Session 5.6
In vitro approaches for determining acute systemic toxicity

Poster

Pulmonary irritancy potential determination using an *in vitro* human airway epithelium model

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The occurrence of asthma and Chronic Obstructive Pulmonary Disease (COPD) in industrialised countries has been increasing for the past 30 years. Local respiratory tolerance is a critical issue since lung irritation is a common acute side effect for inhaled drug. An *in vitro* airway epithelium model was implemented to determine irritancy potential of new chemical entities when given by the inhalation route to humans.

The Calu-3 model consists of human bronchial epithelial cells of the airway tissue of the respiratory tract. Calu-3 cells when placed at the air-liquid interface form a 3-dimensional epithelium model, that develop tight junctions and functional barrier properties. It also induces ciliogenesis and produces mucus in our culture conditions.

To determine the relevance and the reliability of this *in vitro* model, 8 chemicals intended for the treatment of COPD/asthma were tested in the Calu-3 model. A multiple endpoint analysis approach was used by measuring cell viability (MTT), inflammatory responses (interleukines and chemokines), tight junction disruption (trans-epithelial resistance) and production of mucin secretion on the apical side of the model. *In vitro* and *in vivo* animal data correlated suggesting that the Calu-3 model could be used to address lung irritancy potential of new chemical entities.

Poster

Relationship between total antioxidant activity of plasma and parameters related to oxidative stress induced by hepatotoxic drugs in rats

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Introduction: Antioxidant defense system encompasses the enzymatic and non-enzymatic factors some of which are often measured in tissues. Measurement of all these parameters is not feasible. Total Antioxidant Capacity (TAC) of plasma is a single assay that represents the balance between pro- and antioxidants factors. In this study the reliability of TAC of plasma as an index of oxidative stress was assessed in relation to formation of lipid peroxidation and changes in individual antioxidants.

Methods: Rats were treated with different doses of acetaminophen or menadione, blood was collected and ferric reducing ability of plasma (FRAP) was determined as a measure of TAC. The rate of lipid peroxidation products were measured in plasma. The relationship between FRAP and antioxidants such as blood glutathione, plasma bilirubin, plasma uric acid and total protein together with catalase and superoxide dismutase (SOD) activities in erythrocytes were assessed.

Results: FRAP was markedly increased (5-6 fold) in rats following administration of a single i.p dose of APAP to rats. Elevation of FRAP was observed to be highest, 4-12 h after APAP injection. FRAP was increased depending on APAP dose given. Elevation in FRAP was inversely related to the rate of lipid peroxidation in liver. Interestingly, in growing rats among the enzymatic and non-enzymatic factors measured, plasma bilirubin and erythrocyte's superoxide dismutase (SOD) were correlated with changes in FRAP.

Discussion: FRAP is a simple and reliable assay for assessment of whole body antioxidant capacity. FRAP changes due to hepatotoxins is correlated with certain antioxidant factors namely bilirubin and SOD.

Poster

Novel *in vitro* exposure techniques for toxicity testing and biomonitoring of airborne contaminants

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Exposure to air toxicants is a major contributor to human health problems. The aim of this study was to develop practical and reproducible *in vitro* techniques for assessing the toxicity of airborne contaminants. Two methods were developed based on the physiochemical properties of test chemicals: static and dynamic direct exposure techniques at the air/liquid interface. Xylene, Toluene and Nitrogen dioxide were chosen as a model test compounds. Human cells including A549 (lung derived), HepG2 (liver derived) and skin fibroblasts were grown in porous membranes. For the static method, test atmospheres of volatile organic solvents were generated in glass chambers (322 ml) and cells were exposed to airborne concentrations for 1 hour at 37ºC. For the dynamic method, cells on membranes were placed in horizontal diffusion chambers and exposed to dynamic flow (25 ml/m) of test gas for 1 hour at 37°C. Cytotoxicity was investigated using the MTS (tetrazolium salt; Promega), NRU (neutral red uptake; Sigma) and ATP (adenosine three phosphate, Promega) assays. Xylene (e.g. IC_{50} = 5,350 ± 328 ppm, NRU; IC_{50} = 5,750 ± 433 ppm, MTS in fibroblasts) was found to be more toxic than Toluene (e.g. IC_{50} = 10,500 ± 527 ppm, NRU; IC_{50} = 11,200 ± 1044 ppm, MTS in fibroblasts) in all cells tested. Dose dependant effects of NO_2 were observed in human cells tested. Our findings suggest that the static direct exposure is a practical technique for assessing the toxicity of volatile compounds. Further, dynamic direct exposure offers the potential for respiratory toxicity studies and as an advanced technology for biomonitoring of airborne contaminants.

Poster

The third FRAME Toxicity Committee: Alternatives in toxicity testing

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Substantial progress has been made since the first FRAME Toxicity Committee was established in 1979, particularly with regards the successful validation and regulatory acceptance of non-animal replacement methods. A third FRAME Toxicity Committee (FTC) was established in 1999 and comprised 18 experts from industry, academia, the legislative, regulatory bodies and animal welfare. The primary objective of the FTC is to review and make recommendations about the use of Three Rs approaches in the research, development and safety evaluation of medicines, biological products, cosmetics and chemicals. The FTC has more recently been restructured in the form of a Standing Committee and a smaller Steering Group which together will guide the activities of five working parties. The first of these working parties specifically addresses issues relating to risk assessment and, later this year, will hold a focused scientific workshop entitled 'Toward a better way to assess risk of toxic exposure'. The workshop will address, among other issues, how the risk assessment process can be improved to cope with the demands of legislation such as the REACH system. A separate working party is looking at the practicalities of data-sharing with particular reference to the REACH system and assessing the reliability of read-across within (Q)SAR-driven intelligent testing strategies. Two further working parties are addressing more specific areas namely, carcinogenicity testing and endocrine disruption, whilst a final working party is more directly involved in organising workshops. This presentation summarises the main activities and recommendations of the third FTC.

