often associated with many adverse effects including endocrine disruption. This type of endpoints cannot be studied only by chemical analyses because the environmental samples contain very complex mixtures of chemicals and their overall biological effects result from interaction among the pollutants. Thus, it is necessary to employ bioassays to detect the real toxic effects of the mixtures.

In our study, we described specific biological effects of air samples from eight localities in Central and South Eastern Europe. Sampling sites were located in Serbia, Romania, Lithuania and Slovak Republic, each country described by two sites. The localities were chosen to include industrial, urban as well as background sites. Endpoints were assessed using five month composite samples from passive sampling campaign in 2006. Unlike active air sampling, passive air sample collection allows relatively cheap long-term monitoring even in areas without developed infrastructure. In our work, we selected two specific effects that have been described in several studies to be related to air contaminants and areassociated with the increased occurrence of diseases. Dioxin-like activity and estrogenicity with antiestrogenicity were assessed using H4IIE-*luc* (rat hepatocarcinoma) and MVLN (human breast carcinoma) reporter gene cell models, respectively.

The dioxin-like activity data indicate that samples from industry- and traffic-burdened sites produced greater dioxin-like activity than samples from background sites. The activity was elicited mainly by non-persistent compounds (more than 90% of the effect). Toxic equivalents calculated from chemical data on28 PAHs representatives indicate that these compounds were responsible for relatively low fraction of dioxin-like activity assessed with bioassay. This fact suggests that the assessed PAH representatives were not the most important dioxin-like compounds in the samples. We did not observed significant estrogenic potential in none of the samples. However, antiestrogenicity was detected in five of eight samples. Interestingly, this type of effect was produced also by some background-site samples.

Results demonstrate utility of *in vitro* biotests in assessment of specific biological effects of environmental air samples obtained by passive sampling.

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# P 4.4 EFFECTS OF THE DEOXYNIVALENOL ON HUMAN LUNG EPITHELIAL CELLS: CELL CYCLE ARREST AND INFLAMMATORY RESPONSE

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Toxicological effects of mycotoxins, secondary metabolites of moulds and responsible for mycotoxicoses in humans and animals, have been investigated mainly in relation to alimentary exposure. However, inhalation of mycotoxins may pose health risks as well for exposed categories of workers such as farmers and limited research has been done in this regard so far. Deoxynivalenol (DON), a trichothecene mycotoxin produced by Fusarium species, is a common natural contaminant of cereal grains and feedstuffs and it has been detected also in airborne dust. The aim of the work was thus to define the toxicity and the pro-inflammatory potency of DON in two lung cell lines. A549 cells are representative of the alveolar epithelium while BEAS-2B cell line of the bronchiole. Cells were exposed for 24 h to different concentrations of DON (10-1000 ng/ml). Cell viability was assessed by MTT and Alamar-blue assay, the pro-inflammatory potency was determined by the measurement of IL-6 and IL-8 release (ELISA) and the cell cycle by flow cytometry. DON resulted to be cytotoxic in both A549 and BEAS-2B epithelial cell lines. A549 cells were more responsive in term of release of cytokines while in BEAS-2B the pro-inflammatory effects of DON were not significant. However BEAS-2B cells exposed to DON (500µg/ml) were arrested in G2/M phase while no such effect was observed in A549 cells. The data here reported underline the importance to further improve the knowledge on the effect of DON, also in combination with other fusariotoxins, on the lung with particular regard to its ability to induce pro-inflammatory effects and cell cycle alterations.

# P 4.5 SEASONAL DYNAMICS OF SPECIFIC ACTIVITIES OF POLLUTANT MIXTURES IN POLLUTED RIVER-BASIN ASSESSED BY A BATTERY OF *IN VITRO* BIOTESTS

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Chemical compounds occur in the environment as complex mixtures that can act through specific modes of action in organisms. Instrumental analysis can be used to identify and quantify known pollutants; however, hazard evaluation based on chemical monitoring and known toxic potencies of compounds is complicated because other unknown contaminants or degradation products can be present in the mixture. Moreover, compounds may not exert only additive effects as usually assumed in this approach. On the contrary, *in vitro* bioassays in which specific biological effects are directly estimated are suitable tools to estimate total toxic potency of complex mixtures in environmental samples.

The present research focuses on contamination of a model river ecosystem in south-eastern part of the Czech Republic. Aim of our one-year long study was to characterize temporal and spatial variability of contamination and toxicity of complex mixtures in river compartments (sediments and water samples) collected from five sampling sites. A battery of *in vitro* bioassays based on transgenic cell lines was applied to assess the AhR-mediated (dioxin-like), anti/estrogenic and anti/androgenic activities of all samples. Our results revealed strong seasonal fluctuations of contamination especially in sediment samples and also spatial variability of contamination among the sampling sites. Further, specific activities measured in sediments and particles transported by the river (fresh sedimentary material) were much higher than those evaluated in aqueous phase, which may be attributed to pollutants associated with solid materials. *In vitro* biotests applied in this study were proven to be a useful model for environmental pollution screening.

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# P 4.6 CHARACTERIZATION OF THE ENVIRONMENTAL QUALITY OF TWO ESTUARINE AREAS (SOUTH PORTUGAL) BASED ON DIFFERENT *IN VITRO* BIOASSAYS

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Several studies have shown that fish cell lines are useful screening tools to evaluate the potential toxicity of aquatic pollutants. In this work we used the fish hepatoma cell line (PLHC-1) in order to characterize the cytotoxic potential and presence of CPY1A inducing agents in sediments collected from two estuarine systems in South Portugal, including areas affected by urban effluents and harbour activities as well as potential reference sites. Sediments were extracted with dichloromethane/hexane (1:1, v/v) followed by dichloromethane/ acetone (1:1, v/v) and the combined extracts applied on PLHC-1 cells to evaluate their cytotoxic potential by using Alamar Blue (AB) and Carboxyfluoroscein Diacetate Acetoxymethyl ester (CFDA-AM). About 60% of the extracts showed cytotoxicity when tested at 60 mg eQsed/mL. Exposure of PLHC-1 cells to non-toxic concentrations of the sediment extracts in the range of 0.1 to 60 mg eQsed/mL lead to a significant induction of 7-Ethoxyresorufin-*O*-Deethylase (EROD) activity after 24 hours exposure. Additionally, the ability of sediment extracts to induce the generation of reactive Oxygen Species (ROS) in PLHC-1 cells was assessed by using 2',7'-dichlorohydrofluorescein diacetate as a probe; those extracts being cytotoxic to cells lead to a 20 to 60% induction in ROS production. Overall, the study highlights the usefulness of PLHC-1 to assess the toxic potential of coastal sediments and the environmental risks for aquatic organisms inhabiting those areas.

# P 4.7 ROLE OF P53 IN HUMAN HEPATOMA CELLS (HEPG2) EXPOSED TO CADMIUM: *IN VITRO* AND *IN SILICO* APPROACHES

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Cadmium (Cd) is a widespread environmental contaminant that causes toxicity and accumulates in target organs such as kidney, and liver with a long biological half-life (20-30 years). Cd toxicity has been associated to the deregulation of cell homeostasis and interference with essential metals (such as $Zn^{2+}$, $Cu^{2+}$). The International Agency for Research on Cancer classified Cd and Cd compounds as human carcinogens (group I). Cd-induced carcinogenicity is likely due to multiple distinct mechanisms, but the debate is still opened to elucidate molecular aspects and pathways involved in the activity of this trace element. We applied several approaches to get insight into the mechanism of Cd carcinogenicity, by integrating *in vitro* and *in silico* methods, and focusing on p53 pathway. This zinc-finger protein is well known to regulate DNA damage-induced cell cycle arrest, triggering apoptotic responses to genetic damage.

