Overview

Introduction to the assay
Transgenic models
OECD TG 488 assay design
Practical considerations
Specific considerations for germ cells
Mechanisms of Genotoxicity

**GENOTOXICITY**

- Single gene
  - Point mutation
  - Single base changes/deletions
- Chromosome
  - Multiple genes
  - Rearrangement/loss
  - Numerical changes

**HUMAN HEALTH EFFECTS**

**HERITABLE CHANGES**
Detection of *In Vivo* Genotoxicity

**Chromosome Aberrations**
- Micronuclei or Chromosome aberrations
- Blood, bone marrow, liver, GI tract, skin

**Gene Mutations**
- Transgenic rodent - Any somatic tissue
- Mouse/Eye Spot tests
- *Endogenous genes with detectable phenotypes (Hprt, Aprt, Tk)*

**Indicator Tests**
- Unscheduled DNA Synthesis, Comet, *Sister Chromatid Exchanges*

**Germ cell tests**
- Transgenic rodent - Male germ cells
- Dominant lethal, Spermatogonial chromosome aberrations, Mouse heritable translocations
Transgenic Rodent (TGR) Gene Mutation Test

Detection and quantification of mutations
- Somatic and germ cells from same animals

Several rodent models
- Each with its own advantages and disadvantages

All follow the same principles
- Look for mutations in a transgenic reporter gene
Basic Principles of the TGR Test

- **Enzyme restriction/phage packaging recognition sites**
- **Transgene**
- **Vector-specific genes for packaging / plasmid selection**

Multiple copies of the vector chromosomally integrated

- **Treat animal**
- **Collect tissues**
- **DNA extraction**

**Phage packaging / Plasmid recovery**

**Infection / Electroporation into host bacteria**

**Colorimetric**

- Normal
- Mutant

- All plaques
- Mutant plaques
- Positive

- **Selection**
Data Interpretation

Measurements
- Total titre (efficiency)
- Number of mutants
- Animal results = combined result of multiple reactions

Acceptance criteria for packaging
- No OECD defined criteria
- Concurrent positive control DNA response
- At least 1 mutant
- Consistently low titre (poor quality DNA) used with caution

Total number of plaques / plasmids
- Defined by TGR model
- Based on spontaneous mutant frequency (SMF)
Data Interpretation

Total number of plaques/plasmids per animal

- If SMF = 3x10^{-5}
- Minimum of 125,000 to 300,000 plaques/plasmid required

Reported measure

- MUTANT Frequency (MF)

\[ MF = \frac{\text{Number of mutant plaques / plasmids}}{\text{Total number of plaques / plasmids recovered}} \]
Data Interpretation

Positive result

- Dose related increase in MF
- Clear increase in MF in a single dose group compared to concurrent vehicle control

Statistics

- Often highly variable
- Use non-parametric statistics

Historical data

- Useful in interpretation
- Sufficient database for the tissue examined
Data Interpretation

FOLLOW-UP ANALYSIS

Sequencing of mutants

- Identify mutation spectrum
- Rarely used in a regulatory setting

Uses

- Identify mechanism
- Identify clonal expansion
- Comparison of mutagenesis at different loci
Advantages & Disadvantages of the TGR Test

**PROS**
- Gene mutation
- Multiple tissues, same animal
- Somatic & germ cells
- Multiple routes of administration
- Site of contact assessment
- Good reproducibility
- Small number of animals
- Neutral transgene

**CONS**
- Poor sensitivity to clastogens
- Detection of insertions / deletions limited by size
- High spontaneous mutant frequency
- Time
- Cost
- Animal supply
- Commercial expertise
Animal Models

Phage vectors

- Muta™Mouse
- Big Blue® Rat or Mouse
- gpt delta Rat or Mouse

Plasmid vector

- LacZ plasmid mouse
## Comparison of Models

<table>
<thead>
<tr>
<th></th>
<th>MM</th>
<th>BB</th>
<th>gpt Δ</th>
<th>LacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial availability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Mouse</td>
<td></td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Well characterised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Availability: animals / CRO labs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large deletions/insertions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive Selection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Technical Complexity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutations genetically inert</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
OECD TG 488 Study Design

**Animals**
- Generally test males
- ≥5 animals per group

**Dose groups**
- ≥3 dose levels
- MTD or maximum recommend dose
- 1000 mg/kg/day for >14 days dosing

**Controls**
- Concurrent negative (vehicle) control
- Concurrent positive control
- Animals
- Tissue matched DNA

**Dose route**
- Any with justification
- Human exposure
- Target tissue
OECD TG 488 Study Design

Treatment time

- Tissue specific
- Additive
- Allow sufficient time to detect weak mutagens without inducing false effects
- Clonal expansion, genome instability, oxidative damage, chronic toxicity
- 28 days generally a good compromise

Sample time

- Last treatment to necropsy
- Tissue specific
- Influenced by Uptake, Metabolism, Repair, Cell turnover
- 3 days generally accepted for most somatic tissues
Treatmet and Sample Time Considerations

28 DAY DOSING WITH 3 DAY SAMPLE TIME

**LIVER**

- Mutation event occurs during early administrations
- Mutations become fixed over following weeks reaching a peak frequency within 1 month
- Mutations appearing early in treatment phase are lost due to rapid turnover of tissue

