Session I-10: Safety testing for carcinogenicity and genetic toxicity: Recent 3Rs advances

Session I-10: Oral presentations

I-10-670

In vitro versions of the Muta™Mouse Transgenic Rodent (TGR) Mutation Assay for hazard identification of chemicals

P. A. White, C. L. Lemieux and A. S. Long

Health Canada, Ottawa, Canada

Paul.white@hc-sc.gc.ca

Transgenic rodent (TGR) mutation assays, such as the Muta™ Mouse assay, provide the ability to quantitatively assess mutagenicity in virtually any tissue of an exposed experimental animal. An OECD guideline for proper conduct of the assay (no. 488) is pending final approval. However, the costs associated with this *in vivo* assay, and the desire to reduce, refine and replace laboratory animals in toxicity testing, has promoted interest in the establishment of *in vitro* alternatives. In 2003 we introduced the Muta™ Mouse FE1 cell line. The cells, which retain several characteristics of alveolar epithelium, are cytogenetically stable, metabolically-competent, contain wild-type p53, and yield reproducible responses upon exposure to a variety of mutagenic substances. We investigated the utility of the FE1 assay in a regulatory context by examining 9 non-carcinogens that have been highlighted for their ability to induce "irrelevant positives" in a traditional mammalian cell assay (i.e., Mouse Lymphoma Assay). In addition, we are conducting simultaneous *in vitro* (i.e., FE1) and *in vivo* exposures to selected compounds in an effort to investigate the mechanistic and empirical relationships between the different endpoints. More recently, we have integrated other genotoxicity endpoints into the FE1 assay; including micronuclei in binucleate cells and stable DNA adducts. We also successfully demonstrated the ability to culture and expose primary hepatocytes from Muta™ Mouse. The *in vitro* hepatocyte assay has been employed to examine mutagenic aromatic amines that generally require metabolism and activation by cyp1A2, a P450 isozyme that is almost exclusively hepatic.

I-10-134

In vivo Comet Assay: update on the ongoing international validation study coordinated by JaCVAM

Y. Uno[1], H. Kojima[2] and M. Hayashi[3]

[1]Mistubishi Tanabe Pharma, JEMS/MMS, Chiba, Japan; [2]NIHS/JaCVAM, Tokyo, Japan;
[3]Biosafety Research Center, Shizuoka, Japan

Uno.Yoshifumi@ma.mt-pharma.co.jp

The *in vivo* rodent alkaline Comet assay is used worldwide for detecting genotoxic chemicals. The assay, however, has not been formally evaluated for its reliability and relevance. Thus, the Japanese Center for the Validation of Alternative Methods (JaCVAM) has been coordinating an international validation study to evaluate the *in vivo* rodent alkaline Comet assay as a potential predictor of genotoxic carcinogens. Our goal is to establish an OECD guideline. The study protocol has been finalized as version 14 based on the results of the 1st to 3rd phase validation studies conducted in four or five lead laboratories. A daily administration regimen of three doses per day of test chemicals to the animals has been adopted in the study protocol to combine the Comet assay with the micronucleus assay, resulting in both reduction and refinement of animal use. The 4th phase of the (definitive) validation study began in 1Q/2009 in accordance with the study protocol. In the first step, data obtained from 13 laboratories indicated good reproducibility of the assay results among laboratories when the assay was conducted with four coded test chemicals: 2-acethylaminofluorene; ethyl methanesulfonate (EMS); D-mannitol; and N-methyl-N-nitrosourea. The 2nd step of the 4th phase validation study is now on-going in 14 laboratories with many more coded test chemicals and a positive control, EMS, in order to investigate the predictive capability of the assay against the known carcinogenicity of the test chemicals.

I-10-265

ECVAM-coordinated pre-validation study of three cell transformation assays for the carcinogenicity testing of chemicals

P. Phrakonkham, C. Griesinger and R. Corvi

European Commission – IHCP/ECVAM, Ispra, Italy

pascal.phrakonkham@ec.europa.eu

The *in vitro* Cell Transformation Assay (CTA) has been proposed as a valuable alternative to the traditional rodent carcinogenicity bioassay since it recapitulates critical stages of *in vivo* carcinogenesis, generates transformed cells that can be tumourigenic *in vivo* in suitable hosts, and can detect both genotoxic and non-genotoxic carcinogens. Following past activities of the European Centre for the Validation of Alternative Methods (ECVAM) concerning the use of the CTA, and to complement the findings of the OECD detailed review paper (DRP) on CTAs for the detection of chemical carcinogens, ECVAM coordinated a formal pre-validation study on the Syrian hamster embryo (SHE) and BALB/c 3T3 CTAs. The study objective was to address issues of protocol standardization, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. Three protocol variants (SHE pH6.7, SHE pH7.0 and BALB/c 3T3 CTAs) were evaluated in a multi-laboratory trial with six chemicals per assay. In October 2010, the study results were submitted to the ECVAM Scientific Advisory Committee (ESAC) for scientific review, the conclusions of which will serve a basis for the ECVAM recommendation on CTA. In agreement with the study Validation Management Team (VMT), the ESAC concluded in February 2011 that the study succeeded in generating standardised protocols, which appear transferable and reproducible for the SHE CTAs. Although promising, further optimisation of the BALB/c 3T3 protocol was recommended and use of the refined protocol, including the modifications suggested by the VMT and the ESAC, was encouraged to expand the data on assay reproducibility. These results, together with the extensive database summarised in the OECD DRP, support the utility of the CTA for the assessment of carcinogenicity potential. Moreover, the ESAC made detailed suggestions regarding the next steps considered to be necessary for the possible routine use of the CTA.

Session I-10: Poster presentations

I-10-170

Using high content imaging to automate the *in vitro* micronucleus assay: analysis of CHO-K1 and Balb/3T3 in the presence and absence of cytochalasin B

D. M. Miller, A. M. Peters and J. R. Haskins

Thermo Fisher Scientific, Pittsburgh, USA

donna.miller@thermofisher.com

The Micronucleus (MN) assay is a commonly used tool to identify the genotoxic potential of various agents. The OECD guideline for this assay (#487) allows for several variations (length of compound exposure, blocking of cytokinesis or not, metabolic activation, etc.) of the MN assay, along with the potential to screen with multiple cell types, tissue models or mammalian blood samples. Each of these variations requires flexibility of the automated analysis of the assay and is perhaps why attempts to develop an automated assay have proven to be difficult. In this study, we used high content imaging analysis to evaluate the genotoxicity of six compounds in two cell lines (CHO-K1 and Balb/3T3-a31) with and without cytokinesis block to determine whether compounds had different effects on the cell lines and/or whether cytochalasin B (cyt B) had an effect on the results.

The compounds tested exhibited dose-dependent changes for MN frequency, but the cell lines and cyt B test methods varied in the cells' sensitivity to each compound. The automated analysis allowed six compounds in seven-point dose-response curves, six replicates per dose, in two cell lines and two treatment schemes to be evaluated in approximately 36 h without any hands-on time required. High content imaging analysis of the MN assay is a flexible tool that offers a significant improvement in speed and throughput over manual methods, eliminating subjectivity and hours of manual scoring time.