Here, we measured p53 levels in the human hepatoma cell line HepG2, as model of target organ, exposed to low human relevant Cd concentrations that caused DNA single-strand breaks, as visualized by comet assay.

The micro array expression profile and the immunochemical analysis showed that p53 is not up-regulated even at Cd concentrations (2 and 10 μM) that triggers DNA damage.

In parallel, based on existing evidences of Cd interference with essential metals, the effects of the replacement of the $Zn^{2+}$ ion coordinated to the DNA binding domain of p53 with the $Cd^{2+}$ ion has been investigated by Molecular Dynamics simulations. Analysis of the trajectories shows that the replacement of Zn with Cd does not lead to major changes in the conformation of the domain, but only to subtle modifications in the region around the metal coordination site. These conformational changes significantly affect the formation of hydrogen bonds involved in the interaction between p53 and DNA, decreasing the protein/DNA binding energy compared to the *wild type* (zinc not replaced) domain. These results suggest that replacement of Zn with Cdmay reduce its ability to bind the DNA thus altering the transcriptional activity of the protein.

Taken together, our data suggest that the conformational modification of p53 along with a lack of up-regulation could contribute to the mechanisms of Cd-induced carcinogenicity. In addition, the integrated *in vitro* and *in silico* methods, herewith used, stress the importance of a multidisciplinary approach in the comprehension of complex mechanisms.

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# P 5.1 EFFECTS OF METAL NANOPARTICLES ON AIRWAY EPITHELIAL CELLS AND MACROPHAGES

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Airway epithelium is the first barrier encountered by inhaled materials and the interaction between them and the epithelial cells markedly influences the exposure outcome. On the other hand, interaction of nanomaterials with lung macrophages may elicit inflammatory responses or macrophage activation. Therefore, *in vitro* tests to evaluate the respiratory toxicity of nanomaterials are badly needed. Metal-based Nanoparticles (NP) are widely used, but their effects on airway epithelium and macrophages are incompletely characterized. To elucidate this issue, cultures of airway epithelial cells and macrophages were incubated with $CeO_2$, $TiO_2$ and Cu NP (mostly consisting of CuO) of comparable size.

The experiments with epithelial cells were performed with confluent human Calu-3 cells, grown on permeable filters, where they form a high-resistance monolayer, providing an *in vitro* model of airway barrier. The widely used murine line Raw264.7 was used as a macrophage model. Metal NP, obtained from industrial sources, were characterized under the conditions adopted for the biological tests. Epithelial barrier permeability was monitored measuring the Trans-Epithelial Electrical Resistance (TEER) and cell viability was assessed through the resazurin method. ROS production was assessed from 2',7'-dichlorofluorescein diacetate ($H_2$-DCFDA) fluorescence.

In the range of nominal doses from 10 to 100 $\mu g/cm^2$ of monolayer, CuO NP produced a marked decrease of TEER, when applied to apical side of the epithelial monolayer for 7 days, indicating that the permeability was increased and the epithelial barrier impaired. TEER decreased by 30% at 10 $\mu g/cm^2$, in the absence of effects on cell viability, and was abolished at doses $\geq$40 $\mu g/cm^2$, where the viability was completely suppressed. At the same doses, neither $CeO_2$ nor $TiO_2$ NP had significant effects on cell viability or TEER.

Exposure to CuO NP caused an increased production of ROS and loss in cell viability in subconfluent monolayers of Raw264.7 cells, whereas other NP were substantially inert. At equal nominal doses of Cu, CuO NP seemed endowed with higher toxicity than solutions of $CuCl_2$. These results indicate that (1) CuO NP exert marked toxic effects on both epithelial cells and macrophages; (2) CuO NP markedly increase airway barrier permeability even at relatively low doses; (3) on the contrary, $TiO_2$ and $CeO_2$ NP are poorly toxic for airway epithelial cells and macrophages and do not perturb significantly TEER. It is concluded that TEER measurements may provide a simple and rapid method to assess the toxicity *in vitro* of nanomaterials on the cells of the airway barrier.

# P 5.2 SIZE-DEPENDENT CYTOTOXICITY BY UPTAKE OF GOLD NANOPARTICLES IN BALB 3T3 AND THEIR GENOTOXIC AND CARCINOGENIC POTENTIAL EFFECTS

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Due to the increased presence of nanoparticles in consumer products and industrial applications, it is a compelling issue to study their potential risks for health and safety. Although much research is currently devoted to biomedical applications, potential toxicological effects pose fundamental limitations to the widespread use of nanoparticles. The aim of this study was to determine if differences in gold nanoparticles sizes might lead to different cytotoxicity effects and the mechanisms of gold nanoparticles cell interaction.

Citrate-stabilized gold nanoparticles of 5 and 15 nm were synthesized and characterized according to well-known protocols and tested on Balb 3T3 cell lines at different concentrations (10, 50, 100, 200 e 300 μM) for 2, 24 and 72h of exposure times.

Cytotoxicity was evaluated by Colony Forming Efficiency (CFE) assays and trypan blue test. Our experiments did not show toxicity at lower concentrations for both sizes of gold nanoparticles tested using the two tests, while significant toxicity was found for 5 nm nanoparticles at higher concentrations (greater than 200 μM) and at the exposure times of 24 and 72 h only by CFE.

Genotoxicity was assessed by Micronucleus Assay and cancerogenic potential by Cell Transformation Assay. Both tests demonstrated no genotoxic and no cancerogenic damage.

In addition, we verified that nanoparticles were up-taken by cells by Transmission Electron Microscope (TEM) and we quantified them by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis. The mechanisms of nanoparticles cell interaction was studied by Western Blotting and we observed that the uptake was not mediated by Clathrin as the protein expression did not change in the range of concentrations and exposure times used.

# P 5.3 A SIMPLE METHOD FOR TESTING THE TOXICITY OF NANOMATERIALS ON 3D AIR-LIQUID INTERFACE HUMAN AIRWAY EPITHELIA (MUCILAIR™)

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We developed a simple method to deliver nanoparticles to Air-Liquid Interface (ALI) culture systems. This patented method (PCT/IB2010/053956) uses Dextran as carrier, which allows testing a wide range of doses of nanoparticles. Briefly, the nanoparticles were diluted and mixed with the Dextran powder; small pellets were made and then applied onto the apical surface of the ALI culture. We tested the toxicity of several nanoparticles, such as ZnO and $Fe(IO_3)_3$, on an *in vitro* cell model of the human airway epithelium (MucilAir™). MucilAir™ closely mimic the morphology and functions of the normal human airway epithelium. Moreover, it has a unique shelf-life of one year, allowing chronic/long term toxicity testing. Using multiple endpoints, like Trans-Epithelial Electrical Resistance (TEER), cell viability assay (LDH), cilia beating frequency, morphology, cytokine release, etc, we determined the dose response curve of ZnO and $Fe(IO_3)_3$ nanoparticles on MucilAir™. Toxicity of ZnO (9 nm) was observed at doses higher than 0.1% (9 $\mu g/cm^2$). Interesting, at 0.1% of ZnO, the epithelia had the potential to recover/repair after the exposure, while at 0.5% (45 $\mu g/cm^2$) of ZnO, it was not the case. Effect of two forms of $Fe(IO_3)_3$ from 10 to 20 nm were also compared: namely spheroids and nanoneedles.

