Session 5.2
New approaches to risk assessment (ESTIV-Symposion)

Lecture

An *in vitro* cytotoxicity study of the interactive effect of 24 binary and ternary mixtures from the GHS classification groups

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While conventional toxicology testing focuses on single chemicals, human exposures are usually to more than one chemical. Most regulatory agencies use the default assumption that the risk of exposures to more than one chemical is treated in an additive manner. This approach may under- or over-estimate the risk of chemicals depending on their different modes of action or interaction. Therefore, one objective of combination toxicology is to establish whether exposure to a mixture of chemicals will result in an effect similar to that predicted on the basis of additivity. In this study, human skin fibroblasts were used in the colourimetric MTS (tetrazolium salt; Promega®) and the NRU (Neutral Red Uptake; Sigma) assay to investigate combination toxicology phenomena. Individual IC_{50} toxicity values for 24 chemicals whose *in vivo* toxicity was spread over the five Globally Harmonised System (GHS) categories for acute oral toxicity were used to create 18 binary and 6 ternary chemical mixtures. Concentrations of individual chemicals in mixtures were chosen based on an estimation of equitoxicity by applying the concentration addition concept, ensuring that no chemical contributed disproportionately to the overall combination effect. Both MTS and NRU assays were similar and consistent in estimating the interactive effect. The toxicity of the total mixture (IC_{50}(tot)), was then compared with the calculated value (IC_{50}(calc)). 37% of studied chemical combinations resulted in additive (IC_{50}(tot)=IC_{50}(calc)), 45% antagonistic (IC_{50}(tot)<IC_{50}(calc))and 18% in synergistic interactions. These results suggest that while additivity covered some of the studied interactions, both antagonism and synergism cannot be excluded from chemical risk assessments.

Poster

Prevalidation study on testing percutaneous absorption via reconstructed human epidermis

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Introduction: Toxicological testing is of increasing importance for risk analysis. The recently approved OECD guideline 428 and guidance document 28 standardise experiments on *in vitro* percutaneous absorption using human and animal skin. Five laboratories plus the ZEBET run a prevalidation study funded by the German Ministry of Education and Research (BMBF) to qualify Reconstructed Human Epidermis (RHE) for *in vitro* testing of percutaneous absorption.

Methods: A test protocol was set up, thoroughly tested and refined in the partner laboratories. OECD standard substances caffeine and testosterone were applied to human epidermis sheets, pig skin and RHE (EpiDerm™, EPISKIN® and SkinEthic®) mounted into Franz cells.

Results and Discussion: Epidermis sheets and pig split skin are clearly less permeable compared to RHE. With respect to permeability the order of RHE was as follows: testosterone: SkinEthic®>EpiDerm™, EPISKIN®; caffeine: SkinEthic®, EPISKIN®>EpiDerm™. This, however, needs to be verified by a broader validation study including more substances of widely varying physicochemical characteristics.

Lecture

Possibilities for assessing risk to humans from chemical exposure by using non-animal test data

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The use of data from non-animal toxicity methods in risk assessment has mainly been limited to hazard identification and for elucidating mechanisms of toxicity. However, there is a need to extend the use of *in vitro* tests to hazard characterisation and risk assessment. For a test to be useful for HC it must: a) be mechanistically based with a biologically plausible relationship between the endpoint measured and the phenomenon being modelled; b) have been validated against human data (ideally); c) have a relevant endpoint (one occurring in the target species); and d) have a prediction model related to toxicity in the target species. This might be realised by: a) increasing the use of human cells; b) better maintenance of differentiated cells; c) use of genetically-engineered cells; d) development of organotypic cell systems; e) use of co-cultures of different cell types; and f) development of techniques for long term culturing, repeat dosing and assessment of recovery. Also, it will be necessary to obtain more information on the differences between cells in culture and *in situ* in tissues, and on the effects of dosing *in vitro* and *in vivo*, to develop realistic and meaningful uncertainty factors to allow *in vitro* information to be used for risk assessment in its own right, and in conjunction with animal data. These issues and a suggested proposal for using *in vitro* data in risk assessment by implementing the above strategies to facilitate the extrapolation from tissue culture to the whole animal, are discussed.