Lecture

The role of biokinetic information in the interpretation of *in vitro* cytotoxicity data: An essential part of estimating acute toxicity in the ACuteTox programme

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In previous programmes, the feasibility of the use of *in vitro* cytotoxicity data for the prediction of *in vivo* lethal doses was tested. In these studies it was shown that basal cytotoxicity gave good estimates for about 70% of the compounds, i.e. these chemicals could be classified in the appropriate LD_{50} classes.

An important drawback of the use of cytotoxicity data is the difficulty of extrapolating a toxic concentration in the *in vitro* system to a toxic dose in the *in vivo* situation. Deviations from a simple linear relationship between effective concentrations *in vitro* and toxic doses *in vivo* can be the result of the fact that the effective concentrations *in vitro* are irrelevant for the concentrations that may cause toxicity in target organs *in vivo*. These deviations may be caused by the processes in the biokinetics of the compound under study. For instance, the absorption of the compound may be minimal, thus leading to low systemic concentrations. Moreover, the processes of distribution, metabolism and elimination may lead to lower or higher concentrations in target organs than could be expected from an even distribution of the compound over the body.

Efficient tools to estimate the biokinetic processes can be found in biokinetic modelling. Therefore, one of the work packages in the ACuteTox programme is focusing on biokinetic processes: *in vitro* determination of metabolism and transport and biokinetic modelling.

In conclusion: the incorporation of biokinetic information will highly improve the possibilities of estimating the *in vivo* toxic dose on the basis of *in vitro* basal cytotoxicity data.

Poster

Use of the Balb/c 3T3 mouse fibroblast Neutral Red Uptake cytotoxicity assay and the Normal Human Keratinocyte (NHK) Neutral Red Uptake cytotoxicity assay to predict the maximum tolerated dose for anticancer drugs – stage 1, cytotoxicity data

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An ECVAM, FRAME, Cancer Research-UK collaborative project commenced January 2004 to evaluate the predictive capacity of *in vitro* basal cytotoxicity assays to determine human toxicity of ten coded anticancer drugs. Testing employed the Neutral Red Uptake (NRU) protocol, from the ICCVAM/ECVAM validation study (http://iccvam.niehs.nih.gov/methods/invitro.htm) which was compared with the Kenacid Blue (KB) total protein assay.

NR uptake is modified by chemical alterations of the cell surface and/or the lysosomal pH. Other mechanisms, including cell membrane damage, are involved in the inhibition of cell proliferation, resulting in a decrease in total protein content. Therefore a chemical could cause a change in NRU without affecting total protein. Results: the KB assay rankings (IC_{50} values) matched the NRU rankings for both cell types. NRU IC_{50} values ranged from 0.0115 to >1750 µg/ml for NHK cells and from 0.00248 to 1022 µg/ml for 3T3 cells. The chemicals were differentially ranked for the two cell types, with the lowest IC_{50} values first. For NHK cells; Ben Lomond > Gulvain > Ben Nevis > Lochnagar > Ben Cruachan > Ben Lawers > Ben Macdui > Cairn Gorm > Schiehallion > Sgurr Mor. For Balb/c 3T3 cells: Ben Lomond > Gulvain > Schiehallion > Lochnagar > Ben Nevis > Ben Cruachan > Ben Lawers > Ben Macdui > Cairn Gorm > Sgurr Mor. Schiehallion was significantly more toxic to the Balb/c 3T3 cells. Upon code disclosure the significance of this difference can be explored. Despite the use of passage 2 keratinocytes from different donors the positive control fell within the acceptance criteria set for the NRU assay.

This work was funded by the EU grant F1ED-ISP-GB-21468-03-12

Lecture

Analysis of the correlation between *in vitro* cytotoxicity data and acute toxic effects in humans

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Several studies have shown that *in vitro* cell systems can predict acute toxic effects *in vivo*. NICEATM and ECVAM recently conducted a multi-laboratory validation study to assess the predictive capacity of two *in vitro* basal cytotoxicity assays primarily for predicting rodent, but also human acute toxicity. Seventy-two coded chemicals were tested in mouse 3T3 fibroblasts and normal Human Epidermal Keratinocytes (NHK) using the Neutral Red Uptake (NRU) assay. Forty-one chemicals used in the study are MEMO chemicals, i.e. chemicals for which relevant human toxicity data exists. The collection of human toxicity data for four other chemicals, for which these data were lacking, was commissioned by ECVAM. A preliminary analysis conducted with twelve chemicals showed a good correlation for both cell types (3T3 cells: R^2=0.787, NHK: R^2=0.886) between *in vitro* IC_{50} values (i.e. the concentration of a chemical that inhibits cell growth by 50%) and peak serum concentration in humans derived from time-related lethal and sub-lethal concentrations curves. However, the *in vitro* IC_{50} values for cadmium chloride and ethylene glycol under- and over-predicted human toxicity by more than 10-fold respectively. The evaluation of the correlation between human data and the IC_{50} values for the complete set of chemicals is ongoing.

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Poster

High-resolution respirometry – an important tool to evaluate energy metabolism of isolated hepatocytes

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Mitochondrial system of energy provision plays important role in many basic biological functions, including cell death and cell proliferation. It is also a target of many toxic substances. Therefore detailed information about energy metabolism is essential to elucidate mechanism of toxic action of xenobiotics and to evaluate stimulatory or inhibitory effect of various substrates. Recently new method to assess energy status of mitochondria is available.

High-resolution respirometry (Oxygraph 2K, OROBOROS, Austria) is very sensitive electroanalytical method, which allows measuring oxygen consumption in isolated cells or mitochondria. Specific substrates and inhibitors of respiratory chain enzymes and ATP formation enable to evaluate function of individual complexes of oxidative phosphorylation. In this study we compared respiratory rate of intact and digitonin-permeabilised hepatocytes. Permeabilisation allows better accessibility of energy substrates for mitochondria and offers conditions more close to the situation *in vivo*. Hepatocytes were isolated from male albino Wistar rats (220-250 g) by collagenase perfusion of the liver. Oxygen consumption was measured in suspension of intact hepatocytes incubated in Krebs-Henseleit medium, digitonin-permeabilised hepatocytes were incubated in potassium-medium.

