Mutagenesis vol. 25 no. 6 pp. 631–658, 2010 doi:10.1093/mutage/geq090

Abstracts of the 33rd Annual Meeting of the United Kingdom Environmental Mutagen Society, 12th - 14th July 2010 at the University of Derby, UK.

1. Photo*geno*toxicity – science without application?

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In 1999 the International Workshop on Genotoxicity Test Procedures prepared recommendations for photogenotoxicity testing (1). In addition, photogenotoxicity endpoints have subsequently been included in regulatory photosafety guidances. Several in vitro and in vivo models have since been improved or were newly developed. However, with growing concern about possible triggers for photogenotoxicity testing as well as about the validity of individual assays, the focus of the discussion has moved from implementing adequate assays towards the strategic role of photogenotoxicity testing as part of an overall photosafety risk assessment. Recently, an expert panel convened from Regulatory, Academic and Industrial scientists (as part of the 5th International Workshop on Genotoxicity Testing (IWGT), Basel, Switzerland, 2009) reappraised the initial recommendations and reviewed the progress made in photo(geno)toxicity testing over the past decade. The performance of photogenotoxicity assays (old and new) was discussed, particularly in view of reports of "pseudo"photoclastogencity (2) . The expert panel finished with an assessment of the positioning of photogenotoxicity testing within a photosafety testing strategy. In parallel, during the revision of the ICH M3 guidance, a chapter on photosafety has been integrated indicating that photocarcinogenicity testing is no longer warranted, since chronic administration of phototoxic drugs is per se considered to be associated with an increased risk of developing skin tumours. In summary, the IWGT expert panel concluded that – despite having appropriate test systems available – there appears to be no added value of photogenotoxicity testing integrated into a standard photosafety battery to be used during drug development. However, this conclusion is currently not reflected by any of the regulatory documents addressing photosafety. Therefore, it remains open whether all regulatory authorities will acknowledge this position in the near future. Specifically, during the development of a dedicated ICH Photosafety Guidance the role of photogenotoxicity assays needs to be clarified. In addition, the role of photogenotoxicity testing beyond drug development, e.g. in the context of cosmetics or chemicals might be considered differently.

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2. Update and ongoing issues regarding risk assessment of genotoxic impurities in pharmaceuticals

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The EMEA guideline on limits of genotoxic impurities in new drugs, published in 2006 (1), together with a more recent EMEA Question and Answer document (2), recommends limits for such impurities based on the duration of dosing in man, often referred to as the staged Threshold of Toxicological Concern (TTC). More recently in late 2008, the FDA released a draft guideline on the same topic (3). While these and other publications provide useful summaries and overviews, there are several inconsistencies across the different documents and in some cases, insufficient guidance in some specific areas. It is also the case that currently there is no available guidance from Japan. Largely as a result of this, a concept paper was released in November 2009 (4) laying out a proposal for a new ICH guideline for Genotoxic Impurities that would provide internationally harmonised guidance for this important topic. Examples of some of the key issues that remain regarding the current EMEA and draft FDA guidelines include the following:

a) Use of Structure Activity Relationship (SAR) tools to identify genotoxicity alerts. Many SAR tools are available and it is clear that the pharmaceutical industry and Regulatory Authorities do not necessarily use the same tools or assessment strategies, making it difficult to ensure the same conclusion is reached on any given structure. Use of a common system would address this concern.
b) Implications for control of genotoxic impurities if same structure is present as a metabolite or degradation product. An assessment of total dose/exposure to a potentially genotoxic impurity/metabolite/degradant should be performed but the complexity suggests that worked examples would be useful.
c) Use of compound-specific risk assessments or default limits for genotoxic impurities. Current guidelines suggest compound specific cancer risk assessments can be used where data are available but further information and examples are needed on the specific method(s) that are considered acceptable.
d) Limits of genotoxic impurities for drugs used to treat life-threatening diseases. ICH S9 (5) provides useful clarification in this area but further support in the new ICH Genotoxic Impurities Guideline would help to ensure appropriate and consistent use by industry as well as acceptance by Regulatory Authorities.

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3. Medical device testing

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Medical devices are diverse, ranging from skin electrodes to artificial heart valves. The biocompatibility of each device is assessed using existing information and/or new studies [1]. The range of tests that should be considered depends on the duration and invasiveness of the patient contact. Genotoxicity should be considered for devices that will be in direct contact with tissue, bone or circulating blood for more than 24 hours and for most other types of device when the patient contact will exceed 30 days.

Two alternative genotoxicity testing strategies are described [2]:

a) Ames + *in vitro* gene mutation + *in vitro* chromosome aberration
b) Ames + *in vitro* mouse lymphoma thymidine kinase gene mutation assay.

In vivo testing is not normally required. Note that this guidance is currently under review. The OECD test methods are used.

As it is not possible to prepare suitable formulations of most medical devices, extracts in polar and non-polar solvents are usually tested [3]. The extraction should be 'exaggerated' e.g. by using a high ratio of the amount of device to the volume of solvent, or by increasing the extraction time and/or temperature. Ideally, the extracts should contain sufficient extracted material to comply with the usual OECD criteria for the highest test concentration, but achieving this is usually challenging. The guidance on extraction procedures is under review.

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3. ISO10993 Biological evaluation of medical devices – Part 12: Sample preparation and reference materials. 3rd edition (2007, corrected 2008), *International Standards Organisation, Switzerland.*

4. Regulatory genotoxicity testing – what have we learned and how will things change?

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Around 30 years ago there were >40 test systems for detecting mutagenic and genotoxic effects. Through various collaborative trials and other initiatives, less than half of these survived to be included in the first published regulatory guidelines, though many only advised what tests to do, not how to do them. The UKEMS guidelines committee provided the first clear advice on the best practices for performing the most commonly used tests. However, guidelines in different parts of the world required different tests and different ways to perform them. The first International Workshop on Genotoxicity Test Procedures (IWGTP) succeeded in reaching global consensus on how to perform the most commonly used tests, and then, in subsequent workshops, addressed the up-coming tests for which guidelines had not (and some still have not) been published. The emphasis of the workshops then expanded to include strategy for testing, including how to interpret test results, and IWGTP became IWGT. These discussions have proven considerably more difficult as recommendations are based more on opinions than data. However, which tests we perform, how we perform them and how we interpret the biological relevance of the results has changed over the years. Now we are confident that the tests we use are sufficiently sensitive that we will not miss any important genotoxins, the specificity of the tests is being challenged, and several initiatives have recommended modifications which will not reduce the ability to detect "real" positives, but can help reduce the incidence of "misleading" positives. Thus, the choice of cell type, measures of cytotoxicity, top concentration for testing etc. are likely to mean further changes in the way we perform the tests. Furthermore, questions are now being asked regarding which are the best tests to use in a battery, and this may result in further changes to regulatory testing in the future.

5. Genetic toxicology: ahead of the game but not at the final whistle

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Genotoxicity testing in the pharmaceutical, chemical and cosmetic industries is currently undergoing major reviews to change/ improve testing strategies and reliability of the assays for the detection of carcinogens. Whilst the current battery of *in vitro* mammalian cell tests has been questioned for its specificity, *in vivo* assays are under pressure to adopt the 3R's principle (reduce, refine, replace). Consequently, a number of initiatives have been started addressing these issues. In particular for the comet assay, studies have focused on the development and improvement of the assay for incorporation into the regulatory test battery. By undertaking such investigatory work, the practicalities and limitations of a new assay become apparent, as well as understanding the types of damage that it can detect. The results of these initiates have illustrated the importance of study design, issues with evaluation criteria and how the comet assay can be used to elucidate DNA damaging mechanisms. In addition, our current testing strategies may need to adapt and/ or change to make them appropriate for certain product types, in particular those that are DNA-based. Testing requirements for these products raises a whole new set of questions due to differences between the potential spectra of damage. No doubt new systems will be required but, hopefully, by addressing the issues now we will be ready to support the research areas of the future.

6. Is a triple-helix forming oligonucleotide targeting a genomic locus capable of sequence specific mutagenesis?

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Duplex DNA has been shown to be capable of binding a third oligonucleotide strand in a sequence specific manner to form

a triple-helix (triplex) structure. Such structures have been shown to induce mutation as determined using plasmid-based reporter constructs (1). The European Medicines Agency (EMEA) have highlighted the putative binding of biotechnology derived pharmaceuticals to genomic DNA consequently resulting in heritable sequence alterations. Thus, we have sought to examine the mutagenicity of a non-conjugated triplex forming oligonucleotide (TFO) targeting a genomic locus. We have engineered a TFO, TFO27, targeting the hemizygous *hypoxanthine-guanine phosphoribosyltransferase* (HPRT) locus in the human lymphoblastoid TK6 cell line, and assessed mutagenicity through 6-thioguanine resistance ($6TG^r$). Electrophoretic mobility shift assays were used to demonstrate triplex formation by TFO27 at the target motif at nanomolar concentrations. A control oligonucleotide, SCR27, failed to form a triplex at the target motif. Efficacy of a range of transfection reagents were evaluated for facilitated delivery of TFO27, resulting in variable cellular toxicity. Treating a population of $3x10^6$ cells with TFO27 appeared to induce mutation at the targeted HPRT locus in a sequence specific manner, but SCR27 did not. Furthermore, TFO27 failed to induce mutation at the non-targeted *thymidine kinase* locus suggesting locus specificity for the mode of action of TFO27. Treating a larger population of cells (10^7) failed to induce mutation. We suggest that an apparent toxicity shift that is transfection facilitator dependent could be the contributory factor in the contradictory result obtained. Sequencing of isolated $6TG^r$ clones will inform the sequence context of mutation to validate a triplex mediated mechanism.

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7. An *in vitro* model of chemotherapeutic damage to mesenchymal stem cells

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Mesenchymal Stem Cells/Stromal Cells (MSC) form the bone marrow microenvironment and are essential in supporting haemopoiesis. Following stem cell transplantation (SCT), whilst haemopoietic cells are replaced, MSC remain of recipient origin. However, MSC have previously been shown to be damaged by chemotherapeutic treatment, administered prior to SCT. If damage is severe it may be implicated in lack of engraftment following some SCT, a major cause of mortality, occurring in 10% of allogeneic peripheral blood transplants. To elucidate this damage, a physiologically relevant *in-vitro* model is needed as many chemotherapeutic agents are extensively metabolised by hepatic cytochrome P450 enzymes.

An *in-vitro* co-culture model utilising HepG2 liver spheroids as a source of metabolic enzymes has been developed, enabling study of chemotherapeutic damage. Several cytotoxic effects in MSC have been observed *in vitro* following chemotherapy treatment with alkylating prodrugs such as cyclophosphamide using this model. These include altered morphology, decreased expansion ($p<0.01$), and reduced expression of CD44 ($p<0.05$), an adhesion molecule involved in haemopoiesis. Similarly, treatment with active chemotherapeutics, e.g. vincristine, leads to grossly altered morphology, reduced CD44 expression ($p<0.01$) and decreased expansion ($p<0.001$). In the presence of liver spheroids, however, these effects are reduced ($p<0.05$ and $p<0.001$, respectively), indicating detoxification, as would occur *in vivo*.

These results are comparable with effects seen in patients previously treated with chemotherapy, where CD44 expression is decreased ($p<0.05$), MSC survival *in vitro* is reduced ($p<0.01$) and ability to support haemopoiesis *in vitro* is diminished ($p<0.05$). Using this co-culture model and age- and sex-matched untreated and chemotherapeutically-treated patient samples, genotoxic damage to MSC *in vitro* and *in vivo* is currently under investigation, using ^{32}P post-labelling to separate and identify DNA adducts. In conclusion, a physiologically relevant model has been developed to study cytotoxic and genotoxic chemotherapeutic damage, with results comparable with effects seen in patients who have undergone chemotherapy treatment for malignancy.

8. Impact of long-term exposure to chemical agents on exposure to ionising radiation

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A wealth of information is available on the health risks from ionising radiation. However, in practice, everybody is exposed to a complex mixture of chemical, biological and physical agents. Investigations of interactions between different types of agent are therefore important.

The aim of this study is to investigate the impact of chronic, low level exposure to certain chemical agents having different modes of action (sodium arsenite, benzo[a]pyrene, 4-nitroquinoline-1-oxide (4NQO) and N-nitroso-N-methylurea) on radiation responses. Human lymphoblastoid cell lines are cultured in the presence of low levels of chemicals for up to six months. The exposure regimes are designed to mimic in a very simple way chronic environmental exposure to chemicals followed by radiation exposures as a consequence of medical procedures or accidents. Following chemical exposure, challenge doses of X-rays are given. The effects of such treatments are monitored by the standard cytogenetic methods of scoring dicentrics, translocations, micronuclei and chromatid damage. Acute exposure to sodium arsenite alone induced significant levels of chromosome damage at concentrations >8 ng/ml. No modification of radiation responses has thus far been observed in cells chronically exposed to 8 ng/ml sodium arsenite. Acute exposure to >20 ng/ml 4NQO alone was toxic and induced chromosome damage. Cells chronically exposed to 20 ng/ml 4NQO showed elevated chromosome damage that was additive with radiation-induced chromosome damage. In summary, our initial cytogenetic analysis of the effect of combined exposure of cell cultures to chemical agents and ionising radiation suggests an additive, rather than synergistic, response.

9. Oxidative stress and gene expression alterations by single walled carbon nanotubes

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Nanotechnology is without doubt a fast growing industry with major benefits in the fields of engineering, environmental remediation and healthcare. Single walled carbon nanotubes (SWCNTs) are a part of this growth and yet numerous questions regarding their genotoxicity and the mechanisms involved in such processes remain unanswered. Therefore, in this study the influence of SWCNTs of three different lengths (400-800nm, 1-3μm, 5-30μm) at a dose ranging between 1μg/ml to 100μg/ml on oxidative stress and gene expression were studied in human bronchial epithelial cells (BEAS-2B). The 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorometric assay revealed a concentration and time-dependent increase in reactive oxygen species (ROS) in the 1-3μm sample. The same was true for the 400-800nm sample up to 20μg/ml. Interestingly, the only significant increase in ROS was observed at the lowest doses (1μg/ml and 5μg/ml) in the 5-30μm sample possibly due to larger agglomerates sedimenting out of suspension. There was a remarkably significant decrease in these levels when 4mM antioxidant N-acetyl cysteine (NAC) was added to the reaction. These results were consistent with data obtained from oxidative damage pathway gene expression profiling by RT-PCR. Exposure to all the sub-cytotoxic doses of 1-3μm SWCNTs, lower doses of 400-800nm and lowest dose of sample 5-30μm sample resulted in $\geq$2 fold up-regulation in the expression of six genes. These results were comparable to alterations seen in this study from treatments with crocidolite asbestos fibres. The up-regulated genes were; DUOX1, EPHX2, GPX2, NCF1 SEPP1, and SFTPD, which are involved in the removal of H_2O_2 and other reactive oxygen species in cells. Similar results were seen at the lower doses in the 400-800nm sample and the lowest dose in the 5-30μm sample. These findings suggest that exposure to SWCNTs can lead to adverse cellular responses in mammalian cell lines through the activation of genes involved in the protection and possibly molecular signaling associated with oxidative stress.

10. Investigations into the Pig-A mutation assay

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We are currently validating a new mutation assay based on the endogenous Pig-A locus, along with Litron, the International Life Sciences Institute (ILSI) and other pharmaceutical companies, with the intent of integrating genotoxic endpoints into general toxicology studies. We have been focusing on the following;

1. Assessing the portability of the Pig-A mutation assay.
2. Investigating the low dose region to assess possible thresholds for alkylating agents.
3. Understanding Pig-A expression in tissues other than bone marrow.
4. Extending the Pig-A assay in vitro.

