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1. Defining the chemistry associated with DNA binding to allow for prediction of mutagenicity by grouping and read across

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Grouping similar chemicals together is an increasingly popular technique to allow for the computational prediction of toxicity. This technique allows for read-across within a rationally formed chemical category. In order to group chemicals successfully, robust methods are required which provide mechanistically relevant approaches to form categories. For mutagenicity, robust categories can be formed using chemistry relevant to DNA binding. The aim of this study was to review known chemistry based mechanisms of action and compile this knowledge. This chemistry was then developed into novel structural alerts, allowing for category formation. The literature relating to the ability of chemicals to bind to DNA was searched. Relevant structural alerts were compiled. For each alert, an unambiguous mechanism of action was established. These mechanisms were for each alert were schematically documented. The alerts were then assigned to one of the following mechanistic domains: Michael acceptor; Schiff base formation; S_NAr; S_N (which covers S_N1 and S_N2 mechanisms); radical; unclear (i.e. unknown or not established). The analysis reviewed five structural alert compilations related to mutagenicity and genotoxic carcinogenicity in order to define the electrophilic reaction chemistry domains. In addition, mechanistic information was gathered from data related to idiosyncratic drug toxicity. This resulted in a DNA profiler featuring 85 structural alerts corresponding to a wider coverage of the mechanistic chemistry related to covalent DNA binding than has been previously published. The resulting mechanistic chemistry has been subjected to expert review and incorporated OECD QSAR Toolbox. The Toolbox, including the updated profiler, is freely downloadable from: www.qsartoolbox.org (or from the authors). Version 3.0 of the OECD QSAR Toolbox (October 2012) has further organised the mechanistic chemistry into different levels of granularity on the basis of mechanism of action. The funding of the European Chemicals Agency (EChA) Service Contract No. ECHA/2008/20 /ECA/203 is gratefully acknowledged.

2. SAR models for mammalian genotoxicity

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Several expert- and statistically derived models have been created that predict mammalian genotoxicity, both *in vitro* and *in vivo*. In general, such models show promising predictivity for the data from which they have been derived, but lower predictivity against 'new' data. Whilst it may be beneficial to use these models as surrogates for *in vitro* or *in vivo* testing in certain circumstances, the perception of low predictivity is a block to acceptance. Current usage, therefore, is generally limited to either prioritising testing within a batch of chemicals or adding supporting information to data produced within the existing genotoxicity test battery. The barriers to improved predictivity are threefold. Firstly, the limited pool of publically available micronucleus and cytogenetic test data used to train models has not been generated using standardised conditions and contains well-reported artefactual results. Secondly, there is not a single mechanistic interaction that is a prelude to *in vitro* clastogenic or aneugenic responses. Thirdly, the techniques used to create models have inherent limiting factors, either restricting the speed of development or their ability to synthesise discordant data into a rational and holistic SAR. To make a significant improvement in predictivity, shared proprietary data could be used to improve and expand the training sets used for model development. Alternatively, the focus could shift to the compilation and modelling of *in vitro* mechanistic data, such as the inhibition of topoisomerase II or microtubule dynamics, which can be reliably extrapolated to the prediction of mammalian genotoxicity. In either case, the first step is the assembly and curation of data.

3. Predicting aromatic amine mutagenicity: past, present and future

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Aromatic amines are key components of many pharmaceutical drugs, food additives and cosmetics, yet a significant number of this chemical class show mutagenic activity in the Ames test. An ability to predict which aromatic amines would be free of such activity would reduce unnecessary testing and allow resources to be prioritised on those compounds that are a genuine risk to human health. A structure–activity study highlighted the importance of electronic and steric factors for promoting and mitigating mutagenic activity, the knowledge from which was used to improve the predictive performance of the expert system, Derek for Windows. Subsequent evaluations of Derek with external test sets, however, demonstrates that additional data, by itself, are not sufficient to improve *in silico* systems. Further improvement can be gained by understanding, from a mechanistic perspective, why some aromatic amines are devoid of mutagenic activity. Following a Precompetitive Workshop at the Royal Society of Chemistry (RSC), a cross-pharma project has been initiated with the aim of creating a database of aromatic amine mutagenicity data that could be used to support regulatory submissions and to improve the performance of predictive models. An evaluation of these data demonstrate the importance of sharing proprietary, yet non-sensitive data, between commercial organisations.

4. Molecular modelling of the 3D structural differences in TP53 and RAS isoforms when carcinogens are bound to nucleotides at mutation hotspots

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The *TP53* tumour suppressor gene and the RAS oncogene isoforms (*KRAS*, *HRAS* and *NRAS*) are mutated in many cancer types. In lung cancer, G>T transversions are the common mutation signature for mutation hotspots at codons 157, 158, 245, 248, 273 in *TP53* and codon 12 in *KRAS* (1). These hotspots are adduct sites for the cigarette smoke carcinogen benzo[a]pyrene diol epoxide (BPDE) and regions of slow nucleotide excision repair. Because of the molecular and clinical consequences of differential site-specific repair, it is essential to gain an understanding of the structural factors that determine the rate of excision in response to a particular DNA lesion. Such distortions include kinks in damaged DNA, impaired Watson-Crick hydrogen bonding, flipped out nucleotide, enhanced local dynamics and thermodynamic destabilisation. We want to determine the relationship between the mutation frequency and the types/degree of distortion of local sequence when an adduct has formed. We have previously used *in silico* modelling (2) and multivariate analysis (3) to correlate carcinogen and cancer gene mutation patterns. We have developed a new molecular dynamics pipeline using high performance computing (HPC Wales) to predict the three dimensional structural changes and degrees of DNA distortion when chemicals bind at a particular nucleotide. Using our pipeline we show that DNA distortions, dependent on local sequence context, at codons 157 in TP53 and 12 in KRAS are greater than at other sites in the same genes/isoforms when BPDE is bound due to increased stretch, stagger, shift, buckle, opening, roll and tilt within the local DNA helix.

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5. How are points of departure derived for mutagenicity and clastogenicity following exposure to alkylating agents?

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The scientific and regulatory community are challenging the default assumption of linearity for DNA-reactive genotoxic agents. Our work in Swansea helped to initiate this paradigm shift using the model alkylating agents, methyl- and ethylmethanesulphonate (MMS and EMS), as well as methyl- and ethylnitrosourea (MNU and ENU). Consequently, there is now a wealth of data for these chemicals for both gene mutation and chromosome damage endpoints *in vitro* and *in vivo*. These high power data sets can be used to calculate point of departure (PoD) metrics using statistical modelling packages, hence showing non-linearity. These extensive statistical analyses have been carried out by the ILSI-HESI *In Vitro* Genetic Toxicity (IVGT) Quantitative Subgroup, and clear PoD have been defined for all agents both *in vitro* and *in vivo* (1). However, to accept a range of low doses as biologically irrelevant by defining a PoD, a plausible mechanism of action must be shown experimentally. We are addressing this data gap for these four alkylating agents, which are known to induce specific DNA adducts. Our recent work has been to investigate the roles of DNA repair in relation to their genotoxic PoD. Specific DNA repair enzymes have been shown to be up-regulated by low doses of alkylating agents, and knocking down specific DNA repair enzymes *in vitro* alters the shape of the dose response e.g. to EMS and MNU. In conclusion, DNA repair has been shown to be a mode of action for the PoD exhibited by mono-functional alkylating agents.

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6. The role of p53 in genotoxicity testing in human lymphoblastoid cells

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The choice of cell type is important for genotoxicity testing. p53 deficiency in cell lines used in genotoxicity testing may lead to positive results, therefore limiting the use of these cells in genotoxicology test assays. The human lymphoblastoid cell lines TK6, AHH-1 and MCL-5 are commonly used in genotoxicity testing. These cells differ in that TK6 cells are heterozygous at the thymidine kinase locus, but contain the wild-type p53 gene, whereas MCL-5, which is derived from AHH-1, and AHH-1 carry a heterozygous mutation in the p53 locus. Therefore, in order to explore whether impaired p53 function plays a role in low dose response studies these cell types were treated with mitomycin C (MMC) for 4 h and cytosine arabinoside (araC) for 24 h at a low dose range of 0–0.1 μg/ml. Chromosome damage was investigated using the cytokinesis-block micronucleus (CBMN) assay and cytotoxicity was measured using relative population doubling (RPD) analysis. These studies used the newly installed automated micronucleus detection system Metafer (MetaSystems), which allowed scoring of ~10 000 cells per dose. p53 activity was investigated through p21 gene expression analysis using Real-time PCR as well as Western Blotting-based assessments of total and phospho-p53 (Ser15) levels. Results of ongoing studies have revealed that TK6 cells are more sensitive to cytotoxicity and DNA damage than AHH-1 and MCL-5. Furthermore AHH-1 and MCL-5 showed much lower p53 activity compared with TK6 cells.

Thus, these data suggest that impaired p53 function may play a role when investigating dose relationships of genotoxins at low doses. Finally, it is possible to confirm that TK6 cells are a robust cell line for use in genotoxicity testing.

7. Development of the *in vivo* comet assay JaCVAM

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The single-cell gel electrophoresis or comet assay is a technique for quantifying DNA damage (double strand and single strand breaks) and repair and is rapidly gaining importance in the field of Genetic Toxicology. Although at present no OECD guideline is available GLP studies are being performed to widely accepted IWGT guidelines/protocols. In 2006 the Japanese Centre for the Validation of Alternative Methods (JaCVAM) enlisted the help of several labs from around the world in order to establish a working protocol. Blinded compounds which were known genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens and non-carcinogens were sent out to labs for testing and the results analysed. Throughout the four phases of the trial the protocols were improved step by step in order to produce a protocol which gave consistent results. The trial produced reliable results, a working protocol and promise of an OECD guideline which is expected to be signed off in 2013/2014.

8. Antigenotoxic effects of curcumin in conjunction with piperine on benzo[a]pyrene-induced DNA damage in murine model

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The use of curcumin in chemoprevention of malignancies remote to the gastro-intestinal tract has been limited due to its poor systemic bioavailability. Piperine an alkaloid derived from black pepper (*Piper* sp.) is known to increase the bioavailability of curcumin. In this study, the antigenotoxic effects of coadministration of piperine with curcumin and curcumin alone on benzo[a]pyrene-induced DNA damage and carcinogen biotransformation enzymes was investigated in liver and lung of mice. Male Swiss albino mice received curcumin (100 mg/kg body weight) and piperine (20 mg/kg body weight) separately as well as in combination orally in corn oil for seven days as pretreatments and thereafter 2 h, BaP (125 mg/kg body weight) was administered orally in corn oil. A single dose of BaP to normal mice increased the activities of ethoxyresorufin *O*-deethylase (EROD), pentoxyresorufin *O*-depentylase (PROD) and levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG) content, benzo[a]pyrene-diol epoxide–DNA adducts (BaPDE–DNA adducts) and % DNA in the comet tail in both the tissues. Quinone reductase (QR) activity was also elevated in the BaP treated group in both liver and lung when compared with normal control group but no significant change was assessed in glutathione *S*-transferase (GST) activity. Pretreatment of curcumin and curcumin plus piperine before administration of a single dose of BaP significantly decreased the activities of EROD, PROD and the levels of 8-oxo-dG content, BaPDE–DNA adducts and % DNA in the comet tail with consequent increase in QR and GST activities. The study indicates that curcumin when given in combination with piperine is more effective in modulating benzo[a]pyrene-induced genotoxicity.

9. Dose-response of alkylating agents in DNA repair-proficient and -deficient Ames tester strains

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Mutagenic and clastogenic effects of some DNA damaging agents such as methylmethanesulphonate and ethylmethanesulphonate have been demonstrated to exhibit a thresholded dose–response *in vitro* and *in vivo*. However, no threshold was apparent for the induction of adducts to DNA, indicating that cells are capable to repair alkylations to a certain dose without apparent error. It is interesting to investigate the dose–response relationship in cells lacking the repair mechanisms involved in error free removal of alkylations. The best characterised systems are in bacteria, where two alkyltransferase systems have been identified. Mutant strains of the *Salmonella typimurium* reversion tester strain TA1535 are available. Furthermore, the ease to score large numbers of mutations in the Ames assay allows defining the dose–response relation with high statistical power. We assessed several alkylators in the *S. typhimurium* assay with four different strains: the DNA repair proficient (TA1535, ogt+/ada+) and the methyltransferase-deficient strains (YG7104, ogt–/ada+, YG7100, ogt+/ada–, YG7108, ogt–/ada–). Ethylsulphonate, methylsulphonate, ethylnitrosourea and methylnitrosourea were tested in a series of 22 concentrations. Dose–response curves were fitted by the PROAST benchmark model and the Lutz and Lutz 'hockeystick' model. For EMS, a clear sublinearity was seen in TA1535 and YG7100 with a BMDL of 200 µg/plate and a threshold ~700 µg/plate by the hockeystick model. In YG7014 and YG7108 the threshold was completely abrogated, indicating that ogt is mainly responsible to repair EMS induced damages. In response to MMS, a BMDL of 100 µg/plate was found for TA1535 and a threshold of 817 µg/plate. For the deficient stains linear dose responses were observed. YG7100 was the least and YG7108 the most sensitive. For the nitrosourea compounds no clear sublinearity of the dose–response curves was evident even in the repair proficient strain, concordant with the investigations in mammalian cells.

10. The use of micronucleus assays in environmental and public health genomics: enhanced impact via improved understanding of mechanisms and better diagnostics

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Micronuclei (MNi) predominantly arise from lagging chromosome fragments or whole chromosomes during anaphase/telophase in mitosis. MNi were originally described by haematologists in red blood cells of patients who were deficient in folate and/or vitamin B12. Subsequently it became evident in mice that they may also be induced by chemical genotoxins *in vivo* and, later *in vitro*, in human lymphocyte cultures exposed to ionising radiation. The relative ease with which MNi can be quantified and their strong biological association and statistical correlation with chromosome aberrations has resulted in their widespread use for *in vitro* and *in vivo* genetic toxicology testing across species including humans. Over the recent years there has been a great advance in our knowledge on the physical, chemical, nutritional and life-style factors that cause

DNA damage as measured by the micronucleus (MN) assay in humans. Furthermore there have been several cross-sectional or prospective studies showing significant associations of this biomarker with infertility, pregnancy complications, obesity, diabetes, kidney failure, cardiovascular disease, neurodegenerative disorders and cancer. These observations have been underpinned by a deeper understanding of the molecular mechanisms and defective DNA damage response networks that lead to the chromosome instability events that cause MNi and associated nuclear anomalies such as nucleoplasmic bridges and nuclear budding. The use of the MN assay in its various modes, including techniques to identify once-divided cells in which MNi are preferentially expressed or quantified, has received great support from new developments in automated scoring using flow, image and laser scanning cytometry. Further developments using high content analysis techniques for measuring other multiple DNA damage biomarkers simultaneously within MN assays are currently underway to allow a more comprehensive analysis of genome damage. Standardisation of visual and automated scoring criteria, quality control and acceptable performance characteristics via international collaborations such as the HUMN (www.humn.org) and the MultiBioDose (www.multibiodose.eu) projects have also advanced to a stage that we can be more confident of the possibility of more reliable mass screening using MN assays at lower cost and of realising the implementation of MN assays in public health strategies aimed at DNA damage diagnosis and prevention in human populations.