I-10-185

A validation study on a Bhas 42 cell transformation assay using 96-well micro-plates

A. Sakai[1], S. Arai[1], K. Sasaki[1], D. Muramatsu[1], N. Endou[1], F. Mizuhashi[2], S. Kasamoto[2], M. Nagai[2], M. Takai[2], M. Asakura[3], N. Tashiro[4], N. Ishii[4], S. Yamazaki[1], M. Umeda[1] and N. Tanaka[1]

[1]Food and Drug Safety Center, Hadano, Japan; [2]Biosafety Research Center, Foods, Drugs and Pesticides, Iwata, Japan; [3]Japan Bioassay Research Center, Hadano, Japan; [4]Mitsubishi Chemical Medience Corporation, Kamisu, Japan

sakai.a@fdsc.or.jp

The Bhas 42 cell transformation assay (Bhas 42 CTA) is a short-term system for predicting chemical carcinogenicity. The Bhas 42 cells were derived from the BALB/c 3T3 cells transfected by v-Ha-ras gene. The assay protocol of Bhas 42 CTA consists of two components, the initiation assay and the promotion assay, to detect tumor-initiating activity and tumor-promoting activity, respectively, of chemical carcinogens. In the promotion assay the Bhas 42 cells do not require to be pre-treated by an initiator such as 3-methylcholanthrene, showing that these cells are initiated. The Bhas 42 CTA was applied to 98 chemicals in-house and its proficiency in predicting carcinogenic potential and its capability of detecting Ames-negative and Ames-discordant carcinogens were previously reported in *Mutation Research*. Validation studies have recently proven that the Bhas 42 CTA is reproducible between and within laboratories and applicable to the prediction of chemical carcinogenicity (manuscript in preparation). These studies were carried out using 6-well micro-plates (6-well method). We have since developed the Bhas 42 CTA using 96-well micro-plates (96-well method) that has the potential to be utilized for high throughput automated applications. The present study was performed to validate this 96-well method. Four laboratories participated and the study was forwarded stepwise (pre-validation phase, phase I and phase II). All the test chemicals were coded and a total of 25 chemicals were tested including duplicate chemicals between phases. The study results proved that the 96-well method is transferable between laboratories, reproducible both within and between laboratories, and applicable to the prediction of chemical carcinogenicity. The 96-well method and the 6-well method gave the same judgment for 15 out of the 17 chemicals duplicated between validation studies.

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

I-10-207

The use of human 3D epidermal models for genotoxicity testing: Results with the Comet assay

A. Reus[1], K. Reisinger[2], T. Downs[3], S. Pfuhler[3] and C. Krul[1]

[1]TNO, Zeist, The Netherlands; [2]Henkel, Düsseldorf, Germany; [3]Procter & Gamble, Cincinnati, USA

astrid.reus@tno.triskelion.nl

EU policy with regards to the safety evaluation of cosmetic ingredients marks the need for a broad replacement of animal tests due to the Seventh Amendment to the Cosmetics Directive. For many chemicals and cosmetic ingredients, the skin is the route of exposure (first site of contact). The *in vitro* genotoxicity assays that are currently used result in many false positives and there is no follow-up assay available in the target tissue skin to further evaluate a positive response obtained from *in vitro* assays. Therefore, genotoxicity (comet and micronucleus) assays using human 3D epidermal models were developed, representing a relevant test system with regards to barrier function of the stratum corneum and the possibility to apply formulations (realistic exposure conditions).

For the assay discussed here, the comet assay, EpiDerm™ models (MatTek) were cultured in an air-liquid interface and topically exposed to test compounds for 3 h, followed by cell isolation, lysis, electrophoresis, and preparation of slides. Inter-laboratory reproducibility of the 3D skin comet assay was demonstrated for MMS and 4NQO and results showed good concordance with *in vivo* data. Phase 1 of the pre-validation of the 3D skin comet assay was performed with 5 coded compounds at 3 different laboratories. Results of the collaborative study will be presented.

Results thus far indicate that the comet assay in 3D skin models is a relevant model for safety evaluation of compounds that penetrate the skin.

This work was sponsored by the European cosmetics industry (COLIPA) and the European Center on Validation of Alternative Methods (ECVAM).

I-10-214

Novel *in vitro* genotoxicity assays using reconstructed human tissues

G. Ouédraogo[1], N. Flamand[1], F. Nesslany[2], S. Simar[2] and D. Marzin[2]

[1]L'Oréal, Aulnay-sous-Bois, France; [2]Institut Pasteur de Lille, Lille, France

gouedraogo@rd.loreal.com

The poor specificity (tendency to yield a high number of positive results whereas *in vivo* genotoxicity results are negative) of *in vitro* genotoxicity assays (especially the mammalian cell based ones) is of concern in the context of the European legislation (7th amendment to the European Cosmetics Directive, REACH).

An *in vitro* micronucleus assay using human reconstructed skin tissues and target cells grown beneath the skin was developed. The purpose was to improve the relevance of exposure conditions in *in vitro* genotoxicity assays for dermally applied compounds. Previous results have shown that this method was reproducible and could be transferred to other laboratories. In addition, clastogens as well as aneugens could be detected. The system has now evolved to combine both the comet assay and the micronucleus assay. A set of 12 chemicals has been tested with this system. The results obtained will be shown. So far, they look promising. Most of the "irrelevant positives" yielded negative *in vitro* results using this system.

I-10-223

Comparable metabolite patterns of benzo[a]pyrene and cinnamic aldehyde in human skin *ex vivo* and human skin models *in vitro* indicate comparable metabolic capacities

J. Brinkmann[1], C. Hutzler[1], S. Trappe[1], U. Bock[2], M. Liebsch[3], F. Henkler[1] and A. Luch[1]

[1]Federal Institute for Risk Assessment, Berlin, Germany; [2]Across Barriers GmbH, Saarbrücken, Germany; [3]Federal Institute for Risk Assessment (BfR), Center for Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

Joseph.Brinkmann@bfr.bund.de

In vitro skin models are widely recommended in toxicological test guidelines for addressing penetration, as well as corrosion and irritation. However, the suitability of these test systems for other toxicological endpoints, such as genotoxicity or skin sensitization depends on their metabolic competence to transform non-toxic parental compounds into relevant metabolites of toxicological concern. Initial studies on metabolizing enzymes in skin models have focused on expression patterns of cytochrome P450 (CYP) monooxygenases and selected detoxifying phase II enzymes. Besides gene expression, comparatively little is known about enzyme activities present in skin models. Both CYP1A1 and 1B1 are thought to initiate the biotransformation of benzo[a]pyrene (BP), thereby resulting in a complex pattern of metabolites due to various parallel or alternate pathways of BP conversion.