Incorporating a gene mutation endpoint (Pig-A locus) into 28-day repeat dose studies was investigated using *N*-methyl-*N*-nitrosourea (MNU). MNU (2.5-10mg/kg) resulted in dose dependent increases in Pig-A mutants in both reticulocytes and red blood cells (see poster by Giddings *et al*). An additional 28 day study was conducted with MNU in order to better characterise induced Pig-A phenotypic mutations in the low dose region. Male SD rats (n=3) were dosed orally for 28 days MNU (at 0.1, 0.3, 0.6, 0.9, 1.25 and 2.5 mg/kg/day). Blood samples were collected and analysed on Days -1, 15 and 29 for Pig-A mutation and Days 4 and 29 for micronucleated-Ret determination. Analysis showed that doses of MNU $<$ 0.9 mg/kg/day did not generate a significant increase in the %MN RETs compared with controls.

Using real time PCR analysis, Pig-A gene expression was observed in various rat tissues i.e. bladder, kidney, lung, liver, stomach, ileum and blood at levels of 0.0042 - 0.015 and 0.00039 – 0.0045 by copy number relative to GAPDH and Actin expression, respectively. Tk6 cells were experimentally shown to express Pig-A, using CD58 as the phenotypic cell surface antigen, at a ratio of 40% positive cells. To optimise cell populations for mutation analysis, the frequency of Pig-A positive cells was enriched using a streptavidin – biotin Dynabead™ positive selection method. This resulted in 98% Pig-A positive Tk6 cells, based on CD58 labelling. We now plan to expose this enriched cell population to MNU and look for Pig-A phenotypic mutations using flow cytometry.

11. p53 in Mouse Lymphoma L5178Y $tk^{+/-}$ clone 3.7.2c cells is functional and can mediate apoptosis

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Apoptosis is the process of cell death characterised by cell shrinkage and retention of cellular constituents (1) and utilises caspases – cystein-aspartic proteases which cleave cellular proteins at sequence specific points (2). Apoptosis can be initiated intrinsically (p53-mediated) or extrinsically (non p53-mediated) with both pathways culminating at caspase 3&7 (3). L5178Y cells have only one p53 allele that codes for a complete, although mutant, protein and have been speculated to be unable to undergo apoptosis (4,5). 24 hour treatments of L5178Y cells were performed with carbendazim (CBZ), colchicine (COL), staurosporine (STAU) and methyl methanesulfonate (MMS). CBZ, COL and STAU initiated caspase 3&7 activity and this effect was still observed up to 72 hours post treatment, as was disruption of the cell cycle. CBZ, COL and STAU had also increased intracellular p53 levels when analysed by flow cytometry at the end of treatment. RNA interference experiments were designed to investigate p53's potential involvement in the apoptosis responses observed. Comparative treatments were performed in the absence of and

presence of knocked-down p53 (achieved through transient transfection of p53-targeted short interfering RNAs) and the apoptosis endpoints of caspase 3&7 and annexin V were assessed. Reduction in intracellular p53 correlated with reduction of the apoptosis responses to STAU (known to cause p53?mediated apoptosis (6) but did not affect responses to COL. These data show that p53 is involved in apoptosis in L5178Y cells, thus demonstrating that the endogenous p53 is functional. This has important implications when using this cell line for regulatory testing.

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12. The protein composition of mitotic chromosomes determined using multi-classifier combinatorial proteomics

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Despite many decades of study, mitotic chromosome structure and composition remain poorly characterized. Proteomic analysis of metazoan chromosomes is difficult because many cytoplasmic components bind to the highly charged chromosomes following nuclear envelope breakdown. Here, we have integrated quantitative proteomics with bioinformatic analysis to generate a series of independent classifiers that describe the ~4,000 proteins identified in isolated mitotic chromosomes. Integrating these classifiers by random forest and cluster analyses uncovers functional relationships between protein complexes in the context of intact chromosomes and reveals which of the ~560 uncharacterized proteins identified here merits further study. Indeed, of 34 GFP-tagged predicted chromosomal proteins, 30 were chromosomal, including 13 novel centromere-associated proteins. Of 16 GFP-tagged predicted non-chromosomal proteins, 14 were confirmed non-chromosomal. Our integrated analysis predicts that up to 97 novel centromere-associated proteins remain to be discovered in our data set. These methods allow to study the impact of perturbations of the kinetochore in its native context of the entire chromosome. Such an unbiased analysis of the whole chromosome proteome from *Ska3/Rama1* genetic knockout cells revealed that the APC/C and RanBP2/RanGAP1 complexes depend on the Ska kinetochore complex for stable association with chromosomes. This may have particular impact for the study of environmental agents when trying to understand their role in mitosis.

13. Chromosomal and nuclear genome architecture

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In recent years, some evolutionarily conserved principles of nuclear architecture were discovered. Recent technical developments in the quantitative analysis of 3D nuclear genome organisation by fluorescence in situ hybridisation to 3D-preserved interphase nuclei (3D-FISH) include multi-colour FISH with up to six different fluors, the use of quantum dots, combined RNA-ish/DNA-FISH to cryosectioned tissue samples and immuno-FISH. There are several examples of the interplay between the linear organization of vertebrate genomes, metaphase chromosome structure and the non-random spatial arrangement of entire chromosomes, of chromosome sub-regions and of individual loci in the cell nucleus: a) Chromosomes occupy distinct regions in animal and plant nuclei, so-called chromosome territories. These chromosome territories show a non-random, gene-density correlated radial arrangement, with gene-dense chromatin orientated towards the nuclear interior. b) At least at the level of chromosome arms, the structure of metaphase chromosomes appears to be preserved also in interphase nuclei. c) In addition, the spatial positioning of individual loci is determined by replication timing, by local GC content and by gene density. d) The impact of transcriptional activity of a gene on its nuclear positioning remains a controversial topic, since some genes change their nuclear address when expressed, whereas others remain stably positioned. In this context, changes in structural genome organisation and in gene expression impacts on the nuclear topology of affected loci In the case of a fusion and a reciprocal translocation, preferential nuclear side-by-side arrangements of breakpoint flanking loci during meiosis may predispose to non-random breakage and repair of chromosome rearrangements.

14. DNA damage-induced centriole disengagement

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Centrosomes organise an astral microtubule network during interphase and a bipolar mitotic spindle in dividing cells. Their numbers increase after ionising radiation (IR) and in tumour cells, so that centrosome amplification is implicated in genome instability. Centrosomes consist of two centrioles, which contain nine microtubule bundles arranged in a cylinder, within an amorphous pericentriolar material (PCM). The centrioles are

arranged at right angles to one another. This orthogonal arrangement is lost when the centrioles are disengaged late in mitosis, prior to the next round of centriole duplication. Current models invoke centriole disengagement as a key licensing step for centrosome duplication. The duplicated centrosomes remain linked until mitosis onset, when NEK2 kinase activity causes dissociation of the proteinaceous linker that maintains centrosome cohesion. Premature centriole disengagement also arises after DNA damage and may permit centrosome overduplication.

We examined IR-induced centriole disengagement in non-transformed human cells using fixed and live cell microscopy. We found that irradiation causes highly mobile centrioles and that these disengaged centrioles carry mature/ mother centriole markers. However, the PCM associated with IR-disengaged centrioles differs from that normally associated with the mother centriole. Centriole disengagement was greatly increased by siRNA knockdown of components of the centrosome cohesion apparatus, indicating that centrosome cohesion and engagement are regulated by similar activities. Our findings show that centrosome cohesion controls the outcome of DNA damage-induced centriole disengagement, which may contribute to centrosome overduplication and spindle multipolarity after genotoxic stress.

15. Aneuploidy as a biomarker of cancer risk in pre-malignant gastric cancer

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Aneuploidy is one of the most common genetic defects found in the cells of solid tumours. Indeed, the chromosome instability (CIN) theory states that aneuploidy is a major driver for tumourigenesis. Hence, it follows that aneuploidy might occur early in neoplastic development and might even be predictive of cancer development. We aimed to investigate this possibility using gastric cancer as a model. Gastric cancer has been linked to infection with the bacterium *H. pylori*. Here, the chronic inflammation induced in the epithelium drives the gastric tissue through a series of distinct histological stages (the Correa pathway) leading to gastric adenocarcinoma. These pre-malignant histological stages are ideally suited to assessing the early drivers of neoplastic development. We have utilised this histological series to assess the role of aneuploidy in gastric carcinogenesis. We have studied chromosome copy numbers in early histological stages (gastritis) and intermediate histological stages (atrophic gastritis, intestinal metaplasia) using FISH in order to investigate if step wise increases in aneuploidy frequency occur in parallel to histological progression. We have also collected gastric biopsies from these patients to assess gene expression abnormalities of mitotic checkpoint genes (e.g. Mad2, Aurora kinase). These gene products are thought to play a crucial role in maintaining normal chromosome segregation, with dysregulation of these factors being linked to aneuploidy. We have observed that the earliest histological stages of gastric neoplasia (gastritis, or inflammation of the stomach) show increased levels of aneuploidy relative to histologically normal gastric tissue. Furthermore, the level of aneuploidy rises in parallel with the histological progression thereafter. We have also shown that mitotic checkpoint gene abnormalities occur in parallel to aneuploidy and to histological progression, although the source of these abnormalities (the aneugen(s)) are not as yet known.

This data supports the CIN theory of carcinogenesis and suggests that aneuploidy may be a clinically useful biomarker of cancer risk.

16. Aurora kinases: a therapeutic target

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The Aurora kinases (AK) play a critical role in mitosis and have been suggested as promising targets for cancer therapy due to their frequent overexpression in a variety of tumours. Several AK inhibitors are advancing in various stages of development including AZD1152, a selective Aurora B kinase inhibitor [1]. AZD1152-hydroxy quinazoline pyrazol anilide [hQPA], the active form lacking the phosphate ester, has a novel antitumour mechanism of action *in vitro and in vivo,* leading to prolonged antitumour activity in a variety of solid [2] and hematological [3] preclinical cancer models. These data suggest that selectively targeting Aurora kinase B may be a promising therapeutic approach to treat a range of malignancies. In addition to histone H3 phosphorylation suppression, determination of tumour cell polyploidy and apoptosis may be useful biomarkers in the clinic for this class of therapeutic agent.

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17. Predicting small molecule properties and biological behaviour using genomic data and bioinformatic analysis

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For more than 10 years genomic technologies have been generating global gene expression data on many types of chemicals including mutagens and environmental pollutants. These data have been analysed in a variety of ways with two favourites being phenotypic anchoring, where the data are linked to a measured parameter such a pathological change,

and pathway mapping where pathways where more genes are differentially regulated than would be expected by chance. However where a compound has mixed biological activity, for example genotoxicity and gene induction these methods can find it difficult to distinguish one interaction from another, particularly where one is dominant. Thus, for example genotoxicity may be missed in a compound whose predominant activity is receptor mediated gene induction. Furthermore neither of these methods link chemicals together using a database. They work only within one experiment. In 2006 Justin Lamb *et al* [1] published in Science on another method called connectivity mapping that could be used to discern similar properties in molecules. This method was connected to a database of gene expression profiles for many chemicals and its utility was proven with estrogen, the signature of which recovered from the database other estrogenic-like chemicals. Additionally estrogen receptor antagonists were selected by their complete opposite connections to the estrogen signature. The original method was improved by Zhang and Gant [2] and the software made available. For the analysis presented in this talk data was obtained from the public data set E-MEXP-2209 (Arrayexpress at ebi.ac.uk), a time course of gene expression data derived from mouse primary hepatocytes at time points after exposure to various gene and non-genotoxic chemicals [3]. The connectivity analysis of these data demonstrates how connectivity mapping can identify genotoxic activity in molecules that have several biological properties and in particular where genotoxicity is not the major interaction of the compound in cells. Finally as genomics technology advances it is necessary to ask 'what next'. There are two technologies in particular that may impinge on the testing for mutagens, global analysis of copy number variation and high throughput sequencing.

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18. Genotoxic measures in molecular epidemiology

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A variety of methods has been developed which can be used to study the impact of dietary, lifestyle and environmental factors and of occupational exposures on DNA stability in humans. Classical chromosomal aberration analyses are nowadays partly replaced by less time consuming and cost efficient micronucleus (MN) assays. The cytome MN assay with lymphocytes is well standardised and validated and it has been shown that MN are a reliable marker for human cancer risks. At present attempts are made within the frame of an international initiative to establish guidelines for MN assays with exfoliated oral cells. This approach enables to monitor in addition nuclear aberrations that reflect acute toxic effects (pyknosis, caryorrhexis) as well as DNA instability (nuclear buds, broken eggs) and has the advantage that the cells can be collected with noninvasive methods. This holds also true for assays with nasal cells which are promising for the detection of air contaminations but are methodologically less developed. Another cluster of endpoints which require shorter intervention or exposure times is based on the determination of DNA migration in an electric field. Single cell gel electrophoresis assays enable the measurement of singe and double strand breaks and apurinic sites, endogenous formation of oxidised DNA bases, alterations of repair processes and changes of the ROS sensitivity. Recently we developed protocols which enable to study the impact of physiological conditions, gene polymorphisms and dietary factors on the induction of DNA damage by occupational and dietary carcinogens (metals, nitrosamines, HAs, PAHs, mycotoxins, ect.). Overall, the recent findings show that these multiple endpoint approaches provide valuable information on the impact of factors that affect DNA integrity. The combination with animal models and advanced OMICS technique offers the possibility further to elucidate the underlying mechanisms and health consequences for humans.

19. Metabolomics, human health and the environment: big opportunities for small molecule profiling

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Improving exposure assessment will give a clearer picture of how specific risk factors interact with genotype to affect human health. Metabolic profiling allows the study of an individual's metabolic phenotype, which is influenced by a number of factors including genotype, diet, lifestyle and xenobiotic exposure. It is a systematic and efficient approach to biomarker discovery to aid in the prediction of disease and defining mechanisms of toxicity. We have used NMR-based metabolic profiling (metabonomics/metabolomics) to investigate changes in human systemic metabolism from volunteers living near a point source of environmental pollution: a disused zinc smelter, with elevated levels of environmental cadmium. Five specific urinary metabolites were identified as potential biomarkers of systemic cadmium exposure, but several of these were also correlated to potential confounding factors such as age, gender, renal damage and smoking status. After controlling for these indirect associations, urinary citrate levels retained

a significant correlation to both cadmium body burden and current or previous smoking status. Smoking is one of the major sources of cadmium in human populations; although citrate was correlated with smoking, there was still an additional response which could be ascribed to the effects of environmental cadmium exposure. This study shows for the first time that a metabolic profiling study in an uncontrolled human population is capable of identifying intermediate biomarkers of response to environmental toxicants at realistic concentrations relevant to actual environmental exposure levels.

20. Epigenomic analysis in common complex disease

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Epigenetic marks are stable, somatically heritable changes in the genome that occur without alteration in DNA sequence. These can modify gene expression and consequently phenotype. It is this mechanism that enables cells, although they possess identical genomes, to differentiate into more than 200 distinct types within the human body and subsequently perform vital individual organ functions. True genome-wide assessments, or epigenomic profiling, of these epigenetic alterations, such as DNA methylation (methylomes), or chromatin modifications are now possible, either through high-throughput arrays or increasingly by second-generation DNA sequencing methods. The ability to collect these data, at this level of resolution, facilitates interrogation for levels of genetic control and changes that occur due to development, lineage and tissue-specificity, and significantly those caused by environmental influence, including aging, stress, diet, hormones or toxins. The epigenome therefore plays the pivotal role as interface between genome and environment. Common complex diseases are under variable levels of genetic and additionally epigenetic effect and the integration of genomic and epigenomic data will allow more powerful analyses. Furthermore, the detection of pathological epigenetic alterations will reveal novel insights into disease aetiology.