11. Analysis of *in vivo Pig-a* gene mutation and chromosomal damage potential of 13 reference compounds

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The ability to monitor gene mutation at the *Pig-a* locus and micronucleated reticulocyte (MN-RET) frequency in short-term and repeated dosing schedules was investigated with nine genotoxicants (chlorambucil, melphalan, thiotepa, cyclophosphamide, azathioprine, 2-acetylaminofluorene, hydroxyurea, methylmethanesulphonate, benzo[a]pyrene) and four non-DNA reactive compounds (*o*-anthranilic acid, sulphisoxazole, sodium chloride, pyrene). Male rats were treated for 3 or 28 days with several doses, including MTD. Serial blood samples were collected up to day 45, and were analysed for *Pig-a* mutation with a labeling method that facilitated mutant cell frequency measurements in total erythrocytes and the reticulocyte subpopulation. A mutant cell enrichment step based on immunomagnetic separation was used to increase statistical power. Day 4 blood samples, and day 29 in the case of the 28-day study, were evaluated for MN-RET frequency. The four non-DNA reactive compounds did not induce *Pig-a* or MN-RET responses. Besides hydroxyurea, each genotoxicant increased mutant reticulocyte and erythrocyte frequencies. Significant increases in MN-RET frequency were observed for each genotoxicant at both time points. Whereas the highest *Pig-a* responses tended to occur in the 28-day studies, the highest induction of MN-RET was observed in the 3-day studies. There was no clear relationship between chemicals' maximal *Pig-a* and MN-RET responses, despite the fact that both endpoints are based on the same cell lineage. These data demonstrate the value and feasibility of integrating *Pig-a* and micronucleus endpoints into *in vivo* toxicology studies, thereby providing information about mutagenesis and chromosomal damage in the same animals from which toxicity, toxicokinetics and metabolism data are obtained.

12. Advances in Fourier-transform infrared spectroscopy analysis to characterise chemical-induced alterations in the Syrian hamster embryo assay—towards biomarkers stability

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The SHE assay (pH 6.7) is being considered as a '3Rs' alternative in animal laboratory studies (1). We have previously developed a protocol to conduct Fourier-transform infrared spectroscopy in the Syrian hamster embryo (FTIRS-SHE) experiments, and corresponding software to build up a FTIRS-SHE database. Subsequently, we applied machine learning and statistical methods to analyse our datasets towards chemical-treatment classification, morphological transformation classification, and extraction of biomarkers (i.e. spectral wavenumbers) related to chemical treatment (2). In the present study, we set out to validate and develop further our biomarker extraction techniques. Biomarker validation is of extreme importance, for it was found that depending on different biomarker extraction methods (i.e. computational algorithms), there was marked variability in the subsequently identified discriminating biomolecular entities and this would inevitably give rise to different mechanistic interpretations. Furthermore, currently a number of techniques used for such biomarker extraction purposes employed in a variety of fields were never initially conceived with this intention. In this work, we compare different techniques used to extract biomarkers and present rationales for their possible disagreement. We recommend an analysis framework that can derive robust biomarkers for the FTIRS-SHE assay based on pattern classification. The application of our framework can be extended to other studies that use FTIR or Raman spectroscopy. This work was funded by Unilever and the SHE assays were conducted at BioReliance, USA.

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13. Lowered oxygen concentration produce quantitative differences in genotoxicity dose–response data in the GADD45a–GFP assay

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The *in vitro* genotoxicity assays do not entirely mimic the conditions found inside mammalian tissue. One difference is that CO_2 incubators used for cell culture routinely contain 20% oxygen,

which is far higher than that within tissues *in vivo* (1–5%) (1). We hypothesised that this might be sufficient to overwhelm oxidative stress responses, and hence contribute to the generation of misleading positive genotoxicity results from pro-oxidants. Long and Halliwell (2) previously reported that these can produce peroxide from interactions with common growth media. The question was addressed by assessing whether genotoxicity and cytotoxicity results from the GADD45a–GFP reporter assay were affected by incubation at different oxygen concentrations. Twenty-six compounds including pro-oxidants, mechanistically diverse genotoxins and non-genotoxins were tested using the GADD45a–GFP assay. They were incubated at 20% and at 5% oxygen concentrations. Oxidative stress was also assessed using a fluorescein-based assay. Nineteen of the 26 compounds produced positive genotoxicity results in both atmospheric and lowered oxygen conditions in the GADD45a–GFP assay. However, differences in the magnitude of GADD45a–GFP induction were observed between 20% and 5% oxygen. Induction levels were elevated, for positive compounds, at 5% oxygen compared with those observed at 20%. Unexpectedly, 13 of 16 antioxidants produced positive genotoxicity results at both oxygen concentrations. Results suggest solely reducing the oxygen concentration to 5% is not sufficient to reduce pro-oxidant positive results in the GADD45a–GFP assay and may increase the magnitude of response for some compounds.

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14. The O^6-alkylguanine response: mechanisms and implications for thresholds and cancer therapy

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Alkylating agents are widely distributed environmental carcinogens and, moreover, are being used in cancer therapy. They induce different DNA lesions, some of them have been identified to be carcinogenic, genotoxic and cytotoxic. A critical DNA adduct induced by methylating agents used in glioma and melanoma therapy is O^6-methylguanine (O^{6}MeG), which is not only a potent inducer of mutations, but also of apoptosis, autophagy and cellular senescence. O^{6}MeG is repaired by MGMT, which very likely provokes a threshold for mutations and cancer. MGMT also causes a no-effect threshold for cell death. We have studied how death is triggered following O^{6}MeG in glioma and melanoma cells and showed that it is executed via both the death receptor and the mitochondrial apoptosis pathways. In glioma cells, the efficiency of O^{6}MeG in triggering the p53 dependent death receptor pathway is higher than the mitochondrial apoptosis pathway, which explains the high sensitivity of p53wt glioma cells to temozolomide. Interestingly, p53wt glioma cells are more resistant than p53mt cells to chloroethylating agents that are also applied in cancer therapy. The data obtained with glioma and melanoma cells revealed that p53 has a dual role: up-regulation of the death receptor thus sensitising to methylating agents, and upregulation of DNA repair genes such as *ddb2 and xpc* thus protecting against O^{6}chloroethylguanine-induced apoptosis. O^{6}MeG and O^6-chloroethylguanine triggered apoptosis is bound on DNA double-strand break (DSB) formation and, therefore, DSB repair plays a critical role in determining alkylating drug resistance. The major pathway for repairing them is homologous recombination (HR), and not non-homologous end-joining. The specific players involved in DSB recognition and HR represent potential therapeutic targets, including NBS-1, ATM, ATR, Rad51, XRCC2 and XRCC3, since downregulation of their expression sensitised against O^6-alkylating agents. Further, in melanoma cells silencing of caspase-8 and induction of DNA repair contribute to inherent drug resistance, which can be at least in part abrogated by pretreatment with interferon-ß and valproic acid. Supported by DFG KA724 and Deutsche Krebshilfe.

15. DNA repair and translesion DNA synthesis as possible mechanisms underlying genotoxic thresholds

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Rodent carcinogenicity assays are usually conducted at high doses, which are often 1000- to 10 000-fold higher than the doses at which humans are exposed to chemicals in daily life. It is questionable, therefore, whether the carcinogenicity observed at high doses can be extrapolated to low doses where people are actually exposed to the chemicals. In regulatory toxicology, it is supposed that there is no threshold for genotoxic carcinogens, which induce cancer via DNA damage and mutations. However, humans possess a variety of self defense mechanisms, which may protect humans from genotoxicity of chemicals at low dose and contribute to a threshold of genotoxicity. To examine the possibility, we established Salmonella Ames tester strains lacking DNA repair mechanisms such as *O*^{6}-methylguanine methyltransferase and 8-oxoguanine DNA glycosylase. The repair defective strains exhibited much higher sensitivity to mutagenicity of alkylating agents and oxidative mutagens, respectively, at low doses. The results are consistent with the idea that DNA repair may contribute to the threshold of genotoxicity. We also hypothesised that translesion DNA synthesis (TLS), which is a short DNA synthesis across DNA lesions by specialised DNA polymerases, might contribute to the threshold. To examine the possibility, we established human cell line Nalm-6 lacking DNA polymerase ζ (Pol ζ), which is a specialised DNA polymerase. The cells lacking Pol ζ exhibited much higher sensitivity to the killing effects of various genotoxic agents. The results suggest that Pol ζ is involved in TLS across a variety to DNA lesions and raise the possibility that TLS might contribute to the threshold.

16. Pro-oxidant induced DNA damage in AHH-1 human lymphoblastoid cells: homeostatic mechanisms of genotoxic tolerance

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Oxidative stress contributes to many disease aetiologies including ageing, neurodegeneration, and cancer, partly through DNA damage induction (genotoxicity). Understanding the interactions of free radicals with DNA is fundamental to discern the mutation risks posed. In genetic toxicology, regulatory authorities view most genotoxins to exhibit a linear relationship between dose and mutagenic response. Yet, homeostatic mechanisms exist, including DNA repair, which allow cells to tolerate low levels of genotoxic exposure. Acceptance of thresholds for genotoxicity has widespread consequences in terms of understanding cancer risk and regulating human exposure to chemicals/drugs. Three pro-oxidant chemicals, hydrogen peroxide (H_2O_2), potassium bromate ($KBrO_3$) and menadione, were examined for low dose-response curves in the human lymphoblastoid cell line AHH-1. Cellular DNA repair and antioxidant capacity were assessed as possible threshold mechanisms. H_2O_2 and $KBrO_3$, but not menadione, exhibited thresholded responses, containing a range of non-genotoxic low doses. Levels of the DNA glycosylase OGG1 were unchanged in response to pro-oxidant stress. DNA repair focussed gene expression arrays reported changes in *ATM* and *BRCA1*, involved in double strand break repair, in response to low dose pro-oxidant exposure, however, these alterations were not substantiated at the protein level. Determination of oxidatively induced DNA damage in H_2O_2-treated AHH-1 cells reported accumulation of thymine glycol above the genotoxic threshold. Further, the H_2O_2 dose–response curve was shifted by modulating the cellular levels of the antioxidant glutathione. Hence, observed pro-oxidant thresholds were due to protective capacities of base excision repair enzymes and antioxidants against DNA damage, highlighting the importance of homeostatic mechanisms in 'genotoxic tolerance'.

17. Investigation of the dose–response curve for isopropyl methanesulphonate using the *in vivo Pig-a* mutation assay

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It is generally accepted that DNA reactive compounds exhibit linear dose responses. However, it has recently been demonstrated that at least some mutagens exhibit a sub-linear dose response. Evidence of a sub-linear dose response could be beneficial in the management of pharmaceutical genotoxic impurities if it allowed for daily exposures above the default control level for mutagens of 1.5 μg/day, the Threshold of Toxicological Concern. The aim of this work is to investigate the dose response for a pharmaceutically relevant alkyl ester, isopropyl methanesulphonate (IPMS). The induction of mutations in the peripheral blood of Wistar rats dosed with IPMS either acutely (single doses) or sub-chronically over 28 days was examined using the *in vivo Pig-a* mutation assay. With both acute and chronic administration of IPMS, mutations accumulated over the course of the study. The dose–response curve appeared to be linear in nature at acute doses of 3.5–56 mg/kg, while with chronic dosing only total cumulative doses of 14–56 mg/kg appeared linear based on an ~2-fold increase in mutant frequency with a doubling in dose. Additionally, a comparison of the mutant frequency from acute and 28-day dosing regimens indicated a less than additive dose–response relationships, such that a single dose resulted in a much higher mutant frequency than the same dose fractionated over 28 days. The data suggest a sub-linear dose–response for IPMS.

18. Genotoxic impurities: boring … but important?

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The EMEA and FDA genotoxic impurities guidance was issued to address the widely acknowledged deficiencies regarding impurity assessment outlined in ICH Q3A/B and the subject area is now the subject of an ICH guidance process. The guidance has had a profound effect on the drug design and development process and has also influenced the field of genetic toxicology. It is now widely accepted that there are exposure levels (i.e. <1.5 μg/day) where the majority of non-thresholded direct acting genotoxins represent a negligible risk to human health. Likewise compound specific assessments based on *in vivo* mutagenicity data (1) and or rodent carcinogenicity data (2) have been used to define acceptable daily intakes of non-thresholded direct acting presumptive or known genotoxic carcinogens that are in excess of 1.5 μg/day. Negative (Q)SAR predictions alone are also now considered sufficient to determine an impurities potential genotoxicity without any further follow up *in vitro* or *in vivo* testing. This proposal has resulted in several initiatives to re-examine the robustness and accuracy of the existing *in silico* (Q)SAR tools used to predict mutagenicity (3). However certain subjects remain problematic, for example there is still no consensus on whether any established acute *in vivo* genotoxicity studies can discharge the perceived risk associated with an Ames mutagenicity finding, or whether it can only be discharged by negative carcinogenicity studies. In short, the subject of genotoxic impurities may appear boring, but it has become incredibly important from both a genetic toxicology and drug development perspective.

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19. Should boronic acids be controlled as genotoxic impurities?

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Boronic acids are increasingly common intermediates in the synthesis of pharmaceuticals and as such are potential impurities in

drug substances. Originally non-alerting in any SAR prediction of genotoxicity, an example was found to be a bacterial mutagen when tested for occupational safety purposes. Subsequently 40–70% of all boronic acids tested have been shown to be mutagenic. Typically only strains TA100 and WP2uvrA(pKM101) are reverted but a subset are also detected by TA1537; metabolic activation is generally not necessary. The mechanism of action is not known but ^{32}P-postlabelling studies indicate DNA reactivity may not be responsible (1) and, therefore, boronic acids may be a novel class of bacterial mutagen. Formation of epoxides and 'boronation' have both been suggested to be responsible for the bacterial mutagenicity and initial results indicate that genotoxicity is not seen in mammalian cells *in vitro*. Until the mechanism is fully understood it is prudent to control boronic acids as genotoxic impurities but there would be clear commercial benefit if this could be shown not to be necessary. At this stage it is not clear what data are required to establish whether or not boronic acids represent a real genotoxic risk.

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20. Dose response relations: the impact of non-linearity

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As pointed out in Kroes *et al.* (1) and the EMEA guideline on the limits of genotoxic impurities (2) the calculation of the generic TTC value encompasses a multitude of conservative (worst case) assumptions. Among these the 'low dose linearity' concept for genotoxic carcinogens is possibly the most conservative presumption as demonstrated in two recently published studies: (i) For the exemplary alkylating agent ethylmethanesulphonate (EMS) the dose response relations were shown to be distinctly thresholded (3). Therefore, the carcinogenic dose response of EMS must also be thresholded. (ii) For the potent genotoxin dibenzo[a,l]pyrene (IARC class 2B) a clearly sublinear dose response was demonstrated in a 'mega cancer' study in the rainbow trout (4). Based on these data we estimate the factor by which the 'linear dose response concept' overestimates the 'true' cancer incidence in the very low dose region.

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21. The hormetic dose response

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Hormesis is a dose-response concept that is characterised by low-dose stimulation and high-dose inhibition. The hormetic dose response is the most fundamental dose response, significantly out-competing other leading dose-response models in large-scale, head-to-head evaluations used by regulatory agencies such as the EPA and FDA. The hormetic dose response is highly generalisable, being independent of biological model, endpoint measured, chemical class, physical agent (e.g. radiation) and interindividual variability. Hormesis also provides a framework for the study and assessment of chemical mixtures, incorporating the concept of additivity and synergism. Because the hormetic biphasic dose response represents a general pattern of biological responsiveness, it is expected that it will become progressively more significant within toxicological evaluation and risk assessment practices as well as having numerous biomedical applications.

22. Nanomaterials in the life cycle

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Nanomaterials (NM) are used as major or minor components in many products. Almost exclusively, the risks and hazards of NM have been studied with pristine nanomaterials but how the toxicity of NM is affected when it occurs as a minor or major component in a product has not been studied well at all. In the NanoKem and NanoSustain projects we investigated toxicity of a few nanomaterials pristine and as occurring in sanding dusts of complex matrices. The dusts were generated under conditions that simulate situations where workers sand paint- or composite plastic surfaces. We think that the sanding may also represent abrasive processes in work places or in the environment. We characterised the dust aerosols in detail and collected dust with an electrostatic precipitator (1). The dusts (and the pristine materials in parallel) were tested in mice and in cells in culture for genotoxicity, inflammation, and cytotoxicity (2,3). We argue that the toxicity of TiO_2 is obscured when it is included in a typical paint matrix. However the toxicity of ZnO is conserved when it occurs in a complex window-glass treatment product. We need to know more on different exposure scenarios throughout the life cycle and we need to study biological effects of complex mixtures with NM before we can make valid predictions on the safety of NM. The research leading to these results has received funding from the Danish Working Environment Research Fund (Nanokem, grant #20060068816) and the European Community's Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 247989 (NanoSustain).

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23. Engineered nanomaterials' genotoxic effects—how to better define and find them?