Our results showed that Dextran-carrier method is an easy and efficient way to deliver the nanoparticles *in vitro*. MucilAir™ is a relevant *in vitro* system for assessing the toxicity of the nanoparticles.

# P 5.4 TAILORED PREPARATION OF MODIFIED $TiO_2$ NANOPOWDERS FOR THE STUDY OF THE ROLE OF PHOTO-REACTIVITY IN THE TOXICITY TOWARD KERATINOCYTES

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Titanium dioxide ($TiO_2$) is one of the most promising products of nanotechnology.

$TiO_2$ generates large amount of oxygenated radicals when illuminated with UV light in aerobic and wet conditions. For this reason, $TiO_2$ has been proposed for the remediation of waste waters or as antibacterial and self-cleaning components in coatings and textiles. $TiO_2$ nanopowders are also employed as UV-filters in health care products and in polymeric composites. In this case the photocatalytic activity needs to be suppressed since free radical species may cause skin damage or degradation of the organic components of the cosmetic preparations or composite. Other applications of $TiO_2$ nanopowders include antifogging coatings and photovoltaic cells. A high number of $TiO_2$ nanopowders having distinct physico-chemical properties are therefore currently in commerce, or are expected to be introduced in the next future in the market.

Albeit considered in the past a highly biocompatible material $TiO_2$ raised some concerns on its possible toxicity when in nanometric size. The toxicity of $TiO_2$ nanopowders has been related to different physico-chemical features e.g. surface area exposed, crystalline phase, size and photocatalytic activity. However, a definite correlation among oxidative potential, structural properties and toxic effect elicited is still lacking.

In this study, a series of $TiO_2$ nanopowders purposely modified at the surface to modulate their oxidative potential, leaving unaltered crystalline phase, size and specific surface area, have been characterized by measuring the ability of generate free radicals and the capability to cause damage to lipids, proteins or DNA in the dark and under illumination by means of EPR spectroscopy/spin-trapping technique, UV/Vis spectrophotometry, SDS-page and DNA agarose gel electrophoresis.

To investigate whether the observed photo-activity would relate to the toxicological properties of the different $TiO_2$ powders the cytotoxicity and genotoxicity (oxidative damage) on human keratinocytes HaCaT cells have been evaluated.

# P 5.5 PHYSICO-CHEMICAL DETERMINANTS IN THE CELLULAR RESPONSES TO NANOSTRUCTURED AMORPHOUS SILICAS

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Exposure to crystalline silicashas been associated to silicosis, lung cancer, autoimmune and renal pathologies. On the contrary, amorphous silicas have been regarded so far as non-pathogenic. However, little attention has been given to the potential toxicity of the wide array of amorphous forms: silicas, in spite of their common and simple chemical composition, comprise a large variety of materials. The development of nanotechnology has raised new interest on silica-based materials, including amorphous silica forms, because of their employmentin nanomedicine. There is however concern that when in nanosize amorphous silicas might become hazardous.

In a study devoted to amorphous vitreous silica we have recently shown that both surface reactivity and cellular responses are quite similar to those from crystalline quartz dust, suggesting a potential toxicity of this form. Here we report a study concerning the role of preparation procedures (pyrolysis *vs* precipitation) and the effects of size on cell damage and induction of oxidative stress, upon exposure to nanostructured silicas, i.e. silicas made up by agglomerated/aggregated nanoparticles. Two pyrogenic silicas prepared in the same way but differing in particle size, and one mesoporous silica, prepared by precipitation and exhibiting particle size close to one pyrogenic form, have been compared to a commercial quartz dust widely employed in toxicological studies and to a commercial micrometric amorphous silica previously found inactive. Our results indicate that pyrogenic silicas are remarkably more reactive than the precipitated ones as to cytotoxicity, ROS production, lipid peroxidation, NO synthesis, and production of tumor necrosis factor-α (TNF-α). All the above features indicate that, in the present case, the origin of the dust is more important than particle size.

# P 5.6 USE OF MODEL SOLIDS TO TACKLE THE PREDICTIVE ASSESSMENT OF THE POTENTIAL TOXICITY OF INCINERATOR EMISSIONS

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Urban waste incinerators have the great advantage to produce energy but their use is still controversial as there is a general concern that the emission might cause damage to human health and the environment. Incinerator emissions are composed by a great number of compounds, both organic and inorganic, including inorganic particles in the micron and nano size range. A local multidisciplinary project - NANOSAFE - funded by local government of Regione Piemonte, Italy, focussed a part of its attention on the study of the physico-chemical properties and on the potential toxicity of the particulate fraction of the emission.

Chemical composition, size distribution and morphology of emitted inorganic particles are influenced by the incinerator parameters. These properties may modify the biological activity of the particles. Taking into account that metals are present in the incinerator plants, the particles are not pure but often covered by these metals. It is known that the presence of metal contaminants at the surface of particles may modify the physico-chemical properties and consequently their toxic effects.

Considering the complexity of the emitted particles the first step is to have appropriate reference samples. The emitted particles are mainly made up by metal oxide, silica and carbon-based particles. Therefore, hematite, amorphous silica and carbon particles in different size have been synthesized and characterized. A detailed evaluation of the physico-chemical properties considered relevant for particle toxicity has been carried out and correlated with the potential to induce toxic effects on both lung cell lines (epithelial and macrophage cells) and central nervous system cell line.

# P 5.7 AN IMPROVED COCULTURE SYSTEM MIMICKING THE CELLULAR ORGANIZATION AT THE ALVEOLAR BARRIER TO STUDY THE POTENTIAL TOXIC EFFECTS OF PARTICLES

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Exposure to Particulate Matter (PM) and to engineered Nanoparticles (eNPs) has increased in the last century due to increased combustion processes, road traffic, etc. The exposure to small particles of less than 100 nm in diameter is linked with an increased risk for respiratory diseases, such as asthma or rhinitis. In addition it was shown that, due to their small size, NPs can also cross the alveolar epithelial barrier and affect the underlying cells by inducing oxidative stress. To date, more than 1000 customer products contain eNPs, despite the knowledge about their toxic potential and health effects is still poor. For instance, ultra-fine particles, such as NPs are also supposed to have detrimental effect on human health leading to aggravation of pre-existing diseases, like asthma.

Here we present a complex coculture system consisting of four different cell lines that should mimic the cell response of the alveolar surface *in vitro* after exposure to particles. The system is composed of an alveolar Type-II cell line, differentiated macrophage cells, mast cells and an endothelial cell line. Since the alveolar surface *in vivo* is also lined by a thin surfactant film, *in vitro* systems to study effects of particles should also consider this additional barrier. Surfactant plays an important role as it lowers the surface tension and facilitates particles displacement *in vivo*, but it also prevents the cells from drying. Cells of the 4 types were grown on microporous membranes in a certain ratio under submerged conditions and then cultivated at the air-liquid-interface to force the alveolar Type-II cells to produce the surfactant film.

First preliminary experiments, where hydrogen peroxide was used to induce oxidative stress, have shown that there are differences for the response of the coculture with four cell types compared to bicultures and monoculture. Bicultures with macrophages have shown a higher level of oxidative stress in comparison to the biculture with mast cells. The fourculture system has the highest response, showing a possible synergic effect.