21. Novel omic biomarkers and the exposome in molecular epidemiology

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Omics is the study of the complete set of biological molecules such as the complete set of microRNAs expressed in a cell, the microRNAome, or the complete set of all mRNAs expressed by all known genes, the transcriptome. It is now possible to perform microarray-based analyses of the transcriptome, microRNAome and methylome (CpG island methylation status of all known genes) of essentially any human cell, including peripheral blood mononuclear cells and exfoliated epithelial cells. Even single cells can now be studied. The application of array-based omic methodologies has led to the discovery of signatures related to prognosis, exposure and early effect that are potentially useful biomarkers in the clinic and molecular epidemiological studies. Advances in proteomics and metabolomics have also identified novel biomarkers and the technology for these omic approaches is advancing rapidly allowing them to become much more widely used in epidemiological studies. New omes are constantly arising, such as the adductomes on DNA and proteins, which could provide global biomarkers of exposure. Thus, the field continues to evolve rapidly and its utility in obtaining snapshots of the human exposome, representing all exogenous and endogenous exposures from conception onwards, is the next frontier. Currently researchers use bottom-up exposomics where they assume *a priori* that they know what to look for and try to measure it. In top-down exposomics you take an agnostic discovery-based view and look for patterns, then attempt to find out what those patterns reflect in terms of exposure. Using a combination of omic high-throughput technologies, it should be possible to characterize the human exposome by this approach. Increasing throughput, lowering cost and reducing sample requirements should be feasible through bioengineering approaches, such as lab-on-a-chip devices. If successful, the pay-off in disease prevention from studies that interleaf the human genome with the human exposome would be huge.

22. Genotoxicity of iron oxide nanoparticles and carbon nanotubes

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Over the last 5-years investigation into the toxicity of engineered nanomaterials has gained momentum, but the importance of their genotoxic potential on human health remains poorly understood. This is of concern given the predicted applications of nanomaterials in consumer and medical healthcare products, together with the increasing occupational exposure experienced within the ever expanding nanotechnology industry. We have therefore sought to investigate the genotoxicity of dextran-coated ultrafine superparamagnetic iron oxide nanoparticles (dUSPION) and single-walled carbon nanotubes (SWCNT) based upon subtle difference in physico-chemical properties. Physico-chemical characterisation of the nanomaterials under study was performed using a range of techniques including dynamic light scattering, X-ray photoelectron spectroscopy, energy dispersive x-ray analysis, transmission electron microscopy (TEM), zeta-potential and BET surface area analyses. Cellular uptake of the nanomaterials was assessed by TEM, Prussian blue staining and the ferrozine assay, and subsequent genotoxicity was evaluated using the cytokinesis blocked micronucleus and HPRT forward mutation assays. None of the nanomaterials investigated induced cytotoxicity over the tested dose ranges. The dUSPION were found to induce gross chromosomal damage, but this was reliant on the iron oxidation state with dFe_2O_3 inducing significant levels of clastogenicity, whilst little damage was associated with dFe_3O_4. SWCNT also induced clastogenicity the degree of which was reliant on their length (400-800nm $>$ 5-30um $\geq$ 1-3μm). In contrast, a thresholded dose-dependent increase in mutagenicity was only observed with the 1-3μm length sample and interestingly this was the only SWCNT that induced a significant degree of oxidative stress following exposure, mirroring the induction of point mutations. There is therefore evidence that some nanomaterials do have the capacity to induce genotoxicity but this is highly dependent upon their physico-chemical characteristics and although oxidative stress is likely to play a role, it is alone unable to explain the different genotoxic profiles observed in these studies.

23. Medical implants, wear nanoparticles and biological response

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Total hip replacement (THR) has revolutionised the management of patients crippled with end stage arthritis of the hip. In the 1960's and 1970's total hip replacement was reserved for the elderly and over 75% of hip prostheses succeed beyond 25 years in elderly recipients. Today, ever younger patients seek replacement surgery to deliver quality of life aspirations and approximately 10% of hip replacements are now performed in patients less than 55 years in whom there is a high risk of failure with the need for revision surgery. Two types of joint replacement are currently favoured; metal on ultra-high molecular weight polyethylene (MoPE) and metal on metal implants (MoM). MoPE implants generate predominantly bioinert PE particles. The major consequence of exposure is osteolysis, leading to aseptic loosening at 10-25 years instigated by 0.1 to 1 micrometre sized PE wear particles interacting with macrophages. Modern MoM THR are optimally designed and have extremely low wear rates. Over the last ten years, MoM surface replacements have been increasingly being advocated for young and active arthritic patients. Modern MoM articulations generate CoCr particles in the nanometre size range (typically 20-60nm) giving rise to 10^{12}-10^{14} particles per year. The nanoparticles are small enough to be taken up by endocytosis or pinocytosis and even to cross cell boundaries such as endothelium. The young orthopaedic patients will be exposed to CoCr particles and ions for up to 60 years. The size and number of nanometre metal particles represent an enormous area for the release of metal ions into the body during the wear process. Our studies have focussed upon understanding the biological response to metal wear particles. We have uniquely demonstrated the toxic effects of the clinically relevant nanometre sized metal wear particles generated from orthopaedic alloy on cells in vitro. Sub-lethal concentrations of metal particles to give rise to oxidative stress, DNA damage and genotoxicity. We have shown that CoCr nanoparticles induced more •OH and •O, greater DNA damage, mitochondrial damage, cytotoxicity, aneuploidy and metal dissolution in human fibroblasts compared to micrometer sized particles. Current studies are investigating the consequences of exposure to metal wear particles in a mouse model.

24. Nanospecific genotoxic effects

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The exceptional properties of engineered nanoparticles, utilized in their industrial applications, may also affect the genotoxicity of the material. It cannot, however, be assumed that nanoscale size should generally increase the genotoxicity of the material - this will depend on the material, and nanomaterials are highly variable. Enhanced surface reactivity due to nanosize and mechanical interference in nanoscale are considered features that could contribute to genotoxicity, although the possible modifying effect of nanoparticle coating with proteins in biological systems is not understood. To allow interaction with DNA, nanoparticles will have to get to the nucleus, and this may not occur easily with many nanoparticles. Also interaction with the mitotic apparatus or cytoskeleton requires that the nanoparticles are available and not confined to lysosomes or other organelles. Information on the possible photogenotoxicity of some nanoparticles is presently controversial. A major genotoxic mechanism often attributed to nanoparticles is the generation of reactive species and oxidative stress through, e.g., endocytosis and interference with cellular membranes or mitochondria. Size-specific uptake of particles is an important factor affecting the intracellular dose which may differ greatly among cell types. Ion release from partly soluble metals may be faster with nanoparticles than larger particles, which may modulate genotoxic action if it depends on the soluble form. Access to the body, organ distribution and clearance - and thereby tissue-specific dose - may be different for nano- than larger particles. It is presently not clear to which extent in vitro genotoxicity assays can be applied to test nanoparticles, and how well such data could predict the carcinogenicity of these substances. Information on larger particles suggests that particle persistence, shape and dimensions influence the possible carcinogenic action and that inflammatory mechanisms could be important. It is a challenge to clarify the role of genotoxic events in these processes.

25. Toxicity, genotoxicity and behavioural deviations in fish exposed to nanomaterials

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The rapidly expanding use of engineered nanomaterials (ENPs) within industrial, medical, and remediation processes, coupled with their inclusion in a plethora of personal care products, makes environmental exposure to such materials inevitable. Biological effects following exposure to materials such as carbon nanotubes and metal oxides have already been widely reported. The use of nanosilver alone has raised regulatory and environmental concerns over the paucity of knowledge regarding the risks posed by such materials. As studies into the effects of nanomaterials have expanded from human health protection to environmental protection it has become apparent that nanomaterials act differently in aquatic environments compared with airborne exposure. Using quantum dots coated in methyl-polyethylene glycol to ensure dispersion in aqueous media, Cefas has adopted a tiered testing strategy using *in vivo* and *in vitro* techniques to identify biological effects. This has culminated in identifying alterations in the behaviour, gene expression and pathology of 3 spine-sticklebacks following exposure to nanomaterials. Interpretation of the data remains difficult due to limitations in current metrology and analytical techniques and this has raised questions over the suitability of current assessment strategies to identify and characterise the risk posed by nanomaterials within the aquatic environment.

26. Genotoxic impurities in production batches of drug substances

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The first example was a mutagenic impurity identified because a new production batch of drug substance (DS) was found to be

positive in the Ames test. Repeated purification did not remove the mutagenic activity which appeared to be typical of an alkyl halide. 4-Chlorobutanol was then identified as a possible culprit, but was not mutagenic at the levels present in the DS; bis(chlorobutyl) ether, a much more potent mutagen, was found to be responsible. The learning from this example was that the initial analytical methods were able to detect only drug-related impurities. The second example considered how to qualify an impurity related to a DS which was itself weakly active in the Ames test, but not the mouse lymphoma assay (MLA). It was suggested that Ames test and MLA results showing that the impurity was no more active than the DS should be sufficient, but it was also thought that *in vivo* data to calculate permitted daily exposures might be required. Following from this, the consequences were considered of finding late in the development of a drug that it had a genotoxic impurity resulting from oxidisation of the DS. Establishing levels in batches to which people have been exposed is most important and it is likely any ongoing clinical trials would have to be discontinued; the impurity would obviously have to be controlled to TTC levels in any subsequent formulation. Knowing the levels in batches used in toxicity studies might be helpful, particularly *in vivo* genetic toxicity tests and oncogenicity studies, if completed. If oncogenicity studies are in progress, levels in the formulations would need to be established and decisions made on how to progress; should new studies be started with the impurity controlled and, if so, should the existing studies be stopped or continued?

27. How a modified testing strategy for an insoluble compound caused unexpected problems due to extreme pharmacology

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As part of the pre-clinical safety evaluation of new pharmaceutical a standard genetic toxicology testing battery was planned. The compound was soon found to be completely insoluble in all solvents routinely used in the bacterial reverse mutation assay and human lymphocyte chromosome aberration test. The practicalities of testing this compound were initially overcome by relatively straight forward modifications to the in vitro assays. However, as the assays were potentially compromised by limited test system exposure the testing strategy was modified further to provide more in vivo data than usually required at this stage of testing. Unexpected genotoxicity results were obtained and further work was required to demonstrate that this activity was due to extreme pharmacology rather than inherent genotoxicity.

28. Genotoxic investigation of an Absorbable Surgical Film in the MLA test system to meet the requirements of the ISO 10993-3 recommendations

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Investigation into the genotoxic potential of an absorbable surgical film was performed to meet the requirements of ISO 10993-3. Initially an Ames test (to OECD 471) was performed which gave a negative result. Then using option 2 in the guidance document a gene mutation test was performed (to OECD 476), namely the mouse lymphoma assay incorporating colony number and size determination to cover both endpoints (gene mutations and clastogenicity), this gave a positive response following 24 hour exposure in the absence of metabolic activation with evidence of clastogenicity. Following discussions with the sponsor it was found that the film contained a colouring agent, methylene blue, which was found to have known mutagenic potential in the MLA test system. A revised batch of the surgical film without methylene blue was prepared and tested using only the 24 hour exposure condition, and the result was yet again positive. Investigations using L5178Y cells with alternative endpoints also gave positive genotoxic results. An *in vivo* micronucleus test gave a response that was considered to be negative. Following a retrospective review of the procedures used it may be that the test sample preparation produced a large bolus of genotoxic agents that was exposed to the cells under *in vitro* conditions, whilst in clinical use the production of these genotoxic agents is gradual and handled by the bodies repair mechanisms.

29. Beyond The Standard Genetic Toxicology Battery

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Bacterial DNA gyrase is a homolog of topoisomerase IV and both enzymes are essential for bacterial cell growth and division. Consequently, this has made them a target for antibiotic treatments. Fluoroquinolones are a well known class of compounds which function by this mechanism (1). During the development of our antibacterial DNA gyrase/topoisomerase IV inhibitors we observed signals in the in vitro bacterial and mammalian genotoxicity assays. It is recognised that DNA gyrase/topoisomerase inhibitors can induce mutagenicity in the Ames assay and this is related to their pharmacology. Such compounds inhibit DNA ligation during replication, fixing the transient scission and resulting in a double strand break. The persistence of the double strand break is responsible for the subsequent mutagenicity. We demonstrated that mutagenicity observed with compound 1 and 2 was due to bacterial specific gyrase inhibition and therefore not relevant to humans. The additional bacterial assays conducted included a comparison of compound 1 with other fluoroquinolones in *Salmonella* strains TA1537 and TA102, all compounds tested positive in both strains. Generation of TA1537 DNA gyrase resistant mutants showing compound 1 was less cytotoxic and less mutagenic when tested up to 50 μg/plate. As well as mutagenicity, structural chromosome aberrations (clastogenicity) were observed in the in vitro micronucleus assay with compound 2. It is recognised that clastogenicity observed with gyrase/topoisomerase IV inhibitors is due to mammalian topoisomerase II inhibition, rather than direct DNA reactivity (2). This consideration is particularly relevant to drug development as compounds that exhibit a thresholded genotoxic mechanism can be risk managed and progressed, providing an appropriate safety margin in vivo is demonstrated. Compound 2 appeared to have an in vitro clastogenicity threshold at 14 μg/mL in the micronucleus assay after 24 hour treatment. Additionally, compound 2 did not induce micronuclei in the bone marrow of rats tested up to 1000 mg/kg/day, providing confidence there was negligible in vivo risk. In summary, despite the undesirable

genotoxicity profiles observed with compounds 1 and 2, with scientifically designed and well executed follow-up studies, we were able to support progression of both antibacterial candidates.

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30. DNA damage induced by size fractionated samples of exhaust particulate matter from a heavy duty diesel engine using diesel and rapeseed oil as fuel

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Plant oils are frequently used as biofuels intended to replace conventional diesel, often following transesterification, a process which itself requires a significant energy input. A diesel fuel (DF) additive package added to unprocessed rapeseed oil (RSO) can significantly improve its combustion characteristics [1], thereby reducing the need for transesterification. However the potential toxic effects of the exhaust particles produced from this fuel are unknown. Particulate matter was collected from the exhaust of a heavy duty diesel engine using DF, RSO and RSO with an 800ppm dose of additive (RSOAd) as fuel. Size fractionated particulate samples were collected using Anderson impactors and an ELPI. Total suspended particle (TSP) samples were collected onto a glass fiber filter. For toxicological analysis particles were incubated with A549 cells at a concentration of 50μg/ml for 24 hours. DNA damage was assessed using the comet assay. Analysis of size fractionated collections shows that DF combustion produces a lower mass of particulate material than either RSO or RSOAd. Irrespective of fuel type the majority of the mass collected (>60%) was in the sub-micron range. DNA damage was greater in cells exposed to DF exhaust particles (22.5% tail DNA) than in RSO (14.9%) or RSOAd (12.5%) exposed cells ($p<0.005$). Analysis of size fractionated DF samples showed that smaller particles induced higher levels of DNA damage. RSOAd particle DNA damage appears to be correlated with particle size but not to the same extent as the DF samples. No clear link between RSO particle size and DNA damage was found.

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31. Cytotoxicity of urban dust and diesel engine particulates in Neil1 and Ogg1 DNA repair deficient cells

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Human exposure to genotoxic reactive oxygen species (ROS) is unavoidable and therefore, to prevent ROS induced mutagenesis, cells contain a number of different mammalian base excision repair (BER) such as 8-oxoguanine glycosylase (Ogg1) and endonuclease VIII (Nei)-like-1 (Neil1) that remove oxidised purines and pyrimidines respectively, To further investigate the biological role of these proteins, we have developed a new *Neil1* knockout (*Neil1*$^{-/-}$) mouse model by deleting 101 bases, coding for 33 amino acids, in the helix 2-turn helix DNA binding region of the protein. The genotype was confirmed by PCR and phenotype by reverse transcriptase PCR and western blotting. Murine embryonic fibroblast (MEF) cell lines were then derived from the *Neil1*$^{-/-}$ mouse strain and a previously established *Ogg1*$^{-/-}$ strain and treated with urban dust (UD) and diesel engine particulates (DEP). Cytotoxicity was determined using the MTT assay. *Neil1*$^{-/-}$ mice were found to be viable and fertile and outwardly indistinguishable from wildtype litter-mates. Contrary to previous results (1) no overtly obese phenotype was observed. In all MEF cell lines, exposure to DEP and UD caused a dose-dependent increase in cytotoxicity. However, there was evidence that MEFs derived from *Neil1*$^{-/-}$ and *Ogg1*$^{-/-}$ mice were more resistant to DEP than wildtype cells, whereas there was no difference on UD treatment. Initial results then suggest that loss of Neil1 or Ogg1 increases cellular resistance to DEP induced cytotoxicity, implicating DNA damage as a cause of this cytotoxicity. Further work is required to substantiate these results and to explore the effects of DNA repair deficiency on air pollution induced toxicity both *in vitro* and *in vivo*.