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The unique and diverse bio-physico-chemical characteristics of engineered nano-scale materials (ENMs) suggest that their toxicological properties may differ from the corresponding bulk materials. Therefore, better understanding of (geno) toxic potential of nanomaterials, including nanoparticles (NPs), is required for hazard identification and evidence-based risk assessment of ENMs. The main goal of our research is to perform toxicological profiling of ENMs and NPs. In our studies we apply a tiered experimental approach. First, detailed physico-chemical characterisation of nanoparticles is performed. Consequently, well characterised nanomaterials are tested for their biological effects using *in vitro* systems (e.g. Balb/T3T cells). We examined cellular functions upon exposure to ENMs, in particular to multiwall carbon nanotubes (mwCNTs), SiO_2 and cobalt ferrite NPs using a battery of different methods. This included assessment of NP–cell interactions (electron transmission and fluorescent microscopy), cytotoxicity (colony forming efficiency assay; CFE), genotoxicity (micronucleus and comet assays), carcinogenic potential (cell transformation assay; CTA) and changes in mRNAs expression levels (transcriptomics). In the case of mwCNTs, combinational approach showed negative cytotoxicity and genotoxicity results. However, carcinogenic potential and mwCNT interaction with cells were evident under given experimental conditions. We stressed that assays detecting long-term effects in parallel with a panel of tests for more immediate effects on cell biology must be taken into account when studying nanomaterials. Standard end-points and new biomarkers defining genotoxicity, carcinogenic potential as well as novel genotoxic stress detection methods should be developed, validated and incorporated into nanotoxicological research and risk assessment of ENMs and NPs.

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24. DNA damaging potential of superparamagnetic iron oxide nanoparticles—role of oxidation state

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Superparamagnetic iron oxide nanoparticles (SPION) hold immense potential in a variety of biomedical applications such as, magnetic resonance imaging, targeted delivery of drugs and tumour destruction. Though the influence of physico-chemical features on toxicity of nanomaterials is increasingly becoming evident, data however, are lacking that assess an array of such characteristics in parallel. The aim of this study was to characterise coated or uncoated SPION of different chemical compositions, determine cellular uptake, and their potential cytotoxic and genotoxic effects. Human MCL5 lymphoblastoid-B cells were exposed to different SPION: dextran or PEG-coated maghemite, dextran or PEG-coated magnetite, uncoated maghemite or uncoated magnetite (0–100 μg/ml). Physico-chemical characterisation on all SPION was performed. Ferrozine assay and TEM analysis were used to determine cellular uptake. Genotoxicity was assessed by the micronucleus assay with cytotoxicity measured in parallel. Mass spectrometry was used to determine the levels of five different DNA lesions: 8-hydroxyguanine (8-OH-Gua), thymine-glycols (TGs), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 4,6 diamino-5-formamidopyrimidine (FAPyA) and 6-diamino-4-hydroxy-5-formamidopyrimidine (FAPyG). Dextran or PEG-coated maghemite showed significant cellular uptake and DNA damage in the absence of any cytotoxic effects. DNA lesion quantification showed significant increases in 8-OHGua, FapyG, FapyA and TG only for dextran-coated maghemite. Pre-treatment with *N*-acetyl-L-cysteine (NAC) significantly reduced the micronuclei frequency, suggesting a direct role of oxidative stress in the induction of chromosomal damage. The increased size of PEG-coated maghemite loaded endosomes may modulate actin cytoskeleton/cell division resulting in the formation of micronuclei. All other SPION did not show cellular uptake or genotoxicity; both seemed to be influenced by several factors such as, agglomeration, surface coating and iron redox state. Despite lack of cytotoxicity, the study showed genotoxic response with or without induction of oxidative stress. As human exposure to ferrofluids is expected to increase in nanomedicine-based therapeutics, these findings are important in guiding the fabrication and biocompatibility of SPION.

25. Evaluation of the applicability of standard assays for assessing the genotoxicity of nanomaterials

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The unique properties of nanomaterials may cause adverse biological effects that are different from their bulk counterparts. It is important to evaluate whether the currently used genotoxicity tests are adequate to detect the potential genotoxicity of nanomaterials. Genotoxicity of titanium dioxide nanoparticles (TiO_2NPs) and silver nanoparticles (AgNPs) were evaluated using the Ames test, the *in vivo* and *in vitro* comet assay, the mouse lymphoma gene mutation assay, the mouse *Pig-a* mutation assay, and the *in vivo* and *in vitro* micronucleus assays. The evaluation of 10 nm TiO_2NPs and 5 nm AgNPs using the Ames test showed that the nanoparticles did not enter the bacteria and were consequently not mutagenic. Uncoated 5 nM AgNPs entered and induced mutations in mouse lymphoma cells most likely via an oxidative stress mechanism. Likewise, micronuclei in TK6 cells were increased by AgNPs in a dose-, size- and surface coating-dependent manner. Exposure analysis indicated that 10 nm TiO_2NPs entered cells *in vitro* and damaged DNA, as measured by the comet assay, in TK6 and L5178Y mouse lymphoma cells, yet did not induce mutation. *In vivo*, they induced DNA damage in mouse liver, lung, spleen and bone marrow, but were negative in the *in vivo* micronucleus assay, and the *Pig-a* mutation assay. These investigations underscore the importance of nanomaterial physicochemical characterisation and exposure assessment for interpretating genetic toxicology studies. Our results suggest that the Ames test may not be responsive to the treatment of nanomaterials while the comet assay is very sensitive to the insults of the nanomaterials, and may respond positively to exposures that do not appear to induce mutations or chromosomal damage. More research, using a much wider array of nanomaterials, is required before drawing final conclusions as to the capability of the genetic toxicology assays for assessing the potential genotoxicity of nanomaterials.

26. Effect of benzo[*a*]pyrene metabolism on cells and vice versa

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Although discovered and identified more than 75 years ago, the environmental carcinogen benzo[*a*]pyrene (BaP) is still widely studied and has become a standard test agent for exploring the metabolic capacity of biological systems and the responses of cells or tissues *in vitro* and *vivo* to external genotoxic insult. In all these studies, the principle pathway of activation demonstrated is via the formation of BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) to form an adduct with the N^2 position of guanine (dG-*N*^{2}-BPDE) which can induce gene mutations *in vivo* in transgenic rodent mutation assays. In cells in culture this pathway is mediated by cytochrome P450 (e.g. CYP1A1 and CYP1B1), but it now appears that *in vivo* P450 metabolism acts primarily to detoxify BaP. Gene expression changes *in vitro* can be categorised as either resulting from induction of the Ah receptor, or from causation of DNA damage. In cells that are p53 competent BaP causes accumulation of p53, evident at the protein level but not at the mRNA level. DNA adduct formation by BaP, but not by BPDE, appears to be p53 dependent, indicating that loss of p53 affects metabolic activation. We also found that cells are more susceptible to BaP in a particular phase of the cell cycle. Higher levels of DNA damage occurred in S- and G_2/M- compared with G_0/G_1-enriched cultures and correlated with higher levels of CYP1A1 and CYP1B1 protein expression. In contrast, BPDE did not result in significant changes in DNA adduct levels at different phases of the cell cycle. Meanwhile *in vivo* studies show that BaP forms DNA adducts with equal measure in both target and non-target tissues, while gene expression changes are organ specific. Further insights into the complexities of interactions of BaP with mammalian cells may shed further light on mechanisms of carcinogenicity.

27. Molecular epidemiology of aflatoxin exposure and child health

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Dietary aflatoxin B_1 (AFB_1) exposure occurring through the consumption of contaminated maize and groundnuts has been significantly associated with liver cancer incidence in regions of high aflatoxin exposure and impaired child growth in West Africa (1). The aflatoxin-albumin adduct (AF-alb) is a well validated biomarker of exposure that has been applied to several population studies in Africa, providing a reliable marker of individual exposure for assessing the contribution of aflatoxin to human health effects (2). The AF-alb biomarker has been applied to a number of molecular epidemiology studies, including a recent study in Kenya, in which the biomarker was used to identify a novel association between aflatoxin and hepatomegaly (3), and a recent study of child exposure in three regions of Tanzania. Current work is focused on the application of epigenetic and gene expression methods to explore possible mechanisms of aflatoxin related child growth impairment. We have evidence that AFB_1 can affect IGF gene expression *in vitro* and protein levels *in vivo*, suggesting that a disrupted IGF growth axis may have a role to play in aflatoxin exposure-related child growth impairment. Further research would be necessary to identify other mechanisms and factors that contribute towards aflatoxin-induced child growth retardation.

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28. *In vitro* protective effects of quercetin in MCF-7 cells despite an underlying toxicity profile

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Flavonoids occur in the human diet, generally as glycosylated and sulphated derivatives. They appear to possess bioactive

potential and are often evaluated for their chemopreventative properties. Quercetin is a flavonol found in onions, apples, tea and red wine. Although known for its beneficial bioactivity, it is known to be mutagenic (1) and growth retarding (2) in short-term *in vitro* tests. This study evaluated quercetin (geno) toxicity in MCF-7 cells, with benzo[*a*]pyrene (B[*a*]P) as a positive control. In the clonogenic assay, dose-related reductions [0.01, 0.1, 0.5 ($P \leq 0.05$), 1.0 ($P \leq 0.005$), 10.0 ($P \leq 0.005$) and 100.0 μM ($P \leq 0.005$)] in survival were noted compared with vehicle (DMSO) control following 24-h treatment. That said, lower quercetin concentrations (1.0 μM) appeared to inhibit B[*a*]P-induced toxicity. In line with this, quercetin (≥0.5 μM) was micronucleus-forming, inducing 2-fold increases in micronuclei determined in the cytokinesis-block micronucleus assay. However, it appeared to again reverse B[*a*]P-induced increases in micronucleus formation. These findings were associated with a modulation of B[*a*]P-induced increases in $P21^{WAF1/CIP1}$ expression, as determined by real-time RT-PCR. Our results tentatively support the *in vivo* protective properties of quercetin whilst highlighting discrepancies that may emerge with *in vitro* testing.

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29. Modifications to the human *TP53* knock-in mouse fibroblast immortalisation assay for studying *TP53* mutations induced by environmental carcinogens

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The Hupki (Human *TP53* knock-in) mouse embryo fibroblast (HUF) immortalisation assay (HIMA) is an important model for studying the impact of environmental carcinogen exposure on *TP53* mutagenesis (1). However, the *TP53* mutation frequency of the HIMA remains low (≤25%) and the assay is not selective for only *TP53*-mutated cells. We investigated whether nucleotide excision repair (NER)-deficient HUFs would have a higher frequency of mutation and sought ways to improve the selectivity of the assay. Firstly, Hupki mice were crossed with *Xpa(–/–)* mice to generate (NER)-deficient HUFs. *Xpa(–/–)* and *Xpa(+/+)* HUFs were exposed to the diesel carcinogen 3-nitrobenzanthrone (3-NBA) to assess viability, DNA adduct formation and *TP53*-mutagenesis in the HIMA. Secondly, immortalised clones from the HIMA were screened with the p53-activating compound Nutlin-3a to determine whether it can be used to discriminate between *TP53*-mutated and *TP53*-wild-type clones. We found that 3-NBA-treated *Xpa(–/–)* HUFs had a greater loss of viability than *Xpa(+/+)* HUFs, but had a similar level of DNA adducts. Furthermore, 3-NBA induced the same frequency of *TP53* mutations in immortal clones of *Xpa(–/–)* (21.7%) and *Xpa(+/+)* (23.3%) HUFs, as detected by sequence analysis, with a predominant mutation type of G to T transversions in both cell types. Treatment of immortalised HUFs with Nutlin-3a selectively inhibited the growth of clones containing wild-type *TP53*, thereby improving selection for *TP53*-mutated clones in the HIMA. In conclusion, NER-deficiency did not increase 3-NBA-induced mutation frequency in the HIMA. However, we show that Nutlin-3a can be used to identify *TP53*-mutants prior to sequencing and eliminate p53-wild-type clones, which significantly improves the specificity and efficiency of the assay.

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30. The histone tale: the role of BRCA1-dependent ubiquitylation in dealing with genotoxic stress

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Exposure of cells to genotoxic agents may cause the generation of double stranded breaks (dsb), either directly or through the replication of DNA containing other lesions. To counter this threat cells have developed pathways to repair dsb such as non-homologous end-joining (NHEJ) and homologous recombination (HR). Whilst the repair of breaks by NHEJ is error-prone, the repair of breaks by HR is largely error-free. Therefore, an important question is, how do cells maximise the repair of dsb through HR to promote the maintenance of genome stability? A key component in the signalling and repair of DNA damage by HR is the tumour suppressor protein BRCA1. The function of BRCA1 in HR is mediated through its association with several key partner proteins, the most important of which is BARD1. The interaction with BARD1 serves two purposes: Firstly it retains BRCA1 in the nucleus by occluding a nuclear export signal and secondly, it enhances the only known biochemical activity for BRCA1, as a ubiquitin (E3) ligase. We analysed the ubiquitin ligase function of BRCA1 and demonstrated that an important substrate for this activity is nucleosomal histone H2A. Moreover, we show how ubiquitylation of the histone H2A C-terminal tail has the capacity to alter chromatin folding. We speculate how BRCA1-dependent ubiquitylation of H2A can promote the repair of genotoxic dsb by homologous recombination for increased genome stability and prevent the harmful genomic rearrangements that are associated with the development of cancer.

31. An 'omics' based method for sensitively measuring genetic damage

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DNA damage can occur via a wide variety of genotoxic agents which can compromise a genome's integrity. This DNA damage if left unrepaired can cause the generation of genetic mutations that are often associated with diseases including cancer. Being able to sensitively measure the location and the level of DNA damage induced throughout the genome is crucial to being able

to determine the mechanism of genotoxicity and the DNA repair pathways that promote genome stability. Using ultraviolet light as a paradigm for DNA damage induction, we have developed a novel technique which uses Agilent Technologies' Chip on Chip DNA microarrays that is capable of sensitively measuring the levels and distribution of DNA damage and its repair throughout an entire genome. Our method involves the affinity capture of damaged DNA and its separation from undamaged parts of the genome. By hybridising the captured damaged DNA to Agilent's whole-genome DNA microarrays, we are able to sensitively measure the levels of DNA damage and their precise location throughout the genome. Repeating this process at various times after the induction of DNA damage permits a sensitive and high-resolution estimation of DNA repair capacity throughout the genome. In partnership with Agilent Technologies, we are adapting our technique which was originally developed using a model organism for use in the human context. We aim to develop an in-vitro alternative to existing animal-based genetic toxicology assays for use in the chemical, cosmetic and pharmaceutical industries. Our aim is to improve genotoxicity testing in humans, as well as elucidating the underlying mechanisms of genotoxicity.

32. Development of the PARP inhibitor olaparib to generate new therapies for cancer patients

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Inhibitors of DNA damage response (DDR) pathways offer an exciting new opportunity for identifying targeted cancer therapies. In addition to the potential to enhance the effectiveness of DNA damaging chemotherapies and ionising radiation treatment, DDR-inhibitors also have the possibility for single-agent activity in specific tumour genetic backgrounds. This is exemplified by inhibitors of the DDR protein poly (ADP-ribose) polymerase (PARP). Olaparib is a PARP inhibitor currently in Phase II clinical trials, and has been shown to induce tumour-specific cell death (synthetic lethality) in DNA double strand break (homologous recombination) repair deficient cancers, such as those with BRCA1 or BRCA2 mutations and more recently has been shown to have broader anti-cancer activity. In addition to utility as single agents, PARP inhibitors can be combined with DNA damaging chemotherapies. However, in the clinic increased bone marrow toxicity has been reported. We have set up rodent preclinical models to assess these combination toxicities and identify dose and schedules that may be translated into the clinic.