Our results indicate that succinate-dependent respiration is twofold increased after the addition of ADP ($p<0.001$) and there was a fivefold increase of glutamate+malate-dependent respiration in permeabilised hepatocytes ($p<0.001$). These findings are additional evidence that mitochondria in permeabilised hepatocytes are tightly coupled.

High-resolution respirometry enables monitoring of oxygen consumption in small amounts of biological material ($pmolO_2/s/10^6$ cells) and is thus suitable method for evaluation of energy metabolism.

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Poster

Use of alternatives to animal methods in risk prognostication for hazardous effects of cosmetics preservatives

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Depending on their chemical structure, preservatives have been shown to increase or decrease barrier function of cell membrane and *stratum corneum* lamella. Alternatives to animal methods were used to investigate the effects of cosmetics preservatives (Benzoic acid, Phenoxyethanol, Triclosan, Kathon CG, Dimol, maximum authorised concentrations and lowest concentrations) on the membrane permeability and barrier function of epidermis using a modification of the BRC-test and a biphasic water-lipid model of the epidermis. Membrane permeability in the BRC-test was evaluated by measurement of the leakage of haemoglobin. The barrier function of the biphasic water-lipid model was evaluated by TEWL measurement.

Triclosan, Benzoic Acid and Phenoxyethanol caused a concentration-dependent increase of membrane and *stratum corneum* lamella permeability. Dimol and Kathon CG increased the cells' resistance to cold-induced stress (t=4ºC). Dimol and Kathon CG can be used in cold protective cosmetic products. In the BRC-test Benzoic Acid, Phenoxyethanol and Triclosan caused concentration-dependent membrane alterations when applied under normal conditions of use (maximum authorised concentration, t=37ºC). Exposure of erythrocytes to Benzoic Acid (0.1%) and Triclosan (0.01and 0.3%) induced more than 13-fold (Benzoic Acid) and 3-fold and 120-fold (Triclosan) higher levels of erythrolysis than solvent control. Phenoxyethanol (0.5%) caused a 2-fold increase of the membrane permeability. Treatment of erythrocytes with Phenoxyethanol (0.25%) did not elicit any alteration of membrane integrity. Topical application of Benzoic Acid and Phenoxyethanol on the lipid layer of the water-lipid model caused an increase of the level TEWL by 29 and 40%, respectively. Benzoic Acid and Phenoxyethanol increased the cold-dependent changes in membrane permeability.

Lecture

AcuteTox – Optimisation and pre-validation of an *in vitro* test strategy for predicting human acute toxicity

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Validated alternative test methods are urgently required for safety toxicology testing of drugs, chemicals and cosmetics. ACuteTox is a 5-years integrated project under the EU 6FP with the aim to develop a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes.

The extensive amount of work performed since the 70s has led to a great number of existing *in vitro* models. Many studies have shown good correlation (about 70%) between *in vitro* basal cytotoxicity data and *in vivo* LD_{50} values or human lethal blood concentrations. However, this correlation means that a certain number of misclassifications have to be faced when using the existing tests. ACuteTox aims to improvement of this correlation to a level sufficient enough to ensure a valid prediction of acute toxicity.

This will be performed by evaluating the existing outliers of the correlation in order to introduce further parameters, such as ADE, metabolism and organ specificity, which might improve the correlation. This would allow the integration of alerts and correctors in the prediction algorithm, which together with robust implementation of medium throughput approaches, would allow the establishment of a new testing strategy with a better prediction of classification. In summary, A-Cute-Tox aims to improve the prediction of acute toxicity using *in vitro* methods and, at the same time, to signal, which compounds require further testing because their acute toxicity can not be properly predicted.

Poster

Toxicity of environmental pollutants on GM-CFU: Comparison between species and genders

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In vitro haematotoxicology provides the opportunity to study the effects of toxicants directly on relevant human target tissues. Exposure to environmental pollutants as inorganic Arsenic, Atrazine and Naphthalene in drinking water has emerged as a public health concern since they can be easily transferred to the fetus.

Umbilical cord blood is part of the fetal tissues, and it is possible to estimate the effects of chemicals that can affect the future development of fetus by analysing the main feature of these cells, which is their capability to clone.

In this study we evaluated the effect of Arsenic, Atrazine and Naphtalene on the clonogenic capability of blood progenitors (cord blood cells and bone marrow) belonging to different species (human and murine), different genders (male and female) and through different schedules of treatment.

Our data indicated that Arsenic has a relevant toxic effect, at the same level both in human and in mouse, without any difference between the genders (IC_{50}=0.81 uM in female mice and 0.80 uM in male mice; IC_{50}=0.87 in human). Atrazine treatment affects very poorly only at the maximal dose tested (50 uM) the clonogenic capability of both mouse bone marrow (84% male, 91% female) and human cord blood (85%). Naphatalene treatment relieved a different sensitivity in the two species, being human CBC totally unaffected by the treatment, while the murine bone marrow clonogenicity is reduced of 40%, in both the male and female gender.

Poster

Barriers and potential solutions to the implementation of *in vitro* cytotoxicity testing for acute systemic toxicity

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Novel approaches to assessing the toxicity of chemicals or formulations often follow a tortuous path from initial reflections on scientific plausibility to the dream of regulatory acceptance. Currently, many barriers (both real and perceived) to industry's full implementation of a new toxicity method are in this path. Examples include: 1) Liability concerns – Will courts find the new method to be "state-of-the-art" in providing assurance of safety? 2) Cost concerns – Will the new method take longer to perform, be more expensive, or might submission of data from the method to a regulatory agency not prepared to receive or evaluate the information result in longer approval times for products? 3) Public relations concerns – Will submission of paired data from traditional animal tests and non-animal tests (generally necessary to establish the validity of the new method) draw the attention of the animal protection community to the amount of animal testing done in the past by the company? 4) Utility – Does the new method provide information sufficient to address the specific safety concern? and 5) Regulatory acceptance – Will regulatory bodies approach the method with a proactive attitude, or will there be an uphill battle to bring about movement from the *status quo*? It is important that solutions to these potential barriers (and many others besides) be understood and addressed by all stakeholders interested in the implementation of any non-animal test method. Progress, or lack of it, in the implementation of *in vitro* cytotoxicity testing to estimate acute systemic toxicity will be presented.