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Work supported by CEFIC, the British Cotton Growers Association and UiTM.

32. Understanding the in vitro genotoxicity induced by ultrafine superparamagnetic iron oxide nanoparticles (USPION)

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Ultrafine superparamagnetic iron oxide nanoparticles (USPION) are being widely used for various biomedical applications e.g. magnetic resonance imaging. Although, the potential benefits of USPION are considerable, there is a distinct need to identify any potential cellular damage associated with these nanoparticles. The aim of this study was to characterize a range of USPION particles, determine cellular uptake, effect on iron homeostasis and their potential in vitro cytotoxic and genotoxic effects. Human MCL5 lymphoblastoid B cells and HepG2 hepatocellular carcinoma cells were exposed to dextran-coated USPION (dUSPION; 0μg/ml to 250μg/ml). The Ferrozine assay and Prussian blue staining were used to determine dUSPION accumulation in cells. Real time RT-PCR was used to examine gene expression of the iron regulatory proteins transferrin-receptor 1, ferritin, ferroportin and hepcidin. Genotoxicity was studied by performing the micronucleus assay with cytotoxicity measured in parallel. Full physico-chemical characterization studies on the dUSPION were also performed. dUSPION uptake was time- and dose-dependent and correlated with the induction of genotoxicity in

the absence of any apparent cytotoxic effects. Cellular uptake was influenced by several factors such as particle size, surface coating, iron oxidation state and serum concentration. Ferritin and ferroportin expression changes were absent, but transferrin-receptor and hepcidin were dramatically down-regulated. An understanding of the physico-chemical characteristics of dUSPION was critical in understanding their observed effects and mechanism of action. Interestingly, despite a clear lack of cytotoxicity, dUSPION clearly showed a genotoxic response and perturbation in the regulation of iron homeostasis.

33. Novel genetic polymorphisms in the promoter of the human DNA polymerase kappa gene may play a role in multiple myeloma

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Polymerase kappa (polκ) belongs to the Y-family of DNA polymerases, and carries out translesion synthesis (TLS) past oxidative and aromatic adducts on damaged DNA. Replicative B-family polymerases copy native DNA with high fidelity, whereas polκ has poor fidelity of replication and so is likely to increase the probability of developing a mutator phenotype. When polκ is over-expressed, it competes with replicative polymerases for DNA, which has been suggested to create a drug resistance phenotype and genome instability. One possible reason for over-expression could be genetic polymorphism in the promoter region of the gene. A region of approximately 1Kb of the polκ promoter was examined by PCR single stranded conformational polymorphism (SSCP) analysis, by the use of overlapping primer pairs in order to detect polymorphisms which may induce gene expression. Two polymorphisms were detected at -158 (G/C; freq – 0.016) and +53 (C/T; freq = 0.05). Polymorphisms were confirmed by DNA sequencing. These polymorphisms lie in the promoter region and intron 1 respectively, according to the transcription start site described by Granero and colleagues (1). Whilst there is no clear bioinformatic evidence that transcription factor binding motifs are close to these polymorphisms, the -158 polymorphism may delete a CpG island and the intron 1 polymorphism is a common area of gene expression control and deserves closer investigation. Myeloma patients, who typically demonstrate drug resistance, were assessed for the presence of the polymorphic locus and 4 of 45 individuals were heterozygous at position -518 (freq = 0.044) which was shown to be highly significant ($p<0.0005$). The polymorphism at +53 has yet to be confirmed in the myeloma patients. We conclude that polκ demonstrates genetic polymorphism, but the relevance of this has yet to be confirmed.

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34. Dextran coated superparamagnetic ultrafine iron oxide nanoparticles: compatibility with common fluorometric and colorimetric dyes

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The unique physiochemical properties of nanomaterials and the limited information regarding their reactivity means that when attempts are made to measure parameters such as nanomaterial-induced cyto- and geno-toxicity using fluorometric and colorimetric probes the possibility of nanomaterial altering the optical properties of these dyes, leading to inaccurate readings cannot be ignored. The potential of dextran coated ultrafine superparamagnetic iron oxide nanoparticles (dUSPION) (γ-Fe_2O_3 and Fe_3O_4) to alter the background signal of common probes used for evaluating cytotoxicity: MTS, CyQuant, Casein and EthD-1; and oxidative stress: DCFH-DA and APF was evaluated. The results of this study demonstrated that in an acellular system 100 μg/ml of both forms of dUSPION caused an increase in MTS signal. However, casein and APF signal were decreased when as little as 10 μg/ml of both types of dUSPION was included in the assay. The effect of dUSPION on DCF signal depended on the oxidative state of the nanoparticle, with Fe_3O_4 causing a decrease in signal and γ-Fe_2O_3 causing an increase. This increase was substantially reduced when the antioxidant N-Acetyl Cysteine was added to the system. Neither form of dUSPION had an effect on CyQuant and EthD-1 fluorescence responses. Here the importance of considering and controlling for possible interactions between nanomaterial and fluorometric or colorimetric dyes in experimental test systems is demonstrated. The present study also draws attention to the differences observed between γ-Fe_2O_3 and Fe_3O_4 dUSPION in relation to their interactions with the fluorometric dyes and ROS production, emphasising the importance of considering oxidative state of the nanomaterial when evaluating nanomaterial toxicity.

35. The influence of titanium dioxide and silver nanoparticles on genotoxicity in different cell types of Wistar rats

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The study was designed to evaluate the effects of titanium dioxide (TiO_2) and silver nanoparticles (AgNPs) on Wistar male rats. Rats were injected intravenously with 5 mg/kg of TiO_2, 5 mg/kg or 10 mg/kg AgNPs of size 20 nm or 5 mg/kg of size 200 nm, and sacrificed at 24 h, 1 and 4 weeks thereafter. Cell samples were analysed using the micronucleus test (bone marrow reticulocytes), sperm count, and the Comet assay (germ cells and lung cells). TiO_2 slightly enhanced the frequency of micronuclei at 24 h after exposure and also 1 week later. AgNPs at different doses and particle sizes induced slightly increased levels of micronuclei at 24 h and 4 weeks later. Sperm counts were slightly reduced at 24 h after exposure to TiO_2 and to 5 mg/kg of size 20 nm AgNPs, and after 4 weeks in the group exposed to TiO_2. Ag NPs significantly increased the comet tail moments in germ cells at 24 h after exposure. TiO_2 markedly increased the level of comet tail moments one and 4 weeks after exposure, whereas 5 mg/kg of

size 20 nm of AgNPs gave increased tail moments at one week following the injection. A dose of 10 mg/kg of size 20 nm AgNPs slightly increased comet tail moment in lung cells at 24 h and caused cytotoxicity 4 weeks later. A dose of 5 mg/kg of size 200 nm AgNPs significant enhanced comet tail moments one week following exposure. The results show that both AgNPs and TiO_2 may induce DNA damage in lung and male germ cells of rats.

This work was funded by the Polish Norwegian Research Foundation, project Ref. No PNRF-122-AI-1/07.

36. Using structural alerts to assess the relevance of in vitro cytogenetic tests

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The relevance of the in vitro cytogenetic tests for the assessment of genotoxic risk has been called into question due to the perceived prevalence of "false positive" results generated by such tests and a lack of correlation with in vivo cytogenetic and carcinogenicity assays. In this research, a collection of structural alerts for chromosome damage, based on in vitro chromosome aberration and micronucleus test data, has been used as a surrogate to evaluate the predictive performance of these tests for carcinogenicity. These data have been further analysed to assess whether particular mechanisms of action (for chromosome damage) are well correlated with carcinogenic activity. The carcinogenic potency database (CPDB) was processed against 77 structural alerts for chromosome damage. Overall, 609 chemicals from the CPDB activated a structural alert for chromosome damage in vitro, and 410 of these were carcinogenic. By this surrogate analysis, then, in vitro cytogenetic tests show a positive predictivity of 67.3% for carcinogenicity. When the predictions were grouped by mechanistic class, four classes performed better than the alerts as a whole, whereas most were less predictive. By contrast, all of these mechanistic classes correlated well with activity in the in vitro chromosome aberration test. Overall, in vitro cytogenetic tests displayed a reasonable predictivity for carcinogenicity using the surrogate measure of structural alerts for chromosome damage. In terms of mechanistic contributions, many of the correct positive predictions were related to a single mechanism of action (formation of covalent DNA adducts). It is likely that this reflects the composition of both the CPDB data set and structural alerts used for this analysis, which are still a work in progress. As such, further development of these alerts will focus on "indirect" mechanisms of action that may not be detected in other genotoxicity assays (e.g. the Ames test).

37. TK6 micronucleus test: ready or not?

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The in vitro micronucleus test is a well-established screening assay for assessment of clastogenic and/or aneugenic potential of compounds during early phases of drug development and has its value in different industrial and academic research activities. The assay can be performed in rodent and human cell lines and in primary human lymphocytes. The cell's p53 status is one of the key factors in the capacity of the cells to regulate their cell cycle and DNA repair mechanisms and is therefore also crucial in the generation of misleading positive findings. In addition, the need for appropriate cytotoxicity measurements and limits has been demonstrated. Our aim was to adopt the in vitro micronucleus test for the p53 wild-type human lymphoblastoid cell line TK6 and to evaluate the compliance of our protocol with the OECD guideline (96-well plate treatment and microscopical analysis). A set of reference compounds (e.g. OECD list, "Group 3" compounds [1] and apoptosis inducers) were tested and their micronucleus inducing potential was evaluated in the light of the three cytotoxicity endpoints described in the OECD guideline. The OECD compounds were all correctly identified. The magnitude of response in TK6 cells was usually more modest than what is generally seen in p53 mutated cells and a close inspection of the cytotoxic effects is required for appropriate data interpretation. A limited number of proprietary compounds were concurrently tested in a human lymphocyte chromosome aberration test. As was indicated previously [2], the comparison of such two datasets is not always straightforward. Our results complement other previously reported and point to the need for a data driven discussion on evaluation criteria, cytotoxicity measures and cut-off values, the importance of understanding the mechanism of action of reference compounds we test and the biological effects related to the cell type's characteristics.

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38. Setup of the Litron *in Vitro* MicroFlow® 96 well plate format micronucleus analysis kit for investigation of kinase associated genotoxicity

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The *in vitro* MicroFlow® 96 well plate format MN analysis kit (Litron Laboratories) is a high content, high throughput means of analysing the genotoxic activity of compounds. The high throughput nature of this assay allows 6 compounds to be tested per plate and multiple plates per week. The high content characteristics of this assay allow for the analysis of multiple endpoints from flow cytometric data including percentage apoptotic cells, cell cycle information and mode of action signatures for aneugens and clastogens. This high content data can be particularly useful for determining genotoxic potential of kinase inhibitor compounds [1]. To establish the assay at GSK TK6 cells were treated on a 96 well plate with two direct acting genotoxins and two aneugens: MMS (0.024-25μg/mL), MMC (0.00039-0.4μg/mL), vinblastine (0.0031-3.2ng/mL) and vincristine (0.0039-4ng/mL) for 27 hours. In an additional study cells were treated with the non genotoxins

dexamethasone and phenformin hydrochloride and the artifactual *in vitro* genotoxic positive 2,4-dichlorophenol. An analysis spreadsheet was developed to automatically determine experiment validity for each plate by checking the positive and vehicle controls. The spreadsheet then determines for each compound whether the result is positive. To assist with assay interpretation intrinsic checks and balances have been built into the spreadsheet, for example to ensure that if the %EMA is below threshold. This will be especially important for interpretation of data for kinase inhibitor compounds, where causes of genotoxicity can be uncertain for example by polypharmachology or kinases crosstalk. 96 well plate MN analysis represents a particularly promising development in *in vitro* screening of large compound sets. The large volume of information generated can be used to correlate genotoxic results with other data for instance within the kinome. Data sets could then be used to develop bioinformatic models. We are currently evaluating further use of the high content data generated by this assay and screening kinase inhibitor compounds.

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39. Development and validation of in vivo micronucleus bone marrow filtration with kinetochore staining in rats, for use in preclinical drug development

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The in vivo rodent micronucleus-kinetochore test (1) enables the differentiation of micronuclei according to the type of genetic damage (clastogenicity or aneugenicity). In certain cases, if the positive result is due to aneugenicity, a genotoxic threshold mechanism can be established and development can potentially continue. However, the presence of nucleated cells on bone marrow smears can be problematic for kinetochore staining, therefore we have implemented a bone marrow filtration method to remove 50-70% of nucleated cells prior to slide preparation. To evaluate the in vivo kinetochore staining method, male rats (n=6 per group) were administered vehicle or the known aneugens, carbendazim (1500 or 2000 mg/kg/day po) or vinblastine sulphate (0.25 or 0.75 mg/kg/day ip) on 2 occasions, 24h apart. Animals were killed 2h after the last dose and bone marrow samples were prepared and stained using standard methods. Treatment with vinblastine sulphate resulted in an unequivocal dose related increases in MPCEs (up to 27-fold of vehicle controls) with predominantly kinetochore positive micronuclei (78 %), as expected. Carbendazim treatment also resulted in an increase in MPCEs compared to vehicle control, but the magnitude of the response was not consistent for all animals in each dose group, with only half of the animals showing an unequivocal positive response. This is likely to reflect the metabolic requirement for carbendazim aneugenicity. Additional studies were conducted on two in-house compounds (kinase inhibitors), the first (compound A) positive in the in vitro micronucleus test, and the second (compound B) positive in the in vivo micronucleus test and was re-tested here to include kinetochore assessment, to determine a mechanism of genetic damage. Treatment with compound A was negative, while compound B resulted in a dose related increases in MPCEs (up to 2.65-fold of vehicle controls) with predominantly kinetochore negative micronuclei (81.5 %). These studies indicate that the in vivo rodent micronucleus-kinetochore test can be used to differentiate micronuclei according to mechanism (clastogenicity or aneugenicity) and contribute to risk assessment.

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40. Comparison of micronucleus frequency in peripheral blood detected by flow cytometry and microscopy

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The *in vivo* rodent micronucleus test is widely used in regulatory genotoxicity testing and it is now becoming common to analyze micronuclei in immature erythrocytes (reticulocytes) from peripheral blood rather than bone marrow. The objective of this study was to compare the micronucleus frequencies in peripheral blood measured by flow cytometry after staining with proprietary kit obtained from Litron laboratories with those peripheral blood smears scored microscopically, and in the conventional bone marrow method Male Wistar Han rats, approximately 10 weeks old at dosing were given two oral doses of the vehicle (water),cyclophosphamide (20 mg/kg), methylnitrosurea (12.5 mg/kg) or ethyl methanesulphonate (200 mg/kg) 24 hours apart. Groups of 5 were sampled for bone marrow analysis 24 hours after the second dose. Peripheral blood was sampled from groups of 7 rats 3, 4 and 5 days after dosing and fresh blood smears, prepared for microscopic analysis using supravital staining, were scored for type 1 and type 2 reticulocytes. Replicate blood samples were analysed by flow cytometry either after staining with the Litron kit at AZ or sent frozen for staining and analysis by Litron. Significant inductions of micronuclei were seen with all the test agents but the magnitude of responses varied between the different methods. The incidence detected in the peripheral blood by flow cytomery was similar to that seen in the conventional bone marrow smears. However, the frequency of micronuclei detected by microscopy in the fresh blood smears was about half that seen by flow cytometry.This difference indicates that the type 1 and type 2 reticulocytes identified by supravital staining are not the same population as the CD71 presenting cells measured by the flow cytometry kit.