33. The 3D skin micronucleus assay: international efforts towards validation

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3D models are increasingly used for predictive toxicology including genotoxicity testing. This is driven by ethical, time, and cost considerations, as well as an emphasis on animal reduction. *In vivo* genotoxicity assays were banned for testing cosmetic ingredients March 2009 (7th amendment to the EU Cosmetic Directive). Relying solely on *in vitro* genotoxicity assays that have a high rate of misleading positive results would greatly limit new product development. For this reason, genotoxicity assays in 3D models have been developed for use as a follow-up for positive results from the current *in vitro* genotoxicity battery (1). 3D models allow for more natural cell-cell interactions and demonstrate key '*in vivo*-like' characteristics such as proliferation, differentiation, morphology, gene/protein expression/function. 3D skin models have been successfully established for genotoxicity testing for dermal compounds and are currently involved in an international Cosmetics Europe validation exercise. A detailed protocol and scoring atlas for the 3D micronucleus (RSMN) assay using EpiDerm™ skin models was published by (2). Data from the pre-validation showed strong intra- and inter-laboratory reproducibility (3). The number of coded chemicals was extended to 28 (focused on *in vivo* non-genotoxic non-carcinogens) with results demonstrating > 80% specificity. A limited number of carcinogens were tested of which 5/7 were correctly predicted. Additional genotoxic carcinogens are being tested. Overall, combining the Cosmetics Europe project and previously published data, more than 40 chemicals have been evaluated in the RSMN assay in the EpiDerm™ model and the results demonstrate over 85% concordance with rodent carcinogenicity. The RSMN assay has recently been validated for use as a GLP assay.

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34. Comet assay with reconstructed 3D human skin: results from a multi-laboratory comparison

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Cosmetics Europe (formerly COLIPA) initiated a multi-laboratory project to establish *in vitro* genotoxicity assays (micronucleus and comet assays) using reconstructed 3D human skin tissues. In 'Phase 2' of the project intra- and inter-laboratory reproducibility was assessed using five coded chemicals. MatTek's EpiDerm™ human skin model tissues were topically exposed to these chemicals for 3 h followed by isolation of basal keratinocytes which were then assessed for DNA damage using the alkaline comet assay. Compounds were double-blinded and tested at least twice at three different laboratories, and results were decoded and evaluated by an external statistician using predetermined criteria. All laboratories found clear increases for all three *in vivo* mutagens tested, methylmethanesulphonate and *N*-ethyl-*N*-nitrosourea and 2,4-diaminotoluene. Two non-carcinogens were also tested. For the first, cyclohexanone,

no DNA damage was observed except in one laboratory, and the second, *p*-nitrophenol, was negative in all laboratories as it showed significant DNA damage only at doses exceeding the cytotoxicity cutoff (>30% cell loss). Phase 2 results demonstrated good reproducibility as all three genotoxic carcinogens were correctly predicted in all laboratories and both non-carcinogens were negative except for one false positive prediction in one laboratory. Our data support that the comet assay in reconstructed 3D EpiDerm™ human skin is a relevant model for genotoxicity testing of dermally applied chemicals. For the next phase of the project efforts will be combined with a German project and it is planned to test thirty coded chemicals among five laboratories in two reconstructed human skin models.

35. MucilAir™ versus RAW264.7 cells in nanotoxicology

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Predictive *in vitro* tests are needed to rank nanomaterials according to toxicity and bioavailability, in order to determine priority for subsequent *in vivo* testing. *In vitro* nanomaterials are predominantly studied in A549 (representing alveolar epithelial cells) or RAW264.7 (representing macrophages) cells. Using human 3D airway models opens up new possibilities in testing nanomaterials. The 3D models consist of fully differentiated human respiratory epithelial cells and allow relevant exposure via air as they are cultured at an air-liquid interface. To investigate the applicability of human 3D airway models in the safety assessment of nanomaterials, we compared the toxicity of SiO_2 and CeO_2 nanoparticles on MucilAir™ (EpiThelix Sarl) to RAW264.7 macrophages. MucilAir™ inserts and RAW264.7 cells were exposed for 24 h to the nanoparticles via droplet exposure on the tissue surface and via the medium, respectively. Cytotoxicity was measured by LDH and TEER (MucilAir™) or MTT (RAW264.7). Various cytokines were analysed in culture medium as a measure for inflammation. Oxidative stress and genotoxicity were evaluated by HO-1 expression and Comet assay, respectively. In RAW264.7 cells, SiO_2 and CeO_2 were cytotoxic at similar concentrations, but SiO_2 induced only TNF-α, whereas CeO_2 induced only HO-1 expression and % tail DNA. In MucilAir™, no significant effects were seen on all endpoints tested up to 10-fold higher concentrations. It seems MucilAir™ is less sensitive compared with cell lines towards particle induced toxicity. This may be more realistic, as interaction of particles with mucus is taken into account and the cells are and morphologically similar to human airway epithelium, in contrast to RAW264.7 cells. In future, we will further assess the applicability of human 3D airway models by exposure via different routes and compare the results with both cell culture and *in vivo* inhalation data. Ultimately, these models may be useful in the safety evaluation of engineered nanomaterials.

36. 3D spheroidal fish hepatocytes as an alternative *in vitro* model for environmental toxicology

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The development and validation of reliable *in vitro* methods that offer an alternative to conventional *in vivo* studies is rapidly becoming an important tool to determine the toxicity of chemicals and contaminants. In common with mammalian systems, three-dimensional (3D) cell culture systems that can mimic the micro-environment of target-organ tissue could serve as an alternative *in vitro* model for eco- and genotoxicity testing. Through an iterative process, we established a protocol for the routine formation of 3D rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte spheroids and as a prerequisite, characterised their biochemical organ-functionality, with comparisons to immature aggregate and 2D monolayer cultures (plated from the same individual fish). Spheroids displayed significantly higher levels of glucose production and albumin synthesis compared with 2D cultures ($P < 0.01$), much like the levels detected in organotypic fish liver slices and mammalian spheroids. A significantly lower level of basal lactate dehydrogenase leakage ($P < 0.01$) suggests a high degree of membrane integrity within the spheroids, an important criterion for longevity in culture. Spheroids also maintained (a) the morphological characteristics of *in vivo* liver tissue, with tight cell-cell contacts; (b) histological architecture and (c) F-actin expression. Since it is also possible to culture individual spheroids for up to a month, there is potential for this work to lead towards *in vitro* bioaccumulation alternatives and to conduct high throughput screens of chronic exposure over weeks that will demonstrate the metabolic loss of the parent toxicant, or molecular detoxification in a manner that has not previously been possible in the relatively short exposure times of suspensions (hours) or 2D culture (up to 4 days). We believe the methodology developed has the potential to provide realistic organotypic responses *in vitro*, providing an alternative approach to exposing large numbers of fish to toxicants which could also be translated to mammalian studies.

37. Technical considerations and imaging strategies with the EpiDerm™ 3D human skin model

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The rapid growth of the nanotechnology industry increasingly demands the use of *in-vitro* toxicology assays for cost effective safety assessment. The development of a 3D micronucleus assay utilising EpiDerm™ tissue is therefore of key interest as human skin represents a key barrier/exposure route to environmental and occupational nanomaterial exposures. Optimisation work at Swansea University using paraformaldehyde fixed, paraffin embedded sections stained with haemotoxylin and eosin showed clear growth and differentiation of the tissue model across the assay time period that was morphologically unaffected by inclusion of cytochalasin-B. Resulting binucleate frequencies in control tissues showed excellent reproducibility averaging 56% (SD=6%, $n = 3$). A statistically significant ($P < 0.01$) increase in micronucleus frequency relative to control was observed following 48hr treatment with 6 μg/ml mitomycin-C ($n = 3$). Preparatory work for studying nanoparticles included cryo scanning electron microscopy to assess the preserved agglomerative state and deposition homogeneity of 85 nm amorphous silica and 20 nm polystyrene

latex nanoparticles after topical application to the EpiDerm™ tissues. Tissue sections (1, 3 and 6 microns) were further imaged using transmitted differential interference contrast confocal microscopy: images collected using this method provided excellent structural information and the use of automated tile scanning/image stitching algorithms allowed a large tissue area to be assessed. It is suggested this technique could be well suited to study the ability of fluorescent nanoparticles to penetrate the stratum corneum, or for visualisation of *in-situ* immunohistochemical markers. Our initial work suggests that 3D tissue models such as the EpiDerm™ construct are ideally suited to the challenge of engineered nanomaterial safety assessment.

38. The effect of blood sampling on micronucleus frequency in the Han Wistar rat

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The *in vivo* bone marrow micronucleus test is a widely used short term genotoxicity assay capable of detecting clastogenic and aneugenic activity. The principle of the assay is that micronuclei present in immature erythrocytes form as a result of either acentric chromosome fragments or whole chromosome loss which fail to attach to the mitotic spindle during the erythroblasts' final cell division. The assay has been extensively used over several decades, yet the need to understand factors that influence results remains. The objective of this project was to evaluate the effect of blood sampling on micronucleus (MN) frequency in the Han Wistar rat. Micronucleus assessment was also performed using recombinant human erythropoietin (rhEPO). Animal acclimatisation was also assessed for its affect on micronucleus frequency. The acclimatisation periods investigated gave similar MIE frequencies. As a consequence of this, the reduced period has been introduced into all *in vivo* genotoxicity studies performed at AstraZeneca (AZ), UK. EPO treatment significantly increased the frequency of micronucleated immature erythrocytes (MIEs) and stimulated erythropoiesis in the bone marrow of the Han Wistar rat. However, blood sampling did not significantly increase MIE frequency even with changes in haematology and bone marrow pathology. The differences observed between EPO treatment and blood sampling are considered to be attributable to the action of EPO on the erythroid lineage. Furthermore, it is highly probable that the levels of erythropoietin produced endogenously in response to bloodletting were grossly exaggerated by EPO treatment. Sampling the maximum volume of blood permitted by the home office license held at AZ did not increase the micronucleus frequency in the bone marrow of the 10-week-old Han Wistar rat. Individual laboratories are encouraged to assess the effect of blood sampling on MN frequency using the laboratory's preferred strain.

39. Evidence for a stem-cell lineage in human intestinal adenocarcinoma using synchrotron radiation-based Fourier-transform infrared microspectroscopy

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The epithelial-cell lining of the two anatomically and functionally distinct segments of the mammalian intestinal tract, small and large intestine, is constantly being renewed as a result of continuous proliferation of intestinal stem cells (SCs); this is to accommodate the gut-lining cells' harsh environment. Although intestinal SCs are thought to reside near the base of the crypts, a lack of specific markers has hampered previous attempts to identify their exact location. We obtained tissue sections of small and large intestinal crypts derived from normal or adenocarcinoma (AC) human intestine; these were floated onto BaF_2 windows and interrogated using synchrotron radiation-based Fourier-transform infrared (S-FTIR) microspectroscopy *via* an aperture size of 10 μm × 10 μm. The derived infrared (IR) spectral data were analysed using principal component analysis (PCA) and/or linear discriminant analysis (LDA). The cells within the normal crypts were then classed based on their representative IR spectral scores clustering. A clear cell lineage progressing from SCs to TA cells to TD cells was observed in normal samples. On comparison of AC-derived spectra *versus* corresponding normal, a sub-population of AC-derived spectra exhibited a SC-like spectral fingerprint; the remaining IR spectra were classed mostly as transient amplifying (TA) cell-like with a smaller number exhibiting terminally differentiated (TD) cell-like spectral characteristics. Our findings point to a novel approach towards imaging and identifying the *in situ* location of putative cancer SCs.

40. Aneuploidy levels in pancreatic intraepithelial neoplasia

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Pancreatic ductal adenocarcinoma (PDAC) has the highest cancer mortality rate (survival <4% at 5 years). Carcinogenesis is initiated in precursor lesions like pancreatic intraepithelial neoplasias (PanINs 1–3) and is marked by progressive accumulation of genetic abnormalities, including chromosome copy numbers anomalies (aneuploidy). This is a retrospective 2-year study using archival tissue samples of pancreatic adenocarcinoma containing intraductal premalignant lesions of each stage as well as normal ducts. The technique involves fluorescence in situ hybridisation to quantify chromosome copy numbers for chromosomes 1, 6, 9, 18 (probes Vysis, UK). The deletions and amplifications are analysed in correlation to the histological stage to reveal progressive changes leading to PDAC. Over 100000 cells scored from 40 samples with various histological stages revealed: the deletions levels are subject to bias from tissue sectioning techniques and remain below the threshold level [mean + 3SD] for a significant molecular event for all probes; in PanIN 3 lesions the amplifications levels are significantly higher ($P = 0.039$, 95% CI = 0.49–22.79) compared with normal ducts for chromosome 1 and above the [mean + 3SD] level for all probes. Carcinogenesis in pancreatic cancer is an intricate process and aneuploidy levels in PanIN lesions can provide vital information towards prognosis and survival.

41. Utility of cell cycle changes to analyse nuclear content distribution in bronchial epithelial cells by high content screening

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The cell cycle is controlled by a series of checkpoints and pathways, the deregulation of which can contribute to the development of cancer. High content screening (HCS) is a cell based technology which combines automated fluorescence microscopy with multi-parameter quantitative image analysis to detect and quantify intracellular targets using fluorescent probes. In this study the Cellomics® Arrayscan® VTi platform was used to measure DNA content in various phases of the cell cycle in response to genotoxic and non-genotoxic compounds. Initially, human bronchial epithelial (BEAS-2B) cells were exposed for 24 h to a range of compounds that were either non-carcinogenic (sodium chloride (NaCl)), carcinogenic (potassium bromate (PB), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)) or inhibitors of the cell cycle (nocodazole). Post exposure, cell viability was assessed using the CellTiter-Glo® assay. For each compound, a concentration that gave ≥80% cell viability was selected for further analysis. Fixed endpoint measurement of cell cycle distribution was achieved by staining the cells with the nuclear dye Hoechst 33342, followed by imaging on the Cellomics® platform. Data were exported and analysed using Minitab® and GraphPad Prism®. Treatment with 200 μM NaCl resulted in a large G_0/G_1 peak representative of diploid cells and a shorter G_2/M peak representative of tetraploid cells. Nocodazole (50 μM) treatment gave a single peak at the G_2/M position, confirming its cell cycle inhibitory activity. Both 25 μM MNNG and 1000 μM PB treatment resulted in a reduction in the proportion of diploid cells and an increase in the proportion of tetraploid cells. These data suggest that HCS is a suitable alternative to flow cytometry for the detection of carcinogen-induced cell cycle changes. As the Cellomics® platform can measure an array of endpoints from a single sample, it promises to be a valuable tool for the assessment of the toxicological potential of chemical agents.

42. The investigation of the increase in levels of aneuploidy in progressive stages of gastric cancer: aneuploidy as a cause or consequence?

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Aneuploidy is a chromosome mutation that can occur in somatic cells (mitosis) and gametes (meiosis). This mutation causes the daughter cells upon division to hold a non-multiple of the haploid number, where the number of chromosomes are above or below the normal. One factor that can contribute to aneuploidy is an error at the mitotic checkpoint, for which component proteins have been discovered (1). Gastric cancer, like many solid cancers, commonly contains aneuploid cells. Indeed, we have previously shown that aneuploidy accumulates during the histological progression, being first noted as early as the gastritis stage (2). To further investigate the source of this aneuploidy in inflamed gastric cells, this study has mimicked stomach conditions *in vitro* to aneugenic potential for acidity, bile acids and hydrogen peroxide in NCI-N87 and AGS cells. Chronic and acute *in vitro* studies were performed and 'FISH' used to identify aneuploidy. Bile acids and acidity treated separately or in combination did not show aneuploidy but upon the use of Metafer, chromosome painting and RT-PCR, aneuploidy appears linked. Time-dependent expression of Mad2 protein was also investigated following exposure to hydrogen peroxide to decipher a possible relationship. There was no variance in MAD2 levels with increasing time of hydrogen peroxide exposure from Western Blotting but upon the use of RT-PCR, a significant upregulation of MAD2 between 1 and 24 h treatment was observed whilst a mean upregulation was seen at 4 h exposure. Investigating aneuploidy levels in correlation with mitotic checkpoint gene changes on *in vivo* endoscopy samples will contribute to findings thus far.