Lecture

Is progress being made in replacing traditional rodent studies with *in vitro* approaches for determining acute systemic toxicity?

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Investigations of a potential correlation between a chemical's toxicity to animals (including humans) and its toxicity to cells in culture have been conducted for over 50 years. It has seemed reasonable to many scientists that chemicals are toxic in animals because they cause the failure of one or more organ systems. Since interference with cell function (via cytotoxicity, for instance) in the organ system is the likely cause of organ failure, levels of chemical which might cause such organ failure should be determinable from *in vitro* experiments. Thus *in vitro* measurements alone might provide sufficient information on systemic toxicity. This hypothesis has been constructively explored recently by the publication of the Registry of Cytotoxicity (RC) by Willie Halle and collaborators from ZEBET, and in the MEIC program, championed by Bjorn Eckwall. The RC demonstrated a positive correlation between *in vitro* cytotoxicity values from published manuscripts and rodent LD_{50} values from a standard reference source (347 chemicals). These results have prompted Spielmann et al. (1999) to propose using *in vitro* cytotoxicity results to estimate starting doses for animal studies, and an international study is ongoing to validate this proposal. The MEIC program showed that cytotoxic concentrations of selected chemicals *in vitro* correlated well with lethal blood concentrations in humans; actually better than rodent LD_{50} values did. Recent efforts have begun to add necessary ADME components to the cytotoxicity predictions, hopefully resulting in the construction of a fully *in vitro* prediction system for human acute systemic toxicity.

Poster

Modulation of different stress pathways after styrene and styrene-7,8-oxide exposure in HepG2 cell line and normal human hepatocytes

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Styrene is one of the most important monomers produced worldwide, and it finds major use in the production of polystyrene, acrylonitrile-butadiene-styrene resins and unsaturated polystyrene resins. IARC classified styrene a possible carcinogenic to humans (Group 2B).

Styrene-7,8-oxide is the main reactive metabolite of styrene, and it is found to be genotoxic in several *in vitro* test systems.

We investigated the toxicity of styrene and styrene-7,8-oxide on HepG2 cells, evaluating different endpoints of toxicity such as metallothioneins, heat shock proteins, apoptosis related proteins, as well as the accumulation of styrene within the cells and the expression of two isoforms of cytocrome P450. Moreover, the potential activity of styrene and styrene-7,8-oxide in modulating gene expression has been investigated.

Our data revealed that in HepG2 cells there was an hsp70, metallothioneins, BclX/L, c-myc induction and a decrease in BAX expression after styrene and SO treatments, confirming that styrene and SO activated protective mechanisms and did not induce apoptosis.

In addition, we found an up-regulation of TGFb2 and TGFbRIII in HepG2 after exposure to styrene, while in human normal hepatocytes these genes have been down-regulated after both treatments.

Finally, we found that the styrene and SO treatment induced CYP1A2 protein expression.

In conclusion, both the compounds caused toxic stress in HepG2 cells, being SO directly more toxic; in the meantime, we observed an opposite effects of the two compounds in carcinoma cells and normal hepatocytes regarding their activity in gene modulation.

Poster

An *in vitro* acute and chronic evaluation of NP0361 cytotoxicity in hepatic cells: Comparison with tacrine

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The most widely used pharmacological approach for Alzheimer's disease (AD) is the enhancement of cholinergic transmission with acetylcholinesterase inhibitors (AChEI). Tacrine (THA) was the first AChEI in the market but it is now hardly ever used because of its hepatotoxicity and gastrointestinal side effects.

Neuropharma is working on the development of new drugs for AD treatment and has synthesised a series of dual AChEI with a very potent inhibition of both the esteric site of the enzyme and the peripheral site involved in the aggregation of b-amyloid. NP0361, which activity is about 2.5 10-11M, reduced both plaque load and soluble Aβ peptides and improved cognitive functions in a transgenic h-APP mice model. Here, we present the experiments performed to explore whether NP0361 might present a better safety profile than THA exploring the cytotoxicity of these two compounds in cell models of hepatic origin.

No significant difference was observed between chronic and acute treatment of rat hepatocytes primary cultures with NP0361 in contrast to THA which induced a time-dependent increase in toxicity at similar concentrations. Moreover, unlike THA, the toxicity of NP0361 would mainly rely upon its metabolism, since no toxicity was observed in the poorly-metabolising cells HepG2 or RLEC.

Taken together, these studies show that the mechanism of NP0361 toxicity is different to that of THA in similar cell models. A superior margin of security in these test systems confirms the promise that NP0361 is a good candidate for further development for the treatment of AD.

Poster

Armenia: achievements and problems in development of alternative approach *in vitro*

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Our Department is an only institution in Armenia using animal cells *in vitro* (cultures of normal human and rat fibroblasts, transformed human and animal cell lines) as alternatives to predict acute lethality *in vivo*, to assess risk of new chemicals, to identify potential mammalian cell-based biopharmaceuticals. The techniques applied include acute (cell survival determination by vital dye exclusion and cell clonogenic activity) and chronic (induction of chromosome aberrations and micronuclei, comet-assay and comet-assay-FISH) toxicity evaluation. Earlier we have revealed radioprotective and antioxidant activity of two new Mn-chelates and cytotoxic action of two new metalloporphyrins and extracts of callus culture of medicinal plant Oleander. To introduce alternative approach *in vitro* into practice of research we collaborate with Armenian scientists in biology, organic chemistry and medicine in screening of new compounds' biological and anticancer activities. To disseminate 3Rs' ideas and principles we perform animal-free teaching at the Biological Faculty of the Yerevan State University and educating of researchers from the Yerevan Medical University in cell systems *in vitro* as animal alternatives. Alternative approach *in vitro* in Armenia is on the early steps of development and we strongly need contacts, collaboration with and support of global community to be integrated into international activity.

