Development of chronic and passive dosing systems \textit{in vitro} for genotoxicity assessment.

Professor Gareth Jenkins, Swansea University, UK
**In vitro Toxicology Group, Swansea.**

- Longstanding interest in DNA damage and mutation testing.
- Studying the link between mutation and cancer in patients.
- Designing new, more sophisticated *in vitro* testing strategies for genotoxicity and carcinogenicity.
- Reducing reliance on animals in toxicology.
Genotoxicity

- Used as a surrogate for carcinogenicity *in vitro* and in short term *in vivo* tests.
- A 2-year rodent bioassay still also used for carcinogenicity as an apical endpoint.
- Obviously some sectors (e.g. Cosmetics) cannot perform animal tests any longer.
- Tiered approach for genotoxicity employing *in vitro* and *in vivo* stages.
Regulatory genotoxicity testing

Stage 0:
Structure Activity Relationships (SAR), screening tests and physio-chemical properties (of substance and impurities)

Stage 1:
- Bacterial gene mutation tests (Ames test)
- Clastogenicity and aneugenicity (in vitro micronucleus assay)

- **Negative** results in all tests
- **Equivocal** result in any test
- **Positive** results in any test

Stage 2 (in vivo):
Under take one or more of the following recommended assays:
1. Micronucleus assay or chromosome aberration test
2. Transgenic mutation test
3. Comet assay

- **Negative** after full assessment
- **Positive**: if data is robust consider substance to be in vivo somatic cell mutagen and possible germ cell mutagen

Substance is **not mutagenic**

Insufficient evidence to assess the mutagenicity of the substance
Review available data and make pragmatic conclusions based on case–by-case study
Issues with current testing paradigm.

- *In vitro* tests qualitative not quantitative.
- Binary decisions on genotoxicity. Dose response relationships not fully considered.
- Sensitivity not specificity.
- Heavy reliance on animals, used to de-risk misleading positive results *in vitro*.
- Simple Acute dosing scenarios do not reflect human exposure – lead to misleading positives? Overwhelms cellular defence mechanisms -non-physiological.
The Micronucleus (Mn) assay.

- Standard test for genotoxicity, detects most classes of genotoxins.
- Strict adherence to >50% cell viability, as cytotoxicity is a confounder.
- Usually block cytokinesis and look for Mn in binucleated cells.
- OECD guide available for consistency.
- Automation can increase statistical power enormously.

Mechanistic Influences for Mutation Induction Curves after Exposure to DNA- Reactive Carcinogens

Shareen H. Doak, Gareth J. S. Jenkins, George E. Johnson, Emma Quick, Elizabeth M. Parry, and James M. Parry

School of Medicine, University of Wales Swansea, Swansea, Wales, United Kingdom

Acute v Chronic (subchronic) *in vitro* exposures

- Current OECD guidelines recommend acute exposure scenarios for *in vitro* tests (e.g. 4-6 hours or 24 hours for the Mn test).
- Dose fractionation studies 1970’s and 1980’s done at high dose (100x above LOEL). Mainly focussed on radiation in animal models. Relevance not clear to low dose *in vitro* situation.
- Human exposure is chronic low dose in many cases.
- Need to better model human exposure scenarios *in vitro* to assess hazard (and risk).
Subchronic *in vitro* dosing (TK6 cells)?

- We have been exploring the influence of exposure scenario on genotoxicity. Using simple alkylating agents (MMS, MNU).

- What effect does chronic low dose exposure have on genotoxicity for potent genotoxins.

- Comparison to low dose acute exposure?
Genotoxicity of chronic MMS and MNU *in vitro*

- MMS and MNU delivered at 1/5\textsuperscript{th} the concentration for 5 days or 1/10\textsuperscript{th} the concentration for 10 days.
- Compared to an acute, one-off exposure.
- Each treatment (acute/chronic) 24 hrs.
- High powered studies for Mn induction (large numbers of cells).
- Mononucleate MN assay performed (12,000 cells per dose).
Chronic *in vitro* dosing at low dose level reduces genotoxic hazard.
Mechanisms of action behind the effects.