41. The bile acid impurity methyl cholate (MC) is non-genotoxic, as measured by the micronucleus assay in oesophageal OE33 cells

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De novo biosynthesis of primary bile acids from cholesterol is a multistep process which is controlled by extremely sensitive negative feedback mechanisms. Unfortunately, inherited disorders of bile acid metabolism exist, where children possess

inactivated copies of key metabolic enzymes, leading to the build up of toxic bile acid intermediates. These rare disorders were traditionally lethal until it was noted that supplementation with an external source of primary bile acid shut down the build up of these toxic metabolites, through negative feedback signalling. One type of supplementary primary bile acid often used for this purpose is cholic acid. As a natural biomolecule produced in large quantities in humans, cholic acid is well tolerated and indeed has been shown by several groups to be non-toxic and non-genotoxic (1). However, cholic acid has been noted to be contaminated with the impurity methyl cholate and hence it is important to assess if methyl cholate (MC) is genotoxic. We performed an in vitro genotoxicity study in oesophageal cells (OE33 cells) using the cytokinesis blocked micronucleus assay as described previously (1) to assess if MC was genotoxic. We found no genotoxicity across the dose range employed in this study (25-200μM). Neither did we note any toxicity (as measured by the lack of a change in binucleate frequency). In parallel, we again showed that cholic acid was non-genotoxic across the same dose range, whilst the secondary bile acid deoxycholic acid was genotoxic across this same dose range as previously described (1). In conclusion, the impurity MC is unlikely to be a toxic (or genotoxic) threat to the therapeutic use of cholic acid in patients with inborn errors of bile acid metabolism.

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42. Karyotypic analysis and stability with time in culture of TK6 and L5178Y cell lines

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There is an ongoing ILSI/HESI initiative addressing positive in vitro genotoxicity findings and to consider improvements to existing assays. Good practice in cell culture has been identified as an obvious area for improvement since the commonly used cell lines were all established decades ago and there is increasing evidence that genetic drift may influence their response to genotoxins. Consequently, it was decided to generate accurate karyotypes for the two cell lines used in this laboratory, L5178Y and TK6, and compare them with published data. TK6 is a human B lymphoblastoid cell line derived from WI-L2 which was established from the spleen of a 5 year old male having hereditary spherocytic anemia. The TK-6 cell line was derived as a thymidine kinase heterozygote and contained a normal human karyotype. By 1986 TK6 cells contained 14q marker chromosome der14t(14;20) and an extra copy of chr13 in 100% of cells. The composite karyotype of the TK6 cells by 1996 is 47,XY+13,+14q,+21p.

TK6 cells purchased from Ecacc in 2009 show a modal chromosome number of 47 and composite karyotype of 47 XY,+der3 t (3,21),+der13t(13;22) -14+der14t (14;20). The composite karyotype is from 20 metaphases G banded and confirmed by FISH. We also recorded further changes in chromosome distribution when cultured for up to 4 weeks i.e. drift from modal chromosome number and karyotype. The mouse lymphoma L5178Y line was derived from lymphoma induced by methylcolanthracene in 1952 in a DBA/2 mouse. L5178Y/$tk^{+/-}$ cells, clone 3.7.2c, were obtained from Dr J. Cole, (MRC Cell Mutation Unit). A modal chromosome number of 40 and a composite karyotype 40 X0der 5t(5;15),der9t(9;6) robertsonian fusion 12 and 13,+15(t15;5)+15(t15;18),-15q,der18t(18;6) established by FISH. The cells contain 3.3% tetraploid cells. In addition following 4 weeks continuous culture the number of tetraploid cells increases to 11.5% and stability of cells decreases.

43. Automatic analysis of the micronucleus test in primary human lymphocytes using the ROBIAS system

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The in vitro micronucleus test (MNT) is a well established test for early screening of new chemical entities in industrial toxicology. For assessing the clastogenic or aneugenic potential of a test compound, micronucleus induction in cells has been shown repeatedly to be a sensitive and specific parameter. Replacements for the tedious and time consuming manual slide analysis procedure have been developed either using automated image analysis or flow cytometric approaches. The ROBIAS image analysis system for both automatic cytotoxicity assessment and highly sensitive micronucleus detection in primary human lymphocytes was developed at Novartis, where the assay is used as to confirm positive results obtained in the MNT in TK6 cells, which serves as the primary screening system for genotoxicity profiling in early drug development. The comparison of manual with automatic analysis results showed a high degree of concordance for 27 independent experiments conducted for profiling of 12 compounds. For a concentration series of cyclophosphamide, a very good correlation between automatic and manual analysis could be established, both for the relative division index used as cytotoxicity parameter, and for MN scoring in bi-nucleated cells. Generally, false positive micronucleus decisions could be controlled by fast and simple relocation of the automatically detected patterns. The possibility to analyze 24 slides within 65 hours by fully automatic analysis over the weekend and the high reproducibility of the results make automatic image processing a powerful tool for the micronucleus analysis in primary human lymphocytes. The automated slide analysis for the MNT in human lymphocytes complements the portfolio of image analysis applications on ROBIAS supporting various assays in genetic toxicology and other biomedical areas.

44. An automated scoring protocol for the cytokinesis-block micronucleus assay by fluorescent image analysis

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The induction of micronuclei (MN) in human lymphocytes or cell cultures is a well established cytogenetic endpoint for evaluating structural and/or numerical chromosomal alterations. The cytokinesis-block micronucleus (CBMN) assay is

frequently used for measuring MN frequency in genotoxicity testing. Automation of MN analysis would enhance throughput and reliability of results and reduce scoring subjectivity of MN identification. Working with IMAS Imaging Associates and MetaSystems an automated CBMN assay method, based on fluorescence microscopy and computerized image analysis, has been established in our laboratory. Firstly, the slide preparation methodology was customized to obtain optimal cell density and diffusion, which is critical for quality image analysis. The detection and scoring process was separated into three steps; initially slides were scanned and binucleated (BN) cells were detected; then, MN-positive BN cells were validated microscopically by the operator; finally manual substantiation of MN in the gallery images was performed. There are several obstacles to scoring MN prepared for fluorescent observation, mainly due the loss of cytoplasmic boundaries during slide preparation. The HUMN scoring criteria for the CBMN assay were followed; however allowances for automated slide preparation were required. For example, particular attention should be paid to the size, staining pattern, and staining intensities of the two nuclei in BN cells, as well as distance of MN from the nuclei. Validation of the optimised automated image system was performed by comparing the results of visual and automated scoring of MN-BN cells in cultured human lymphocytes induced by the clastogen mitomycin C, and the pro-oxidants hydrogen peroxide, menadione, and potassium bromate. Although the absolute MN frequencies obtained with automated scoring were higher as compared to those detected by manual visual scoring, similar dose response curves for MN-BN frequencies were observed with both scoring systems, indicating that it is adequate to produce biologically relevant and reliable results.

45. Study of oxidative damage in AHH-1 human lymphocyte cells by the cytokinesis-block micronucleus assay

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The dose response relationship is arguably the single most central concept in toxicology. The demonstration and acceptance of dose response thresholds for genotoxins may have substantial implications for the setting of safe exposure levels. Chemical thresholds (or non-linear dose responses) are defined as critical exposure levels, below which, the experimental concentration of a compound will not produce a statistically significant increase in mutation or chromosomal effects. Application of a threshold mechanism in toxicology is not new, however, the effect has only been demonstrated for a limited number of DNA reactive compounds, such as ethylmethane sulphate (EMS) and methylmethane sulphate (MMS) (1.). Homeostatic cellular defences acting to reduce/repair damage are hypothesized to be involved in thresholded dose-responses of genotoxins. The current investigation aimed to broaden the number of genotoxic chemicals identified with an established threshold, through the investigation of the dose response relationships of pro-oxidants hydrogen peroxide (H_2O_2), potassium bromate ($KBrO_3$), and menadione utilising the cytokinesis- block binucleated micronucleus (CBMN) assay and the HPRT forward mutation assay. Base excision repair (BER) machinery and cellular antioxidant levels were also assessed as possible threshold mechanisms. All three chemicals exhibited nonlinear curves containing a range of non-mutagenic low doses. The lowest observed effect level (LOEL) for induction of point mutations was 0.5mM $KBrO_3$, 18.0uM H_2O_2, and 2.9uM menadione. Induction of chromosomal aberrations also demonstrated dose-response curves with thresholds. Protein and mRNA levels of the BER enzyme 8-oxoguanine DNA glycosylase (OGG1) were not modulated in response to pro-oxidant stress. The shape of the H_2O_2 dose response curve, however, was altered by the intracellular increase and depletion of the antioxidant glutathione.

This observed nonlinearity for pro-oxidant chemicals therefore, appears to be due, in part, to the protective capacity of antioxidants against DNA damage plus basal (non-induced) DNA repair.

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46. A spectral phenotype of oncogenic HPV-infected exfoliative cervical cytology distinguishes women based on age

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Human papillomavirus (HPV) is a sexually-transmitted infection associated with cervical cancer. Cervical cytology can be graded as normal (atypia-free), low-grade (borderline nuclear abnormality/cervical intraepithelial neoplasia [CIN]1), or high-grade (CIN2/3). We employed infrared (IR) spectroscopy as a non-destructive technique that allows the acquisition of a biochemical-cell fingerprint representative of chemical bonds present in the interrogated sample. Exfoliative cervical cytology specimens (n=147) were retrieved, graded by a cytologist and HPV genotyped using Hybrid Capture 2 and the Roche HPV Linear Array. Additionally, the spectral signatures of cervical cell lines C33A, HeLa and SiHa were examined. Cellular material was interrogated using attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy; spectra were acquired from 10 independent locations per sample. Principal component analysis (PCA) was applied to reduce the data dimensionality. Linear discriminant analysis (LDA) was subsequently applied to provide a visual representation of the data (scores plot) and, identification of the corresponding chemical entities responsible for segregation (loadings plot) (1). Immortalised cell lines were readily distinguishable from each other. It was difficult to segregate categories of cytology associated with specific HPV infection types. However, in low-grade cytology infected with high-risk oncogenic HPV 16 or 18, it was possible to segregate women based on age (20 – 29 years and 30 – 39 years) (2). This suggests a spectral phenotype in exfoliative cervical cytology associated with transient *versus* persistent HPV infection.

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47. Clastogenic effect and aneugenic abilities of cadmium and nickel salts and their combinations in male mice

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Cadmium and nickel are important metals in industries, electronics and household products and are used in combination in nickel-cadmium batteries. This study was undertaken to compare the genotoxic effects of cadmium and nickel individually and in combination in bone marrow cells of male mice. Three tests were used to assess genotoxicity, namely, the micronucleus assay, the aneuploidogenicity potentials and structural chromosomal aberrations in metaphases of bone marrow cells. Cadmium chloride (Cd-II) at the following doses: 20, 40 and 60 and nickel chloride (Ni-II) at three doses (40, 80 and 120 μM/kg b.w. were used individually as well as their three mixtures. The incidences of micronuclei, aneuploidy and CA show that Cd-II is a stronger mutagen than Ni-II. And dose response relation for Cd-II and Ni-II. Treatment with heavy metals increased significantly the incidences of (MNPCEs). Also the ratio of (PCE/NCE) suggests that treatment with higher doses of the two metals increased cytotoxicity. NCA increased hypoploidy with the treatment which reached 2-3 folds of the frequency of hyperploidy. The results show that both Cd-II; Ni-II are aneugenic and act on kinetochores and cause malsegregation of chromosomes as well as being clastogenic. Both heavy metals increased single break aberrations which are in agreement with MN results. Mixtures of Cd-II and Ni-II at low doses resulted in incidences of MNPCEs, aneuploidy and CA insignificantly different from their expected effects. However, mixture of higher doses produced actual effects less than their expected additive effect. The results obtained confirm that both cadmium and nickel are clastogenic and aneugenic *in vivo* showing dose dependent relationships.

48. Optimization of culture conditions to give a population of T-lymphocytes in exponential growth

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Peripheral blood lymphocytes have long been used as a source of human primary cells in the *in vitro* mammalian chromosome aberration test (IVC) and, more recently, for the *in vitro* mammalian cell micronucleus test (MNvit). The cells are routinely obtained from healthy volunteers and stimulated to divide in whole-blood or separated mononuclear cell cultures by the addition of phytoheamagglutinin (PHA). In these standard protocols, the cells are incubated for 48 hours and then treated with test agents. However, at this time, the majority of the mononuclear cell population is not dividing. In the IVC, mitotic index is routinely used as the cytotoxicity measure, however it has been recognized that this is an imperfect measure of toxicity and population doubling has been proposed as a better estimate (1). However, this measure cannot be applied to lymphocytes in the standard protocols because the population is not in exponential growth. Work has been conducted to optimize conditions for lymphocyte culture including, PHA concentration, media type, culture vessels, cell volumes and density, feeder cells and pooling cells from different donors. In conclusion, cultures of T-lymphocytes that are in approximately exponential growth can be achieved 96 hours after stimulation with PHA. Using this protocol, it is possible to use population doubling to estimate cytotoxicity in concurrent IVC and MNvit tests.

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49. The use of Salmonella typhimurium TA1538 expressing mouse sulphotransferase 1D1 to detect aromatic amine mutagens

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Sulphonation is an important phase II reaction in the metabolism of numerous xenobiotics which, although most commonly associated with detoxification, can generate DNA-reactive metabolites from hydroxylamines, benzylic and allylic alcohols (1). However, the bacterial strains and mammalian cell lines routinely used in genetic toxicity testing have no capacity for sulphonation so several strains and cell lines expressing sulphotransferases (SULT) have been developed (2). The S9 dependent mutagenicity of the heterocyclic amine, MeAαC, has been shown to be greatly enhanced in TA1538 expressing human SULT1A1 (3). Subsequently, TA1538 has been transfected with mouse SULT1D1 which has substrate specificity similar to hSULT1A1, including high affinity for hydroxylamines from 1-3 ring aromatics (Prof. H.Glatt, German Institute of Human Nutrition, Potsdam). The objective of this study was to use TA1538-mSULT1D1 to examine *o*-anisidine and *p*-cresidine, both potent bladder carcinogens in rats and mice but which are notoriously difficult to detect in genotoxicity assays (4). Modified liquid pre-incubation tests using TA1538-mSULT1D1 (kindly supplied by Prof. Glatt) were performed with 2-aminoanthracene which showed substantially greater activity than with TA1538 tested in parallel. Subsequently, *o*-anisidine and *p*-cresidine were also shown to give indications of activity with TA1538-mSULT1D1, but not TA1538. These results indicate TA1538-mSULT1D1 shows increased sensitivity to some aromatic amine mutagens.

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50. The effect of storage on TPM stability

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Total particulate matter (TPM) from mainstream cigarette smoke is used as the test article for the *in vitro* testing of combustible tobacco products. TPM is the particulate fraction of the smoke aerosol. No published data are available on the stability of stored TPM, apart from a published paper that states: "For the *Salmonella* reverse mutation assay, the total particulate matter (TPM) was collected... and stored immediately after preparation at -75°C for up to 1 month until use. Mutagenicity data (not given here) for mainstream smoke condensate (MSC) of reference cigarette types indicate stability under these storage conditions when compared to fresh condensate" [1]. A study has been commissioned by British American Tobacco to quantify the effect of storage of TPM on mutagenicity, genotoxicity and cytotoxicity over a 2 year period. Four *in vitro* toxicity assays (Ames, mouse lymphoma, micronucleus and neutral red uptake assays) were carried out in the study, but only data from the Ames assay for 3 months storage are represented here. TPM from two reference cigarettes, 3R4F (a blended product) and M4A (a flue cured product) were tested in three strains of *Salmonella typhimurium* (TA98, TA100 and TA1537), in the presence of metabolic activation. These strains are known to be the most responsive to tobacco smoke condensates. TPM was stored at -80°C for 1 and 3 months. Comparisons were made between stored and freshly prepared 3R4F and M4A TPM to determine whether storage had an effect on the mutagenic potential in the Ames assay. Data show that all TPMs resulted in clear concentration dependent responses. Freshly prepared 3R4F and M4A TPMs were tested at each timepoint to estimate the variability of repeat testing. On the linear part of the concentration response curves, small differences were observed, some of which were statistically significant ($P<0.05$). Stored 3R4F and M4A TPMs were also tested at each timepoint, and the responses were comparable to those of the freshly prepared TPMs ($P<0.05$). This suggests that, as the statistically significant differences were maintained, they are more likely to be due to the variability of the assay and TPM preparation than an effect on storage, as the differences were observed in both freshly prepared and stored TPMs. The data indicates that, after 3 months storage, the relative bacterial mutagenicity of the 3R4F and M4A TPMs were stable when stored at -80°C. The study will continue over a 2 year period.