Poster

Assessment of the Halle Register for classifying and labelling new chemicals according to the EU toxicity classes

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To reduce the number of animals used in acute oral toxicity testing, cytotoxicity data (IC_{50}) can be used to predict the *in vivo* acute toxicity of chemicals by using statistical modelling approaches between the IC_{50} values and acute oral LD_{50} values in the Register of Cytotoxicity (RC).

Here, we propose strategies for using *in vitro* data as a basis for classifying and labelling new chemicals representative of all 3 EU toxicity classes as well as the unclassified category and evaluate the probabilities of correct classification depending on the true LD_{50} and depending on the neighbourhood of class limits.

Main topics will be the quality of data, *in vitro* as well as *in vivo*, and its impact on modelling approaches. IC_{50} (RC) and LD_{50} values (ICCVAM validation study using 72 chemicals) and associated 95% confidence limits were calculated for 347 resp. 72 chemicals. LD_{50} values of the RC, listed in the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS) and LD_{50} values selected by ICCVAM will be compared.

Lecture

Cell culture models of the air-blood barrier for the evaluation of aerosol medicines

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Introduction: The pulmonary route is of increasing interest for the development of new medicines, not only for the treatment of lung diseases (e.g. asthma, COPD) but also for the fast efficient delivery of drugs into the systemic blood circulation. Advanced drug carriers, such as nanoparticles or liposomes, however, require the use of polymers and other excipients, the effects of which on the airway and respiratory epithelia are still relatively unknown, especially with regard to their safety.

Methods: We have been evaluating the pulmonary epithelial cell lines, Calu-3, 16HBE14o- and A549, as well as primary cultures of human alveolar epithelial cells (HAEpC). Typically, cells are grown on permeable filter supports, allowing to form monolayers with functional tight junctions and pharmaceutically relevant transporter proteins. This setup can be used to perform transport, cytotoxicity and irritancy studies of drugs, excipients, particles and other xenobiotics.

Results: While the cell lines Calu-3 and 16HBE14o- appear useful to model the bronchial epithelium, the cell line A549 develops only week barrier properties. Therefore, it still appears necessary to use primary cultured cells to model the alveolar epithelium.

Discussion: Cell culture models of pulmonary epithelia offer excellent opportunities to study transport processes of drugs and other xenobiotics across the air-blood barrier, as well as to assess the inhalation safety of new polymers and other chemicals. After adequate characterisation and validation, such systems may be valuable alternatives to inhalation experiments on small rodents or dogs.

Poster

Evaluation of *in vitro* effects of 50 toxic reference chemicals using an electronic cell counting and sizing system versus MTT- and NRU -assay

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According to the guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity (NHI publication No.: 01-4500 from 2001) the performance of the electrical current exclusion method (ECE) was studied for its suitability as an *in vitro* cytotoxicity test. In a comparative study two validated *in vitro* assays based on quantification of metabolic processes necessary for cell proliferation or organelle integrity (MTT/WST-8 assay and NRU-assay) and two cytoplasma membrane integrity assays (trypan blue exclusion and electrical current exclusion) were performed.

IC_{50} values were evaluated for 50 chemicals from low to high toxicity, 46 listed in Halle's Registry of Cytotoxicity (RC, Halle and Goeres, 1988). High correlation between IC_{50} values obtained in this study and the IC_{50} data published in the RC was found. The sensitivity of the assays was highest for the electrical current exclusion method and decreased from MTT/WST-8 assay to NRU to trypan blue (TB) assay. The consistent results of the electrical current exclusion method (ECEM) are based on technical standardisation, high counting rate and the ability to combine cell viability and cell volume analysis for detection of first signs of cell necrosis and subsequent damage of the cytoplasmic membrane caused by cytotoxic agents.

Poster

Protocol optimisation during a validation study to evaluate *in vitro* cytotoxicity assays for estimating rodent acute systemic toxicity

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Previous studies have identified a correlation between *in vitro* cytotoxicity and acute oral toxicity. NICEATM and ECVAM subsequently initiated a three-phase multi-laboratory validation study to evaluate the usefulness of two standardised *in vitro* basal cytotoxicity assays for estimating acute rodent toxicity and the extent that they may reduce animal use. Seventy-two coded chemicals (12 from each of five acute oral hazard categories and 12 unclassified/non-toxic chemicals) were tested in mouse 3T3 fibroblasts and in normal human epidermal keratinocytes (NHK) using neutral red (NR) uptake assays. Phase Ia established the historical databases for sodium laurel sulfate, the positive control, for each of three laboratories. Three chemicals were tested in Phase Ib and nine chemicals were tested in Phase II. Protocols were optimised after each of the first two phases to minimise intra- and inter-laboratory variation prior to testing 60 chemicals in Phase III. Technical challenges arose in Phases Ia/Ib (i.e., formation of NR dye crystals; uneven growth of NHK cells; slow growth of 3T3 cells) that were resolved with Phase II protocols. Significant variation in NHK growth in Phase II attributable to different lots of media and supplements required pre-qualification of medium. The optimised final protocols were used for Phase III testing. These studies demonstrate the value of using a phased approach during validation to optimise and standardise a final test method protocol that can then be used for the final validation phase.

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Poster

ECVAM key area on systemic toxicity: Summary of ongoing activities

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The overall aim of this key area is to validate *in vitro* tests relevant for target organ and target system-specific toxicities to be incorporated into optimal test batteries for the estimation of human systemic toxicity.

In the area of acute toxicity an ECVAM workshop report has recently been published. An ongoing international validation study aims to reduce the number of animals used in oral acute toxicity testing. An Integrated Project, A-Cute-Tox, aims to achieve full replacement for predicting human acute systemic toxicity.

In the area of haematotoxicology the main achievements are the peer review of the validation of the GM-CFU assay to predict *in vivo* acute neutropenia, the pre-validation of the *in vitro* CFU-MK assay to predict thrombocytopenia, and the refinement of the clonogenic assays for high throughput testing.