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43. Genotoxicity assessment of pro-toxicants using a high content screening *in vitro* assay for γH2AX

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H2AX is a histone that is rapidly phosphorylated to become γH2AX after exposure to DNA-damaging agents that cause double-strand DNA breaks (DSBs). γH2AX can be detected and quantified to give a direct correlation with the number of DSBs present in the DNA. Here, we investigated the effect of individual pro-toxicants on the formation of DSBs in human bronchial epithelial BEAS-2B cells both in the presence and absence of Aroclor-induced rat liver S9. DSBs were identified by immunostaining using a fluorophore-coupled γH2AX-specific antibody followed by image acquisition and software analysis on the Cellomics Vti Arrayscan. S9 was intended to provide exogenous metabolic activation of the pro-toxicants benzo[a]pyrene (BaP), aflatoxin B1 (AB1) and 2-acetylaminofluorene (2AAF) into reactive toxicants. Treatment with BaP (7.8 μM) for 3 h with S9 (3h+S9) produced a significant increase (≥1.5-fold increase) in γH2AX levels relative to control. In the absence of S9 no increase in γH2AX levels was observed. The same effect was observed when BaP (15.6 μM) 3h+S9 treatment was followed by a 24 h recovery period. Treatments with AB1 3h+S9 (2 μM) and 3h+S9 followed by 24 h recovery (0.5 μM) produced significant increases in γH2AX levels. In the absence of S9, AB1 treatments (500 μM for 3 h) and (250 μM for 3 h followed by 24 h recovery) both produced significant increases in γH2AX levels. In contrast, treatment with 2AAF had no effect on γH2AX levels at any concentration with or without S9. These results suggest that this high content screening method incorporating rat liver S9 has potential to detect pro-toxicants causing DSBs. However, there are limitations to the use of S9 including inherent toxicity and the fact that not all phase I biotransformation enzymes are present. We propose that additional metabolic competency should be incorporated to reduce the incidence of false negatives, such as illustrated here for 2AAF.

44. Colorectal cancer: do phytophenols in the human diet prevent genomic instability?

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Colorectal cancer is the third most common cause of death in the world (1). Diet plays an important role in the prevention of malignancy (2). The aim of this study was to determine whether phytophenols (from fruits and vegetables) and their metabolites, at concentrations detected in the human colon, effect genomic instability in normal human colon cells *in vitro*. Men and women (40–55 years, BMI 18–30) were split into two age- and sex-matched groups. The control group (n = 24) maintained their habitual diet (up to 160 g of fruit and vegetables/day) and the intervention group (n = 20) consumed an additional 500–600g of fruit and vegetables/day for 12 weeks. Analysis (LC-MS/MS) of faecal water (FW) showed that 18 compounds increased significantly (e.g. phenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, 1,2,3-trihydroxybenzene) while three compounds (*p*-hydroxybenzaldehyde, indole-3-acetic acid and protocatechualdehyde) decreased due to intervention ($P < 0.05$). Normal human colon cells (NCM460) were exposed to FW from either control or intervention subjects (dilution 1:100 for 24 h) and cell proliferation (total cell number), viability [Trypan Blue; TB and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] and DNA-strand breakage (endogenous and in response to H_2O_2; SCGE) were measured.

Biomarker	Control	Intervention
Cell proliferation (total cell number $\times 10^5$)	64.4 ± 6.5	66.2 ± 4.1
Cell viability (%)		
TB	86.6 ± 1.0	83.5 ± 1.0
MTT	90.9 ± 2.6	91.1 ± 2.6
DNA strand breakage (arbitrary units)		
EndogenousOxidative	12.3 ± 0.977.9 ± 3.5	11.4 ± 1.083.8 ± 3.8

Results are mean ± SEM. Significance was tested by t-test post-intervention.

Increased fruit and vegetable intake significantly changed phytophenol concentration in FW from human volunteers after 12 weeks. However, this did not alter several biomarkers of genomic stability in human colon cells. Funders: Rank Prize Funds and Scottish Government (RESAS)

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45. Validation of the *in vitro* mammalian cell micronucleus test (MNvit) using human peripheral blood lymphocytes in exponential growth

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The *in vitro* mammalian cell micronucleus test (MNvit) is a widely used short term genotoxicity assay capable of detecting both clastogenic and aneugenic activity. The premise of the assay is that micronuclei (MN) present in the cytoplasm of interphase cells are formed as a result of either acentric chromosome fragments or whole chromosome loss due to their inability to attach to the spindle at cell division. Although the assay has been around for many years, it is only recently that an OECD test guideline has been fully adopted. The objective of this work was to evaluate the suitability of separated primary human lymphocytes (PHL) in exponential growth phase for use in an MNvit. The ethos behind using exponentially growing lymphocytes for the assay was to enable population doubling (PD) to be used as an alternative measure of cytotoxicity to replicative index (RI). Of the four reference chemicals selected for testing only two gave clear results, those being mitomycin C, whose clastogenic activity was readily detected by both short and extended treatment protocols and sodium chloride which was correctly identified as negative. The genotoxic activity of colchicine was not detected despite repeat testing using the extended treatment protocol. Cyclophosphamide did induce MN, but at concentrations in which there was no measurable increase in cell numbers and in which there were substantial levels of apoptotic cells. The intrinsic property of exponentially growing PHL to readily apoptose is a major drawback in their suitability for incorporation into an MNvit assay. This propensity results in the cells being vastly over sensitive, not only to toxic insult by the test agent but also to manipulation during the assay and to the incorporation of S9 into the test system. Furthermore, these characteristics resulted in RPD being an impractical cytotoxicity measure for this cell type.

46. Impact of *TP53* status on the metabolic activation of environmental carcinogens

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The tumour suppressor p53 plays a key role in cancer prevention. Over 50% of human tumours contain a mutation in the *TP53* gene and exposure to several environmental carcinogens is linked to characteristic mutations in *TP53*. Recently, cellular *TP53* status was found to influence the metabolism of different environmental carcinogens, suggesting a role for *TP53* in regulating xenobiotic-metabolising enzymes (XMEs). One class of carcinogens for which this has been indicated is the polycyclic aromatic hydrocarbons (PAHs) that are bioactivated by cytochrome-P450-dependent monooxygenases (CYPs) to form diol-epoxides which can bind to DNA, forming DNA adducts. To investigate the role of *TP53* in CYP-mediated metabolism of PAHs, a panel of isogenic colorectal HCT116 cells was used, differing in their *TP53* status. Cells having wild-type *TP53* [HCT116 *TP53(+/+)*], heterozygous *TP53* [HCT116 *TP53(+/–)*], knock-out *TP53* [HCT116 *TP53(–/–)*], or mutant *TP53* [HCT116 *TP53(R248W/-)* or *TP53(R248W/+)*] were treated with benzo[*a*]pyrene (B[*a*]P), dibenz[*a,h*]anthracene (DB[*a,h*]A) and dibenzo[*a,l*]pyrene (DB[*a,l*]P) and the corresponding diol-epoxides and subsequently analysed for DNA adducts by ^{32}P-postlabelling. Expression of relevant XMEs was investigated using Western blotting.

Parent PAHs formed significantly higher DNA adduct levels in *TP53(+/+)* cells compared with the other cell lines whereas exposure to diol-epoxides induced similar adduct levels in all cell lines. The genotoxic potencies of the parent PAHs increased in the order: DB[*a,h*]A << B[*a*]P << DB[*a,l*]P. Western blotting

showed that CYP1A1 protein expression after PAH treatment was induced to much greater extent in *TP53(+/+)* cells than in the other cell lines, whereas AHR expression decreased relative to controls in all cells to the same extent, independent of *TP53* status. Since PAH diol-epoxides, unlike parent PAHs, do not require metabolic activation to form DNA adducts these results show that cellular *TP53* status is linked to the CYP1A1-mediated bioactivation of PAHs. Other classes of environmental carcinogens and XMEs remain to be tested.

47. An investigation into non-disjunction with aneugenic chemicals

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Aneuploidy can result from chromosome loss or non-disjunction due to mis-segregation at anaphase. To measure non-disjunction chromosome segregation in binucleated cells can be examined using chromosome-specific centromeric FISH probes. The purpose of this work was to determine if non-disjunction is seen at lower doses than chromosome damage as determined by micronucleus production. This has previously been shown in human lymphocytes for microtubule inhibiting aneugens including colchicine (1). In order to investigate this, a number of aneugens (colchicine, vinblastine, diethylstilbestrol and chloral hydrate) and a reference clastogen, azacytidine, were analysed in the human lymphoblastoid cell line TK6. Non-disjunction and micronucleus frequencies were estimated in binucleated cells in an *in vitro* micronucleus assay. In addition a pan-centromeric probe was used to assess the proportion of micronuclei produced by the aneugens that contain whole chromosomes. Preliminary results suggest that, for colchicines, non-disjunction can be detected at a lower concentration than micronucleus production. Diethylstilbestrol was shown to induce centromere positive micronuclei but not non-disjunction. Vinblastine also was not shown to induce non-disjunction and chloral hydrate showed significant aneugenic effects only at concentrations with high cytotoxicity. Azacytidine showed no significant effect on non-disjunction although it did seem to produce an increase in the number of cells with aberrant chromosome sets which was unexpected for a clastogen. Results so far suggest that the relationship between the induction of non-disjunction and chromosome loss varies for different types of aneugen.

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48. Effect of drinking water disinfection by-products in human peripheral blood lymphocytes and sperm

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Drinking water disinfection by-products (DBPs) are generated by the chemical disinfection of water. An important class of DBPs, haloacetic acids (HAAs), are formed following disinfection with chlorine, which reacts with iodide and bromide in the water. The HAAs are cytotoxic and genotoxic DBPs when analysed in the Ames test, and in the mammalian CHO cell system. HAAs have also been found to be cytotoxic in human lymphocytes but not genotoxic in the micronucleus assay (1,2). What has not been shown is the effect of HAAs in human germ cells as well as somatic cells and whether oxidative stress is involved in the mechanism of genotoxic action. In the present study we have examined both somatic and germ cells as peripheral blood lymphocytes and sperm. We have investigated the effects of three HAA compounds: iodoacetic acid (IAA), bromoacetic acid (BAA) and chloroacetic acid (CAA). After determining appropriate dose responses in the cells, we investigated oxygen radical involvement with the anti-oxidants, butylated hydroxanisole (BHA) and the enzyme catalase, in the single-cell gel-electrophoresis (comet) assay under alkaline conditions, >pH 13. This was to determine the mechanism of action of HAAs in these cells. BHA and catalase were able to reduce DNA damage in both cell types, suggesting oxygen radicals play a role. These observations are of concern to public health since both somatic and germ cells show similar responses.

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49. Matrix ranking for prioritising testing of veterinary medicine residues

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Farmed animals including fish, game and bees can suffer from diseases. Farmers have a duty to protect the health and welfare of their animals, so may use medicines to treat or prevent disease. Withdrawal periods are set after the end of treatment with a medicine before that animal can be slaughtered, or an animal product is taken, for human consumption. The surveillance schemes measure the concentrations of any residues of veterinary medicinal products and certain other substances in foods of animal origin. The Non-Statutory Surveillance Scheme (NSS) concentrates on imported and processed animal products and is relatively small and flexible. It complements the much larger National Surveillance Scheme for domestic produce. The NSS does not have a legal basis. Therefore, the Veterinary Residues Committee (VRC) has greater freedom to recommend the substances and foods which should be included. The scheme is funded by Defra. With limited funds available, choices have to be made. Therefore, the VRC has developed a matrix ranking system to ensure that the funds are used to best effect, by prioritising the substances of greatest concern. Each substance is assessed against specific criteria and weightings to arrive at a score. The criteria include the nature of the hazard, the potency, estimated

exposure and evidence for detectable residues. The higher the score, the greater the level of concern, and the more likely it is to be included in the plans for surveillance. This evolving matrix ranking system may have wider applications in risk assessment.

50. Inducible nitric oxide may be an important mediator of DNA damage in oesophageal cells exposed to bile acids

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Barrett's Oesophagus (BO) is a pre-malignant condition affecting the lower oesophageal tissue. The molecular mechanisms underlying the metaplastic process of change from squamous to columnar mucosa are not fully understood, but an inflammatory effect driven by acid and bile in patients with gastro-oesophageal reflux with induction of reactive oxygen species and the transcription factor NF-κB have been strongly correlated. Inducible nitric oxide synthase (iNOS) and the subsequent generation of nitric oxide (NO) have been implicated in DNA damage mechanisms through single and double strand breaks, increased deamination and inhibition of DNA repair mechanisms. Its role in the neoplastic process of conversion of Barrett's mucosa to oesophageal adenocarcinoma (OA) may offer potential for better understanding the disease process and directing future screening mechanisms as well as therapeutic strategies. Confirmation of iNOS protein levels in BO and OA tissues was performed using immunohistochemistry. Scoring of staining intensity and distribution demonstrated increased iNOS staining in OA tissue compared with BO and normal squamous tissue ($P = 0.034$). *In-vitro* investigations using the OA cell line OE33 assessed for an effect on NF-κB by the bile acid deoxycholic acid (DCA) using nuclear extraction and ELISA techniques. Furthermore induction of iNOS mRNA by DCA and subsequent protein transcription were assessed by real time-PCR and Western blotting. 300 μM DCA causes a 2-fold increase in NF-κB p-65 and this is not affected by iNOS inhibition ($P = 0.50$), suggesting the relationship is not a linear one. iNOS mRNA and protein levels are both increased by bile acids—43-fold and 2.4-fold, respectively ($P < 0.05$), confirming its role in the carcinogenesis of OA, although slightly higher concentrations of DCA (450 μM) were required to demonstrate protein translation. *Ex-vivo* work is currently being undertaken to investigate whether the *PIG-A* gene may prove to be a potential biomarker for NO induced mutagenesis in oesophageal cancer.

51. Genotoxicity of low doses of hydrogen peroxide in *Saccharomyces cerevisiae*: analysis of dose–response relationships and an adaptive response

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This study analyses low-dose effects and an adaptive response to hydrogen peroxide (H_2O_2) in yeast. The discussion of low doses of toxicants and radiation is complicated by the fact that low-dose data are often compatible with more than one dose-response model. The expectation for most toxicologic endpoints is usually a threshold model, whereas linearity with no threshold is often assumed in genetic toxicology. An alternative model proposes hormesis, a dose–response relationship in which effects at low doses are opposite to those at high doses, thus giving a biphasic curve. Another complication concerning low doses is that effects of sequential exposures are often not independent of one another. In adaptive responses to ionising radiation and alkylating agents a small priming exposure leads to a diminished biological response to a larger subsequent exposure. We have analysed an adaptive response to H_2O_2 using the induction of mitotic gene conversion in *Saccharomyces cerevisiae* strain D7 as an indicator of genetic damage. Using a broad range of doses (0.000975 to 2 mM), we saw a biphasic induction of an adaptive response, such that very low doses are insufficient to induce an adaptive response, low doses are protective, and higher doses contribute to damage. The adaptive response is expressed within 10–20 min of the initial exposure, and the yeast remain in the adapted state for ~6 h. Given the similarity of the biphasic induction to the hormesis dose-response model, we extended our study of low doses to single H_2O_2 exposures. Multiple replicates were used to discern small differences in convertant frequencies. The responses are more compatible with a threshold model than with low-dose linearity or a hormetic model.

52. How genotoxic compounds behave in binary mixtures, a quantitative and qualitative perspective

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Humans are exposed to a cocktail of genotoxic chemicals at low doses, such as environmental pollutants, cigarette smoke and pesticides. It is postulated that at low doses, mixtures of genotoxic agents significantly elevate the risk of cancer. However, health risk assessments regarding the mixture toxicity are based on hypothetical assumptions of additivity. Additivity assumes that compounds with similar modes of action should work by dose addition and no complex interactions amongst the test chemicals or biological system occur that could cause a serious health concern. This default assumption of additivity has been debated by the scientific community with a view that DNA damaging mechanisms of compounds in mixtures may play a vital role in risk assessment i.e. components within the mixtures may interact (synergism) or oppose each other (antagonism) which could have a significant impact on carcinogenic effect induced by the compound in mixtures. The project aims to challenge the default assumption of dose addition. Cytokinesis-block micronucleus assay was used to characterise DNA damage. Where, DNA damaging events were scored in the form of micronuclei in Tk6 human lymphoblastoid cells treated with EMS, MMS, benomyl, carbendazem and their binary combinations using the automated Metafer system. Dose response relationships of EMS, MMS, benomyl and carbendazem were analysed individually to allow expected mixture responses to be calculated. Non-linear dose responses were observed in the lymphoblast cells treated with EMS, MMS, benomyl, carbendazim. Analysis of the mixture effects of EMS+MMS was compatible with dose addition ($P < 0.05$) as predicted. The experimental results helped us to eliminate the possibility that EMS+ MMS in combination elevate the risk of carcinogenesis in a synergistic fashion, as well as giving support to our novel study design for binary mixture analysis. Future work involves testing mixture effect of compounds with different modes of action, e.g. EMS plus benomyl.