The presented system will also be used to study the potential of NPs to induce oxidative stress and a potential inflammatory response. To provide a uniform exposure to particles a state-of-the-art aerosol chamber will be used to expose the cells under more *in vivo* like conditions than in a submerged culture. It is expected that the outcome of these experiments will contribute to our mechanistic understanding about particle-induced inflammation. Nevertheless this *in vitro* system is in an early stage and further improvement and standardization are still required.

# P 6.1 THE SILVIE PROJECT: AN INTEGRATED *IN SILICO-IN VITRO* APPROACH TO STUDY THE ENDOCRINE ROLE OF PESTICIDES ON HUMAN REPRODUCTIVE TISSUES

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The SILVIE project – "A tiered, integrated *in SILico-in VItro* approach to screen Endocrine interference in human reproductive tissues by environmental chemicals" - is a newly supported proposal by the French National Agency for Water and Aquatic Environments within the framework of "Plan Ecophyto 2018". The aim of the project is to study the effects of a dozen of pesticides/biocides/plasticizers belonging and representing different chemical classes of these chemicals whose role has endocrine active compounds/EACs, particularly as antiandrogens, has emerged in the last decades. Indeed, evidence suggests that prenatal and early- life exposure to pesticides/biocides may be causative factors in a variety of human disorders including a widespread decline in male reproductive health and disturbances of the developing endocrine system starting from the gestational period. Hence, within SILVIE project will be developed an integrated approach aimed to investigate *in vitro* the gene expression profiling of all 48 human nuclear receptors/NRs in two different reproductive tissues: the prostate and the placenta.

SILVIE will be performed in a stepwise manner with a strategy that simultaneously will: i) develop new QSAR models for quantitative predictions of potential EACs impinging on the 48 human NRs; ii) study their combined effects such as additive effects and antagonism; iii) obtain dose - response curves for both models of prostate and placenta, by checking the general cytotoxicity and the specific functional assays.

Overall, the project will develop alternative methods and deliver *in vitro* data about human cell lines which represent the two above mentioned functional tissues, prostate and placenta, amidst the most sensible and less analyzed targets, at molecular level, related to the environmental risks for human health.

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# P 7.1 EVALUATION OF THE POTENTIAL SKIN IRRITATION OF PLANT DERIVATIVES IN CELL CULTURES AND RECONSTITUTED HUMAN EPIDERMIS MODELS

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Information on potential skin irritation of bioactive botanicals is important for general safety assessment purposes, and the detection of acute skin irritation is included in international regulatory requirements for the testing. To assess the safety for topical use of *Melissa officinalis* (lemon balm), *Lannea microcarpa* (paudre de feuille), and *Kigelia africana*, theircrude extracts and major compounds were tested on cells (J774.A1, WEHI-164, HEK-293) grown in Monolayers (ML) or in reconstituted human epidermis (Episkin$^{SM}$ or RHE-SkinEthic$^{®}$). The 3D reconstitued tissues are validated replacement alternative to animal testing for primary skin irritation (OECD 439). The active have been assessed at the concentrations of 0.01-1.00% for pure compound and 1.00-10% for the extracts. On cell monolayer the actives were not cytotoxic (MTT assay). After exposure on Episkin, 15 min followed by 42 h of recovery, tissue viability by MTT test, release of pro-inflammatory cytokine IL-1α, and TEER Trans-Epithelial Electrical Resistance (TEER), measuring the efficiency of tight junctions related to barrier function were determined compared to PBS-treated epidermis. The viability was not significantly reduced; no increased release of IL-1α, expressed as pg/mL in the medium underneath, was observed in tissue treated with all products. The positive control SDS 5% impairing the barrier integrity of the epithelium caused a release of IL-1α release to 300 pg/mL and a reduction of -14 kΩ $cm^2$ in TEER values. Although some tested compounds possess a surfactantlike structure (triterpenes), they showed neither cytotoxic nor pro-inflammatory effects or affected TEER. On the basis of the absence of skin-irritant effects, extracts and compounds (triterpenes and rosmarinic acid from lemon balm, myricetin 3-*O*-α-L-rhamnopyranoside from paudre de feuille, and verminoside from Kigelia) appear to be safe for use in dermopharmaceutical formulations according to their traditional use and well established bioactivity. In conclusion, an integrated approach have been used to investigate complementary parameters of the irritation mechanism and to have a screening of the topical ingredients efficacy. *In vitro* tests using both monolayer cell cultures and 3D models offer the possibility of an integrated approach to drug discovery screening in botanical derivatives with interesting results i) concordance of MTT viability data obtained both from ML and RHE; ii) possibility to evaluate the cytotoxicity or cutaneous compatibility of both complex plant extracts or simple biomolecules at the same concentrations that could be used in topical formulations *in vivo*.

# P 7.2 EXPRESSION LEVELS AND INDUCIBILITY OF SELECTED CYTOCHROME P450 ISOFORMS IN TRIDIMENSIONAL AGGREGATING BRAIN CELL CULTURES AS *IN VITRO* MODEL TO MIMIC THE METABOLIC COMPETENCE OF BRAIN TISSUE

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Among the *in vitro* brain models, aggregating brain cells are considered as very promising model to study of organ-specific toxicity. Indeed, these 3D cultures, prepared from mechanically dissociated fetal brain and grown in serum-free chemically defined medium, closely represent the multicellular architecture, maturation, and functions of the *in vivo* brain tissue. However, no information are available on their metabolic capability. This is a relevant data gap, since brain P450 enzymes (CYPs) can metabolize *in situ* centrally acting drugs and neurotoxins. Brain CYPs are inducible: nicotine and lindane administration at low doses has been reported to induce P450 in rat brain *in vivo*.

The aim of the present study was to characterize the mRNA expression of a panel of CYPs in the control rat aggregating brain cells or after treatment for 4h, 24h and 48h with nicotine (50, 100, 200 and 800 μM), or for 24h and 48h with 10 μM lindane. Levels of mRNA expression of CYP1A1, CYP1A2, CYP2B1, CYP2B1/B2, CYP3A1, CYP3A2, CYP2E1, CYP2D2 and CYP2D4 were measured. RNA extracted from aggregating brain cell cultures was retro-transcribed into cDNA and analyzed by Real Time PCR using TaqMan or SYBR-green chemistry. The appropriateness of three housekeeping genes (18S RNA, Cyclophilin and Beta-actine) has been evaluated: cyclophiline has been selected for its stability.

CYP2B1, CYP1A1, CYP3A1, CYP2D2, CYP2D4 and CYP2E1 expression was clearly detectable both in controls and in nicotine/lindane treated samples, whereas levels of CYP2B1/B2, CYP1A2 and CYP3A2 were under the limits of detection. CYP450 mRNA relative content with respect to the total CYP mRNA in control 3D cells was higher for CYP1A1 (55%) and CYP2B1 forms (26%).

CYP1A1 and CYP2B1, the most abundant CYPs, were the only inducible isoforms: their expression were induced by nicotine or lindane in all samples (up to 20-fold for 2B1 and up to 2.5-fold for 1A1, with respect to controls) although no time-dependence nor dose-dependence of induction was observed.

To further characterize the system, the functionality of the expressed enzymes will be checked.