- Some evidence for hormetic effect with MNU – induced repair?
- P53 status. Isogenic cells (NH32) deficient in p53 show altered chronic response. Chronic exposure shows same results as acute TK6 system.
- Losses of chemical in system, allowing cells to tolerate higher exposures in total.
Chronic dosing effects for Non-genotoxic carcinogens

- Non-genotoxic carcinogens, negative in standard *in vitro* genotoxicity tests.
- Nickel Chloride shown here.
- But, can show some +ve results under chronic exposure conditions (5 day exposure to 1/5\(^{th}\) acute level)
- Linked to ROS induction, which rises daily.
Passive dosing

• An interesting extension to this line of investigation is the use of passive dosing (PD).

• Here a hydrophobic genotoxin (from an inert polymer source) is passively contributing a low but constant exposure to a cellular system and the mutational endpoints assessed.

• Exposure level can be varied by differential saturation of the loading solution.

• Sampling allows monitoring toxin concentration (or can be estimated from partitioning coefficient).
Advantages of PD

• Constant (low) level exposure to toxin.
• Partitioning coefficient from polymer allows prediction of behaviour.
• More reproducible data as exposure more reliable.
• No toxic co-solvents.
• More realistic exposure scenarios to some toxins.

Disc delivery system.

- B[a]P a model hydrophobic genotoxin
- MCL5 human lymphoblastoid cells (P450 activity).
- Mn (mononucleate) automated method, 6000 cells per replicate x3 replicates = 18,000 cells.
- 24 well plate format.
- 48 hours exposure to loaded PDMS discs.
- Media contains serum (10%).
Establishing a PD system for B[a]P induced micronucleus formation

Day 1:
- Seed cells
- Pre-equil medium o/n

Day 2:
- Cut PDMS discs
- 1x wash ethyl acetate
- 2x wash methanol
- Store in ddH₂O
- Add disc + preconditioned medium to cells

Day 4:
- Sample medium at 0, 24h
- Sample medium (48h)
- Methanol extraction
- Fluorescence HPLC
- Remove disc + change medium

Day 5:
- Fix cell nuclei;
- Score for MN using automated Metafer system

Day 18,000 cells per dose
B[a]P induces Mn in MCL5 cells, confirming metabolism (spiked).

- MCL5 cells can metabolise B[a]P to genotoxic metabolites.
- Mn induction and cytotoxicity (reduced RPD) evident.
- Dose dependent effect.
- TK6 cells (no metabolic activity) showed absolutely no effect (not shown).

Passive dosing of B[a]P in an in vitro MCL5 culture system

Serum presence increases B[a]P levels in media
Shaking during cellular exposure?
24 hr v 48 hr?
CYP450 expression induced by PD?
Conclusions

• In order to explore and refine the *in vitro* exposure to test genotoxic agents, we should investigate more sophisticated *in vitro* exposure scenarios.

• Chronic dosing can inform us about mutational hazards related to typical human exposure scenarios.

• Passive dosing linked to genotoxicity as an extension of this approach can be informative in the context of hydrophobic compounds.

• Can be used in IVIVE studies

• These approaches will reduce the need for routine *in vivo* studies.
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Diolch  Thank you
B[a]P passive dosing data.

- Inserts
- Steel posts
- Free discs

B[a]P concentration in medium after 48h (µM)

- Av. MNMN frequency
- Av. RPD

RPD (%)

Av. MNMN frequency (%)

Av. RPD (%)

B[a]P concentration in medium after 48h
The micronucleus (Mn) test

- The Mn test detects mutagens very sensitively. Most mutagens +ve in Mn test (clastogens and aneugens).
- Automation aids dose response analysis, by providing large data sets (10,000 cells per dose analysed).
- Use these dose responses to select LOEL doses for other endpoints (3-4 doses around the LOEL).