53. In vitro micronucleus data from cell type comparison study for improving existing assays

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Accumulated evidence has shown that *in vitro* genetic toxicology assays have a high rate of reported positive results that are not confirmed in *in vivo* and/or carcinogenicity studies (1), raising the question of relevance to human risk assessment. A recent study (2) compared the use of commonly used p53 deficient cell lines (Chinese Hamster Lung/Ovary (CHL, CHO), and V79) with human p53-competent cells (blood lymphocytes, TK6 and HEP-G2 cell lines). Comparisons were made with 19 so-called 'false' positive chemicals [non-DNA reactive, Ames and *in vivo* negative; (3)] of which 9 were found to be reproducibly positive in at least one cell line, with hamster cell lines showing a higher positive rate than human. This raised the question of whether these differences were due to p53 status or species origin. This present study (coordinated and funded by the IVGT Project Committee of ILSI Health and Environmental Sciences Institute remit to improve the scientific basis for interpretation of *in vitro* genetic toxicology data) compared human versus mouse and p53 competent versus p53 mutated function. The 9 reproducibly 'false positive' chemicals were tested in a micronucleus study using mouse lymphoma L5178Y (mutated p53), human TK6 (functional p53) and WIL2-NS (TK6 related with mutated p53) tested both with and without Cyto-B (two independent experiments). Of the nine chemicals tested, five provided clear positive results in at least one cell type with the remaining being weak or equivocal. Rodent (L5178Y) cells were more likely to yield a clear positive response than either TK6 or WIL2-NS, though origin rather than p53 functionality seemed the relevant factor. Further investigations measuring caspase-3/7 activity indicated clear differences between mouse and human cells although further work is needed to investigate the kinetics, relevance and any link with MN induction.

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54. Improvement of *in vitro* risk assessment for carcinogenic potential: validation of defined genotoxic thresholds

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The demonstration and acceptance of dose-response thresholds for genotoxins at low doses may have substantial implications for establishing safe exposure levels for certain agents (1). However, due to use of high, non-physiological doses delivered as single dosing events, the current battery of *in vitro* genotoxicity tests fails to distinguish reliably between genotoxins and non-genotoxins (2). As most human exposure to genotoxic compounds is low-dose and chronic, such high doses often induce 'false positives', preventing chemicals with safe, or even beneficial, exposure levels from being utilised in products and treatments. To investigate chronic, low-dose effects *in vitro*, methylating agents methylmethanesulphonate (MMS) and *N*-nitroso-*N*-methylurea (MNU) were used to compare the effects of acute (1+2 day) and chronic (5+2 day) dosing in human lymphoblastoid cell line TK6, using a dose-fractionation approach. Substantial differences in the Lowest Observed Effect Level (LOEL) were observed for chronic dosing relative to acute dosing, using the *In Vitro* Micronucleus Assay. However, mRNA levels for selected DNA repair genes did not show significant changes during the 5+2 day studies, suggesting homeostatic maintenance by DNA repair enzymes. If low-dose, chronic *in vitro* studies, therefore, can predict safe exposure levels for genotoxicity, the number of *in vivo* tests required to follow up *in vitro* results may be reduced.

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55. Reduction of misleading ('false') positive results in mammalian cell genotoxicity assays. III. Sensitivity of p53 functional human cells

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Current *in vitro* genetic toxicology assays have a high rate of reported positive results, when compared with negative rodent carcinogenicity data. Post 7th amendment to the EU cosmetics directive *in vitro* models need to be more predictive for the risk assessment of cosmetic ingredients as animal tests are no longer acceptable. As part of a framework for improvements to *in vitro* genetic toxicology assays the performance of currently used cell lines has been investigated using the *in vitro* micronucleus assay (1). The compounds selected are accepted as producing misleading positive results in *in vitro* clastogenicity assays (2). The p53 compromised rodent cell lines; CHO, CHL and V79, demonstrated poorer predictivity than the p53 functional human cell types; TK6, HepG2 and human peripheral blood lymphocytes (HuLy). In order to assess whether a reduction in detection of misleading positives (greater specificity) did not decrease the ability of cells to detect true genotoxins (sensitivity), TK6, HuLy and HepG2, were tested with a panel of 17 genotoxic chemicals from the list detailed in Kirkland et al (2). TK6 and HuLy both gave positive responses in the micronucleus assay for the vast majority of genotoxic chemicals (88% for both) thus reinforcing their suitability for *in vitro* genotoxicity testing. HepG2 on the other hand detected significantly less genotoxic chemicals as positive in these studies. In conclusion, we have shown that by

careful selection of the cell type, the number of false positives can be reduced without compromising the ability to detect true positives. TK6 and HuLy are more suitable cells than HepG2 cells for use in the *in vitro* micronucleus assay.

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56. *In vitro* assessment of benzo[a]pyrene induced point mutations in the human HPRT gene

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Benzo[a]pyrene (BaP) is a well known environmental pollutant that has been shown to cause cancer in humans. It is a procarcinogen that requires metabolic activation (involving CYP 450 enzymes) to cause genotoxicity. Differences in the expression of key CYP enzymes appear to play an important role in the metabolic activation of BaP and the observed genotoxicity. In this work TK6, MCL5 and AHH-1 (human lymphoblastoid) cell lines with different CYP 450 activity were used to assess the role of metabolic activation on BaP induced genotoxicity. The higher expression of CYP1A1 enzymes in MCL5 cells was reflected in the increased cytotoxic and genotoxic responses seen in these cells, which was slightly greater than those observed for AHH-1. TK6 cells showed no difference in genotoxicity because of the absence of CYP1A1. The HPRT forward mutation assay was used to assess the point mutations generated by BaP in MCL5 and AHH-1 cells and the mutants were selected by 6-thioguanine (6TG) resistance. Further investigations were performed to analyse the mutation spectra generated by BaP in these cell lines. A linear increase in the mutation frequency was observed in MCL5 cells following 4 h and 24 h exposures in the 1–10 μM dose range. No safe exposure levels could be established even at the lowest (1 μM) dose following 4 h exposure in MCL5 cells. Most of the mutations observed in AHH-1 and MCL5 cells were base substitutions, specifically GC→CG and GC→TA transversion mutations within exon 3 of the *hprt* gene. A significant number of these alterations accounted for deletions, consisting of 30% tandem TG and ~40% long (one or more base pairs) deletions found in MCL5 and AHH-1 cells, respectively. Further replicates are in progress to allow a more robust statistical analysis of the mutation spectra in the two cell lines.

57. Application of biospectroscopy to investigate low-dose genotoxic risk assessment

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Traditional toxicological risk assessments often estimate low-dose risk by assuming that dose-response curves derived from high concentration ranges can be extrapolated down to environmentally relevant exposure levels. This practice still remains standard in risk assessment despite the growing number of studies investigating low-dose–response relationships (1). The direct investigation of low-dose responses is critical for accurate risk assessment. Biospectroscopy techniques have been previously shown to identify low-dose effects induced by test agents in target cell populations through the derivation of fingerprint infrared (IR) spectra (1). These approaches are capable of detecting subtle changes in underlying molecular structure. They not only indicate the presence of effect alterations but also provide information about mechanism of action. In this study, attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy was coupled with multivariate-analysis to compare low-dose (10^{-10} M) and high-dose (10^{-6} M) effects of benzo[*a*]pyrene (B[*a*]P) in oestrogen-responsive MCF-7 cells concentrated in S- or G_1/G_0-phase (2). Multivariate-analysis allowed for data reduction in order to facilitate visualisation of spectral points within their respective categories. Significant separation of IR spectra derived from 10^{-10} M-treated cells compared with corresponding vehicle controls ($P \leq 0.05$) was observed. In addition, B[*a*]P-induced alterations were associated with DNA-associated wavenumbers (1080 and 1225 cm^{-1}), suggesting a genotoxic mechanism of action. These findings point to a novel approach capable of determining low-dose effects of test agents.

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58. Can lowest unoccupied molecular orbital (LUMO) energies predict the mutagenicity of boronic acids?

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Aryl boronic compounds are commonly used as building blocks in organic synthesis and as such are frequently seen in synthetic routes at GSK. These aryl boronic structures have been found to induce mutagenicity in the Ames test, although many produce only weak positive results and are usually only mutagenic at high concentrations in *Salmonella typhimurium TA100* and *TA1537* and *Escherichia coli WP2 uvrA* strains. The mechanism for the mutagenicity of aryl boronics is presently unknown, therefore the scope of the recently developed alert in DEREK for this class of compound is broad. Hypothetically, the most likely mechanism is direct reactivity due to the electrophilic potential of the electron deficient boron atom interacting with DNA or nucleophilic sites in proteins. Thus the more electrophilic an aryl boronic structure, the more likely it is to give a positive result in the Ames test, however

there are insufficient Ames data currently available to confirm that this is the case. To investigate this hypothesis, an in-house (Q)SAR model was developed based on the energy of the lowest unoccupied molecular orbital (LUMO) of aryl boronic structures. A number of aryl boronic acid exemplar compounds subdivided into hydroxy substituted aryl boronic acids (Class 1, $n = 4$), carboxy substituted aryl boronic acid (Class 2, $n = 5$), aryl boronic acids with bulky groups in the ortho position (Class 3, $n = 3$) and other related classes of boron containing compounds (Class 4, $n = 1$) were tested in the Ames test. While the *in silico* tool correctly predicted the mutagenic potential of Classes 2–4, the model showed poor prediction for Class 1 structures. An alternative mechanism is therefore being explored to explain the mutagenicity of hydroxy substituted boronic acids. Data from this project will be provided to Lhasa for the purpose of rule refinement.

59. The impact of experimental variabilities in the liver comet assay

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Over recent years the comet assay *in vivo* has increasingly been used for regulatory genotoxicity testing, to assess DNA damage in various organs in rodents. In the pharmaceutical industry the comet assay is mainly carried out to follow up on *in vitro* positive genotoxicity results, focusing on the liver as the major metabolising organ unexpected proliferative findings in chronic toxicity studies, to elucidate the contribution of potential organ-specific genotoxicity or organ specific genotoxicity. Currently an international validation study coordinated by the JaCVAM is ongoing. It is hoped that these activities will result in a standardised protocol which is a key element to limit the known experimental variability of the comet assay. It has been demonstrated that several technical parameters, such as electrophoresis and scoring, have an impact on the assay variability. In contrast, little is known about the influence of the initial steps, i.e. from tissue sampling to slide preparation, on assay variability. In a systematic evaluation of the experimental variability brought by different key steps of organ sampling, tissue storage and slide preparation, we closely examined the influence of (i) the time between euthanasia of the animal and tissue sampling; (ii) the size of the sampled tissue; (iii) the composition, temperature and volume of the sampling buffer; and (iv) the time of the tissue or single-cell suspension in the sampling buffer etc. Overall, the liver comet assay seems to be a robust system, regarding initial work-up of tissue sampling and cell preparation. Among the different experimental variabilities examined only an increase in temperature was identified to have a significant impact on comet assay parameters. These data will help to further define a common standardised protocol for the comet assay *in vivo*.

60. Application of the TT21C strategy to a real life safety assessment using genetic toxicology as a case study

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Assuring the safety of consumer products without animal testing is of paramount importance to Unilever but remains a considerable challenge. We describe the output of a joint study undertaken by Unilever and The Hamner Institutes which applies the toxicity testing in the 21st century (TT21C) vision and strategy (1) to a real life safety assessment. Using genotoxicty data and changes in the p53 DNA damage/repair network as a prototype toxicity pathway, we explore how a risk assessment could be performed. Quercetin, a flavonoid material that produces a positive response in genetic toxicology *in vitro* assays but is negative in *in vivo* studies was used as a case study chemical. In addition to the comet assay, two high throughput *in vitro* micronucleus assay methods (Cellomics and Litron flow cytometry) were used to examine the quercetin dose response in the human epithelial cell line HT-1080. Data from high content assays examining p53 pathway responses were interrogated and the combined data used to determine a point of departure for use in the risk assessment. Additional data from Physiologically Based-Pharmacokinetic (PBPK) modelling describing exposure to quercetin enabled us to perform an *in-vitro* to *in-vivo* extrapolation, and so compare changes in the p53 pathway with expected human exposure in tissues and plasma arising from consumer use of the product. Chemical analysis of the amount of free quercetin available was also used to inform the risk assessment decision.

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61. Development of a mass spectrometric method to detect the glycidamide DNA adduct *N*7-(2-carbamoyl-2-hydroxyethyl)-guanine

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While roasting, frying or baking starch containing food, such as crisps, chips, biscuits and bread the chemical acrylamide (AA) forms in a Maillard reaction mechanism. After ingestion AA gets metabolised to the epoxide glycidamide (GA) via a cytochrome P450 enzymatic reaction. The more reactive metabolite GA reacts with biomolecules such as proteins and DNA (1). Animal studies revealed AA to be a carcinogenic compound and the IARC classified it as a possible human carcinogen. Exposure to AA can be assessed by measuring AA or GA adducts of haemoglobin but this does not necessarily correlate to DNA damage and genotoxicity. AA exposure has been shown to produce GA DNA adducts *in vitro* and *in vivo*, with the most abundant adduct formed being *N*7-(2-carbamoyl-2-hydroxyethyl)-guanine (*N*7-GA-Gua) (1). *N*7-GA-Gua could be a valuable biomarker of exposure to dietary/environmental

AA. The aim of this project is to develop a method for the detection and quantification of the *N*7-GA-Gua adduct in human lymphocyte-DNA and urine using online column-switching LC-MS/MS. Method development using synthesised standards, provided good chromatographic separation with a limit of detection for *N*7-GA-Gua at 5 fmol on column with a linear response up to 4 pmol. In seven human lymphocyte DNA (100 µg DNA on column) and two urine (6 ml urine) samples measured, no *N*7-GA-Gua adduct was detected; whereas a treatment of human whole blood with GA resulted in a linear dose response. The adduct recovery of the method was evaluated using blood (49%), cultured HCEC cells (47%) and urine (8–18%) samples spiked with the *N*7-GA-Gua standard.

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62. The resolving power of *in vitro* genotoxicity assays for cigarette smoke particulate matter

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The *in vitro* genotoxicities of tobacco smoke particulate matter from different cigarettes are often compared quantitatively (1). This requires appropriate statistical methods and replication levels to support the comparisons in terms of statistical significance and associated power. We demonstrated previously (2) that a 30% difference between tobacco smoke particulate matter genotoxicity could be detected in the Ames test. Similar resolution can be achieved in the micronucleus test with four replicate cultures per dose and similar or better resolution in the mouse lymphoma assay with six replicate cultures per dose. The statistical methods used previously (2) were also extended, to all three *in vitro* assays. An iterative process was used to identify the linear part of the dose response. Different significance tests were applied for slope, intercept or individual dose comparisons, depending on the linearity of the responses.

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63. Cytotoxic and genotoxic effects of quantum dots with variation in surface charge

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Over the last decade inorganic nanoparticles such as colloidal semiconductor quantum dots (QD) have been gaining increasing interest due to their unique electrical and optical properties making them valuable for future applications in biomedical imaging. However, very few studies have considered the genotoxic effects associated with these nanoparticles. This study provides a fundamental understanding of the way in which carboxyl (negative), amine-PEI (positive) or HDA (neutral) coated cadmium-selenide/zinc-sulphide (CdSe/ZnS) QDs are internalised and distributed in mammalian cells and their cyto- and geno-toxic effects. The size, colloidal charge, purity and intracellular localisation of the QDs were determined. Results showed agglomerate sizes of 8–13 nm in 1 or 10% horse serum and up to 200 nm in 2 or 15% fetal bovine serum. All particles had a slight negative zeta potential in water except for the carboxyl dots which had stronger negative charge. All QDs appeared to be localised in endosomes (confocal microscopy). Our findings, to date, have shown that QD uptake varies with surface charge. Highest uptake levels were observed with the carboxyl-QD at the highest dose (15 nM) in the human lymphoblastoid TK6 cells in 1% serum (ImageStream analysis). This correlated with a significant increase in the micronucleus (MN) and mutation frequencies (*hprt*) detected at this dose in these cells. Cytotoxicity was only evidenced in TK6 and foreskin fibroblasts (HFF1) cells exposed to the neutral HDA-QD at doses ≥7.5 nM. The order of potency for the induction of gross chromosomal damage observed with all QDs was: HDA>carboxyl>amine-PEI coated QD in TK6 cells. However, cyto- and geno-toxicity of QDs was dependent upon the cell line examined and thus this order was: carboxyl>amine-PEI>HDA coated QD in the HFF1 cells. In summary, this study demonstrates that QD induced genotoxicity is dependent not only on physico-chemical characteristics of the nanoparticles, but also the cell type under study.