**BONE MARROW**

- Detectable mutation events occur during final administrations
- Mutations rapidly fixed reaching a peak frequency within a few days
Treatment and Sample Time Considerations

28+3 DAYS DOSING/SAMPLE REGIMEN

Applicable to a broad range of somatic tissues

• Compromise to allow investigation of multiple tissues with a range of proliferation times

Different treatment times can be considered

• Sufficient time for enzyme induction
• Adjust sample time or use multiple sample times for slowly proliferating tissues

Sample time

• Critical variable
• Need to extend beyond 3 days for some slowly proliferating tissues and possibly germ cells
Practical Considerations and Limitations

GENERAL CONSIDERATIONS

- **Laboratory competence**
  - Experience is essential
  - Historical Control Data
  - Proficiency testing for new tissues

- **Positive controls**
  - Concurrent animals vs Positive control DNA
  - GLP records
  - Shelf-life of DNA/tissues
  - Colour controls

- **Animal availability**
  - MM and BB commercially available
  - TG colonies are small due to low historical demand

- **Age of animals**
  - Background frequency of mutation increases with age
  - OECD TG 488 specifies 8-12 week old animals

- **Tissue selection**
  - Any tissue, with practical limitations
  - No female germ cell analysis
  - Tissue size and DNA yield
Practical Considerations and Limitations

GERM CELL SPECIFIC CONSIDERATIONS

Rats: 10 weeks
Mice: 7 weeks

Sample mature sperm @ Day 31

Only late stages evaluated

Early stages are not evaluated
GERM CELL SPECIFIC CONSIDERATIONS

28 Day Dosing + Extended Sample Time

- 10 weeks rat
- 7 week mice
- Only analyse mature sperm
- May need separate groups of animals if somatic tissue analysis also required

Standard 28 Day Dosing + 3 Day Sample Time

- Optimal for most somatic tissues
- OECD TG 488 requires 2 types of germ cells
- Developing germ cells from seminiferous tubules
- Mature sperm cauda epididymis / vas deferens
Germ Cell Specific Considerations

CHALLENGING THE OECD TG REQUIREMENTS

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose level (mg/kg/day)</th>
<th>Number of animals / Sample Day (DX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D31</td>
</tr>
<tr>
<td>1  Water</td>
<td>0</td>
<td>7M</td>
</tr>
<tr>
<td>2  Ethynitrosurea</td>
<td>10</td>
<td>7M*</td>
</tr>
</tbody>
</table>

* Day 3 data from historical positive control treatments

Groups of 7 male mice (Muta\textsuperscript{TM}Mouse)

28 days oral gavage administration

Sampled 3 days or 3, 4, 5, 6 or 7 weeks after completion of dosing

Liver*, bone marrow*, developing germ cells, mature sperm
Germ Cell Specific Considerations

LIVER ANALYSIS

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>ENU</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>0.00</td>
<td>200.00</td>
</tr>
<tr>
<td>49</td>
<td>400.00</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>600.00</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>800.00</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>1000.00</td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>1200.00</td>
<td></td>
</tr>
</tbody>
</table>

Mutant Frequency x10^-6

*** = significant ANOVA at P<0.001
Germ Cell Specific Considerations

BONE MARROW ANALYSIS

**Bone Marrow**

Mutant Frequency x10-6

Day 31 control  Day 31 ENU  Day 49 control  Day 49 ENU  Day 56 control  Day 56 ENU  Day 63 control  Day 63 ENU  Day 70 control  Day 70 ENU  Day 77 control  Day 77 ENU

*** = significant ANOVA at P<0.001
Germ Cell Specific Considerations

DEVELOPING GERM CELLS

** = significant ANOVA at P < 0.01
*** = significant ANOVA at P < 0.001
MATURE SPERM ANALYSIS

Mature Sperm

Mutant Frequency x10^-6

* = significant ANOVA at P<0.05
Germ Cell Specific Considerations

CONCLUSIONS

ENU toxicity to the germ cells

- DNA recovery and packaging efficiency
- Compromised analysis of the germ cells

Standard 3 day sample time (D31)

- Increased MF in liver, bone marrow, developing germ cells
- No effects in Mature sperm

Extended sample times

- Increased MF in liver and bone marrow stable over all time points
- MF in developing germ cells reach steady state at D56+ (4 weeks)

Mature sperm

- Increased MF only detected at D70 or D77 (6 or 7 weeks)
Concluding Remarks

<table>
<thead>
<tr>
<th>Several models available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generally similar in terms of sensitivity, technical requirements, pros and cons</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Commercial supply issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limited colony sizes</td>
</tr>
<tr>
<td>Limited CRO options</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>More amenable to routine testing than other <em>in vivo</em> mutation assays</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Treatment and sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical for tissue-specific mutation events</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OECD TG 488 general design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximises sensitivity</td>
</tr>
<tr>
<td>Reduces risk of false positive effects</td>
</tr>
<tr>
<td>Applicable to most tissues</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Germ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum sample times and specific cells still under scientific debate</td>
</tr>
</tbody>
</table>
Acknowledgements

Germ cell ENU data – Covance Teams
Genetic Toxicology Staff
  Gareth Pearce
  Luke Foster
  Robert Mortimer
  Tina Downend
  Sarah Percy
Animal Unit
  Agata Fierka
  Vicky Brown
  Hayley Parker
Dispensary
  Andrew Baranyai
Questions?