In the area of immunotoxicology two task force meetings and a workshop have been held. A multi-laboratory study is underway to evaluate the most promising endpoints for immune-suppression.

At ECVAM, an intensive search for *in vitro* models for developmental (DNT) and adult neurotoxic hazard assessment is ongoing. ECVAM in collaboration with CEFIC and CAAT organised a DNT workshop to assess the available models and endpoints relevant for validation of alternative approaches.

In the area of chronic *in vitro* toxicity a task force has been created and a workshop was held. ECVAM is involved in two FP6 projects, Predictomics and Pulmonet.

The final goal in the field of systemic toxicity is to provide cheaper, more ethical and more scientifically based testing strategies.

Lecture

A European pharmaceutical company initiative to challenge the requirement for conventional acute toxicity studies in rodents: Do these studies add value prior to first dose in man?

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Conventional acute toxicity studies in rodents are usually conducted to support the registration of any pharmaceutical intended for human use. The main objective of these studies is to estimate the minimum dose causing lethality. The information may be used to set the starting dose in the first studies in man and/or to give an indication of the likely effects of acute overdose. However, these studies do not usually include clinical pathology, histopathology or toxicokinetics and their clinical usefulness is questionable. In addition, data may be available from other study types that don't have lethality as an endpoint.

A working party representing the pharmaceutical industry was formed in 2003. It aims to:

- Review how acute toxicity studies are conducted within the industry.
- Assess the value of acute toxicity data in a clinical setting and establish whether information from other study types could be used.
- Agree a short term harmonised industry approach focussing on reduction and refinement.
- Develop a strategy for challenging the guidelines on the requirement for conventional acute toxicity where lethality is a defined endpoint.

This presentation will describe the working party's progress in assessing the value of conventional acute toxicity data prior to the first clinical trials in man. The results of a data sharing exercise will be described and a proposal that these studies should not be a mandatory requirement prior to first trials in man, leading to a significant reduction in the numbers of animals used due to compound attrition during the development process.

Poster

Use of alternative methods to animal experimentation for assessment the effect of heavy metals compounds on barrier function of cells' membrane and *stratum corneum* lamella

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Alternative methods to animal experimentation were used to investigate the effect of heavy metal compounds ($Zn(CH_3COO)_2$, $ZnSO_4$, $Pb(CH_3COO)_2$, $CoCl_2$, $CoSO_4$, $SrCl_2$, Pyrithione zinc, concentration from 0.00625 to 0.1%) on the membrane permeability and barrier function of epidermis: modification BRC-test and biphasic water-lipid (liquid-crystal) model of the epidermis. Depending of chemical structure, heavy metal compounds have been shown to increase or decrease membrane permeability in BRC-test. $Zn(CH_3COO)_2$, $ZnSO_4$, $Pb(CH_3COO)_3$ caused a concentration-dependent membrane alterations at t= 37ºC. All of these compounds at highest concentration (0.1%) led to strong damage of cell's membrane. Exposure erythrocytes to low concentration of these compounds (0.0125%) caused, respectively, 3.85-, 2.35-, and 1.5- fold higher levels of erythrolysis than control solvent. At minimum concentration (0.00625%) these compounds did not effect on membrane permeability. In all experiments, erythrocytes treated with $CoCl_2$, $CoSO_4$ did not differ significantly from control group in their membrane permeability. Pyrithione zinc have the ability to increase cells' membrane permeability with high efficiency. Topical application of $Pb(CH_3COO)_2$ at concentration 0.1% on lipid layer of the biphasic water-lipid model of the epidermis did not effect on the level TEWL. At the similar conditions $CoCl_2$, $SrCl_2$, and $ZnSO_4$ demonstrated a tendency to protect water losing, respectively by 30, 19, and 18%. Our *in vitro* results suggest that both inorganic and organic heavy metals' compounds such as $Zn(CH_3COO)_2$, $ZnSO_4$, $Pb(CH_3COO)_2$ and Pyrithione zinc can induce changes in barrier function of biological membrane.

Lecture

Progress in refinement: The oral Acute Toxic Class method is a successful alternative to the oral LD_{50} test

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The oral Acute Toxic Class method (ATC method) was developed as an alternative to replace the oral LD_{50} test. The ATC method is a sequential testing procedure using only 3 animals of one sex per step at any of the defined dose levels (5, 50, 300, 2000 mg/kg b.w.). Depending on the mortality rate 3 but never more than 6 animals are used per dose level. The reduction of numbers of animals used in comparison to the LD_{50} test is 40% to 70%. The oral ATC method is based on the probit model and biometric evaluations were conducted before a national (6 participants) and subsequently an international (9 participants from 5 countries) ring study were conducted. An excellent agreement was demonstrated between the toxicity and the animal numbers predicted biometrically and observed in the validation studies. The oral ATC method was adopted as an official test guideline by OECD in 1996 and was slightly amended in 2001. The oral ATC method has been successfully used in Germany for many years and in 2004 >90% of all tests on acute oral toxicity testing was conducted as oral ATC tests. In member states of the European Union the ATC method is used in the range of >50% of all tests conducted. Since the deletion of the oral LD_{50} Test by member states of OECD and EU the use of alternatives to the oral LD_{50} Test is mandatory. So far, the oral ATC method is the most widely used alternative.

Poster

In vitro immunotoxicity induced by mercury on avian lymphocytes

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Introduction: Mercury, a common heavy metal is an environmental pollutant. Immunosuppression has been recorded in birds due to *in vivo* treatment with mercury. The present study was conducted to investigate apoptosis and T- and B-cell blastogenesis on avian lymphocytes treated with mercury *in vitro*.

Methods: Avian Lymphocytes isolated from the spleen of apparently healthy birds were treated with No Observable Effect Level (NOEL)x10-2, NOELx10-3, NOELx10-4, NOELx10-5, NOELx10-6 and NOELx10-7 concentrations of mercuric chloride for 30, 60, 90 and 120 min. Apoptosis was detected by transmission (TEM) and scanning electron microscopy (SEM), agarose gel electrophoresis of isolated DNA and immunoperoxidase staining. T- and B-cell blastogenesis was assessed by lymphocyte stimulation test using Concanavalin-A (Con-A) and Lipopolysaccharide (LPS) as mitogens, respectively.