Poster

Cytotoxicity of the derivatives of adamantan and its prevention with some antioxidants

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Toxicity of the most antiviral preparations is a major problem of their usage. We studied the cytotoxicity of two derivatives of adamantan – Rimantadine®: 1-(1-adamantyl) ethylamine hydrochloride and Polyrem®: the ionic complexe of Rimantadine with the copolymere of vinylic alcohol and vinylamidosuccinic acid in the cell cultures – A-549 and MDCK. Cytotoxicity was assessed by: 1) reduction of MTT or rezazurine; 2) leakage of LDH; 3) neutral red uptake.

The results of the short (2 h) exposure with the tested compounds were close to mammalian acute LD_{50}. A study of the metabolic indices after 48 h exposure revealed a higher susceptibility to tested antivirals of the system of endocytosis (NR uptake) in comparison to the other indices. Binding of the active compound to the polymeric complex significantly diminished a degree of its cytotoxicity: the toxicity of Polyrem was approximately twice lower comparing to the equimolar concentration of Rimantadine and the mixture of Rimantadine with the polymeric carrier.

The assessment of possible cytoprotective effect on Rimantadine-induced cytotoxicity of the reduced and oxidized glutathione and Hypoxene® (derivative of ubiquinone) applied in pharmacologically adequate concentrations revealed a significant effect only of the later compound – the enhancement of IC_{50} 2,3 times in comparison to pure Rimantadine. Hypoxene exhibited its inherent significant antiviral activity diminishing the cytopathic effect on the cells of influenza A (H3N2) virus.

Our results demonstrate the perspective of the application of at least some antioxidants to diminish the toxic effect of antivirals and to boost their specific activity.

Lecture

TestSmart – developmental neurotoxicity

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Introduction: In the United States federal regulations aimed at testing compounds to determine developmental neurotoxicity rely heavily on animal models to examine gross morphological changes or behaviour modification. They are time consuming, animal intensive and difficult to interpret. Advocates, researchers and regulators who strive to eliminate developmental neurotoxicants (DNTs) from the environment are united in their desire to establish a scientifically credible, reliable and humane testing methods that can detect DNTs.

Methodology: This presentation will examine the current United States DNT testing protocols, chart the sources of dissatisfaction among advocates, regulators and the scientific community, and discuss efforts to improve the DNT testing protocol, especially efforts based on the 3Rs, such as utilising *in vitro* toxicology tests, or animal tests in non-mammalian species.

Results: Genetic and cellular mechanisms that underlie the biological processes leading to developmental neurotoxicology are important in understanding how conditions develop that cause or are responsible for neurological disease or developmental impairment. *In vitro* and non-mammalian tests hold great promise as parts of a 3Rs based system to evaluate DNTs. Adoption of such testing protocols will substantially improve the evidence base for evaluating chemical risks.

Discussion: Working together, using CAAT's TestSmart approach, advocates, researchers and regulators examine current toxicology tests and develop approaches to build batteries of tests that will improve public health by identifying developmental neurotoxicants for strict regulation.

Poster

Improvement of experimental setup for cutaneous penetration screening studies with reconstructed skin

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Percutaneous studies are usually performed on human skin samples set up on Franz® Cell device but they depend on the availability of skin samples. Reconstructed skin is an interesting alternative to overcome such limitations but it cannot be easily mounted on diffusion cell. However previous studies showed that Episkin® model could be set up on Permgear Cell device and provide a highly performing model, yet time consuming and unsuitable for screening purposes. Then the use of the insert of Episkin® model *per se* had to be reconsidered. The goal of this study was to compare cutaneous penetration of compounds when applied onto Episkin® samples either on Permgear cell or in their own insert.

Seven compounds having widely different chemical structure and penetration potential were applied in the same vehicle and evaluated in triplicate on two Episkin® batches in both devices (Permgear Cell *vs*. insert). After 4 hour exposure time, receptor fluids were analysed by LC/MS or LC/UV.

No leak was detected in both experimental conditions. For six compounds, the penetrated dose was similar in both devices. For the last one, the penetrated dose was decreased by a factor of two using Episkin® samples still in their insert as compared to sample in Permgear cells.