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51. Validation of a multi-endpoint assay in rats: bone marrow micronucleus, comet and flow cytometric peripheral blood micronucleus

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With the publication of revised draft ICH guidelines (Draft ICH S2), there is scope and potential to establish a combined multi-end point *in vivo* assay to alleviate the need for multiple *in vivo* assays, thereby reducing time, cost and animal usage. Presented here are the results of a validation trial in which the bone marrow and peripheral blood (via MicroFlow® flow cytometry) micronucleus tests (looking at potential chromosome breakage and whole chromosome loss) in developing erythrocytes or young reticulocytes were combined with the comet assay (measuring DNA strand breakage), in stomach, liver and blood lymphocytes. This allowed a variety of potential target tissues (site of contact, site of metabolism and peripheral distribution) to be assessed for DNA damage. This combination approach was performed with minimal changes to the standard and regulatory recommended sampling times for the stand-alone assay designs. 2-Acetylaminofluorene, benzo[a]pyrene, carbendazim, cyclophosphamide, dimethylnitrosamine, ethyl methanesulfonate, ethyl nitrosourea and mitomycin C which are known to act via a variety of different modes of action (direct and indirect acting clastogens, alkylating agents, gene mutagens, cross-linking and aneugenic compounds) were tested. Animals were dosed at 0, 24 and 45 hours, and bone marrow and peripheral blood (micronucleus endpoint), liver, whole blood and stomach (comet endpoint) were sampled at 3 hours post the last dose. Comet and bone marrow micronucleus responses were similar to those found in the conventional (acute) stand alone assays. All compounds were detected as genotoxic in at least one of the endpoints. The importance of evaluating both endpoints was highlighted by the uniquely positive responses for certain chemicals (benzo[a]pyrene and 2-acetylaminofluorene) with the comet endpoint and certain other chemicals (carbendazim and mitomycin C) with the micronucleus endpoint. In conclusion, the data generated from these investigations demonstrate suitability of the multi-endpoint design.

52. Validation of a reduced volume peripheral blood human lymphocyte micronucleus assay (micro HLM) using the draft OECD 487 guideline reference chemical list

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Following issue of the latest draft OECD test guideline (487 – November 2009 [1]) on the conduct of the in vitro Mammalian Cell Micronucleus Test, we have assessed the performance of the assay in human peripheral blood lymphocytes using a reduced volume protocol design (1.2 mL cultures by comparison with a standard culture volume of 10 mL).

Eight of the reference chemicals listed in Annex 3 of guideline 487 (1) were tested using appropriate experimental designs (3 hour treatment in the absence and presence of rat liver metabolic activation (Aroclor induced S-9) with 21 hours recovery and / or 24 hour treatment in the absence of S-9 with 24 hours recovery. Cytosine arabinoside, mitomycin C, benzo(a)pyrene, cyclophosphamide, colchicine and vinblastine which are active via a variety of differing modes of action (including clastogens requiring metabolic activation, direct acting clastogens and aneugenic agents) were tested. Pyrene and sodium chloride (known to be non-DNA reactive) were also included. Maximum concentrations analysed were either limited by cytotoxicity or limited to a maximum of 10 mM. The results confirmed the acceptable performance of the assay using the reference chemicals and the data were comparable with the standard volume assay protocol used in this laboratory. This assay may therefore be used as either a screening or regulatory test in which compound usage may be reduced by more than 8-fold by comparison with the standard assay protocol.

Reference

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53. Evaluation of U. S. National Toxicology Program (NTP) mouse lymphoma assay data using International Workshop on Genotoxicity Tests (IWGT) criteria

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The mouse lymphoma assay (MLA) is widely used to identify potential mutagens. An extensive MLA database created by the NTP during the 1980s is publicly available on the NTP website and often used to develop structure-activity relationships (SAR) and draw correlations to animal carcinogenicity findings. There have been significant procedural and data-evaluation enhancements in recent years to the MLA under the auspices of IWGT. Therefore, it is important that data be reexamined according to the current criteria in order to build a curated database to better inform chemical-specific evaluations and SAR models. Accordingly, NTP data from more than 1900 experiments representing 342 chemicals were examined against pre-defined criteria for the acceptance of background mutant frequency, cloning efficiency, positive control values and appropriate dose selection. Approximately 17% of the experiments met all of the acceptance criteria. This analysis revealed that approximately 60% of the NTP "positive" calls did not meet the current global evaluation factor (GEF) criteria for a positive response. It should be noted that "positive" determinations were made in this re-analysis if a response met the GEF criteria, even if the assay did not pass all acceptance criteria. Strict adherence to the current acceptance criteria would have resulted in a vast majority of studies being considered as unacceptable and therefore uninterpretable. Furthermore, the lack of colony sizing data precluded a judgment of "negative". Overall, more than 60% of the chemicals could not be classified as positive, negative, or equivocal. Of the 265 chemicals from this list evaluated by Kirkland et al (1) using NTP and other data, there was agreement between Kirkland calls and our calls for 32% of chemicals. This evaluation revealed the importance of expert review of primary data for conformance to current standards.

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54. Use of genotoxicity endpoints to determine the genotoxic potential of PI3-kinase inhibitors

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Genotoxic damage and repair are used as measures of drug safety by the pharmaceutical industry and their regulatory authorities. Kinases are key tools of academic research, due to their prevalence in the cell and role in various key cellular pathways, such as DNA repair, metabolism and apoptosis. This versatility makes kinases potential therapeutic targets for disease as they are involved in conditions such as cancer, inflammation and asthma. A battery of tests, consisting of the Ames assay, the in vitro micronucleus assay and the GreenScreen HC assay were used to test the genotoxicity of commercially available phosphoinositide 3-kinase inhibitors. Four inhibitors were used; PI-103, LY294002, PI3K Alpha IV inhibitor and PI3K Gamma inhibitor. Our data indicate these compounds, targeted to the same kinase, show variation in the results produced in these genotoxicity assays. The inhibitors were tested up to 1mM, or their limits of solubility. When tested, PI-103 was negative for genotoxicity in all three tests, whereas LY294002 and PI3K Gamma Inhibitor were negative in the bacterial Ames assay but positive in the mammalian tests (the in vitro micronucleus assay and the GreenScreen HC assay). The PI3K Alpha IV Inhibitor was positive throughout the tests – implying PI3K Alpha IV inhibitor is genotoxic by a direct acting mechanism. None of the compounds produced a computational alert for toxicity when run through Derek for Windows. These findings indicate that inhibition of phosphoinositide 3-kinase is not the sole aspect that influences an inhibitor's potential to cause genotoxic damage. Other factors, such as the chemical structure, specificity for the desired target and other kinases, polypharmacology and the potential for off target effects need to be considered, particularly at the higher concentrations mandated to test to by regulators and where compounds are generically targeted to the ATP domain.

55. Low dose exposure to MNU in Human Lymphoblastoid cell line AHH-1

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Before pharmaceuticals are approved for human usage, their potential to cause point mutations and chromosome damage is assessed at increasing doses using *in vitro* cell culture systems and animal models. The resulting dose-response is a critical

factor in establishing safe exposure limits. It has long been assumed that DNA reactive chemicals cause adverse effects at every dose. However, experimentation into the low dose region has revealed a threshold mechanism where, up to the threshold dose, DNA reactive chemicals can be tolerated without incurring more damage than that naturally occurring within the cell. MNU, a model alkylating agent has been shown to illicit DNA damage in a linear fashion even at low doses, whereas similar acting compounds MMS and EMS have been shown to have thresholds (1). In this study we find a putative threshold dose for point mutation induction at 0.00075 μg/ml MNU when quantified through the HPRT assay in the AHH-1 Human Lymphoblastoid cell line. Sequence analysis of the resultant mutants will give mechanistic information regarding point mutation induction over this thresholded dose range. Through use of the Metasystems metafer software optimised for the micronucleus assay, we aim to evaluate MNUs ability to induce chromosome breakages over a wider dose range than previously tested. Further work will identify the mechanism of the threshold dose-response for genetic damage hypothesised to involve DNA repair of pre-mutagenic lesions.

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56. Quantitative aspects of DNA reactive genotoxins assessed *in vitro*

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Genotoxic agents that are DNA reactive and direct acting have long been assumed to display linear dose responses. However, our group in Swansea University has explored this issue and we have pioneered the *in vitro* studies to determine a threshold for DNA reactive genotoxic carcinogens. This has led to a paradigm shift, and the scientific community now accept that certain alkylating agents do have thresholds. This shift originated from our *in vitro* data set, and some experts appreciate that *in vitro* data has the potential to have more quantitative use for hazard and risk assessment than previously thought. Of particular interest is the case of ethyl methane sulphonate (EMS) for which there are now the first *in vitro* and *in vivo* threshold studies for a DNA reactive compound. This robust and powerful data set has allowed the *in vitro* genetic toxicology (IVGT) quantitative subgroup, of the Health and Environmental Sciences Institute (ILSI-HESI), to start developing a database of *in vitro* and *in vivo* exposure-response relationships in test systems used for genotoxicity testing, to use as a basis for analysis of exposure response relationships. It will also be used in the development of recommendations for extrapolation of results across test systems, and approaches to improve exposure-based estimates of *in vivo* risk. The first extrapolation calculation is very promising, and some preliminary results were presented by its developer, Jim MacGregor in EEMS 2009 in Florence. Our group has normalised *in vitro* data from 3 different classes of DNA reactive compounds and all of them produce a similar NOEL/toxic dose 50 ratio to that presented by Jim MacGregor.

57. Azidothymidine induces DNA damage in rats after repeated treatment over seven days

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Azidothymidine (AZT), a nucleoside analogue reverse transcriptase inhibitor, was approved by the FDA in 1987 as the first drug to treat HIV. AZT is known to be integrated into host nuclear and mitochondrial DNA, leading to gene mutation, chromosomal damage and micronuclei induction in vitro. In the present study the time-and dose-related toxicity, particularly hemato-and genotoxicity of AZT was investigated. AZT was administered orally (gavage) twice daily for seven days to male Wistar rats at 0 (vehicle), 250, 500 or 1000 mg/kg body weight. Additional animals of the vehicle and high dose group were monitored up to a 42 day treatment-free period. Blood for hematology, Comet assay and the detection of micronuclei was taken during the treatment and/or recovery periods. Genotoxicity of AZT was investigated by different endpoints, e.g. the Comet assay in liver, blood and stomach and the Micronucleus assay in blood and bone marrow. First results showed that AZT caused a dose-dependent DNA damage in liver, blood and stomach (Comet assay) and a dose-dependently increased number of micronucleated cells (Micronucleus assay). Hematologic investigations showed dose- and time-dependently and markedly lower levels of red blood cell counts, hematocrit, hemoglobin concentration, mean corpuscular hemoglobin and mean corpuscular volume, hemoglobin distribution width as well as severely lower counts of absolute reticulocytes in AZT treated rats, when compared to the vehicle control. These alterations fully recovered within the first 14 days of the treatment-free period. The geno- and hematotoxic potential of AZT in rats was thus demonstrated in this study using multiple endpoints.

58. What is the real incidence of positive results in the mouse lymphoma TK assay (MLA) in pharmaceutical screening?

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Since the mid 1980's, the MLA has been criticised for its lack of specificity (1). Some of the original censure was found to be groundless once protocols that excluded testing to excessive toxicity, pH or osmolality were introduced (2). However, as recently as 2005, a retrospective review suggested a 'false positive' rate of 61% for incorrectly identifying non-carcinogens (3). Whilst these reviews were not focused on pharmaceutical agents there is a perception that the 'over sensitivity' of the MLA is a general phenomenon. Since 2001, 352 pharmaceutical compounds have been tested in the MLA in this laboratory, the vast majority of which were not bacterial mutagens and considered unlikely to be genotoxic carcinogens. Of these, only 55 (16%) gave positive results in the MLA. Of the positive compounds, 31 were independent of S9 activation and had primary pharmacological targets e.g. DNA repair, kinase, protein and topoisomerase inhibitors that were quite likely to be responsible for their activity in the MLA. A further 4 of the positive MLA responses were also positive in an Ames or *in* vivo bone marrow micronucleus test. This means that

only 20 out of 352 (6%) of compounds tested gave unexpected positive results but, obviously, it is not known whether or not these are carcinogenic. Accordingly, the 'false positive' rate of the MLA seen with pharmaceuticals in early development appears to be far less than that previously reported from retrospective reviews of published data.

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59. Reduction of misleading ("False") positive results in mammalian cell genotoxicity assays: Choice of cell type

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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. Moreover, the rate of misleading positive results with a combination of assays was found to be at least 80% (1). Poor predictivity was expected to be worst in p53-deficient cell lines of rodent origin, particularly long established and widely used Chinese hamster cell lines. Since *in vivo* models have been banned by the EU Cosmetics Directive since March 2009, *in vitro* models need to be more predictive for the risk assessment of cosmetic ingredients. As part of a larger framework for improvement of *in vitro* genetic toxicology assays the performance of currently used cell lines is being investigated and compared with p53-competent cells (2). Comparisons have been made between Chinese hamster Lung, Chinese hamster Ovary, V79, TK6, HepG2 and Human peripheral blood lymphocytes. These comparisons were made using the *in vitro* micronucleus assay to evaluate clastogenic potential and highlight any differences in sensitivity between cell lines, with a selection of compounds that are accepted as producing misleading positive results in i*n vitro* clastogenicity assays (3). For those misleading positive chemicals where positive responses have been reproduced, this has been seen predominantly in certain cell types, and not in others. These positive responses were mostly found in V79 cells, frequently in CHL and CHO cells, far less frequently in TK6 cells, rarely in p53-competent HuLy and almost never in HepG2 cells. Thus it may be concluded that use of p53-competent cells such as HuLy, TK6 or HepG2 cells would reduce the frequency of misleading positive results.

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This work is funded by the European Cosmetic Industry Association COLIPA, ECVAM and NC3Rs.

60. Reduction of misleading ("False") positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement

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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. Moreover, the rate of misleading positive results with a combination of assays was found to be at least 80% (1). Predictivity was worst in p53-deficient cell lines of rodent origin, particularly long established and widely used Chinese hamster cell lines (see poster by Fowler *et al*). Since *in vivo* models have been banned by the EU Cosmetics Directive since March 2009, *in vitro* models need to be more predictive for the risk assessment of cosmetic ingredients. In this study, comparisons have been made between different measures of estimating toxicity after exposure to curcumin, resorcinol, ethyl acrylate, eugenol, p-nitrophenol and propyl gallate which have previously been shown to give misleading positive results in an in vitro micronucleus assay. Emphasis was placed on chemicals that have a steep toxicity profile and a maximum testing concentration limited by toxicity. Comparisons were performed between relative cell counts (RCC), relative increase in cell counts (RICC), relative population doubling (RPD) and replication index (RI). Intracellular caspase levels were also evaluated as an indicator of apoptosis. Our results demonstrate that certain measures, in particular RCC and RI can potentially seriously under estimate toxicity, the implication of which is a higher maximum testing concentration which can contribute to the generation of misleading positive results with *in vitro* genotoxicity assays.