Results: TEM revealed shrunken cells, chromatin margination, karyorhexis, budding and phagocytised apoptotic bodies and SEM showed ultrastructural alterations on cell surface such as formation of apoptotic bodies and budding. Fragmentation of DNA was observed by agarose gel electrophoresis. Apoptotic lymphoid cells exhibited brown colour by immunoperoxidase staining. There was significant reduction in delta OD of the mitogen stimulated lymphocytic cultures. The alterations were maximum in cells treated with NOELx10-2 dose of mercuric chloride for 120 min while minimum at NOELx10-7 concentration for 30 min.

Discussion: The present *in vitro* investigation indicated that mercury exerts its deleterious effects on avian lymphocytes via apoptosis even at very minute concentrations and short exposure time. Therefore, dose and time dependent studies on immunotoxic effect of heavy metals can be done *in vitro* on lymphocyte cell culture system.

Poster

Results of the final phase of a validation study to evaluate *in vitro* cytotoxicity assays for estimating rodent acute systemic toxicity

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Recent studies have identified a correlation between *in vitro* basal cytotoxicity and *in vivo* acute oral toxicity. NICEATM and ECVAM subsequently initiated a three-phase multi-laboratory validation study to evaluate the usefulness of two standardised Neutral Red Uptake (NRU) assays for estimating acute rodent toxicity and to determine the extent that they may reduce animal use. Seventy-two coded chemicals (12 from each of five acute oral hazard categories and 12 unclassified/non-toxic chemicals) were tested using the NRU endpoint with mouse 3T3 fibroblasts and Normal Human Epidermal Keratinocytes (NHK). Three chemicals were tested in Phase Ib, nine chemicals in Phase II, and 60 chemicals in the final Phase III. Based upon preliminary analyses, the results for the positive control, sodium laurel sulfate, were reproducible over the entire study. IC_{50} results from all phases were used with rodent oral LD_{50} values to calculate linear regressions for each lab and assay. Although the NHK data were more reproducible than the 3T3 data, the 3T3 data yielded a better regression fit. Comparison of the regressions for both assays to the Registry of Cytotoxicity (RC) regression indicated that the new regressions were statistically different from the RC, but that their predictions of toxicity category were generally similar. The new regressions will be used in conjunction with computer simulations to determine animal savings that may result by using *in vitro* data as the basis for starting doses for acute toxicity studies. Supported by: N01-ES-35504, N01-ES-75408; EPA IAG DW-75-93893601-0; European Commission 19416-2002-04 F2ED ISP GB.

Poster

Data collection and analysis for an *in vitro* cytotoxicity validation study

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A multi-laboratory validation study designed by NICEATM and ECVAM evaluated two *in vitro* basal cytotoxicity test methods using 72 coded chemicals with a wide range of acute oral toxicity. The study was designed in three phases to allow for refinement of the protocols, and data collection and evaluation procedures. An Excel® template was distributed to the participating laboratories for entry of the raw data, identification of outliers among the six concentration replicates, documentation of materials and procedures, graphical analysis of dose-response, and formatting of data for further analysis. A Hill function analysis with GraphPad Prism® software was used to calculate IC_{20}, IC_{50}, and IC_{80} values and associated 95% confidence limits, and graph the data and fitted model. Initial criteria for an acceptable dose-response for individual tests included one data point between 10 and 50% viability, one data point between 50 and 90% viability, and r2≥0.8. A Prism® template was distributed to the laboratories to automate and provide uniformity of analysis. To increase the speed of data collection and evaluation by the Study Management Team (SMT) and consulting biostatisticians, the laboratories submitted the Excel® and Prism® files by e-mail. Results compiled by the SMT were returned to the originating laboratories for audit to ensure accurate transmission of data. Implementation of these procedures demonstrated that automated data collection in relatively common, easy-to-use electronic formats facilitates uniformity of data collection and analysis.

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Poster

Use of alternative to animal methods for prognostication the effect of benzalkonium chloride on membrane permeability

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Alternative to animal methods were used to investigate the effect of preservatives on the membrane permeability and barrier function of epidermis: modification BRC-test, cells *Escherichia coli*, biphasic water-lipid (liquid-crystal) model of the epidermis, lyophilised pig skin Xenografts. Membrane permeability in BRC-test was value by means of the flow out of haemoglobin. Penetration of plasmatic membrane of the isolated cells of the *Escherichia coli* was value by means of the flow out of the intracellular components (E260). The barrier function of the biphasic water-lipid model and lyophilised pig skin Xenografts was value by means of TEWL. In BRC-test Benzalkonium Chloride at concentration 0.1, 0.001, 0.00075%, caused, respectively, a 7.5-, 6.0-, 3.2-fold increase of the erythrolysis. In cells *Escherichia coli* test Benzalkonium Chloride at concentration 0.001 induced 8,3-fold higher levels of lysis of cells than control solvent. Additionally, quaternary ammonium compound caused inhibition of dehydrogenase activity of *Escherichia coli*. Inhibition observed with a 0.001-, 0.00075- or 0,0005% concentration of Benzalkonium chloride was 79, 68 or 34%, respectively, compared with control group. Topical application of Benzalkonium Chloride (concentration 0,1%) on the lipid layer of water-lipid model don't cause the increasing the level TEWL. Topical application of Benzalkonium Chloride (concentration 0,1%) on the lyophilised pig skin Xenografts caused increasing the level TEWL by 24 %. Our experimental data indicate that Benzalkonium chloride caused a concentration-dependent increase of cells' membrane and lyophilised pig skin Xenografts permeability.