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# P 7.3 CONJUGATED LINOLEIC ACIDS (CLA) AFFECT BASAL INSULIN SECRETION IN THE RAT BETA CELL LINE RIN-38

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Conjugated Linoleic Acids (CLAs) are conjugated dienoic isomers of linoleic acid mainly contained in meat and dairy products. CLA supplementation is known to exert beneficial effects on human health, such as weight loss, anti-inflammatory and anti-carcinogeniic activities, but adverse effects have also been described (mainly hyperglycemia and insulin resistance).

Free fatty acids have been implicated in both positive and detrimental effects on pancreatic beta cell physiology: acute exposure leads to increased insulin secretion in response to glucose, while chronic exposure impairs the insulin response as well as beta cell viability. Such negative effects, collectively referred to as lipotoxicity, may be involved in the onset of type II diabetes in obese subjects.

Activation of signal transduction mediated by binding of medium and long chain fatty acids to the fatty acid G-protein receptor GPR40, highly expressed in beta cells, has recently been demonstrated to lead to increased insulin secretion. GPR40 was also shown to bind CLAs, thus suggesting a link between the adverse effects of CLA administration and insulin resistance. For all these reasons, GPR40 has received much interest as a potential key actor in lipotoxicity.

In the present study we sought to investigate the effect of CLAs on beta cell functions, focusing on the potential undesired side effects in response to CLA supplementation.

We employed the rat insulinoma cell line RIN-38, capable of glucose stimulated insulin secretion (GSIS) and representing therefore a suitable *in vitro* model to study beta cell function. RIN-38 cells were incubated with either CLA isomers or with linoleic acid for 4 and 48 hours, to assess the effect of these molecules on GSIS. Since pancreatic beta cells contain high intracellular concentrations of zinc and this metal is involved in the process of insulin storage and secretion, experiments were also performed under conditions of mild zinc deprivation or zinc supplementation. Intracellular localization of labile zinc ions was performed using the zinc fluorescent probe Fluozin-3.

Our results show that short-term fatty acid exposure (4h) does not affect GSIS in RIN-38 cells, while long-term incubation with linoleic acid or CLAs leads to increased insulin secretion only in basal (no glucose) conditions. As expected, cell staining with Fluozin-3 highlights strong fluorescence concentrated in vesicle-like structures. Neither zinc depletion nor zinc supplementation could affect GSIS in the experimental conditions tested.

These data indicate that CLAs can interfere with basal insulin secretion in pancreatic beta cells, implicating a potential increase of circulating insulin *in vivo* in the absence of glucose stimulation. Such an effect might explain the occurrence of insulin resistance reported in CLA supplementation studies *in vivo*. The role of the long chain fatty acid receptor, GPR40 is presently being analysed.

# P 7.4 CARDAM, CENTER FOR ADVANCED RESEARCH AND DEVELOPMENT ON ALTERNATIVE TEST METHODS

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CARDAM (http://www.cardam.eu/) was founded by VITO, in order to promote and develop new alternative methods for animal testing. CARDAM is a science-based centre of excellence offering profound expertise and innovative tools in the field of safety assessment. The CARDAM team of multi-disciplinary scientists performs R&D and does offer services for the implementation of test methods and strategies that limit animal use while supplying key toxicological information and guaranteeing compliance with high quality standards (GLP, GCCP).

CARDAM focuses to four activities: research on alternative methods in the fields of eye irritation, skin- and respiratory sensitization, embryo toxicity and developmental neurotoxicity. *In vitro* cell culture technology is combined with toxicogenomics, complex data mining and biological pathways analysis to set up *in vitro* screens and mechanism-based models to predict toxicity. The VITOSENS® model can distinguish skin sensitizers from non-sensitizers, and is based on gene expression changes in human cord blood derived dendritic cells. A similar approach is under development for respiratory sensitisation. In the area of reproduction toxicology, zebrafish embryo is used to detect embryotoxicity, early developmental and behavioural effects of compounds. CARDAM is also redesigning and optimizing the opacitometer for the bovine cornea opacity-permeability test (Grant from the Stavros Niarchos foundation).

Development and validation of new promising methods towards an industrial applicable method in line with internationally accepted procedures. CARDAM did participate in various international validation programs: e.g. pre-validation of haematopoietic colony forming assays, hER and hAR transformed cellular assays, 3 fish cell lines for acute aquatic toxicity, the SkinEthic 3D-eye irritation model and the slug mucosal irritation test. CARDAM is currently initiating an international multi-center pre-validation study for skin sensitisation with the VITOSENS®. We further participate in the ECVAM coordinated, COLIPA sponsored validation project on 3D-HRT-based eye irritation methods.

Testing service to industry to meet the regulatory requirements using an extended spectrum of validated methods for compound registration. For this purpose, CARDAM obtained its GLP accreditation. In addition, CARDAM fine tunes newly developed methods for screening purposes to support industry with selecting the most promising compounds.

Training and education on the use of alternatives for animal testing for scientists or representatives from industry and regulatory agencies.

# P 8.1 BIOLOGICAL EFFECT OF ORGANOSELENIUM COMPOUND ON HUMAN TUMOR COLORECTAL CELLS *IN VITRO*

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Colorectal cancer is among the most common cancers. Diet, its specific component, is one of the risk factor in the development of the disease.

Selenium as an essential trace element play role in enzymatic functions in organism, it is also important antioxidant. Selenium is involved in many biological processes as selenocysteine incorporated to enzymes.

In present, there are many studies, which are engaged in chemoprevention effect of selenium, which encompass stimulation of protective cellular mechanisms in control cells and inhibition of proliferation in malignant cells. Cellular and molecular effects of selenium compound depends on chemical form, dose and type of *in vitro* model.

The aim of our study is to examine the effect of organoselenium compound 1,4-phenylenebis(methylene)selenocyanate (p-XSC) on two colorectal carcinoma cell lines HT 29, which has mutation in gene p53 and APC gene and HCT 116 +/+ with wild type p53. Our results show first information about cytotoxic and growth-suppressing effect of p-XSC. Cytotoxic and growth-suppressing effect of p-XSC was measured by WST-1 assay, which indicate the activity of mitochondrial dehydrogenase. Cell proliferation was measured by EdU assay. The method is based on incorporation of thymidine analogue to DNA during its synthesis. Cell cycle progression and apoptosis were evaluated by flow cytometry,using propidium iodide,which binds to DNA, one dye per 5 base pairs of DNA. In all cases, cells were incubated 24 h with tested compound.

In future, we will focus on western blott analysis of selected apoptotic and proliferating markers.

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# P 8.2 AFLATOXIN $M_1$ *IN VITRO* PASSAGE INTOHUMAN INTESTINAL CACO-2/TC7 CLONE CULTURED ON SEMI-PERMEABLE INSERTS

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Aflatoxin $M_1$ ($AFM_1$), a hydroxylated metabolite of Aflatoxin $B_1$ ($AFB_1$), is excreted into the milk of all mammals.

Despite the potential risk of human exposure to $AFM_1$, data reported in literature on the metabolism, toxicity and bioavailability of this molecule is limited and out of date. The aim of this research is to investigate the absorption profile of $AFM_1$ and the damage it may cause to tight junctions of intestinal Caco-2/TC7clone cultured on microporous filter supports. These semi-permeable inserts allow for the separation of apical and basolateral compartments, corresponding to the *in vivo* lumen and the interstitial space/vascular systems of intestinal mucosa respectively.