We have analyzed the induction of CYP1 enzymes, as well as CYP activities in full thickness models, showing that these enzymes are preferentially expressed within the epidermal layers. In order to address the metabolic capacity directly, we have compared the metabolite patterns in BP-exposed 3D skin models, excised human skin and cultured primary human keratinocytes. Importantly, the patterns of eight BP-metabolites detected, including BP-r-7,t-8,t-9,c-10-tetrahydrotetraol, trans-BP-7,8-dihydrodiol, trans-BP-9,10-dihydrodiol, BP-1,6-dione, BP-7,8-dione, 3-OH-BP, 7-OH-BP and 8-OH-BP were comparable between human skin and the MatTek epidermal and full thickness skin models. All analyzed BP metabolites were also detected in primary human keratinocytes. However, these primary cultures appeared to metabolize BP at lower rates than human skin. Further, we also compared the metabolic transformation of cinnamic aldehyde into cinnamic acid and cinnamic alcohol in the skin models and human skin. Our results confirm a sufficient metabolic competence of the selected skin models and support their applicability for replacing *in vivo* tests for genotoxicity and other endpoints that rely on metabolism. The BfR coordinates a German multi-center project (BMBF Funding ID 0315226 A-D) aimed at developing and prevalidating the Comet assay in skin models. Initial observations also confirm that pro-mutagens are converted into their active forms, which can be detected in this assay.

I-10-239

The application and use of cell transformation assays in hazard and risk assessment – experience at a CRO

A. Poth

Harlan Cytotest Cell Research, Rossdorf, Germany

apoth@harlan.com

The prediction and assessment of carcinogenicity of chemical compounds is an essential step in their development. The 2-year bioassay is the standard method for carcinogen detection, which is time and resource intensive. *In vitro* cell transformation tests using SHE-, Balb/c3T3-, Bhas 42- cells simulate the process of animal two-stage carcinogenesis. These tests are suited for the *in vitro* detection of a carcinogenic potential of test compounds in safety and risk assessment. Results from cell transformation assays can provide information, which in combination with data from other testing methods, are useful for identifying the carcinogenic potential of chemical compounds. The cell transformation assays are used: in order to gain additional information when the biological significance of the bioassay results is uncertain; for clarification of the meaning of positive results from genotoxicity assays in the weight of evidence assessment; for compound classes where genotoxicity data have only limited predictive capacity; for investigation of compounds with structural alerts for carcinogenicity; and to demonstrate differences or similarities across a chemical category. New technologies will contribute to a better understanding of the mechanism of these assays and to a more objective evaluation of transformed foci and colonies. We have investigated several biomarkers, characteristic for the carcinogenic process in humans. Promising results were obtained for butylcholin esterase, acetylcholine esterase and alkaline phosphatase. It is time for the cell transformation assays to be accepted as a useful short-term *in vitro* tool for assessing the carcinogenic potential of chemical compounds.

II-10-242

Development and validation of mechanism-based *in vitro* transformation assays for carcinogen screening

H. Yasaei[1], J. C. Pickles[1], M. O'Donovan[2] and R. F. Newbold[1]

[1]Brunel University, Uxbridge, UK; [2]AstraZeneca, Macclesfield, UK

hemad.yasaei@brunel.ac.uk

The use of Syrian hamster (SH) as a model for carcinogen screening carries the advantage (e.g. over mouse and rat) that its somatic cells are, in the absence of carcinogen treatment, extremely resistant to oncogene/stress induced senescence bypass (OSISB), with spontaneous immortalization frequencies well below 10-8/primary cell passaged. Telomerase does not need to be reactivated in SHD cells (a very rare event that is a prerequisite in human cells for immortalization, clonal evolution and malignant progression), making them potentially a sensitive target for use in cell transformation assays. In defining the precise molecular mechanism underlying OSISB in the SHD system, we have produced a near-complete molecular description of the various combinations of mutational and epigenetic alterations leading to carcinogen-induced OSISB (immortalization) in primary SHD cells. Our data indicate that different human carcinogens show a distinct preference for inactivating different elements of ARF-p53/p16-RB senescence effector (human tumor suppressor) pathways by mutational and/or epigenetic means, in some cases leaving a clear molecular fingerprint characteristic of the mechanism of action of the particular class of carcinogen. We are now further developing the *in vitro* SHD immortalization system in order to characterize the extensive panels of non genotoxic carcinogens-immortalized SHD clones. In parallel, the improved understanding we now have concerning the mechanisms of carcinogen-induced OSISB will be applied to a molecular analysis of transformants from the related SH embryo cell morphological transformation (SHE-MT) assay in order to obtain a mechanism-based validation of this promising, but thus far uncharacterized system.

I-10-252

Reconstructed Human Epidermis (RHE) use for genotoxicity testing with fewer false positive results

E. Andres[1], J. Molinari[1], V. Moura Sa-Rocha[2], S. Hurtado[2] and C. Barrichello[1]

[1]Natura Innovation, Paris, France; [2]Natura Innovaçao, Cajamar, Brazil

ericandres@natura.net

According to the Seventh Amendment, Council Directive 2003/15/EC, the use of animals for genotoxicity testing has been forbidden since 2009; consequently, alternative *in vitro* methods have been developed for the safety assessment of cosmetic products. These tests, using cell culture, are now being questioned due to their tendency to give false positive results and because the possibility to replace cell culture by reconstructed human epidermis (RHEs) is being studied. RHEs are particularly adapted for cosmetics testing due to their similarity to the physiological condition of skin.

Here we describe experiments using EPI/001 RHEs for the evaluation of the genotoxicity effect of substances known to give misleading positive results in cell culture tests. All RHEs were pre-treated with 3 μg/ml of cytochalasin B, in order to get binucleated cells, an essential condition for the micronucleus test. Then, dose-ranges of Urea, Eugenol, Resorcinol, Curcumin, Propyl Gallate and Phthalic anhydride diluted in acetone were topically applied (10 μl). Non-treated, acetone-treated and Mitomycine C (MMC)-treated RHEs were used as controls. Keratinocytes were released from the RHE, placed on histology slides and stained by May-Grünwald/Giemsa (MGG) protocol. The binucleated cells and micronuclei rate were evaluated using NIS Element taxonomy software (Nikon).

Pure acetone treatment did not modify the micronuclei rate and viability compared to non-treated RHEs. MMC treatment resulted in a dose-dependent increase of micronuclei scoring, a decrease of tissue viability and a decrease of the binucleated cell rate, while the 6 known false positive genotoxic substances did not give any significant increase of micronuclei scoring.