Poster

Comparison of two cytotoxicity assays with the ICCVAM/ECVAM validation study chemicals

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The ICCVAM/ECVAM validation study employed 72 coded chemicals tested with Normal Human Keratinocytes or BALB/c 3T3 fibroblast cells, using the Neutral Red Uptake assay at 48 hours. Twelve chemicals from each of the six *in vivo* classes had human and rat *in vivo* data.

Neutral red uptake can be influenced by lysosomal pH without affecting cell number or viability, so a Total Protein assay was performed. The Neutral Red desorb solution is the fixative for the Kenacid Blue assay (Riddell et al., 1986) allowing the total protein assay to be conducted on the same cells.

NHKs were seeded onto 60 wells of a 96 well plate. Eight decreasing geometric concentrations of test chemical were applied to 6 replicate wells of the plates, (http://iccvam.niehs.nih.gov/methods /invitro.htm). SLS was the positive control, and the results were required to meet acceptance criteria. The NHK results for the NRU and KB assay were assessed to determine comparability. Chemicals that, at high concentrations, fix the cells do give a different toxicity profile between the two assays.

This approach was also adopted with 11 coded anticancer drugs (see poster by Budworth et al.), which revealed comparability between the IC_{50} values obtained.

This work was funded by the FRAME research programme.

Poster

Toxicity of hexanediones in human neuronal and astrocytic cell lines

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Neurons and astrocytes form a functional unit in the nervous system. The NT2.D1 human embryonal cell line may be differentiated to yield post-mitotic NT2.N neurons, which are the closest model of human neurons currently in use. The CCF-STTG1 astrocytoma line also strongly resembles human astrocytes. n-Hexane is metabolised to 2,5-hexanedione (HD), which reacts with neural proteins to give pyrrole adducts and neurofilament cross-linking. Our group previously found the closely related 2,3-HD and 3,4-HD (used as food additives), also to be neurotoxic *in vitro*. The aim of this study was to investigate the sensitivity of NT2.N, CCF-STTG1 and NT2.D1 cells to toxic insult from 2,5-, 2,3-, and 3,4-HD, for 4 or 24 hours, using MTT turnover to measure cytotoxicity sustained. Comparison of IC_{50} values showed that 2,3- and 3,4-HD were significantly more toxic in all three cell lines, than 2,5-HD. All three cell lines were similarly sensitive to 2,3- and 3,4-HD toxicity and there was no significant difference between toxicity sustained after 4 or 24 hours exposure, whilst 2,5-HD was significantly more toxic following 24 hours than 4 hours exposure, in all three cell lines. After 4 hours 2,5-HD was significantly more toxic to NT2.D1 than NT2.N and CCF-STTG1 cells, which did not differ in their sensitivity. However, after 24 hours exposure, NT2.N cells were significantly more sensitive towards 2,5-HD than were the CCF-STTG1 cells. These data suggest the toxic profile of the 2,3-HD and 3,4-HD isomers differs from 2,5-HD, in rapidity of onset, acute severity and cellular specificity.

Poster

Pyruvate uptake and lactate release by liver spheroids and their changes after exposure to toxicants

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Pyruvate is a key intermediate in energy metabolism. It can be transported into mitochondria to generate ATP, transformed to lactic acid and used for gluconeogenesis to generate glucose. Mitochondrial injury can affect pyruvate metabolism and lactic acid production. Liver spheroid culture has been shown to be a useful model in functional and toxicological studies. This study investigated pyruvate uptake and lactate release by rat liver spheroids and their changes after exposure to toxicants. Rat liver spheroids were prepared by a gyrotatory method. Pyruvate uptake and lactate release were investigated over a period of 15 days. The results showed that liver spheroids took up pyruvate and released lactate across the culture period. After exposure to selected toxicants, diclofenac, galactosamine, isoniazid, paracetamol, m-dinitrobenzene and 3-nitroaniline, pyruvate uptake and lactate release were affected differentially. Diclofenac, isoniazid, paracetamol and m-dinitrobenzene which can cause mitochondria injury significantly ($p<0.05$) reduced pyruvate uptake. Diclofenac, isoniazid, paracetamol and galactosamine significantly ($p<0.05$) decreased lactate release but m-dinitrobenzene increased lactate release ($p<0.01$). The lesser toxic toxicant 3-nitroaniline, a metabolite of m-dinitrobenzene, did not cause significant changes in either pyruvate uptake or lactate release. It is concluded that pyruvate uptake and lactate release are two functions of liver spheroids that should be suitable for use as endpoints in *in vitro* toxicology studies using liver spheroid model.

Poster

Toxicity of hexanediones in a two-tier neurotoxicity system

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In order to develop an *in vitro* high throughput screening system for potential neurotoxins, two assays were evaluated using the SK-N-SH line, an established model of human neurotoxicity. MTT turnover was chosen as a first tier or triage with a simple endpoint (cell death), whilst a second more sophisticated assay utilised flow cytometry with Propidium Iodide dye (PI) to provide more detailed data on toxin-mediated alterations of cell cycle phases. The cells were exposed to 2,3- and 2,5-hexanedione for 48 hrs and assayed for viability using MTT and flow cytometry, with PI. IC_{50} values for 2,3- and 2,5-hexanedione were 3.31±0.13 mM and 20.08±1.93 mM respectively. 2,3-Hexanedione (1.6 mM) caused a significant ($p<0.01$) increase in the percentage of cells in the G2/M phase with respect to control, but apoptosis was not significantly increased until 7.4 mM compared with control. 2,5-Hexanedione (3.4 mM) significantly increased the fraction of cells in the G2/M phase ($p<0.001$), whilst the percentage of cells undergoing apoptosis increased at 17 mM ($p<0.01$). The increases in the G2/M phase specifically occurred at concentrations where no cell death was observed with MTT and before any increase in apoptosis transpired. This suggests that G2/M checkpoint arrest is elicited in response to low concentrations of hexanediones. The MTT assay provides an indication of the broad cytotoxicity of toxins such as 2,3- and 2,5-hexanediones in SK-N-SH cells and is a good basis for an initial neurotoxicity screen. Flow cytometry with PI, offers more detailed information on the effects of 2,3- and 2,5-hexanedione on the cell cycle.