It was demonstrated that percutaneous study on Episkin® samples could be performed easily using insert. Episkin® model has been greatly improved in the recent years. It is now possible to develop screening tests for evaluating skin penetration with a higher reliability.

Poster

Percutaneous absorption test on reconstructed skin: Validation for hydrophilic compounds

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Previous studies demonstrated that human reconstructed skin models could be very useful to evaluate percutaneous absorption. They however involved 3 test chemicals only. The present study extended such validation to 10 hydrophilic compounds.

Ex vivo human skin data were obtained for all compounds in the same laboratory under similar experimental setup using various conditions: whole or dermatomed human skin, two types of cosmetic vehicle (oil/water emulsion or hydroalcoolic gel), with 16 or 24 h exposition time. Despite these differences, data collected could be considered as homogeneous. Two groups of compounds could be distinguished: the first one includes 6 compounds with permeated dose higher than 3% of applied dose and the second one 4 compounds showing permeated doses lower than 0.5%. Reconstructed skin (Episkin® 0.38) model was then used. Each compound was tested on at least 3 batches. The intra- and inter batch variability was generally lower than 30%. The ranking was not modified over these batches. Appropriate experimental setup was used: an application time lowered to 4 hours and an applied dose concentration adapted to the compound solubility in the simplex vehicle. All compounds were studied in one set. To reach such throughput, Episkin® model was used in insert. The Episkin® 0.38 model was able to discriminate the two groups of compounds. The different ranking inside the two groups could be explained by the imperfect barrier function of reconstructed skin model and/or the variability of *ex vivo* data. These results validate reconstructed skin model as an efficient tool for estimating percutaneous absorption.

Lecture

In vitro – in vivo extrapolation of toxic potencies for hazard and risk assessment – problems and new developments

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The aim of toxicological hazard assessment is to characterise the dangerous properties of chemicals for man and the environment. Information on both (a) the toxic potential, i.e. the spectrum of toxic effects a chemical can produce, and (b) the toxic potency, i.e. the quantitative relationship between dose/concentration and toxicity, are essential to characterise the toxic hazard. Toxicological risk assessment comprises hazard assessment and is aimed to characterise likelihood and severity of adverse effects occurring to man or the environment following exposure under defined conditions to a chemical.

Two fundamental problems hamper the application of *in vitro* assays for hazard assessment: Firstly, the endpoints of toxic action detectable *in vitro* are less complex and importantly mostly different from those assessed *in vivo* (toxicodynamic problem). Secondly, toxic concentrations determined *in vitro* are not equivalent to toxic doses or concentrations *in vivo*. This is due to important differences in biokinetics and bioavailability of chemicals *in vitro* and *in vivo* (toxicokinetic problem).

This contribution is focussed on the second aspect. It will be demonstrated, how it is possible to make improved predictions of toxic concentrations in human serum and the aquatic environment, respectively, that are equivalent to cytotoxic concentrations *in vitro*. This can be achieved by the application of a recently developed quantitative extrapolation model taking into account substance and system specific parameters important for the bioavailability of chemicals. It appears that this approach represents a real progress in solving part of the "toxicokinetic problem".

Poster

Application of decision theory to interpretation of *in vitro* tests battery results

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Different *in vitro* tests can give conflicting results. Bayesian decision theory incorporates all, including conflicting, results into one mathematical framework, and formally generates one result. The method allows for a science-based, fully transparent and objective consensus building. It combines strengths of individual tests and minimises influence of weak tests. Bayesian approach allows the framework to function in a tier mode. In addition, Bayesian approach allows to explicitly quantify improvement (reduction of uncertainty). If the target predictivity of a tier is preset the methods allows determining optimal number of tests needed and their minimum predictivity. Battery of genetoxicity *in vitro* tests will be used as a working case study.

Poster

Assessment of the performance of *in vitro* photogenotoxicity assays: Results of a collaborative study with 13 coded test chemicals

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A collaborative study with seven participating laboratories was conducted to evaluate the performance of previously developed test protocols for the photo micronucleus test (PMNT) and the photo Comet assay (PCA) with Chinese hamster V79 cells. Thirteen coded test chemicals were selected based on their ability to absorb UV light of which eight were classified as photogenotoxic and five as non-photogenotoxic (three phototoxic, two non-phototoxic) according to published data. Each compound was tested in two independent runs in both assays by 3-5 different investigators.