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This work was funded by the European Cosmetic Industry Association COLIPA, ECVAM and NC3Rs.

61. In vitro micronucleus method development for whole smoke exposure

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The cytokinesis block *in vitro* micronucleus (CBMN) test detects chromosome breakage and loss. Regulatory guidelines recommend CBMN as a complement to the Ames test in the battery of genotoxicity tests. Total particulate matter (TPM) is routinely used to assess the cytotoxicity and genotoxicity of cigarette smoke. However, whole smoke (WS) exposure is a novel approach that allows for both the TPM and vapour phase (VP) components of cigarette smoke to be tested and is considered biologically more relevant. Therefore, we developed an *in vitro* CBMN method to assess the genotoxicity and cytotoxicity of reference cigarette WS in V79 cells exposed at the air-liquid interface. Cells grown on porous Transwell™ inserts were exposed for 30 minutes to a range of WS dilutions in air (1:350 to 1:3000). Following exposure, cells were incubated with cytochalasin-B for 18 hours, harvested, stained with acridine orange and subsequently scored for micronucleated binucleate (MNBN) cells.

The results show a significant increase in frequencies of MNBN cells (n=7 for dilution up to 1:700, n=2 from 1:800 to 1:3000; $p<0.001$) which was not dose-dependent. When varying the exposure time (5, 10, 20 and 30 minutes) for WS exposure at 1:500 dilution, a trend in increasing MNBN cells over time was observed (n=3; p=0.049). Additionally, a pilot experiment comparing filtered (VP) and non filtered (WS) from reference cigarette showed no differences in genotoxicity and cytotoxicity between WS and VP, suggesting that VP is a major contributor to the cytotoxic and genotoxic effect of cigarette smoke. In summary, this study demonstrated that WS exposure of V79 cells results in a time- but not dose-dependent increase in MNBN cells. The current protocol forms a basis for further optimisation and validation of the method. The present study may assist future investigations using alternative *in vitro* mammalian genotoxicity tests.

62. Advantages of human hepatocyte-derived transformants expressing a series of human cytochrome P450 isoforms for genotoxicity examination

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Metabolites of chemicals can often be ultimate genotoxic species, thus *in vitro* routine testing requires the use of the rat liver S9. However, there is a question as to whether this represents an appropriate surrogate for human metabolism. We have previously demonstrated the usefulness of HepG2 transformants expressing major human cytochrome P450 (CYP) isoforms to assess the genotoxicity of metabolites [1]. We further assessed the advantages of these transformants from the following 3 aspects. Firstly, the sensitivity of these transformants was confirmed with micronucleus (MN) induction by 7,12-dimethylbenz[a]anthracene or ifosfamide in transformants expressing the corresponding CYP1A1 or CYP2B6 and CYP2C9, respectively. Secondly, by using these transformants, β-endosulfan, a chemical for which the CYP isoforms contributing to its genotoxicity are unknown, was found to induce MN through the CYP3A4-mediated pathway. This result was confirmed by the facts that the decreased CYP3A4 activity using a inhibitor or siRNA repressed MN induction by β-endosulfan, and that endosulfan sulfate, one of the metabolites produced by CYP3A4, induced MN in the transformants harboring an empty vector. Thirdly, the interaction between phase I and II drug-metabolizing enzymes was demonstrated by MN induction with inhibitors of UDP-glucuronyltransferases in tamoxifen-treated transformants harboring the corresponding CYP3A4 or with inhibitors of glutathione S-transferase in safrole-treated transformants harboring the corresponding CYP2D6, whereas neither tamoxifen nor safrole alone induced MN in any transformant. These advantages provide the benefits of newly established transformants for *in vitro* genotoxicity testing that reflects comprehensive metabolic pathways including not only human CYP isoforms but the phase II enzymes.

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63. Photo(geno)toxicity of neutral red determined in the ames, comet and mironucleus test

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Over the last years the 3T3 Neutral Red Uptake (NRU) test has become the method of choice to assess the phototoxic potential of new substances *in vitro*. After compound treatment the viability of irradiated versus not-irradiated 3T3 cells is determined by the uptake of neutral red (NR). While within the 3T3 NRU test NR is used as an indicator, we were interested in the photo(geno)toxic potential of NR by itself. NR is structurally similar to the tricyclic aromatic amine 2-aminoanthracene. Both compounds are capable of inducing mutagenic effects in all five standard Ames tester strains (TA1535, TA97, TA98, TA100, TA102) when activated by liver enzymes (S9). In addition, NR can be photoactivated by absorbing light in the visible range (λ_{max} 533 nm). Similarly to the metabolic activation by S9 the photomutagenicity of NR was seen in all five Ames tester strains, albeit with different relative specific activities among these strains. To our knowledge NR is the first photomutagen with such a wide activity in the Photo-Ames test. The reactive species produced by irradiation are short lived as pre-irradiation of an ethanolic solution of NR was not observed to elicit mutagenic effects when added to the bacteria

post-irradiated. We further investigated the photogenotoxicity of NR in the comet assay and micronucleus test in L5178Y cells. A concentration dependent increase in DNA breaks and micronuclei was observed following irradiation. Photocytotoxicity was shown by the Alamar Blue test.

64. DNA damage induced by oxidised PUFA is highly resistant to repair

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DNA oxidation is an inevitable consequence of aerobic metabolism and can also occur following exposure to various exogenous insults, including oxidised unsaturated fatty acids in food for example. To counteract damage, cells possess defence mechanisms including high and low molecular weight antioxidants and DNA repair processes. The spectrum of DNA lesions induced by different oxidative processes varies, as does the repair, with lipid peroxidation producing complex damage profiles. Using modified alkaline comet assay with endo111 and hOGG1 enzymes to detect pyrimidine and purine (8-oxo-7,8-dihydroguanine; 8-oxoGua) derived lesions respectively, we compared the induction and repair of DNA damage in response to different oxidative insults. Human keratinocytes were exposed to different doses of H_2O_2, *tert*-butyl hydroperoxide (TBHP) (up to 500μM) and a buffered extract of heat-oxidized PUFA (linoleic/linolenic acid) to simulate endogenous and exogenous oxidative insults. Following incubation (30 minutes, 4°C) with each treatment and recovery at 37°C for 24h, cells remained viable (MTT assay), notably however, the water insoluble portion of the oxidized PUFA extract was very cytotoxic (<50% viability immediately after exposure). All treatments induced DNA strand breaks/alkali-labile sites (SB/ALS), over the dose range studied. There were differences between each treatment in terms of the relative levels of pyrimidine-dervied lesions and 8-oxoGua generated. For H_2O_2 and TBHP exposure, the repair of lesions initially followed first order kinetics, with repair of oxidised pyrimidines and 8-oxoGua slower compared to ALS. Surprisingly, for oxidised PUFA extract, despite apparent removal of 8-oxoGua (pyrimidine oxidation was not especially evident in this system), >50% of the SB/ALS remained for at least 24h post-exposure, indicating possible failure of DNA repair. Further investigation of this phenomenon is needed to examine whether the DNA repair system itself has been damaged or elements of the damage introduced by oxidised PUFA are particularly resistant to repair.

65. Protection by flavonoids on food mutagen-induced DNA damage in peripheral blood lymphocytes from colon cancer patients compared to healthy controls

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Oxidative stress in cells either originates endogenously from increased enzymatic activity or it can be caused by compounds repeatedly encountered in our food, lifestyle and environment. The food mutagens IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) are heterocyclic amines (HCA), generated when heating proteinaceous food. This study investigates the protective potential of the polyphenolic plant flavonoids quercetin (Q) and rutin (R) against oxidative stress induced *in vitro* by IQ and PhIP using the Comet assay. Both compounds significantly induce DNA damage after *in vitro* treatment of lymphocytes from healthy individuals and colon cancer patients. However, in the presence of up to 500 μM Q and R, the DNA damage resulting from a high dose of PhIP (75 μM) or IQ (150 μM) was significantly reduced ($P<0.001$) to levels comparable to six times lower IQ or 7.5 times lower PhIP doses. Lymphocytes from colon cancer patients had greater baseline DNA damage than those from healthy individuals ($P<0.01$) and this higher level of damage was also observed throughout *in vitro* treatment. Except for the >50 years of age group and male gender, confounding factors such as smoking, drinking and/or dieting habits were not found to be significant. Conclusively, flavonoids were able to reduce oxidative stress caused by food mutagens *in vitro* in lymphocytes of healthy individuals and colon cancer patients. The latter group showed less of a reduction at the highest flavonoid concentration. Thus, supplementation of our daily diet with flavonoid-rich vegetables and fruits may prove very effective in protecting against oxidative stress by decreasing DNA damage.

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66. The effect of quinolones on human type II topoisomerases using the Trapped in AgaRose DNA ImmunoStaining (TARDIS) assay

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Quinolones are a class of drugs that target the bacterial type II topoisomerases DNA gyrase and topo IV. Type II topoisomerases are a family of enzymes that catalyse changes in DNA topology by transiently breaking and rejoining the DNA phosphodiester backbone bonds. Due to similarities between bacterial and human type II topoisomerase in both structure and sequence there is a possibility of cross reaction. The aim of this study was to determine whether quinolones also affect human type II topoisomerases. Human type II topoisomerase-DNA complexes within individual cells are detected using the **T**rapped in **A**ga**R**ose **D**NA **I**mmuno**S**taining (TARDIS) assay. The treated cells are embedded in agarose on microscope slides prior to cellular disruption by detergent and salt extraction to leave DNA and any covalent protein-DNA complexes in the agarose. The slides are probed with a topoisomerase II antibody and then a fluorescently labelled secondary antibody. The topoisomerase II-DNA complexes are detected using a Leica DMCB microscope with a Cool snap HQ2 camera V1.00. Analysis of the image with Volocity software gives an integrated fluorescence value for each cell. Etoposide was used as a positive control and showed the expected dose response.

The quinolones tested in this study were: Ciprofloxacin, Ofloxacin, Enrofloxacin, Levofloxacin, Sparfloxacin, Nalidixic acid and Oxolinic acid. Human K562 cells were treated with the quinolones at a concentration of 1mM for one hour or three hours. At both exposure times, the quinolones did not alter the number of complexes within the K562 cells. This suggests that the quinolones have no or little effect on human type II topoisomerase under these conditions.

67. *Pig-a* mutation assay – 28 Day repeat dose study with MNU

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A novel in vivo mutation assay has been described, [1-2] currently under commercial development by Litron, which measures the frequency of glycosylphosphatidyl inositol (GPI) anchor-deficient erythrocytes in the rat. In this Pig-a assay, the frequency of CD59-negative (i.e. mutant phenotype) erythrocytes and reticulocytes (RETs) is determined via flow cytometric analysis. A 28-Day repeat dose experiment using N-methyl-N-nitrosourea (MNU) was performed in collaboration with Litron to evaluate the performance of this assay in our laboratory. Male Sprague Dawley rats were treated for 28 days consecutively with 0, 2.5, 5 and 10 mg/kg/day (n = 5 per group) MNU via oral gavage. Blood samples were collected on Days -1, 15 and 30 for Pig-a analysis and on Days 4 and 29 for flow cytometric micronucleus (Flow-MNT) analysis. Blood and tissues were also taken for Haematology and Comet analysis on Day 29. Dose-related increases in the frequency of mutant phenotype RETs were observed for all groups on Days 15 (to 60 per million) and 29 (to 198 per million) and an associated increase in erythrocyte mutant frequencies was seen on Day 29 (to 36 per million). Increases were also seen in micronucleated RETs on both Day 4 (to 16-fold) and Day 29 (to 22-fold). Haematology analysis on Day 29 showed a marked dose-dependent decrease in total white blood cell counts. Comet analysis showed increases in Tail moment and % Tail DNA in the liver, kidney and blood at all doses tested, with doses of 10 mg/kg/day showing excess toxicity in the kidney. Pig-a and MNT results obtained were consistent with those generated by Litron, demonstrating good laboratory performance with this new assay.

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68. Visualisation and quantitation of DNA adducts using the Trapped in Agarose DNA immunostaining (TARDIS) assay

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The TARDIS assay was first developed as a means of detecting and quantifying melphalan and cisplatin DNA adducts at the single cell level(1) and has since been adapted and extensively used to quantify topoisomerase II DNA adducts(2). Type II DNA topoisomerases generate a transient enzyme-bridged DNA double-strand break through which a second duplex can be passed. At this stage in the reaction cycle the enzyme is covalently coupled to the DNA through a phospho-tyrosine linkage. After strand passage the break is rejoined. But the rejoining step is blocked by a class of compounds known as topoisomerase poisons leading to accumulation of topoisomerase II-DNA adducts. Topoisomerase II poisons including etoposide, mitoxantrone, mAMSA, daunorubicin, doxorubicin and epirubicin are important cytotoxic anti-cancer agents and topoisomerase poisons can also be encountered in the environment in the form of dietary components such as flavonoids. The ability to detect and quantify specific DNA adducts such as topoisomerase II - DNA complexes is of value in genome stability research, pre-clinical drug development studies and potentially in the evaluation of environmental mutagens. The TARDIS assay quantifies covalent adducts after extraction of agarose-embedded cells under harsh salt and detergent extractions. Genomic DNA and any covalently attached molecules remain within the agarose, while other cellular constituents are removed. Immuostaining for the component of interest and quantitative immunofluorescence allows adduct quantification at the single cell level.

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69. Elevated levels of oxdatively damaged DNA in patients with selenoprotein deficiency

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Selenium (Se) is a vital dietary element for eukaryote and prokaryotic cells. In humans, its biological role is mediated principally by incorporation of selenocysteine, into selenium containing proteins. About 30 different selenoproteins have so far been observed in human cells and tissues, performing a variety of different functions including removal of cellular reactive oxygen species, reduction of oxidised methionines in proteins, metabolism of thyroid hormones, transport and delivery of selenium to peripheral tissues, protein folding and ER stress, plus proteins whose precise function is unknown. One of the best-known functions of selenoproteins is displayed by the family of selenium-dependent glutathione peroxidases which reduces hydrogen peroxide, and damaging lipid and phospholipid hydroperoxides to harmless products such as water and alcohols. This function helps to reduce the risk of induction of further oxidatively generated damage to biomolecules and accordingly reduces risk of a variety of damage-related pathological conditions. In this study, we examined the baseline levels of oxdatively damaged DNA in two individuals,

one adult and one child with compound heterozygous defects in the selenocysteine insertion sequence-binding protein 2 (*SECISBP2*) gene. As part of a complex phenotype, using hOGG1-modified comet assay, we noted significantly elevated baseline levels of 8-oxoguanine (1.95 and 1.65 fold increase in the child and adult respectively) and alkali labile sites (7.4 fold increase in the child and 6.5 fold increase in the adult) in dermal fibroblasts versus age/gender match controls in the presence and absence of exogenous H_2O_2 (50 μM, 30 min). Using the same assay, we demonstrated that whilst DNA repair processes were unaffected, antioxidant defences were impaired, the likely source of the elevated baseline levels. Similarly, there was a 1.92 fold increase in the level of 8-oxoguanine following exposure to 10 J/cm^2 UVA in the affected adult, accounting for the pronounced photosensitivity seen in this subject. These results demonstrate the central role of selenoprotein in cellular antioxidant defence.