Caco-2/TC7 cells were treated with $AFM_1$ concentrations (from 10 to 10,000 ng/kg) for short (40 min) and long periods of exposure (48h). $AFM_1$ influx/efflux transport and effects on tight junctions, evaluated by Trans Epithelial Electrical Resistance and tight junction protein (Zonula occludens-1 and Occludin) localization, were investigated.

The results of the experiments show that: i) $AFM_1$ is poorly absorbed by Caco-2/TC7 cells after exposure to both apical and basolateral compartments; its transport across the monolayer, however, occurs very quickly ($P_{app}$ 105±8 cm/s x $10^{-6}$); ii) the integrity of the tight junctions is not permanently affected by mycotoxin treatment. Viability impairment or barrier damage are not caused either. Further studies need to be conducted for an in-depth analysis of the passage of $AFM_1$ through this *in vitro* model.

# P 8.3 CHEMOSENSITIVITY TESTING OF HUMAN OVARIAN CANCER CELLS AND ITS *IN VITRO* MODEL

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Ovarian cancer is the most common cause of death from gynecological mallignities in Europe and North America, being the third most frequent and the first as to the mortality. Standard first-line chemotherapeutic treatment involves the administration of carboplatin, in advanced stages combined with paclitaxel. Introducing chemoresistance testing of human ovarian cancer cells may help to choose optimal drug and customize the individual chemotherapeutical regimens in patients. One of approaches of individualization of chemotherapy is *in vitro* chemosensitivity testing.

In our study, we evaluated the cytotoxic effects of six selected cytostatics - cisplatin, paclitaxel, carboplatin, gemcitabine, topotecan and etoposide - in cells isolated from ovarian tumours and ascites of individual patients.

Effects of selected cytostatics on cell viability were determined by MTT assay. In our group of clinical samples the highest sensitivity showed cells to topotecan, sensitivity to cisplatin was higer than to carboplatin and paclitaxel used in clinical practice showed the most often only the marginal reactivity. Resistance to carboplatin and most of the time to gemcitabine and etoposide was commonly present.

We realized the same *in vitro* chemosensitivity testing in a model system, human ovarian cancer cell line A2780. In these cells the highest sensitivity was repeatedly detected to topotecan, gemcitabin, paclitaxel and cisplatin. The lowest sensitivity showed A2780 cells to carboplatin.

# P 8.4 ONCASTATIN M: INDUCTOR OF PSORIATIC EPIDERMIS LIKE

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For several decades the research on genetic diseases has been taken a more and more important place in scientific research, in order to identify exactly the origin of these diseases. At present six to seven thousand diseases are identified.

Psoriasis is a badly known inflammatory skin disease, which gets one to three percent of world population, as well women as the men. This dermatological affection is driven by aberrant interactions between the epithelium and the immune system. Anti-psoriatic drugs can therefore target either the keratinocytes or the immunocytes.

In order to test new anti-psoriatic drugs on keratinocytes, we sought to develop an *in vitro* reconstructed skin model that would display the characteristics of psoriatic epidermis in a controlled manner allowing the screening of anti-psoriatic drugs and providing a model to study the biology of this disease.

Oncostatin-M (OsM) is a member of a cytokin subfamily that includes IL6, IL11, LIF, CNTF and cardiotrophin. These molecules are known to induce modifications during the maturation of *stratum corneum*, similar to that of a psoriatic epidermis.

Also we have studied the effect of OsM on our reconstructed human epidermis (RHE) developed with normal keratinocytes on defined medium at the interface air liquid. During the reconstruction, we applied OsM directly on medium to a final concentration of 5ng/mL, until the end of the tissue reconstruction. Then modifications were observed by histological staining and immune-histochemical analysis.

OsM triggered the hyperplasia of the keratinocytes layers leading to an increase in the overall of RHE: untreated tissue 80±5 µm and treated tissue 160±5 µm. In addition, we observed on the RHE induced by OsM a decrease of keratohyaline granules in the granular layer. This phenomenon was confirmed by the immune-histochemical analysis which shows a lack of filaggrin labeling. Consequently, the use of OsM inductor allows to obtain a relevant study model of psoriasis.

# P 8.5 VALIDATION STUDY ON THE OCULAR IRRITECTION® ASSAY FOR EYE IRRITATION TESTING

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A validation study was undertaken to obtain additional prospective data and assess the relevance and reliability of the Ocular Irritection® assay according to the OECD principles for validation and the ECVAM modular approach. The primary goal of the study is to evaluate the ability of the Ocular Irritection® test to reliably discriminate non-classified substances from classified ocular irritants (categories 1 and 2) as defined by the UN Globally Harmonized System for classification.

The assay is based on a macromolecular reagent that when rehydrated, form an ordered matrix mimicking the highly ordered structure of the transparent cornea. Irritant substances produce a turbidity of the reagent by changes in protein conformation and degree of hydration that mimics the disruptive effects irritants may have on the corneal proteins and carbohydrates. Because of its nature, Ocular Irritection® presents the advantage of having long shelf-life (years), and to be easily shipped around the world.

An international Validation Management Group was formed to manage and oversee the study conduct comprising independent chairman, co-chair, biostatistician and chair of the chemicals selection, in addition to the sponsor representatives (INT.E.G.RA and *InVitro* International) and the lead laboratory representative. A challenging set of 60 coded substances for which *in vivo* data are available were selected in collaboration with ECVAM and will be tested in 3 independent laboratories from Europe and the USA, including one naïve laboratory. The testing phase is planned from May to September 2011 after the training and transferability of the test method takes place.

# P 8.6 CHARACTERIZATION OF THE UNFOLDED PROTEIN RESPONSE (UPR) PATHWAY IN A NOVEL MURINE HEPATIC CELL LINE

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The effort to establish a differentiated hepatic cell line able to express *in vitro* the most important liver functions (such as the synthesis of specific serum proteins, the metabolism of carbohydrates and lipids, and the modification and excretion of endogenous and exogenous molecules) has long been pursued in several laboratories and different model systems have been described to date. Such *in vitro* systems are essential to test a wide panel of molecules with beneficial (nutrients, bioactive molecules) or detrimental (environmental pollutants, metals, toxins) effects on the organism, and to study the mechanisms that govern their absorption, metabolism, secretion and catabolism in the liver. Primary hepatocytes are still the closest *in vitro* model for the liver. However, they have scarce and often unpredictable availability, limited growth activity and lifespan, and undergo early phenotypic alterations. Conversely, hepatoma cell lines such as HepG2, despite representing a widely used model characterized by indefinite proliferative capacity, lack several important regulatory mechanisms and crucial liver functions.

In order to have an *in vitro* model that presents the morphological and functional characteristics of hepatocytes, we have characterized a new cell line (called *3A*), isolated from 14,5 dpc embryo of a wild type mouse strain that underwent spontaneous immortalization.

We have characterized 3A cells morphology by fluorescent localization of F-actin and β-catenin, the expression of specific genes and proteins essential to liver function, the capability to excrete molecules in extracellular spaces resembling functional bile canaliculi, the glycogen storage activity and the ability to control Retinol-Binding Protein 4 (RBP4) secretion in response to retinol deprivation. We have also characterized their response to the exogenous stress stimulus induced by Tunicamycin (TM) treatment, through the analysis of the Unfolded Protein Response (UPR) pathway.