Results obtained showed a good reproducibility, both within and between laboratories. Sensitivity in detecting the photogenotoxic compounds (8-methoxypsoralen, chlorpromazine, lomefloxacin, ciprofloxacin, methylen blue, proflavine, dacarbazine, doxycycline) was higher in the PMNT (98%) than in the PCA (77%). Specificity of both models appears to be low as the three phototoxic compounds assumed to be non-photogenotoxic based on literature data (promazine, ketoprofene, acridine) showed predominantely positive findings. However, these results most likely suggest that the available published data were inadequate for a correct pre-study classification.

In summary, the data provide a secure foundation for future evaluations of both assays and for their eventual validation as models for the prediction of photogenotoxicity and potential photocarcinogenicity. An agreed standard list of calibration chemicals is considered key for any further evaluation/validation studies.

This work was supported by the German Federal Ministry of Education and Research, BMBF-project No. 0312916A/B/C/D.

Lecture

In vitro strategies to investigate the potential pro-arrhythmic effects of compounds during the drug discovery and development process

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Interaction of compounds with the hERG potassium channel have been detected as the main source of cardiotoxic events, in particular QT-prolongation and torsade-de-pointes. Therefore, pharmaceutical companies test their compounds at different stages of the discovery and development process on this ion channel. Evotec uses and combines a variety of technologies to detect potential hERG interaction. During discovery and early development, large numbers of compounds (1000-100,000) are tested using a fluorescence assay based on detection of changes in the membrane potential. This assay gives a first assessment of hERG liability. Later during the lead optimisation process measurements using automated patch-clamp are performed with reduced numbers of compounds (10-1000). Selected compounds are investigated using the manual patch-clamp methods, either under GLP or non-GLP to obtain relevant information and documentation for the legal approval of the compounds.

The activities of the compounds on hERG have to be discussed in relation to several factors including active concentration, onset and reversibility of a potential block. Information on other ion channel targets like SCN5A, the sodium channel of the heart, and the L-type calcium channel may be beneficial for a complete evaluation of the side-effect potential of the compounds.

In this presentation an overview of the cardiac ion channels and the combination of *in vitro* screening technologies will be given.

Lecture

DNT testing in the name of children's health: A case for precautionary safety factors

Troy Seidle
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Evidence that infants and children may be differentially susceptible to chemical insult during critical periods of development has led to a marked increase in animal testing for reproductive and developmental endpoints. The US EPA has recently proposed amendments to its data requirements for conventional pesticide chemicals that could make 2-generation reproduction and two-species developmental toxicity studies absolute, rather than conditional, requirements for both food- and non-food-use chemicals. Developmental neurotoxicity testing (DNT) would also become a newly codified, conditional requirement. Although a variety of DNT protocols have existed for many years, no standardised study design has ever been subjected to formal validation according to modern standards, as evidenced by the many published studies that report profound differences in species sensitivity for this endpoint – up to10,000-fold in some cases. From a regulatory perspective, an EPA retrospective analysis revealed that DNT studies rarely produce lower "no-effect levels" than studies within the existing database for a pesticide. DNT data have yet to be used as a basis for lowering any pre-existing reference dose; in fact, the EPA has done the opposite, by removing a statutory 10x "children's health" safety factor for 30 organophosphate pesticides, replacing it with factors of 1x and 3x, respectively. Evidence to date suggests that DNT testing is significantly less protective of sensitive sub-populations than the application of a precautionary safety factor. Thus, in the interests of children's health, DNT testing should be discontinued, and associated regulations and test guidelines should be repealed.