70. Application of infrared spectroscopy to biochemically signature the effects of chemical mixtures in mammalian cells

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Target organisms are continuously and variously exposed to chemical mixtures. Predicting in target-cell populations the biological responses to low-level exposures of mixtures based on adverse effects induced by high concentrations is difficult. As biomolecules absorb in the mid-infrared (IR) depending on their chemical composition, we examined cells treated with environmentally-relevant levels of chemical mixtures with attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy. This approach is based on the notion that depending on the physiological change of cells induced by a chemical agent, a detailed "biochemical-cell fingerprint" in the form of an IR spectrum relating to chemical structure alterations can be detected (1). MCF-7 cell cultures were treated with polybrominated diphenyl ethers (PBDEs) and/or polychlorinated biphenyls (PCB) congeners at low-levels (10^{-12} M) for 24-h and fixed with ethanol prior the integration of cellular material with ATR-FTIR spectroscopy. Principal component analysis (PCA) and/or linear discriminant analysis (LDA) was carried out on the derived IR dataset in order to identify wavenumber-associated biomarkers of effect. Treatment of MCF-7 cells with a binary mixture of PBDE congener plus PCB126 induced less distinctive effects compared to corresponding control cell populations than those for single-agent treatment. In contrast, treatment of cells with a PBDE congener plus PCB153 markedly increased their effects compared the alterations induced by either compound individually. This study points to a non-destructive approach capable of tracking the biological effects of contaminants and shedding light on the complexity of the mechanistic consequences of exposures to chemical mixtures relevant to environmental levels.

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71. Developing a drug resistant model of human multiple myeloma cell lines

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Multiple Myeloma is a malignancy of plasma cells and accounts for 1% of cancer incidence and 10% of haematogical malignancies. Melphalan with prednisone has been a gold standard therapy for more than 40 years, but almost all patients show resistance to the therapy despite their initial response. The aim of this study was to create a model of myeloma cell lines resistant to melphalan which can be used to investigate the mechanisms of drug resistance in myeloma. Three human myeloma cell lines (RPMI8226, U266 and JIM-3) have been selected and have been exposed to gradually increasing doses of melphalan starting from 0.5μM. Melphalan has been added to the culture whenever the cells have been fed. Based on normal morphological appearance and cell count the maximum tolerated dose currently is 2μM in U266, 1.5μM in JIM-3 and 0.5μM in RPMI. Viability of the treated and untreated cells has been measured in the absence and presence of 2, 5 and 10μM melphalan at 72 and 96 hours and suggests a tolerance to melphalan with an inverse relation with dose. The doubling time of the treated cells at the beginning of exposure was up to ten times longer than untreated cells, but as resistance develops the doubling time approaches that of the untreated cells (eg; U266; 5.99 vs 3.12 and JIM3 6.11 vs 3.95 days respectively). By gradual dose increases we have attempted to induce tolerance rather than select for resistant mutants. Our focus of assessment is on genotoxicity processes including increased DNA repair as well as lesion tolerance via the translesional synthesis pathway.

72. The hunt for misleading positives: can non-genotoxic regulation of GADD45a interfere with GreenScreen HC?

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The GreenScreen HC (GSHC) genotoxicity assay has demonstrated high levels of specificity (95%) and sensitivity (87%) when predicting genotoxic carcinogenicity. Accordingly, the frequency of misleading positive GSHC results is low when compared with other mammalian *in vitro* genotoxicity assays. The GSHC assay employs the response of the DNA damage-inducible gene GADD45a as a marker of genotoxic stress, using a GADD45a-GFP reporter containing key regulatory elements. In order to rigorously assess the specificity of the GSHC assay in light of the current knowledge of the regulation of GADD45a, chemicals with the potential to induce a GADD45a response in the absence of genotoxic stress were sought to expose the assay to challenging new biological mechanisms. Examples of such chemicals included activators of signalling pathways known to positively regulate GADD45a, such as p53. Chemicals that inhibit negative regulators of GADD45a, such as NF-κB and Bcl-2 family members, were also included. A third group included chemicals known to activate apoptosis by various cytotoxic stress signalling pathways. This group was chosen to investigate if the activation of these pathways has the potential to cross-talk with those controlling GADD45a activation. Stimulation of an apoptotic response by these

chemicals was assessed by monitoring of levels of caspase 3/7 activation and annexin V binding. Six of 15 chemicals assessed so far were positive in the GSHC assay, though comparative genotoxicity data for these chemicals are either sparse or non-existent. Other genotoxicity data have been generated in this study using an *in vitro* micronucleus test (MNT) and comet assay, as well as *in silico* alerts from the DEREK for Windows SAR software. DEREK SAR assessment revealed that 2 of the 6 chemicals could plausibly cause chromosome damage in mammalian cells. Two of the 4 chemicals without DEREK alerts tested positive in either the MNT or comet assay.

73. N-Methylpurine DNA glycosylase and its potential role in the mechanism behind the genotoxic threshold dose response elicited by monofunctional alkylating agents

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The linear dose response model, used to describe the carcinogenic activity of many direct acting DNA damaging compounds was dispelled by recent evidence that revealed biological tolerance to low levels of monofunctional alkylating agents, with the degree of threshold response being compound class specific. The mechanism pertaining to alkyl-induced genotoxic threshold response is as yet unknown. *N*-methylpurine DNA glycosylase (MPG), an initiator glycosylase of the short-patch base excision repair pathway, typically repairs alkyl-induced DNA adducts many of which are associated with genomic instability and tumorigenic risk. Here we demonstrate the involvement of MPG in modulating the genotoxic threshold response induced by the alkane sulfonate, ethyl methane sulfonate (EMS), and suggest the lack of N7eG adduct repair as a key factor attributable to an observed increase in EMS-induced chromosome breaks. Such findings were substantiated by an increase in MPG mRNA levels in response to EMS doses administered below the low-observed effect level (LOEL). An unexpected dose-dependent decrease in mutation frequency (MF) was observed in the MPG-deficient cell line M09B exposed to ENU and attributed to a pronounced dose-dependent increase in the number of apoptotic cells relative to wild type, which masked the *bone fide* MF induced. O^2- and O^4-eT lesions are generated by ENU and not EMS. With little change in the cytotoxicity and MF of M09B cells challenged with EMS, a role for MPG in preventing the cytotoxic effects induced by exo-O-thymine adducts miss-repair is thus proposed. Our studies implicate the differential involvement of MPG-directed BER, as a primary mechanism of action for the clastogenic threshold response and cytotoxicity induced by alkane sulfonates and *N*-nitrosourea compounds.

74. Why do some validation studies produce different performance figures for the GADD45a-GFP assay?

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For a new *in vitro* genotoxicity test to be adopted, it should be demonstrably better than existing tests. It should produce more accurate predictions of compound carcinogenicity, particularly for genotoxic carcinogens, and systematic reproducibility and transferability studies are required. Published peer-reviewed validation (1,2) and transfer (3,4) studies demonstrate that the GADD45a –GFP assay has greater sensitivity and specificity that existing *in vitro* assays. It is widely used in biotech and pharmaceutical screening and safety laboratories. A recent paper from Olaharski *et al*. (5) reported lower sensitivity for the GADD45a assay than the validation studies. However, assay sensitivity was assessed by its prediction of positive results in Ames and MNT tests rather than genotoxic carcinogenicity. There are confounding consequences to this approach. New assays with greater sensitivity and specificity for genotoxic carcinogens should yield very few positive results for non-carcinogens that produce 'misleading' positive results in Ames and MNT. The seminal review paper from Kirkland *et al*. (6) illustrated the potential scale of distortion that the Olaharski approach produces, reporting that 88% of non-carcinogens were positive in Ames, MNT or both. A new genotoxicity test generating positive data for 88% of non-carcinogens would quite rightly be considered of little practical use. It is therefore reassuring that Olaharski *et al*. reported a low correlation between positive results from the GADD45a assay and the combination of Ames and MNT results. Neither concordance with carcinogenicity data nor correlation with other *in vitro* results can provide useful information on the performance of a new *in vitro* test, unless positive prevalence (PP) of genotoxic carcinogenicity in the test set is known. Positive results from an improved test should be expected to correlate with positive Ames data in collections with known PP values.

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75. Frequency of micronucleated immature erythrocytes along the length of the rat femur

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The *in vivo* rodent bone marrow micronucleus test is widely used in regulatory genotoxicity testing and it is common to

analyse immature erythrocytes (IE) in bone marrow tissue. Erythrocytes (E) are produced in erythroblastic islands which are disrupted in smear preparation (1). There is a belief that erythrocyte production may be focussed at the growing ends of the femur and, if so, micronucleated erythrocytes (MIE) may be more frequent at distal ends of the femur than in the middle. To investigate whether MIE frequency really does vary across the length of the rat femur, bones were taken from existing studies following treatment with four genotoxic compounds (hexamethylphosphoramide, cyclophosphamide, ethylmethansulphonate and methylnitrosourea) and vehicle control. Femurs were sectioned into three and the number of MIE together with the ratio between the IE and E was determined in each section and compared to that from intact femurs. In femur preparations from mice, there was little difference between paint brush to smear sampling techniques, and the most important variable was to score sufficient numbers of cells. (2). In both control and treated groups, counts did vary between the mid and end sections of the femur for individual animals but, when taken as group means there was no significant difference between MIE formation in sections of the femur compared with the values obtained in suspensions from the whole femur. Therefore, these data indicate that MIE may be more frequent at the growing ends of femurs in some individuals but do not alter significantly the mean value from groups of 5-7 rats.

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76. Expression of bioactivating enzymes in normal human prostate suggests a capability to activate pro-carcinogens

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The bioactivating capacity of human prostate may play a role in adenocarcinoma developing in this tissue (1). Expression of candidate enzymes that convert exogenous and/or endogenous agents into DNA-damaging species would suggest the potential to generate initiating events in prostate cancer (CaP). Normal prostate tissues from UK-resident Caucasians (n=10) were collected following either radical retropubic prostatectomy or cystaprostatectomy. An analysis of gene and protein expression of metabolizing enzymes, including cytochrome P450 (CYP)1A1, CYP1A2, CYP1B1, *N*-acetyltransferase 1 (NAT1), sulfotransferase (SULT)1A1, SULT1A3, NAD(P)H:quinone oxidoreductase (NQO1), prostaglandin H synthase 1 (cyclooxygenase 1; COX1) and CYP oxidoreductase (POR) was carried out. Quantitative real-time RT PCR, Western blot and immunohistochemistry were conducted. Except for CYP1A1 and CYP1A2, the metabolizing enzymes examined appeared to be expressed with minimal inter-individual variation (approximately 2- to 5-fold). CYP1B1 and NQO1 were readily identifiable in human prostate. Immunohistochemistry showed that most expression is localized to epithelial cells lining the glandular elements *i.e.*, the cells from which CaP might arise. Constitutive expression of bioactivating enzymes confers the potential to convert exogenous and/or endogenous agents to DNA-reactive species (2). These findings suggest an organ capability for pro-carcinogen activation in the aetiology of human CaP.

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77. Aryl boronic acids: potentially mutagenic synthetic intermediates

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A boronic acid is an alkyl or aryl substituted boric acid containing a carbon-boron bond. Belonging to the larger chemical class of organoboranes, they are used extensively in organic chemistry and pharmaceutical synthesis, predominantly in the Suzuki coupling reaction. Structure-based assessment using the *in silico* tool DEREK (Deductive Estimation of Risk from Existing Knowledge) reveals that boronic acids do not fire structural alerts for mutagenicity or carcinogenicity. Structural alert assessments are routinely performed for intermediates and impurities in pharmaceutical synthetic routes to classify compounds as carcinogenic, mutagenic, potentially genotoxic or non-mutagenic based on alerting substructures. This process identifies impurities in the active pharmaceutical ingredient (API) that require control to very low levels to minimise cancer risk to humans as per regulatory genotoxic impurity guidelines. As boronic acids do not fire any structural alerts, they are currently not flagged as potential genotoxic impurities. Data mining Lhasa's Vitic database, containing public and proprietary toxicity information on a large number of chemicals and particularly synthetic intermediates, revealed 3-fluorobenzeneboronic acid (CAS number 768-35-4) was mutagenic in *Salmonella typhimurium* strains TA97a and TA100, and *Escherichia coli* strain WP2 uvrA with and without metabolic activation. No further experimental details were available. We present the mutagenicity results of several aryl boronic acids tested in the standard plate Ames test with at minimum *Salmonella typhimurium* strains TA100 and TA1537 and *Escherichia coli* strain WP2 uvrA pKM101. Each compound was tested up to maximum concentration of 5 mg/plate with and without metabolic activation. Our preliminary results suggest that aryl boronic acids represent class of synthetic intermediates that have the potential to be mutagenic in the Ames assay. Therefore, it may be necessary to consider threshold of toxicological concern (TTC) based controls for such intermediates. Additional work is needed to fully understand the mechanism of mutagenicity and structure activity relationship.

78. Boronic acids – a novel class of bacterial mutagen

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Boronic acids and their esters have recently become important building blocks in organic syntheses and key intermediates in the preparation of candidate drugs but, as far as can be determined, there is no published report of any test for genotoxicity or carcinogenicity with an example. Consequently, boronic acids as a class do not trigger alerts in any publicly available quantitative structure activity relationship (QSAR) program and there was no *a priori* reason to them. It was surprising, therefore, when positive results were obtained in an Ames test with a boronic acid performed at AstraZeneca for Occupational Safety purposes. About 20 boronic acids have now been tested at AstraZeneca using S.typhimurium TA1535, TA1537, TA98 and TA100 and E.coli WP2uvrA(pKM101), and the majority have been found to be mutagenic. All are active only for TA100 and/or WP2uvrA(pKM101), do not require S9 activation and produce relatively weak responses i.e. no more than 5x concurrent control values at >1000 μg/plate. Initial results with two mutagenic compounds showed no evidence of DNA adduct formation detectable by ^{32}P-postlabelling. It appears that boronic acids represent a novel class of bacterial mutagen. The mechanism of action is not yet understood and it cannot be determined whether or not they present a genotoxic risk.

79. A mathematical framework for spectroscopy data analysis to characterize chemical-induced alterations in the SHE assay

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Acquisition of IR spectra often generates complex datasets that are not readily interpretable for the purposes of deriving biomarkers. From a computational perspective, this raises the question of what multi-step processing is required and, whether there is a well-defined sequence of steps that can be applied to objectively shed insight into a biological question. To generate a dataset to investigate this, we set up an *in vitro* transformation assay (pH 6.7) using Syrian hamster embryo (SHE) cells (1). SHE cells were interrogated by ATR-FTIR spectroscopy. Derived mid-IR spectra ($n_{spectra}$ @14,000) were inputted into a computational framework designed for outlier removal, multivariate analysis and validation of the robustness of analysis, and biomarker identification. Biomarker identification methods were independently applied and compared to identify common discriminating chemical entities. Stable biomarkers of chemical-induced alterations or transformation were identified and confirmed. The analysis framework was implemented in the form of a user-friendly graphical user interface using a programming toolkit designed for research on computational methods. The database platform developed to store our dataset is scalable and can facilitate a data-sharing inter-laboratory process towards end-user applications for IR spectroscopy.

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80. In vitro micronucleus assay –increase in binucleated cells in mononucleate assays

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Work at Swansea University with AHH-1 cells revealed increased numbers of binucleated cells with concentration of methyl methanesulfonate (MMS) in mononucleate micronucleus studies using AHH-1 cells. Consequently, TK6 cells were treated with MMS in this laboratory to see if the same effect was observed.

In both cell lines, the increase in binucleates was only seen in 24 hour treatment with 24 hour recovery. In TK-6 cells, at a concentration of MMS (2ug/ml) causing 50% relative population doubling (RPD), there was a significant increase in binucleate cells, a significant percentage of which contained micronuclei. In AHH-1 cells at the same concentration of MMS a significant increase in micronucleated binucleates (MnBN) was observed. The increased incidence of binucleates raises the question whether scoring micronuclei (Mn) only in mononucleated cells underestimates the total incidence of Mn. When MnBN were included in the totals, the Mn frequency rose by 68% in TK-6 cells and 102% in AHH-1 cells. The increase in binucleates and MnBN was also seen with cytosine arabinoside, mitomycin C and methylnitrosourea.

The increased number of binucleates after 24-hour treatment might be due to slowed mitosis during the second cell cycle. For example, stalls in the replication fork resulting from mismatches formed in the first cell cycle have been shown to be induced by alkylating agents [1].

It is important to understand the effect of binucleate induction in micronucleus assays that do not use cytokinesis block with cytochalasin B. As MnBN are not usually included in mononucleate micronucleus assays it is possible that real increases in MN incidence are underestimated.

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