3A cells display several features of hepatocytes. Furthermore, these cells activate the UPR following a typical stressful event, indicating that this model could be further exploited to investigate hepatic reaction to different injuries and toxic insults.

3A cells can be considered a useful hepatocyte model that preserves several important liver characteristics, particularly suitable for studies related to ER stress response.

# P 8.7 EFFECT OF ACETAMINOPHEN ON INTRACELLULAR GLUTATHIONE LEVEL AND REDOX STATUS IN HEPATOCYTES ISOLATED FROM STEATOTIC RAT LIVER

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Acetaminophen (APAP) overdose is the most common cause of acute liver failure in humans. The mechanisms participating on its toxic effect are glutathione depletion, oxidative stress, redox changes and mitochondrial dysfunction. Non-Alcoholic Fatty Liver Disease (NAFLD) is the most frequent chronic liver disease in the world. The pathogenesis of NAFLD is not completely understood, but accumulating data suggest that oxidative stress and altered redox balance play a crucial role in its pathogenesis. The aim of our work was to compare the effect of APAP on intact rat hepatocytes and hepatocytes isolated from steatotic liver in primary cultures. Male Wistar rats were fed by standard diet (ST-1, 10% energy from fats) and High-Fat Gelled Diet (HFGD, 70% energy from fats) for 6 weeks and then hepatocytes were isolated from liver by two-step collagenase perfusion. Isolated cells were cultured in William´s E medium in a gassed atmosphere (5% $CO_2$) on collagen-coated Petri dishes and 96-well plates. After cell attachment, APAP at concentrations of 1; 2.5; 3.75 and 5 mmol/l was added to culture media for up to 24 hours. The statistical significance was analysed using one-way ANOVA followed by Tukey-Kramer's post-hoc test. APAP causes more severe dose-dependent damage of steatotic hepatocytes (compared to non-steatotic cells) in primary culture as documented by increased release of Lactate Dehydrogenase (LDH) to culture medium and LDH leakage, decreased activity of cellular dehydrogenases (WST-1 test) and reduced albumin production (marker of hepatocytes function, ELISA). MDA production (TBARS) and ROS formation (DCFDA) were also significantly higher in steatotic hepatocytes treated with APAP. Intact steatotic hepatocytes contained lower amount of GSH ($p<0.05$, vs. control non-steatotic cells). Treatment with APAP (1; 2.5 and 3.75 mmol/l) caused more pronounced decrease in GSH in steatotic hepatocytes compared to non-steatotic hepatocytes. GSH-GSSG ratio was not altered in both control groups (ST-1 and HFGD), but was significantly decreased in the presence of APAP in steatotic hepatocytes using lower concentration of APAP (3.75 mmol/l vs. 5 mmol/l in non-steatotic cells).

Our results indicate that steatotic hepatocytes exert higher sensitivity to the toxic action of APAP. This sensitivity may be caused by lower content of GSH in intact steatotic hepatocytes and by more pronounced APAP-induced decrease in intracellular concentration of GSH, higher lipoperoxidation and higher production of ROS in steatotic hepatocytes.

*Acknowledgement: The work was supported by the Ministry of Education Research Project - MSM 0021620820.*

# P 8.8 *IN VITRO* INTESTINAL ABSORPTION PROFILE OF IBUPROFEN AFTER ACUTE AND REPEATED EXPOSURE

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In the field of pharmacological research the *in vitro* model of intestinal epithelium developed from Caco-2 cell line has been mainly used in acute conditions. The aim of acute treatments is a preliminary evaluation of the absorbed amount of a drug candidate. Different algorithms have been developed to build up prediction models for human *in vivo* fraction absorbed by comparing the behaviour in the *in vitro* absorption kinetics with those of reference compounds.

Repeated treatments, likely for drugs, have been poorly used for the characterization of Caco-2 model, although it would be interesting to study the influence of repeated treatment on absorption kinetics mimicking the actual way of exposure to a drugand possibly depending on the mechanism of absorption involved (passive, carrier-mediated etc.) and/or the compound characteristics.

The aim of this study was to set up an *in vitro* experimental protocol for intestinal absorption after repeated exposure and to check differences in the absorbed amount of Ibuprofen (IBU) as a model compound. Ibuprofen is known to be readily absorbed by passive mechanism through the intestinal epithelium *in vivo*.

Composition of the medium was firstly studied to find optimal conditions in order to avoid protein binding of the tested compound, interferences with analytical procedures and induction of cellular suffering. Non-toxic IBU concentrations after acute and repeated exposure (2 hrs, followed by 6 hrs of recovery for 3 consecutive days) and the absorption profile was determined. The results showed a progressive rising in the $P_{app}$ (Apparent Permeability Coefficient) values in the repeated treatment protocol, resulting in a two-fold higher value with respect to the acute exposure after the third treatment. The intracellular concentration was low (<0.2%), with a Mass Balance >90%, indicating no cell bioaccumulation after repeated exposure. By comparing the $P_{app}$ values obtained for IBU with reference compounds, the predicted *in vivo* absorption fraction was ≈80**%:** the estimate fits with the *in vivo* IBU oral absorption.

*The work has been partially supported by the FP7-EU funded Project Predict IV Grant n° 202222.*

# P 8.9 COMBINATION OF *SELAGINELLA DEODERLEINII HIERON* AND DOXORUBICIN TO INHIBIT PROLIFERATION OF T47D BREAST CANCER CELL LINES

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Conventionally, doxorubicin was used as a treatment for breast cancer. Apparently the results of recent studies indicate that breast cancer has begun resistant to the drug. Many researchers are developing the natural product for cancer prevention.

In Indonesia, people use *Selaginella deoderleinii Hieron* plants to treat cancer traditionally. The aim of the research is to scientifically prove that the potential of extracts increase the activity of doxorubicin to inhibit proliferation of cancer cell, espessialy breast cancer cell lines. This research used T47D breast cancer cell lines with AgNor counting method.

The results showed that the combination of ethanol extract of *Selaginella deoderleinii Hieron* dose of 10µg/ml and doxorubicin dose of 5 ng/mlwas able toinhibit cancer cell proliferation by 23% compared to control.

It can be concluded that the extract is effective to increase the doxorubicin activities in inhibiting T47D breast cancer cell line.

# P 8.10 ASSESSMENT OF ACETAMINOPHEN-GLUTATHIONE CONJUGATE TOXICITY IN ISOLATED MITOCHONDRIA

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***Introduction.*** Acetaminophen has been widely used as an analgesic and antipyretic drug. After overdose, it may cause centrilobular liver necrosis and acute liver failure; however, the particular causation of the cell death remains unknown. The toxicity of acetaminophen is likely related to glutathione depletion caused by a reaction with acetaminophen oxidation product rising acetaminophen-glutathione conjugate (APAP-SG). This compound has been considered as a detoxification product so far. However, the mail goal of our study was to estimate the possible toxic effect on isolated mitochondria.

***Methods.*** We used mitochondria from male Wistar rats and male ICR mice that were isolated by gradient centrifugation. To test APAP-SG toxicity, we synthesized the APAP-SG conjugate de novo, purified by HPLC technique and confirmed the purity (>99%) and structure by HPLC/MS. We assessed the toxic effect by measurement of mitochondrial ROS production (CM-DCFDA, Molecular probes, USA) and rate of respiration (high resolution respirometry, Oroboros 2K, Austria) at complex I and II.