Poster

The dog as test species for the toxicological evaluation of pesticides – present status

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The German Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments, ZEBET, reviewed the toxicological data on pesticides obtained in studies using the dog as test species. The study was sponsored by the German Foundation for the Promotion of Research on Replacement and Complementary Methods to Reduce Animal Testing, SET. SET initiated a retrospective analysis of toxicological data on pesticides from the files of the German Agency for the Regulation of Pesticides, which was conducted by the German Federal Institute of Risk Assessment, BfR, and published in two parts in 1998 and 2001. The outcome of this study has been compared with the results of other published retrospective analyses as well as preliminary results of the Agricultural Chemicals Safety Assessment project initiated in 2001 by the International Life Sciences Institute, ILSI, in which data from the database at U.S. EPA's Office of Pesticide Programs are used. All studies support the conclusions of the SET study that toxicological safety testing in dogs can be restricted to sub-chronic studies of 13 weeks and that studies of longer duration do not provide additional essential information. Similar conclusions were drawn from retrospective analyses evaluating data on therapeutic drugs and incorporated in the recommendations of the International Conference on Harmonisation of Registration of Pharmaceuticals, ICH. The present survey thus shows that, the routine conduct of 12-month studies in dogs is no longer required for agricultural chemicals. Changing international regulations accordingly would be beneficial both in economical and animal welfare terms.

Poster

SC Johnson's consumer product hazard evaluation program using alternative assays

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SC Johnson is a global consumer product company that manufactures a variety of household products that must be evaluated for human health hazards. For over 10 years, SC Johnson has conducted the necessary research to spearhead efforts to reduce the use of animals in the hazard assessment process. We routinely use alternative approaches such as the eye and skin irritation assays in a weight of evidence approach for hazard classification and labelling purposes for a variety of products. Assay choice and protocol considerations are defined so as to address possible modes of action on the target tissues. Specific benchmark formulations have been employed with each study to facilitate interpretation of the results. The Bovine Corneal Opacity and Permeability Assay (BCOP) has been used to assess ingredient synergies and the impact of various formulation components on the irritancy potential of the end-use products. The overall safety evaluation approach will be illustrated using two case studies. Alternative assays, especially the BCOP, are indispensable tools for assessing the potential irritancy of our products distributed worldwide while reducing the use of animals.

Poster

Skin irritation testing: A correlation study between *in vitro* and *in vivo*

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Skin irritation is the most common adverse reaction in humans. For reasons of human risk assessment, formulations have to be assessed for putative irritant side effects. Therefore, the human patch test is an appropriate method. But, individual differences in the analyses of the read-out parameters and the test panels make a distinct assessment often difficult.

Aim of this study was to investigate the feasibility of an *in vitro* approach for human risk assessment concerning irritancy of surfactants. Test samples were kindly provided by the German Society of Cosmetic Chemists (DGK), which conducted a human patch test study with the same set of samples in parallel. By this, we had the unique chance to correlate *in vitro* and *in vivo* data.

In order to assess irritant effects *in vitro*, reconstructed human epidermis was exposed to 8 coded test samples, consisting of individual anionic surfactants, blends of surfactants and controls. A Multiple Endpoint Analysis was established to comprise the viability, cytotoxicity, histology, cytokine release and differential gene expression. As results, a high level of correlation was determined for our *in vitro* assessment of skin irritancy to an in theory ranking and the human patch test data.

Here, we presented the good predictability of the Multiple Endpoint Analysis for assessing irritant potentials of formulated surfactants. Further investigations are necessary to evaluate the potential of this *in vitro* method to assess also other classes of irritants and more complex formulations.

Lecture

Replacing animal testing for consumer safety – is it feasible?

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The proposed EU Chemicals Regulation (REACH) and the 7th Amendment to the EU Cosmetics Directive challenge the use of animal tests for evaluating the safety of chemicals and consumer products. Unfortunately today much of the data derived from the existing alternative (non-animal) tests cannot be used for human health risk assessment.

The approach we suggest is based on the concept that consumer safety aims at preventing harm and disease in man. Animal tests served their purpose since technologies used in the animal studies to generate data for risk assessment are similar to the technologies used in clinical medicine. This allows interpretation of the animal data in terms of harm and disease in man.

New molecular biology and informatics technologies are continually being introduced in science and medicine. Using "systems biology" approaches in both experimental biology and medicine should support the integration and interpretation of the large amounts of complex data now being generated, by providing better understanding of the underlying biological complexity. We postulate that this will enable new experimental models and risk assessment paradigms to be developed that do not require the use of animals.

This new approach underpins our experimental projects in skin allergy and inflammation. The overall objective is to be able to make consumer safety decisions for these effects, with an acceptable level of confidence, without the use of new data generated in animal tests.