I-10-256

Genotoxicity evaluation of molecules possessing antifertility potential: Testing in microorganisms as an alternative to animal testing

N. Sharma[1], V. Sharma[2], M. A. Akbarsha[3] and K. K. Sharma[2]

[1]Department of Microbiology, MDS University, Ajmer, India; [2]Department of Zoology, MDS University, Ajmer, India; [3]MGDC, Bhartidesan University, Tiruchirappalli, India

nehaniharmicro@gmail.com

A large number of molecules, synthetic as well as natural, have been discovered for their use as antifertility drugs. Newly discovered molecules of antifertility drugs have to undergo genotoxicity studies to evaluate their mutagenic and carcinogenic activity. To test the genotoxicity of one drug on five different genotypic strains, approximately 3,000 animals are used. Response of the drug is observed in F1 hybrids after five weeks, with an efficacy ratio of 7.2%. Animal testing in preliminary trials should be avoided in view of the principles of the 3Rs. Genotoxicity evaluation using the Ames test in the discovery of various antifertility drugs can reduce the sacrifice of a large number of rodents and other mammals used in laboratories in preclinical research procedures. Among the numerous genetic bioassays tested in our laboratory, the Ames test has been found to be the most effective as a preliminary screening bioassay. Two recombinant strains of *Salmonella* bacteria, *Salmonella typhimurium* TA 1535 and TA 1538 that carry both frame shift and point mutations in the genes required to synthesize histidine, have been used. The antifertility molecule possessing mutagenic potential may restore the gene function and allow the cell to synthesize histidine, resulting in growth of both of the strains. The specially designed F334 strain of mouse (rodent) and *Schizosaccharomyces prombe* ade 6 and ade 7 (yeast) give results similar to *Salmonella* TA 1535 and TA 1538. This study shows that recombinant strains of microorganisms can be used as alternatives to animal testing in genotoxicity studies on molecules to be used as antifertility drugs.

I-10-264

Comparison of *in vitro* versus *in vivo* transcriptomics data of hepatocarcinogens

T. Y. Doktorova[1], H. Ellinger-Ziegelbauer[2], M. Vinken[1], T. Vanhaecke[1], H.-J. Ahr[2] and V. Rogiers[1]

[1]Vrije Universiteit Brussel, Brussels, Belgium; [2]Bayer Healthcare Pharmaceuticals, Wuppertal, Germany

tatyana.yordanova.doktorova@vub.ac.be

Currently, there are substantial efforts to develop "omics-based" *in vitro* models for identifying genotoxic and non-genotoxic carcinogenic substances as an alternative to the classical 2-year rodent carcinogenicity bioassay. An important issue, however, is the *in vivo* relevance of the *in vitro* obtained data. In the current study, we compare the gene expression profiles generated after oral administration of hepatocarcinogens to rats with those derived after *in vitro* exposure of either epigenetically stabilized or conventional primary rat hepatocyte cultures. Three genotoxic hepatocarcinogens (aflatoxin B1, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 2-nitrofluorene), three non-genotoxic hepatocarcinogens (methapyrilene.HCl, piperonylbutoxide and Wy-14643) and two non-carcinogens (nifedipine and clonidine) were tested. After applying comparable statistical tools for data analysis, *in vivo*- and *in vitro*-derived gene expression profiles per compound were extracted and functionally classified. The major descriptive cellular pathway found *in vivo* for genotoxic carcinogens was DNA damage response, whereas for non-genotoxic carcinogens it was cell cycle progression. Those characteristic *in vivo*-derived gene groups were further compared with the *in vitro* data. In the conventional hepatocyte cultures, two out of the three *in vitro* tested genotoxic carcinogens mimicked the *in vivo* relevant DNA damage response, whereas only one genotoxicant was responsive in the epigenetically stabilized system. Exposure to the non-genotoxic hepatocarcinogens triggered a relatively weak response in both *in vitro* systems, with no clear similarities to *in vivo*. This study might be indicative for the importance of the "*in vivo* relevance of *in vitro* data" when prediction of genotoxic/non-genotoxic potential of chemicals is based on toxicogenomics techniques.

I-10-271

Alternative approaches for the evaluation of carcinogenicity and its use for quantitative risk assessment of cosmetic ingredients

J. van Benthem[1], S. Felter[2], S. Pfuhler[2], T. Heinonen[3], A. Poth[4], R. Serafimova[5], J. van Delft[6], E. Benfenati[7], P. Phrakonkham[5], A. Worth[5] and R. Corvi[5]

[1]National Institute for Public Health and the Environment, Laboratory for Health Protection Research, Bilthoven, The Netherlands; [2]Procter and Gamble Company, Cincinnati, USA; [3]University of Tampere, Tampere, Finland; [4]Harlan Cytotest Cell Research GmbH, Roßdorf, Germany; [5]ECVAM, Ispra, Italy; [6]Maastricht University, Maastricht, The Netherlands; [7]Instituto di Ricerche Farmacologiche, Milan, Italy

raffaella.corvi@jrc.ec.europa.eu

In 2010 a panel of experts was tasked with assessing the availability of alternative methods to animal testing for five toxicological areas, including carcinogenicity, in view of the full marketing ban anticipated in 2013 for cosmetic products and ingredients tested on animals in Europe.

The evaluation of cancer hazard and risk assessment is rarely done in the two-year cancer bioassay for cosmetic ingredients. Rather, a combination of shorter-term *in vitro* and *in vivo* studies have been used, including *in vitro* and *in vivo* genotoxicity assays to assess genotoxic potential and repeated dose toxicity studies to assess the risk of non-genotoxic chemicals. It is clear that the animal testing bans will have a profound impact on the ability to evaluate and conduct a quantitative risk assessment for potential carcinogenicity of new cosmetic ingredients, which is mainly due to the ban on *in vivo* genotoxicity testing, any repeated-dose toxicity testing, and other tests such as *in vivo* toxicokinetics studies and *in vivo* mechanistic assays.

Although several *in vitro* tests, which are at different stages of development and acceptance, are available to support conclusions on cancer hazard identification beyond the standard *in vitro* genotoxicity assays, the available *in vitro* tests are focused on hazard evaluation only and cannot currently be used to support a full safety assessment by adequate dose-response information. However, for some chemical classes the available non-animal methods might be sufficient to rule out carcinogenic potential in a weight of evidence approach.

Taking into consideration the present state of the art of the non-animal methods, the timeline for full replacement is expected to extend past 2013.

I-10-286

Genotoxicity testing using the micronucleus and Comet assays in normal human cell-based 3D epithelial models

Y. Kaluzhny, M. Klausner, P. Hayden and H. Kandarova

MatTek Corporation, Ashland, USA

ykaluzhny@mattek.com

Safety assessment of new products for human use requires genotoxicity testing. Current *in vitro* assays have low specificity (high rate of false positives) and previously *in vivo* assays were conducted. However, the 7th amendment to the Cosmetics Directive banned *in vivo* genotoxicity testing in 2009. 3D human tissue models, which have *in vivo*-like barrier function and metabolism, and which allow for topical exposure, are predicted to have improved biological relevance. Toward this end, the Reconstructed Skin Micronucleus (RSMN) and Comet assays (CA) that utilize the MatTek™ EpiDerm™ model have been adapted for use with tracheal, vaginal, oral, and corneal tissues.

EpiDerm is a 3D normal human cell-based epidermal model that contains *in vivo*-like barrier and biotransformation capabilities. RSMN assay results show statistically significant, dose-dependent increases in cells containing micronuclei (MNC) for 9 direct genotoxins and 6 genotoxins that require metabolic activation, and no increases for 4 non-genotoxins. CA results show increases in %tail DNA after treatment with model genotoxins. Utilizing the RSMN protocol with tracheal, vaginal, oral, and corneal tissue models, increases in MNC (0.3 to 1.2%) were observed after treatment with genotoxins. Similarly, CA results with tracheal, vaginal, oral, and corneal tissue models showed increases in %tail DNA. Hence, the RSMN and CA can be applied to other *in vitro* tissue models using real life exposure conditions. Together, RSMN and CA for skin, tracheal, vaginal, oral, and corneal tissue models will identify a wide spectrum of genotoxic hazards, and will increase confidence in the veracity of *in vitro* tests.