64. Effects of dispersion and serum content on cell uptake and toxicity of size differentiated nanoparticles

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Given the rapidly expanding nanotechnology industry, *in-vitro* toxicology assays play an increasingly important role as cost effective screening tools. However, there is often contradiction between studies or comparison with *in-vivo* data. To an extent, these differences may be attributable to insufficient particle characterisation or lack of standardisation of dispersion protocols which in turn influence biokinetics and resultant toxicology (1). Colloidal suspensions of polystyrene-latex nanospheres (20 and 40 nm) in 2% or 10% serum containing media (SCM) were prepared and dispersed by either sonication (288 J/ml) or vortexing (1 min/max RPM) prior to full physico-chemical characterisation. To understand the biokinetics and (geno)toxicology as a function of these characteristics cellular

uptake was assessed by image stream flow cytometry in conjuction with relative population doubling and *in-vitro* micronucleus assays (0–50 nM, 24 h exposure, TK6 lymphoblastoid cell line). No significant ($P < 0.05$) impact on cell viability or genotoxicity was observed, nor was any difference in cell uptake resultant of dispersive method found. Interestingly however, significantly elevated uptake in 2% relative to 10% SCM was observed at all doses for the 40 nm nanoparticle, whilst only at the 50 nM dose for the 20 nm particle. It is suggested the differences in uptake observed in 2% and 10% SCM are attributed to differential nanoparticle agglomerate sizes resultant from concentration dependant serum protein-to-particle interactions. Evidence for this is provided by dynamic light scattering size and zeta potential measurements. The results demonstrate the impact serum protein concentration can play on nanoparticle biokinetics and suggest this impact may go some way to explaining the apparent discrepancies observed between studies.

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65. Mechanistic insights into nanotoxicology in target cell populations employing biospectroscopy techniques

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Nanotechnology has introduced a wide variety of man-made materials designed to nanoscale, especially nanoparticles, into our environment. Because of their unique physicochemical properties, nanoparticles may pose a potential risk to human health and the environment. Most recent investigations assessing the risk of nanotoxicology have mainly focused on high-dose effects. However, classic toxicokinetic or toxicodynamic considerations applied for chemical risk assessment may not apply to nanoparticle exposures. There is also a significant lack of understanding of their mechanism(s) of action. Biospectroscopy techniques have been employed as a novel approach to identify low-dose effects induced by test agents in target cell populations (1). In this study, MCF-7 cells concentrated in S-phase or G_0/G_1-phase were treated for 24 h with short multi-walled carbon nanotubes (MWCNT) at the following concentration range: 0.0025 mg/l, 0.005 mg/l, 0.01 mg/l, 0.025 mg/l, 0.05 mg/l and 0.1 mg/l. Cells were then examined using attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy coupled with multivariate analysis; this approach allows the derived data [i.e. infrared (IR) spectra] to be visualised as spectral points within respective categories, e.g. treatments. Even at the lowest concentrations employed, significant ($P \leq 0.05$) separation of IR spectra representing treatment categories *versus* vehicle control was observed. Dose-related effects were also noted. To identify the discriminating biomolecular entities segregating various spectral categories, cluster vector plots were employed. These showed that the major alterations induced by short MWCNT were associated with the DNA/RNA spectral region [primarily 1080 cm^{-1} ($\nu_s PO_2^-$) and 1225 cm^{-1} ($\nu_{as} PO_2^-$)]. These results suggest that short MWCNT may cross the cell membrane in order to generate a genotoxic mechanism of action.

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66. Cytotoxic effects of silver nanoparticles on DNA repair proficient and deficient mouse embryonic fibroblasts

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Silver nanoparticles (AgNPs) are increasingly being used in the consumer products because of their potential antibacterial property. Adverse effects of AgNPs on useful organisms, such as nitrifying bacteria, aquatic animal cells, as well as various mammalian cells have been reported. Increased intracellular reactive oxygen species (ROS) formation has been suggested to play an important role in AgNP-induced toxicity. This study, therefore, has examined the toxicity of synthesised AgNPs on DNA repair proficient (wild-type) and deficient (8-oxoguanine DNA glycosylase; $OGG1^{-/-}$) mouse embryonic fibroblasts (MEFs) using intracellular ROS formation, the MTT assay and an apoptosis/necrosis assay. Citrate-coated AgNPs were synthesised by chemical reduction methods. The size (mean ± S.D.) of these AgNPs was 7.6 ± 1.2 nm as observed by transmission electron microscope (TEM), and 12.8 ± 0.6 nm as measured by dynamic light scattering (DLS). Intracellular ROS levels increased in both DNA repair proficient and deficient MEFs in a dose-dependent manner. Both 50 μg/ml AgNPs and 2.5 μg/ml silver nitrate ($AgNO_3$) resulted in a maximum increase of ~4-fold and ~6-fold intracellular ROS level in proficient and deficient MEFs, respectively. $AgNO_3$ was more toxic to wild-type MEFs than $OGG1^{-/-}$ MEFs in the MTT assay (IC_{50} 4.5 ± 0.1 and 6 ± 0.5 μg/ml, respectively; $P < 0.05$), whereas there was little evidence of a difference in toxicity between wild-type and $OGG1^{-/-}$ MEFs after exposure to AgNPs (IC_{50} 58 ± 2.8 and 63 ± 10.5 μg/ml, respectively, $P > 0.05$). Also, dose-dependent toxicity was observed in the apoptosis/necrosis assay for both MEFs. A loss of OGG1 in MEFs may result in an altered cellular response after exposure to $AgNO_3$, but not AgNPs. Futher work is required to examine to what extent toxicity pathways differ after exposure to $AgNO_3$ or AgNPs.

67. Examination of five oestrogenic compounds with the comet assay *in vivo*

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Oestrogens interact in various ways showing a protective effect, or by generating reactive oxygen species (ROS) (1). ROS can interact with DNA and disrupt the protective functions of cellular antioxidants and repair mechanisms. For this study five oestrogenic compounds (17β-oestradiol, diethylstilboestrol, genistein, equol and daidzein) were examined in 12-week-old

male hooded-Lister rats (n = 5–8) after intra-peritoneal administration, to ascertain if DNA damage occurred. Furthermore, an investigation was carried out in the same study 10 days after exposure to determine whether one of these oestrogenic compounds elicited behavioural changes *in vivo* (2). Behavioural changes with 17β-oestradiol were examined using the novel object recognition and spatial memory paradigm. Following conclusion of behavioural testing, various cells were taken and analysed using the comet assay. The results showed limited positive responses in cells from the testis and blood, but not from the liver and bone marrow by comparison with the negative control. Cells were examined at this time point as previous studies (3) showed positive responses *in vivo* with alkylating agents, one of which reacted through an oxygen radical mechanism.

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68. Integrated biological responses and tissue-specific expression of *p53* and *ras* genes in marine mussels following exposure to C_{60} fullerenes and/or benzo(α)pyrene

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Whilst there is growing concern over the potential detrimental impact of engineered nanoparticles (ENPs), little is known about their potential toxic impact either alone or in combination with other environmental contaminations. Our work used the marine bivalve *Mytilus* sp., a classic bio-indicator species to assess the biological responses following exposure to C_{60} fullerene (1 mg/l, model ENPs) and/or benzo(α)pyrene (B(α)P, 56 µg/l, a model of polycyclic aromatic hydrocarbons) for 1 and 3 days. A 3-day post-exposure in fresh seawater was also applied to assess the recovery ability of *Mytilus* sp. An integrated approach, which included determination of clearance rates (at individual level), histopathological alterations (at tissue level), DNA strand breaks using the comet assay (at cellular level) and transcriptional alterations of *p53* (anti-oncogene) and *ras* (oncogene) determined by real-time quantitative PCR (at molecular level) was applied for the assessment of impacts following C_{60} fullerene and/or B(α)P exposures. Although there were no significant changes for clearance rate and histopathology of selected organs (i.e. gills, digestive glands, adductor muscles and mantles), significantly increased DNA strand breaks were found after a 3-day exposure. A significant induction for *p53* and *ras* expression was found after exposure and the transcriptional alterations of both genes showed tissue and chemical-specific pattern. Biological responses at different levels showed variable sensitivity with DNA strand breaks and transcriptional alterations showing higher sensitivity. Furthermore, all biological responses at different levels were returned back to control level after the recovery period, suggesting the ability of *Mytilus* sp. to cope with both nanogenotoxicity and ecogenotoxicity induced by C_{60} fullerene and/or B(α)P under experimental conditions. Overall the integrated approach appears to be a useful tool to assess potential impact of ENPs either alone or in combination in a representative marine invertebrate which could be translated for other species and toxicants.

69. Evidence of a chemotherapy-induced bystander effect in human bone marrow: implications of drug dosage

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The recent observation of 'donor-cell leukaemia' (DCL) raises the important question, of how cells from a healthy stem cell donor can become malignant when transfused into a patient treated with chemotherapy. The bystander effect is usually only recognised post-radiotherapy, but could explain DCL if chemotherapy can produce this effect. Bystander describes cellular damage occurring in unexposed cells in response to their irradiated neighbours. This study demonstrates bystander effect in TK6 lymphoma cells from the nitrosourea carmustine. TK6 cells were exposed directly to carmustine (1–10 µg/ml) and assessed for cellular viability by trypan blue exclusion, and for genotoxicity by the micronucleus assay. Indirect exposure was effected by co-culture with bone marrow mesenchymal stem cells (BM-MSC), which had previously been treated with the same doses of carmustine for 2 h, the drug washed off and then either the BM-MSC were cultured for 24 h to 'prime' the culture media before transferring to a TK6 culture, or TK6 cells were co-cultured using culture well inserts. Cellular viability significantly decreased with increasing doses of carmustine ($P < 0.05$) for all treatments (direct, media transfer and co-culture), with the sharpest decrease in viability for doses above 5 µg/ml for media transfer, whilst a linear decrease was observed for co-culture with inserts. Interestingly, TK6 cells in co-culture demonstrated an increased proliferation rate over 24 h relative to the media transfer, suggesting factors promoting growth but not viability. Incidence of genotoxicity increased in the lower doses at both 5 and 24 h, with genotoxicity correlating closely with viability. This is important, as reduced intensity treatment might 'spare' cytotoxicity to the BM, whilst increasing the risk of therapy-related malignancy by genotoxic bystander effects. The mechanism of this effect is currently unknown and is the subject of further research; furthermore, whether this effect is true for all drugs remains to be elucidated.

70. Andrographolide induced multiplication of microtubule organising centres in V-79 chinese hamster lung fibroblast cell line

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Andrographolide is an active phytochemical present in various parts of an annual herb *Andrographis paniculata* which is

popular in traditional medicine system in treating several illnesses. Andrographolide was found to promote multipolarity of microtubule organising centres (MTOCs) in Chinese hamster lung fibroblast V-79 cell line which affected chromosomal segregation fidelity with concomitant increment in the mitotic index. Fluorescently labelled antibodies were employed to visualise MTOCs (γ-tubulin) and microtubules (α-tubulin). V-79 cells that were exposed to increasing concentrations of andrographolide lead to anomalous mitoses where cells failed to undergo cytokinesis due to MTOCs numerical aberrations. Exposure to andrographolide also induced the formation of bi-, tri-, tetra- and multinucleated cells. The phytocompound was also found to cause concentration-dependent necrosis in V-79 cells. This finding possibly explained the previous observation that male rats subjected to andrographolide treatment for 48 days possessed reduced sperm counts, presence of multinucleated cells in the lumen as well as necrosis in Sertoli cells and the seminiferous epithelium (1). Mating of andrographolide-treated rats with untreated females failed to produce any offspring (1). Therefore, andrographolide has the potential to negate the normal process of cell division by inducing numerical aberration of microtubule organising centres *in vitro*.

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71. Mutation accumulation in the whole genome of *Escherichia coli* mutator strains

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The whole-genome sequencing data obtained from analyses with a next generation sequencer can cover comprehensive research of mutation frequency, distribution as well as accumulation in addition to mutation spectra. In this study, we focus on mutator strains of *Escherichia coli*, typical of which is a DNA repair deficient strain, due to their hyper mutation frequency, that is, 100 to 1000 times higher than the wild type, and determined accumulated mutation on their genome. Strains used in this study were YG6156, *mutT*; YG2250, *mutM*/*mutY*; AB1157/pYG782, DinB-overproducing strain. Genomic DNA was prepared from over-night culture of one colony from these strains as well as AB1157, which is their parental strain. DNA sequencing was carried out with Genome Analyzer (GAIIx), Illumina and the reference sequence, *E. coli* K12 substr. W3110 uid58567, was obtained from NCBI site. The specific mutation in mutator strains were extracted as 15 in *mutT*, 24 in *mutM*/*Y*, and 13 in DinB-overproducing strain. The mutation spectra reflected the feature of the deficiency of DNA repair system for each strain, as 40% of mutation (6/15) found in *mutT* strain was A to C, 75% of mutation (18/24) found in *mutM*/*Y* strain was G to T. On the other hand, DinB-overproducing strain seemed to be an exception since –1 frameshift at G's run was not typically observed as reported before. As for distribution of the mutation, more than half of the mutation observed in the case of *mutT* and *mutM*/*Y* were in ORF and most of them are base substitutions of missense mutation. This is possibly because cells might die with nonsense or frameshift mutations, which would disrupt the function of the product. This study suggests a new possibility that such a sequencing analysis of genomic DNA can be a screening test for genotoxicity of chemicals.

72. The development of a mass spectrometric genotoxicity screening system for drug candidates

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A vital stage in the development of new drugs by the pharmaceutical industry is the assessment of toxicology and safety, including testing the genotoxic potential of drugs as part of the cancer risk characterisation. Current methods used to screen drugs for their ability to covalently bind to DNA (such as detection of radioactive bound material or 32P-postlabelling) may provide false positive results, which can unnecessarily halt the development of promising drug candidates. Therefore, there is a requirement for refined and more accurate approaches. We are developing a new genotoxic screening method using LC-ESI-MS/MS to detect DNA adduct formation *in vitro* based on the methodology of Singh *et al.* (1). To establish the analytical methodology, calf thymus DNA has been incubated with a panel of genotoxic agents *in vitro*. Prior to analysis, enzymatically hydrolysed DNA samples are preconcentrated by removal of unmodified deoxynucleosides using solid phase extraction tips, allowing enrichment of the DNA adducts. Preliminary results from experiments using benzo[c]phenanthrene-3,4-diol-1,2-epoxide (B[c]PhDE) as a model compound suggest that >90% of unmodified deoxynucleosides may be removed following preconcentration, with little apparent loss of adducts. Selective concentration of the adducts has enabled tentative identification of a new 5-methyl cytosine adduct of B[c]PhDE. Isomers of other known adducts, B[c]PhDE-dG, B[c]PhDE-dA and B[c]PhDE-dC were also detected. In conclusion, further refinement and validation of this methodology may lead to a novel screening system for detecting the genotoxicity of new compounds, including drugs at an early stage of preclinical development.