***Results.*** We prepared pure APAP-SG conjugate that was used to evaluation of its toxicity. We proved that APAP-SG is able to cause mitochondrial impairment shortly after exposure. The Complex I and II dependent ROS production in 5 mM APAP-SG was increased by 900% and 400%, respectively, compared to control. In addition, we found dose dependent decrease of the mitochondrial respiration rate in the presence of APAP-SG. In the presence of 2 mM APAP-SG, the respiration was reduced to 43% and 20% in Complex I and II, respectively, in both rat and mice mitochondria.

***Conclusion.*** We proved our hypothesis that acetaminophen-glutathione conjugate is able to induce mitochondrial impairment.

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# P 8.11 *IN VITRO* PROTECTIVE ROLE OF SILYMARIN AGAINST ETHANOL INDUCED TOXICITY: A STUDY INVOLVING PRIMARY HEPATOCYTES

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***Background and aims.*** The availability of methods for isolation of large quantities of intact cells had made isolated hepatocytes culture a favorite experiment system for pharmacological, toxicological and biochemical research. Considering the anti-hepatotoxic property of silymarin, a well-knownanti-oxidant (flavonoid); we investigated the *in vitro* hepatoprotective effect of silymarin against ethanol induced toxicity.

***Methods***. The *in vitro* hepatoprotective activity of silymarin was carried on freshly isolated rat hepatocytes. The rat liver was perfused with HEPES buffer and collagenase and the hepatocytes were isolated. In the first step the liver is subjected to non-recirculating perfusion with calcium free buffer or with a calcium chelator like EDTA, causing irreversible separation of desmosomal cell contacts. In the second step liver is perfused with collagenase to dissolve the extra cellular matrix, calcium being added back to ensure maximal enzyme activity. This optimal treatment dissociates the liver completely within 10-15 mins, that is, sufficiently rapid to obviate the need for continuous oxygenation during perfusion. The percentage viability of isolated hepatocytes was determined by tryphan blue exclusion method. The isolated hepatocytes challenged with ethanol (60mM) and were exposed to two different concentrations of silymarin (20 and 40 µg/ml) and incubated at 5% $CO_2$ incubator for 24 hours at 37°C. Hepatoprotective effect of silymarin was studied with help of estimating the different biochemical parameters such as ASAT, ALAT, ALP and LDH.

***Results***. A significant increase in the levels of ASAT, ALAT, ALP and LDH ($P < 0.001$) was observed in hepatocytes exposed to ethanol when compared to normal untreated hepatocytes. These hepatocytes, when pretreated with the silymarin (20 and 40 µg/ml) showed a significant restoration of the altered biochemical parameters towards normal ($P < 0.001$, when compared to ethanol treated cells).

***Conclusion***. Together, these results identify the biological efficacy of silymarin against ethanol induced hepatotoxicity *in vitro*. Hence, silymarin merits further investigation to support its molecular mechanism.

# P 8.12 *IN VITRO* EFFECT OFFUMONISIN B1 ON PROLIFERATION AND STEROIDOGENESIS OF PORCINE GRANULOSA CELLS

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Fusariotoxins, including fumonisins, trichothecenes and zearalenone, produced by *Fusarium* spp. fungi, are common contaminants of cereal grains and feedstuffs that have been implicated in poor reproductive performance in pigs. However, direct ovarian effects of Fumonisin B1 (FB1) have not been reported.

To evaluate the effects of FB1 on porcine Granulosa Cell (GC) proliferation and steroidogenesis, GC from small porcine follicles (1-5 mm) were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 2 days in serum-free medium containing 500 ng/ml of testosterone, 30 ng/ml of FSH, and FB1 at various doses (0, 10, 300, 10,000 ng/ml) in the presence of either IGF1 (30 ng/ml), FGF9 (30 ng/ml) or both. Numbers of GC were determined at the end of the experiments using a Coulter counter and concentrations of progesterone and estradiol in culture medium were determined by radioimmunoassay. FB1 had no significant effect on cell numbers in control cultures, but at 10,000 ng/ml decreased ($P<0.05$) cell numbers in IGF1- and FGF9-treated cultures. In IGF1+FGF9-treated cultures, both 300 ng/ml and 10,000 ng/ml of FB1 decreased ($P<0.05$) cell numbers. After treatment with the highest dose of FB1 (10,000 ng/ml) a stimulatory effect on progesterone production was observed in IGF1-treated cells. In contrast, doses of 10,000 ng/ml of FB1 decreased estradiol production in IGF1-treated and FGF9-treated cells. FB1 at 300 and 10,000 ng/ml also inhibited estradiol production in Control and FGF9+IGF1-treated cells.

# P 8.13 *XANTHIUM STRUMARIUM* DOWNREGULATES SURVIVIN MRNA AND INDUCES APOPTOSIS IN COLON CANCER CELLS

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*Xanthium strumarium* is a weed originated from Central or South America and spread across the world. It is reported to have several pharmacological properties such as hypoglycemic, CNS depressant, antiprotozoal, immunomodulating and hepatoprotective etc. Some plants of *Xanthium genus* are being used as folk medicine in China and India. It is used for the treatment of inflammatory diseases and some malignant disorders by certain tribal groups of India. This plant belongs to Asterace family whose members are well known for the presence of sesquiterpene lactones, a class of compounds showing promising anticancer and anti-inflammatory properties. In the present study, active fractions of the plant *Xanthium strumarium* were tested for their cytotoxic potential using MTT and SRB assay systems against eight cell lines viz., Hela, MCF-7, A 549, COLO 205, HT 29, HCT 15, SW-620 and Vero cells. Chloroform soluble fraction of roots, hexane soluble fraction of fruit, chloroform soluble fraction of fruit and hexane soluble fraction of leaves were found to be promising. They found to induce apoptosis in colon cancer cells, and the apoptotic potential was studied using assays such as Acridine orange/Ethidium bromide dual nuclear staining, comet assay, and DNA ladder assay. Cell cycle arrest and apoptosis was further confirmed by flow cytometry. Effect of these fractions on expression of mRNA of various genes such as bcl-2, bax, cox-2, survivin, were studied. The active fractions were found to down regulate the expression mRNA of antiapoptotic genes and induce cells to apoptotic death on colon cancer cells.

# P 8.14 EFFICIENCY OF CERTAIN PLANT EXTRACTS ON *APHIS CRACCIVORA* KOCH, AND *CHRYSOPERLA CARNEA* (STEPH) IN SOUTHERN VALLEY, EGYPT

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Two plants *Hyoscyamus muticus* and *Ambrosia maritime* were chosen in order to investigate the toxicity of their leaves, stems, flowers or root extracts (using ethanol, acetone, chloroform and hexane) to nymph and adults of *Aphis craccivora* and its predator *Chrysoperla carnea*. The plant extracts were treated using direct spraying on infested bean seedlings. The results showed that for the two plant species, high concentration of leave extracts in ethanol and acetone gave a high mortality rate after 24 and 48h. For the flower extract at high concentration in ethanol and chloroform resulted as well in high mortality rate of the pest after 24 and 48h. As far as the root extract are concerned only at a high concentration in ethanol gave high mortality rate after 24 and 48h. For the stem extracts only at a high concentration in hexane resulted in a high mortality rate after 24 and 48h. Moreover, these high plants extracts concentration were not detrimental for *Chrysoperla carnea*as thus can be considered as safe adjuvant in the prospect of the integrated pest control.

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