I-10-315

Effects of the genotoxic compounds, benzo[a]pyrene and cyclophosphamide on phase 1 and 2 activities in EpiDerm™ models

N. J. Hewitt[1], C. Götz[2], E. Jermann[2], J. Tigges[2], Z. Kohne[2], U. Hübenthal[2], J. Krutmann[2], H. F. Merk[3] and E. Fritsche[2,3]

[1]COLIPA, Erzhausen, Germany; [2]Institut für Umweltmedizinische Forschung, Heinrich-Heine-University, Duesseldorf, Germany; [3]University Clinic RWTH Aachen, Germany

nickyhewittltd@yahoo.co.uk

The micronucleus assay in the 3D human reconstructed EpiDerm™ skin model (RSMN) is a promising new assay for evaluating genotoxicity of dermally applied chemicals. In order to complement the testing of metabolically activated chemicals, we have measured basal phase 1 (ethoxyresorufin O-deethylation and testosterone metabolism) and 2 activities (UGTs and GSTs) in EpiDerm™ models in a study design which mimics the pre-validated RSMN. We have also investigated whether the known genotoxins, cyclophosphamide (CPA) and benzo[a] pyrene (BaP), alter these activities. These studies demonstrated the presence of basal phase 1 and 2 activities of EpiDerm™ models. With the exception of GST (which decreased between 24 h and 48 h), all of the basal activities measured did not change over time. It was possible to measure enzyme induction using this assay design. Of the enzymes tested, EROD activity was significantly induced by BaP but not by CPA. CPA, BaP and the reference chemical, beta-naphthoflavone, all caused a small increase in GST activities, the magnitude of induction being markedly lower than that for EROD, which is consistent with literature findings for hepatic models. Since metabolic enzyme activities have been shown to be present, the RSMN assay does not require an exogenous metabolic activation system and is therefore a good model to reflect the metabolic capacity of human skin.

This work is funded by the European Cosmetic Industry Association COLIPA.

I-10-316

COLIPA validation of the Reconstructed Human Skin Micronucleus Assay (RSMN): Further pre-validation studies and investigations into increasing time efficiency

R. Fautz[1], G. Ouédraogo[2], K. Reisinger[3], M. Aardema[4], B. Barnett[4], B. Faquet[2], G. Mun[5], E. Dahl[5], R. Curren[5], N. Hewitt[6] and S. Pfuhler[4]

[1]KPSS-Kao Professional Salon Services, Darmstadt, Germany; [2]L'Oréal Life Sciences Research, Aulnay-sous-Bois, France; [3]Henkel AG & Co KGaA, Duesseldorf, Germany; [4]The Procter & Gamble Co., Cincinnati, USA; [5]Institute for In vitro Sciences, Inc., Gaithersburg, MD, USA; [6]COLIPA, Erzhausen, Germany

rolf.fautz@kpss-hair.com

The European Cosmetic Toiletry and Perfumery Association (COLIPA) has initiated a multi-laboratory project to establish and evaluate more predictive *in vitro* genotoxicity assays using 3D human tissues as replacements for current mammalian cell *in vitro* genotoxicity assays, which induce high levels of false positives. The reconstructed skin (RS) model, EpiDerm^{TM}, was combined with the micronucleus (MN) and comet assays because the skin is the first site of contact of many different products, including cosmetics. The resulting RSMN model offers the potential for a more realistic application/metabolism of test compounds for evaluating genotoxicity (Curren et al., 2006; Mun et al., 2009). For the assay discussed here, the MN assay, there was a good intra- and inter-laboratory reproducibility with model genotoxins e.g. mitomycin C and vinblastine sulfate (Hu et al., 2009) and studies with coded chemicals also showed excellent prediction of positive and negative chemicals (Aardema et al., 2010). We have extended the number of coded chemicals to 30 as part of the pre-validation process. We have also optimised the viability determination by adopting an image-based analysis of differential staining with ethidium bromide (dead cells) and Acridine Orange (live cells) instead of manual counting using Trypan Blue. The two measurements were comparable and afforded a significant increase in time efficiency. Studies to automate MN scoring are on-going. In conclusion, the RSMN model is a promising alternative *in vitro* method for genotoxicity testing.

This work is funded by COLIPA.

References

Aardema, M. J., Barnett, B. C., Khambatta, Z. et al. (2010). *Mutat. Res. 701*, 123-131.

Curren, R. D., Mun, G. C., Gibson, D. P. and Aardema, M. J. (2006). *Mutat. Res. 607*, 192-204.

Hu, T., Kaluzhny, Y., Mun, G. C. et al. (2009). *Mutat. Res. 673*, 100-108.

Mun, G. C., Aardema, M. J., Hu, T. et al. (2009). *Mutat. Res. 673*, 92-99.

I-10-322

Characterization of molecular events underlying induced morphologically transformed (MT) phenotypes in the Syrian Hamster Embryo (SHE-MT) assay

J. C. Pickles[1], H. Yasaei[1], M. O'Donovan[2] and R. F. Newbold[1]

[1]Brunel University, London, UK; [2]AstraZeneca, Cheshire, UK

jessica.pickles@brunel.ac.uk

Current legislative and ethical pressure will require a substantial reduction in the number of animals used in carcinogenicity testing for safety purposes. The Syrian hamster embryo morphological transformation (SHE-MT) assay is one of the most promising systems used by industry to predict the carcinogenic activity of chemicals. A pre-validation study conducted on behalf of ECVAM has concluded SHE-MT to be a reliable assay for carcinogenicity. It has been shown that the rate-limiting immortalization step in SHE cell transformation requires only bypass of the oncogene/stress-induced senescence barrier (SHE cells have constitutive telomerase activity) and that spontaneous progression towards immortalization is a rare event. Therefore, SHE cells should be further exploitable as a useful *in vitro* cell transformation model for carcinogen screening. However, the underlying molecular events leading to immortalization remain unclear. Recent studies in our laboratory involving a SH dermal (SHD) mass culture system have produced a near-complete molecular description of carcinogen-induced OSIS bypass (immortalization). This has led us to initiate a mechanisms-based validation of the SHE-MT assay. Using quantitative gene expression (qRT-PCR) analysis, High Resolution Melting (HRM) and gene sequencing, we set out to characterize early-events leading to immortalisation in the SHE-MT system. The latest results of this study will be presented. Ultimately, transformation biomarkers will be identified for future use by industry in carcinogenicity risk assessment.