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73. Development of the mammalian *in-vitro Pig-A* gene mutation assay

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The phosphatidylinositol glycan biosynthesis A (*Pig-A*) gene, X-linked, is critically involved in the production of

glycosylphosphatidylinositol (GPI) anchors which tether specific proteins, such as CD59/Protectin, to the extracellular membrane. Loss of these specific surface proteins, due to a mutational event within the *Pig-A* gene, has been exploited as the basis for an *in-vivo* fluorescence flow cytometry based gene mutation assay. I aim to further design and validate the *in-vitro* equivalent assay which will hypothetically supersede the current *in-vitro* mammalian gene mutation assays such as the HPRT and MLA. Using either Fluorescence Activated Cell Sorting (FACS), clonal expansion (>99.8%) or magnetic bead selection (>99.8%) a CD59 positive TK6 cell population was generated and then exposed to a model mutagen, ethylmethanesulphonate (EMS), in order to characterise the low dose response using the *in-vitro* test system. The Amnis Image stream™ platform was used to validate the location and specificity of the anti-CD59 PE antibody and investigate the identity of the 'mutant' population. Following 24 h EMS treatment (0–10 μg/ml), a non-linear thresholded dose response was observed on day 4 mutation analysis ($P < 0.01$, Dunnett's test). Fixatives were shown to increase the stability of the antibody-antigen conjugates, as well as induce a favourable osmotic effect for imaging and allow batch processing of samples. Viability marker investigation implied a lower background mutation frequency than previously reported, increasing assay sensitivity. The current sensitivity, i.e. background mutation frequency (~2000×10^{-6} cells) theoretically will be significantly increased via the incorporation of viability markers within the assay test system allowing greater low dose sensitivity. Further optimisation is required to fine tune the assay design and incorporate all the impending improvements.

74. Diagnostic segregation of human brain tumours using biospectroscopy coupled with discriminant analysis

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During surgery for removal of a brain tumour, it is critical to remove the diseased tissue in its entirety to prevent recurrence whilst minimising damage to normal adjacent tissue. Fourier-transform infrared (FTIR) and Raman spectroscopy has shown promise as a non-invasive approach with diagnostic potential in cancer detection [1]. We set out to determine whether FTIR and/or Raman spectroscopy combined with multivariate analysis could be applied to discriminate between normal and various grades of brain tumours. With ethical approval, 52 paraffin-embedded (FFPE) tissue blocks were obtained. Thin-cut sections were mounted on low-E IR reflective slides or on glass slides (H&E-stained tissue) and dewaxed. Infrared spectra were acquired using a Bruker Tensor 27 FTIR spectrometer or Raman spectra with an InVia Renishaw Raman spectrometer. Towards analysing the data, principal component analysis (PCA) and/or linear discriminant analysis (LDA) was used. Immunohistochemical analysis for IDH1 and P53 was carried out on gliomas to correlate these conventional markers with novel spectral biomarkers. Clear segregation was observed between normal and various grades of brain tumours. In derived PCA-LDA scores plots, marked within-category variation (i.e. heterogeneity) was noted; even so, excellent discrimination between different grades of glioma was also observed (i.e. towards between-category discriminating biomarkers). Additionally, RNA (1121 cm^{-1}) to DNA (1020 cm^{-1}) ratio was significantly altered in meningioma. Spectral biomarkers appeared to be more robust towards identifying aberrant tissue than immunohistochemical markers. This exploratory study indicates that FTIR and/or Raman spectroscopy coupled with multivariate analysis may provide a novel diagnostic approach especially towards identifying tumour margins and has the potential to be used to differentiate brain tumours.

Reference

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75. LiverChip: a 3D perfused platform for stable long-term hepatocyte cultures

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Primary human hepatocyte cultures remain the gold standard for assessment of the hepatic disposition of drugs and xenobiotics. However, the precipitous decline in liver-specific functions complicates the evaluation of hepatically metabolised compounds. Furthermore, drug-induced disregulation of gene expression can be difficult to deconvolve from time-dependent changes in the hepatocyte cultures making the implementation of ´omics approaches for the identification of biomarkers of hepatotoxicity problematic. The current study explored the use of a three-dimensional (3D) perfused bioreactor platform, LiverChip, for the long-term culture of primary hepatocytes. Primary hepatocytes were isolated from male Sprague-Dawley rats (Abcellute Ltd) and cultured for up to nine days in LiverChip plates.(1) Cryopreserved (Life Technologies, USA) and fresh human hepatocytes (Biopredic, France) were cultured according to a procedure modified from Domansky (1) using a Williams' Media E-based culture formulation. LiverChip cultures were compared with human hepatocyte sandwich (SW) cultures (Geltrex™, Life Technologies) and rat hepatocyte SW cultures (Matrigel™, BD Bioscience) which were performed in parallel experiments. Phase I (1A2, 3A4, 2C9, 2D6) and Phase II (7-HC-glucuronidation/sulphation) enzyme activity, albumin secretion, and functional bile canaliculi (CDFDA) were monitored in human LiverChip/SW cultures over nine days. Rat hepatocyte Phase I enzyme activity was monitored using BFC dealkylation and 7-ethoxyresorufin-*O*-dealkylation. These data suggest important liver-specific functions of hepatocytes can be maintained during extended culture (>7 days) when isolated cells are cultured under continuous perfusion in a format that fosters microtissue formation. More detailed characterisation of gene expression, drug transporter activity and drug responses is currently underway to further validate the role LiverChip cultures could play in the identification of highly specific and sensitive biomarkers of liver toxicity.

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76. A Japanese interlaboratory evaluation study on *Pig-a* mutation assay for *in vivo* mutagenicity assessment

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A Japanese collaborative study on *Pig-a* mutation assay has been in progress to establish an optimal protocol to integrate the assay into repeated dose toxicology study since 2010. As a part of the collaborative study, inter-laboratory transferability and data variability of the assay were evaluated in five laboratories. Male SD Rats were administered a single oral dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) or 4-nitroquinoline 1-oxide (4NQO). Blood samples collected at 0, 1, 2 and 4 weeks after treatment were analysed using flow cytometry for CD59 negative cells in total erythrocytes (RBC *Pig-a* assay) or reticulocytes (PIGRET assay). Four laboratories conducted animal dosing and blood sampling separately. Three of four laboratories conducted both the RBC *Pig-a* assay and the PIGRET assay, and a laboratory conducted only the RBC *Pig-a* assay. A fifth laboratory contributed to analyse blood samples from the other four laboratories by the RBC *Pig-a* assay, to evaluate the data variability between laboratories. As for the RBC *Pig-a* assay, a common gating has been set for analysis of CD59-negative erythrocytes initially. Clear time and dose related increases in *Pig-a* mutant frequency were observed in 4NQO and DMBA treated animals from 2 weeks after the treatment in all four laboratories. There are very small differences between laboratories in the *Pig-a* mutant frequency. Furthermore, comparable results were obtained in the fifth laboratory which received the blood samples from other laboratories. In the PIGRET assay, dose-related increases in *Pig-a* mutant frequency were observed from 1 week after the treatment. These results indicate that *Pig-a* mutation assays are easily transferable, data variability between laboratories is small and the PIGRET assay might be able to detect *in vivo* mutagenicity of the test compound earlier than the RBC *Pig-a* assay. This work was supported by Japan Health Sciences Foundation, grant number: KHB1006.

77. Environmentally relevant levels of the genotoxin cyclophosphamide induce alterations in MCF-7 cells detectable by biospectroscopy approaches

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Cyclophosphamide (CP) is a cytotoxic drug used in the treatment of several cancers. It exerts its effect by preventing cell division by cross-linking DNA strands following metabolic bio-activation. However, CP is also a known carcinogen and genotoxin. As CP is found in surface waters at low levels, it is therefore important to determine the effects of environmentally relevant concentrations of this compound in target cells. Infrared (IR) spectroscopy with multivariate analysis has previously been utilised to signature the biological effects of different contaminants found in environmental systems at low levels (1). Using this approach, metabolically competent MCF-7 cells were concentrated in either G_0/G_1 or S phase and exposed to five concentrations of CP (10^{-12}–10^{-6} M) for 24 h; this range included levels found in the environment. Cells were subsequently fixed in ethanol and interrogated using attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy. Resultant spectra were processed using principal component analysis-linear discriminant analysis (PCA-LDA) to identify distinguishing wavenumbers representing alterations between test agent-treated *vs.* vehicle control. Initial results demonstrated that CP treatment at the higher concentrations tested (10^{-6} and 10^{-8} M) was associated with marked alterations in protein, lipid and DNA/RNA absorbance regions, with alterations in lipid and C=O stretching of amino acids associated with the lowest concentration tested (10^{-12} M). These observations suggest that environmental CP exposures could induce alterations in aquatic organisms and future work will assess the relevance of these findings in target tissues from organisms potentially exposed to such contaminants in ecosystems.

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78. Measurement of DNA damage levels in the blood of patients with Barrett's oesophagus

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Some studies have been previously published on the possible impact of mutagenic reflux components in patients with Barrett's oesophagus (BO). Such research studies have not yet investigated the cancer potential in oesophageal tissue by using micronucleus (MN) assay in blood lymphocytes. The aim of this study is to assess the mutagenic potential of reflux components to lymphoblastoid cells and to correlate the measurement of MN levels in the blood of Barrett's oesophagus patients with clinical details of reflux, histology and follow these patients up to assess whether Mn levels might be clinically useful in predicting histology changes. The initial *in vitro* study will investigate the role of mutagenic reflux substances, bile acids and acids in blood lymphocytes. In both cases (*in vitro* and *in vivo*) MN levels will be scored using automated fluorescent microscope Metafer system. In order to optimise the MN assay in the blood lymphocytes in BO patients, the lymphoblastoid cell line TK6 was used. Reflux conditions (and the inflammation induced) were mimicked using hydrogen peroxide (H_2O_2), the bile acid deoxycholic acid (DCA), hydrochloric acid (pH 4 and pH 5) and the +ve control methylmethanesulphonate (MMS). The cell line was exposed to these substances over a range of different times and concentrations. The data show that MN levels increased after exposure to 25 and 50 μM H_2O_2, and acidity (pH 5). MN frequencies ranging from 1.3% to 2.5% in TK6 cells treated with 25 or 50 μM H_2O_2, respectively produce between 2–2.5-fold higher MN frequency whereas TK6 cells exposed to acidity(pH5) gives 2.5% and produce a 2-fold higher in MN levels. In contrast untreated TK6 (-ve) control induce low MN levels between 0.4 and 1.2%. The collection of blood

samples is underway for MN scoring in lymphocytes, allowing correlation with clinical characteristics of BO patients.

79. Epigenetics—relevance to drug safety science

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Epigenetics describes the study of heritable changes in gene expression that occur in the absence of a change to the DNA sequence. Specific patterns of epigenetic signatures can be stably transmitted through mitosis and cell division and form the molecular basis for developmental stage- and cell type-specific gene expression. Associations have been observed that endogenous and exogenous stimuli can change the epigenetic control of both somatic and stem cell differentiation and thus influence phenotypic behaviours and/ or disease progression. In relation to drug safety, DNA methylation changes have been identified in many stages of tumour development following exposure to non-genotoxic carcinogens. However, it is not clear whether DNA methylation changes cause cancer, or arise as a consequence of the transformed state. Toxic agents could act at different levels, by directly modifying the epigenome or indirectly by altering signalling pathways. These alterations in chromatin structure may or may not be heritable but are probably reversible. That said, there are currently insufficient data to support inclusion of epigenetic profiling into pre-clinical evaluation studies. We have proposed a decision testing paradigm to understand when is the right time to include epigenetic screening into toxicity testing and what testing would be required. It will only be when an understanding of chemical mode-of-action is required that evaluation of epigenetic changes might be considered. The current toxicological testing battery is expected to identify any potential adverse effects regardless of the mechanism, epigenetic or otherwise. It is recommended that toxicologists keep a close watch of new developments in this field, in particular identification of early epigenetic markers for non-genotoxic carcinogenicity.

80. Optimisation of the EpiDerm™ 3D human skin micronucleus assay at Swansea University

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With the reduced, refined and ultimate replacement of animals in testing of cosmetic ingredients, emphasis has been placed upon current *in vitro* strategies. However, the ability of such strategies to mimic the *in vivo* situation is questionable. Therefore, alternative approaches to animal testing are needed (1). One such advancement is the development of an EpiDerm™-based 3D skin micronucleus assay (2), which models the complexity of exposures to human skin, the most abundant target for certain occupational and environmental exposures. The following work details the optimisation of the EpiDerm™ micronucleus assay for use on the Metafer automated slide scoring system at Swansea University. This offers increased statistical power over the manual scoring method associated with this micronucleus assay. An increase in micronucleus frequency, over background, was observed following 48 h treatment with 6 μg/ml mitomycin C (MMC). Micronuclei frequencies were shown to be statisitically similar in both methods of scoring (P = 1. Fisher's exact test). The data presented contribute to an inter-laboratory validation of the assay. Further assay and tissue imaging advancements using fluorescent nanoparticles will elucidate the mechanism of skin exposures (e.g. subcutaneous penetration). Such techniques can be transferred to other 3D tissue culture models e.g. Epiairway™ for safety assessment.

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81. Genotoxic potential of quantum dots as a function of their intracellular uptake

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Quantum dots (QDs) are semi-conductor nanoparticles with a range of potential applications in variety of areas largely focused around enhanced imaging. These particles have a core of heavy transition metals such as cadmium selenium or cadmium telerium and are often capped with a protective coating such as zinc sulphide (CdSe/ZnS). In this study the potential cytotoxic and genotoxic effects of QDs with three different surface charges were investigated in human lung epithelial cells. Initially, the physico-chemical properties of the QDs were determined using dynamic light scattering, Zeta potential, transmission electron microscopy (TEM), and confocal microscopy. Genotoxic effects induced by these QDs were established using the cytokinesis blocked micronucleus assay, which enables the determination of both chromosome loss and chromosome breakage. Normal human bronchial epithelium (BEAS-2B) cells were treated with sonicated versus vortexed CdSe/ZnS QDs with neutral (HDA coated), positive (PEG coated), and negative (carboxyl coated QDs) surface charges, at doses ranging between 0 to 20 nM. Experiments were carried out in the presence of 2% or 10% serum-containing media with 24, 48 and 72 h exposure. Initially, cellular uptake studies demonstrated that all three QDs were internalised, but most uptake was observed in cells treated with negative charged QD in the presence of both 10% and 2% serum than with the neutral and positively charged QDs. Despite internalisation of the QD in the cells, no cytotoxicity was observed with any of the QD applied, at any of the doses or time points. Similarly, no significant micronucleus induction was observed with any of the QDs tested after 24, 48 or 72 h in 2% or 10% serum conditions. The absence of cytotoxic and genotoxic effects in these cells demonstrates their resilience to the range of QDs tested in this study.

82. Elucidation of global genome nucleotide excision repair organisation and orchestration in yeast using a novel genome-wide ChIP-Chip technique

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Exposure to genotoxic agents has long been associated with genomic instability. The resulting DNA damage can produce genetic mutations with far reaching consequences for an organism, with many aberrations being linked to diseases such as cancer. For this reason it is imperative that a sensitive measure of the location and level of this genomic damage be developed to enable the elucidation of the underlying genotoxic mechanisms of action and to further our understanding of DNA repair mechanisms in response to this damage and how this maintains the genomic stability of an organism. Attempting to address these questions using UV irradiation as a paradigm for DNA damage induction, we've developed a novel technique utilising Agilent Technologies' ChIP on Chip DNA microarray platform, capable of detecting levels and distribution of DNA damage and repair throughout an entire genome at high resolution. The application of our method involves the affinity capture and separation of damaged regions of DNA from a genomic sample. We are then able to sensitively measure the levels and location of DNA damage throughout the genome by hybridisation of the damaged DNA to Agilent's whole-genome DNA microarrays. This process is repeated at a variety of time points following the induction of damage, which facilitates the sensitive and high-resolution estimation of genome-wide DNA repair capacity. Using our method we show previously unreported binding sites and acetylation profiles for Abf1 and its role as a Yeast regulatory protein, promoting efficient GG-NER repair. As well as demonstrating CPD repair profiles of UV treated cells across the genome. We are currently adapting this technique in partnership with Agilent Technologies for the human context with the aim of developing an alternative to animal-based genotoxicology assays applicable to the clinical, chemical, pharmaceutical and cosmetic industries, improving human genotoxicology testing whilst further elucidating genotoxicity mechanisms.