I-10-352

Applicability and robustness of the Hen's Egg Test for micronucleus induction (HET-MN): results from an interlaboratory trial

K. Reisinger[1], D. Greywe[2], N. Banduhn[1], J. Scheel[1] and T. Wolf[2]

[1]Henkel AG & Co. KGaA, Dusseldorf, Germany; [2]University of Osnabruck, Osnabruck, Germany

kerstin.reisinger@henkel.com

In vivo genotoxicity assays are often performed to clarify the biological relevance of *in vitro* tests because the latter ones exhibit low predictivity. Due to the new EU chemical legislation REACH and the 7th Amendment to the EU-Cosmetics Directive this testing strategy has to be modified, leading to a high demand for improved *in vitro* tests.

The hen's egg test for micronucleus induction (HET-MN) was developed several years ago to provide an alternative test system to the *in vivo* micronucleus test. In order to assess its applicability and robustness, a study was carried out at the University of Osnabrueck (labA) and at the laboratories of the Henkel AG & Co. KGaA (labB). Following transfer of the method to labB, a range of test substances, which had been pre-tested at labA, were tested at labB: the genotoxins cyclophosphamide, dimethylbenz(a)anthracene, methotrexate, acrylamide, azorubin, N-nitroso-dimethylamine and non-genotoxins orange G and myristic isopropylic acid.

In a second phase, additional compounds were examined in both labs: the non-mutagen, ampicillin, the "irrelevant" positives, isophorone and 2,4-dichlorophenol ("irrelevant" means that they were positive in standard *in vitro* tests but were negative *in vivo*), the mutagen, p-chloroaniline, and the aneugens, carbendazim and vinorelbine. All substances were correctly predicted in both labs with respect to their *in vivo* genotoxic properties, indicating that the HET-MN might have an improved predictivity compared to current standard *in vitro* test systems. The results support the promising role of the HET-MN assay as a supplement to existing testing batteries in the future.

I-10-366

Electrophilic reaction chemistry to predict genotoxicity through mechanistically derived grouping and read-across: links to adverse outcome pathways

M. T. D. Cronin, C. M. Ellison, S. J. Enoch, M. Hewitt, J. C. Madden, K. R. Przbylak and J. A. H. Schwöbel

Liverpool John Moores University, Liverpool, UK

m.t.cronin@ljmu.ac.uk

The last few years have seen a vast increase in interest related to chemical grouping approaches to allow for read-across for toxicity prediction. For grouping to be successful, analogues of the target compound must be determined on a rational and mechanistic basis. For a number of endpoints, such as genotoxicity, reactive mechanisms involving covalent bond formation following electrophilic reactions are important for grouping chemicals. This study describes recent work to define the reaction mechanisms associated with individual endpoints and to assess the differences across the electrophilic spectrum of reactivity, as related to different endpoints. For instance, genotoxic chemicals are at the hard end of the electrophilic spectrum. These definitions of chemistry are formed into profilers and are being made freely available through the OECD QSAR Toolbox. The use of the profilers will be illustrated with regard to relevant human health endpoints. These descriptions of chemistry are not predictive tools in themselves; however the groupings they facilitate allow for read-across. Compounds that may be reactive from a structural perspective may not be toxic for a number of reasons. The mechanisms can be rationalised in terms of adverse outcome pathways that attempt to demonstrate the link between the molecular initiating event and the effect on the organism or population. There is a clear need for these approaches to be supported by relevant mechanistic assays to support the grouping and category approaches.

The funding of the EU OSIRIS (GOCE-CT-2007.037017) and COSMOS (Health-F5-2010-266835) projects is gratefully acknowledged.

I-10-384

Opportunities to minimise animal use in regulatory toxicology; a cross-company review

K. Chapman[1], S. Sparrow[2], S. Robinson[3], C. Bruce[4], A. Danks[5], D. Everett[6], R. Hill[7] and H. Palmer[8]

[1]NC3Rs, London, UK; [2]GSK, Ware, London, UK; [3]AstraZeneca, Macclesfield, UK; [4]Pfizer, Sandwich, UK; [5]Charles River, Tranent, UK; [6]Covance, Harrogate, UK; [7]Sequani, Ledbury, UK; [8]HLS, Huntingdon, UK

kathryn.chapman@nc3rs.org.uk

Regulatory toxicology studies required for pharmaceutical development have well-defined scientific objectives. The UK Laboratory Animals Science Association and the National Centre for the Replacement, Refinement and Reduction of Animals in Research established a group of toxicologists from the UK's major pharmaceutical companies and contract research organisations in order to share good practice and identify areas for application of the 3Rs whilst ensuring that the scientific objectives and regulatory requirements of such studies are still met.

A cross company review of numbers of animals used in general toxicology and carcinogenicity studies was carried out. The results showed that there is some variation in the numbers of animals used. The reasons for this have been explored and the information used to develop a series of approaches where small changes in practice may reduce animal use. We recommend these approaches are used where possible but acknowledge that they may not be appropriate for all studies or programmes.

Practical considerations are given on:

– Reducing the number of animals to obtain toxicokinetic (TK) data;
– Incorporating male fertility assessment into the six-month rodent toxicology study;
– Including fewer recovery animals; and
– Using transgenic mice, single control groups and appropriate strains in carcinogenicity studies.

The data collected demonstrate that the largest influence on animal numbers in rodent toxicity studies is for TK profiling. Therefore, the most significant contribution to reducing the number of animals is likely to be the development of analytical techniques which would allow analysis using smaller sample volumes.

I-10-391

In vitro genotoxicity testing using a metabolic competent human 3D bronchial epithelial model

C. Krul[1], B. Klein[2], A. Reus[3], J. van Triel[3] and J. Boei[2]

[1]TNO, Zeist, The Netherlands; [2]LUMC, Leiden, The Netherlands; [3]TNO Triskelion, Zeist, The Netherlands

cyrille.krul@tno.nl

EU policy, including REACH and the Cosmetics Directive, implies an urgent need for reliable *in vitro* strategies with regards to the safety evaluation of chemicals. *In vitro* assays currently used are not sufficiently suitable for the reliable evaluation of substances for which the airways are the primary route of exposure (first site of contact), such as gasses, volatiles and aerosols. The LUMC developed a robust human 3D bronchial epithelial model that is cultured in an air-liquid interface and has several important functional characteristics including mucus production, cilia beating and metabolic activity. Moreover, using this model exposure to gasses, volatiles and aerosols is possible via air, simulating a relevant exposure route.

Human primary bronchial epithelial cells (HPBEC) were isolated from the large bronchi of the unaffected outer layer of resected lung tissue from patients undergoing surgery for lung cancer. Upon culture in an air-liquid interface, the metabolic activity of the human 3D bronchial epithelial models and feasibility of *in vitro* genotoxicity tests was investigated. These human 3D bronchial epithelial models were exposed to chemicals via the medium, a droplet on the tissue surface, or via the air, followed by the measurement of genotoxicity and cytotoxicity parameters, including tissue and membrane integrity, cell viability, micronuclei formation and comet induction. A dose-related response was observed for known positive chemicals mitomycin C and methyl methane sulfonate in the micronucleus test and comet assay, respectively. Results with additional chemicals will be presented. Preliminary results indicate that the model is promising for the safety testing of chemicals, including genotoxicity and acute cytotoxicity.

This work was sponsored by the Netherlands Organisation for Health Research and Development (ZonMw) and the Dutch Ministry of Welfare, Health and Sport.

I-10-403

The COLIPA strategy for animal-free genotoxicity testing

S. Pfuhler[1], P. Carmichael[2], R. Fautz[3], G. Ouedraogo[4] and K. Reisinger[5]

[1]The Procter and Gamble Company, Cincinnati, USA; [2]Unilever, Bedford, UK; [3]Kao Professional Salon Services GmbH, Darmstadt, Germany; [4]L'Oréal, Asnières-sur-Seine, France; [5]Henkel, Düsseldorf, Germany

pfuhler.s@pg.com

The 7th Amendment to the Cosmetics Directive bans the use of animals for genotoxicity testing. This ban has perhaps had less focus from an animal alternatives standpoint because sensitive *in vitro* genetic toxicology assays already exist. However, the regulatory-required battery of these *in vitro* genotoxicity tests has a low specificity (i.e. a high percentage of irrelevant positive results for non-carcinogens). Since it is no longer possible to conduct follow-up *in vivo* genotoxicity tests for cosmetic ingredients with positive *in vitro* tests to further assess the relevance of the *in vitro* findings, valuable ingredients will be rejected.

To address this problem, the EU Cosmetics Association (COLIPA) Genotoxicity Task Force has been funding, directing and conducting a major program to develop approaches for genotoxicity testing of cosmetic ingredients. The program consists of three main projects:

(1) A "False Positives" project performed at Covance Laboratories (UK) to optimize current mammalian cell assays in order to improve specificity, showing that the selection of more relevant cells and toxicity measures can prevent >60% irrelevant positive findings.
(2) A "3D skin model" project focusing on developing and validating new methods based on reconstructed human 3D skin models. Results so far indicate good reproducibility of the assay as well as improved specificity compared to standard *in vitro* tests.
(3) Research into the metabolic capacity of human skin and 3D models which demonstrated the 3D skin models' "human skin like" metabolic competency, confirming their usefulness for testing of compounds with dermal exposure.

The outcome of this program is expected to help enable a sound assessment of the genotoxic hazard of cosmetic ingredients in the absence of *in vivo* data, and should also help to substantially reduce animal use in other sectors, such as the chemical industry.

I-10-414

Spectrophotometric measurements of transformation frequency in Bhas 42 cells using hydrogen peroxide

K. Sasaki, D. Muramatsu, S. Arai, N. Endou, A. Sakai, S. Yamazaki, M. Umeda and N. Tanaka

Food and Drug Safety Center, Hadano, Japan

sasaki.k@fdsc.or.jp

Transformation assays using the focus formation method have widely been applied to study mechanisms of carcinogenesis and predict chemical carcinogenicity. Four decades after development of this system, transformation frequency is still quantified by time-consuming and subjective scoring of transformed foci under a microscope. To assess transformation frequency quickly and objectively, we have developed a spectrophotometric assay to measure transformation frequency in Bhas 42 cells, a clone established by transfection with the v-Ha-ras gene into BALB/c 3T3 cells.

We found that when Bhas 42 cell cultures containing transformed foci were treated with hydrogen peroxide, normal cells were selectively killed. We plated Bhas 42 cells into 96-well plates and treated the cells with 3-methylcholanthrene (MCA) to form transformed foci. At the end of the culture, hydrogen peroxide and WST-8 (a dye monitoring metabolic activity of living cells) were added, and OD at 450 nm was measured with a microplate reader. The cells were then fixed and stained with Giemsa solution, and wells with transformed foci were counted. Wells with transformed foci showed high OD values, whereas OD values of wells without transformed foci showed a blank level. The total OD values and the number of wells containing transformed foci in each plate increased dose-dependently with MCA concentration. The hydrogen peroxide method is a novel approach to quantify transformation frequency and will provide a high-throughput assay by combining automated systems.

This study was supported by the New Energy and Industrial Technology Development Organization (NEDO).

I-10-452

Development of a new genotoxicity assay using proliferating and metabolically active upcyte® hepatocytes

A. Nörenberg, N. J. Hewitt, S. Heinz, A. Nussher and J. Braspenning

medicyte GmbH, Heidelberg, Germany

j.braspenning@medicyte.com

One of the disadvantages of the currently used genotoxicity micronucleus assays is their high "false positive" (FP) rate (i.e. low specificity). Two reasons for the high FP rate are (1) the use of cell lines which lack p53, e.g. the rodent cell lines; and (2) the use of metabolically incompetent cells. Medicyte has investigated a novel technique which causes primary hepatocytes to proliferate whilst maintaining differentiated functions. The resulting "upcyte®" hepatocytes provide a cell model which is both p53 competent and metabolically active during proliferation. Here, we investigated whether upcyte® hepatocytes could be used in the micronucleus assay to differentiate between diverse chemicals from 3 chemical groups, which represent true positives (cyclophosphamide, mitomycin C, etoposide, nitroquinoline), true negatives (ampicillin trihydrate, melamine, tris(2-ethylhexyl)phosphate) and false-positives (2,4-dichlorphenol, benzylalcohol, curcumin and urea). MN scoring was carried out using FACS analysis and the stains EMA and sytox green. Using upcyte® hepatocytes all tested chemicals could be correctly discriminated either as positive, resulting in a dose dependent increase in MN formation, or as negative, resulting in no significant increase in MN formation above the control-treated cells. upcyte® Hepatocytes were able to detect positive genotoxins that need metabolic activation without the help of external CYP enzymes (such as S9 mix) and to correctly identify false positives as being negative. This study supports the use of upcyte® hepatocytes in early ADME/Tox assays such as genotoxicity assays. This will eventually lead to an *in vitro* hepatocyte system in order to reduce or replace animal experiments in preclinical drug development and toxicity testing of chemical compounds.

I-10-464

An efficient approach to carcinogenicity prediction through *in vitro* mutagenicity and cell transformation assays

R. Benigni, C. Bossa, O. Tcheremenskaia and A. Giuliani

Istituto Superiore di Sanità, Rome, Italy

romualdo.benigni@iss.it

The need for tools able to predict chemical carcinogens in less time and at a lower cost both in terms of animal lives and money is still a research priority, even after several decades of effort in that direction. Now, new regulatory requirements (e.g., the Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) recently implemented in Europe) have further increased the pressure to develop new tools in this field. Drawbacks of the present testing strategies have come into the limelight again recently, especially in view of new international regulatory requirements. Among others, there is: a) the lack of alternative assays able to identify non-genotoxic carcinogens; b) the exaggerated rate of misleading ("false") positive results of the *in vitro* mammalian cell-based mutagenicity short-term tests; c) the extremely low sensitivity of *in vivo* mutagenicity short-term tests. Within this perspective, we analyze the contribution of the Cell Transformation assays, and we show that they are a valid complement to tools able to detect DNA-reactive carcinogens. We show as well that a tiered strategy with inexpensive and fast tests in Tier 1 (like the Ames test and the Structural Alerts), and the Syrian Hamster Embryo cells Transformation assay in Tier 2, is able to identify up to 90% of carcinogens.

I-10-484

Quantitative assessment of the effects of low dose ionizing radiation using a human hybrid cell transformation assay

E. Elmore and J. L. Redpath

University of California, Irvine, USA

eelmore@uci.edu

The issue of radiation induced carcinogenesis has received increased interest over the past few years. This concern is due to environmental exposures resulting from nuclear clean-up, nuclear reactor failure, increasing usage of diagnostic scans that use radiation or radioactive isotopes, and concerns raised in the press by scientists who support the linear-no-threshold (LNT) model where "no dose is safe". The LNT model is based on extrapolating dose response data from high doses to zero and assumes that the dose response would remain linear at the low doses. Understanding radiation risks at low environmental or medically relevant doses is difficult due to: the inadequate numbers of exposed individuals to make statistical assessments, the complex environmental exposures, the cost and numbers of animals required for *in vivo* data acquisition, and the availability of quantitative *in vitro* human cell based assays for carcinogenicity. The HeLa x human skin fibroblast neoplastic transformation assay has been shown to be sensitive to ionizing radiation. We have used this assay to study the effects of very low dose ionizing radiation from various sources including: gamma rays, X-rays, protons, iron ion beams, and radioisotopes including ^{125}I. The data clearly demonstrate that at doses <10 cGy, the dose response curve is not linear but has a "J" shape. This is suggestive of an adaptive response that can protect cells against the normally occurring spontaneous events. The data suggest that the LNT model does not reflect reality and that a beneficial effect may occur at low radiation doses.

I-10-565

Application of the threshold of toxicological concern concept in safety assessment of chemically complex matrices

M. A. J. Rennen, S. Koster, P. L. P. van Loo, C. A. M. Krul and G. F. Houben

TNO, Zeist, The Netherlands

monique.rennen@tno.nlnl

Toxicological safety assessment is usually performed as a sequential process, which is often time consuming and expensive. To obtain a more efficient process new concepts would be needed that integrate knowledge and information from different disciplines (i.e. chemical analysis, toxicological research, exposure and risk assessment) right from the start throughout the whole research process to fine-tune the required level of detail. The Threshold of Toxicological Concern (TTC) is one potentially new such concept that was developed to assess substances in food, where the identity is known but where toxicological information is lacking. Application of TTC to chemically complex matrices (CCM) is limited due to a lack of chemical identity.

We drafted a framework to enable application of TTC to such CCM. The approach is based on exclusion of specific groups of compounds following the Kroes et al. (2004) TTC decision tree and modifications as proposed by Munro et al. (2008). We concluded that the highest threshold which can be applied for unknowns after exclusion of specific groups of hazardous chemicals is 540 μg/p/d. To determine the amount of substances above a certain threshold a conversion of response in a chromatogram (visualized as "forest of peaks") into concentrations and subsequently into intake is needed. Those substances which appear above the applicable threshold should be identified, characterized and toxicologically assessed.

The safety assessment framework for CCM and required analytical and test strategy innovations (such as genotoxicity screening methods) that are under development will be presented.

References

Kroes, R., Renwick, A. G., Cheeseman, M. et al. (2004). *Food Chem. Toxicol. 42*, 65-83.

Munro, I. C., Renwick, A. G. and Danielewska-Nikiel, B. (2008). *Toxicol. Lett. 180*, 151-156.

I-10-597

Air/liquid interphase technique as an alternative *in vitro* testing strategy for detecting biological effects of volatile compounds. First results and future perspectives of an ongoing prevalidation study

L. Smirnova[1], R. Pirow[1], M. Liebsch[1], J. Tharmann[1], A. Luch[1], M. Bauer[2], C. Graebsch[2], G. Linsel[3], R. Siemers[3], C. Otto[3], S. Tröller[3], N. Müller[3], E. Berger-Preiß[4], H. Kock[4], A. Oertel[4], D. Ritter[4] and J. Knebel[4]

[1]Federal Institute for Risk Assessment (BfR), Center for Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany; [2]Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany; [3]Federal Institute for Occupational Safety and Health (BAuA), Berlin, Germany; [4]Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM), Hannover, Germany

Lena.Smirnova@bfr.bund.de

The increasing demand for assessing inhalation toxicity hazards calls for new testing strategies comprising both *in vitro* and *in vivo* assays. The aim of this ongoing pre-validation study is to perform a multi-laboratory evaluation of an air/liquid interface culturing and exposure technique for testing the acute cytotoxic and genotoxic effects of gases on a biological cell model. A549 cells, grown on microporous membranes at the air/liquid interface, were exposed to several test atmospheres (NO_2, SO_2, formaldehyde, or ozone). Gas-mediated cytotoxicity was assessed after a one hour exposure via electronic cell counting (CASY® technology). Analysis of dose-response relationships showed a good reproducibility within and between the laboratories for all four gases. Comparison of the derived EC_{50} values with published LC_{50} values revealed a tight quantitative relationship between *in vitro* cytotoxicity and *in vivo* lethality. To evaluate the performance and reliability of electronic cell counting, the cytotoxicity of SO_2 was additionally assessed using two well established viability assays, the Neutral Red Uptake assay and the CellTiter-Blue® assay (Promega). A high correlation was found between EC_{50} values obtained with all three viability assays. The release of IL-6, IL-8, and MCP1 was analysed in order to evaluate the inflammatory effect of SO_2. Genotoxicity assessment via Comet assay demonstrated reproducible dose-response relationships for SO_2 and formaldehyde. No such dose-dependent genotoxicity could be observed for NO_2 and ozone.

The results of the present pre-validation study are promising with respect to the reliability and relevance of the proposed *in vitro* method for inhalation toxicity testing. Extended prevalidation is underway to establish a tested training set of compounds sufficiently large to allow for optimization of the developed prediction model.

I-10-629

Comet assay atlas

J. Tanaka, M. Ueda, S. Masumori, M. Nakajima and M. Hayashi

Biosafety Research Center, Foods Drugs and Pesticides (BSRC), Shizuoka, Japan

t_jin@anpyo.or.jp

This Comet Assay Atlas was designed and edited under supervision of the Validation Management Team (VMT) of JaCVAM and is the initiative of an international validation study on the Comet assay. The object of the Atlas is to provide a standard to identify analyzable images for the image analysis. Although using an image analyzer, which gives data objectively, it is important to avoid bias for selection of nucleus to be analyzed. An operator should choose nuclei randomly from microscopic fields with high quality, taking into consideration conditions such as shape of nucleus, staining condition, cloudiness of nuclei, and existence of scattered debris. For example, peculiar shaped nuclei or much smaller nuclei compared with surrounding ones should not be selected for analysis. The other difficulty is to distinguish so called "hedgehogs" from nuclei with big tails. Hedgehogs may be derived from apoptotic or necrotic cells and not be derived from cells with damaged DNA. This Comet Assay Atlas gives guidance on how to distinguish between hedgehogs and true comet nuclei.