A Toolbox to Estimate Point-of-Departure Metrics in Genetic Toxicology

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Quantitative Dose-response Analyses and PoD Determination in Genetic Toxicology - Brief Timeline

1. 2006-08: Increasing recognition regarding the need for methods to move beyond qualitative “screen and bin” to quantitative dose-response analyses and PoD determination.


5. 2009: 13 papers in special issue of *Toxicology Letters* - *in vivo* PoD for murine mutagenic and clastogenic effects of EMS (i.e., MN in ICR and LacZ in Muta™Mouse).

### Database Feature

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
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<tbody>
<tr>
<td>Number of studies screened</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Number of experiments included</td>
<td>165</td>
</tr>
<tr>
<td>Number of endpoints included</td>
<td>9</td>
</tr>
<tr>
<td>Number of records</td>
<td>2826</td>
</tr>
</tbody>
</table>

Initially only 4 chemicals:
- EMS
- ENU
- MMS
- MNU

- Health Canada – HESI Collaboration
- $130k from Chemical Management Plan
Provide a framework for -

1. Routine quantitative analyses of genetic toxicity dose response data.

2. Efficient and standardized derivation of PoD metrics.

3. Effective use of PoD metric in a risk assessment context.
POD Metrics - Definitions

NOGEL – No-Observed-Genotoxic-Effect-Level. Highest study dose that yields a response that is not statistically different from the unexposed control. Analogous to NOAEL.

Td or BPD – Threshold or Breakpoint dose. Statistically identified dose below which the effect cannot be distinguished from the background observed in the absence of treatment. Piecewise, segmented or hockey-stick regression to identify “point of inflection”.

STD – Slope Transition Dose. Lowest dose in a non-linear dose-response relationship where the first derivative >0 (i.e., slope transition to positive).

BMD – Benchmark Dose. Dose of a substance that yields a predetermined level of response (i.e., the BMR or Benchmark Response). BMR often specified as 10% increase above control (i.e., BMD_{10}). Can also be 1 standard deviation above control (i.e., BMD_{1SD}).
Dose–response Modeling Example for HPRT Gene Mutations Induced by \textit{in vitro} Exposure to MMS (AHH–1 cells)
(Doak et al., 2007. \textit{Cancer Res} 67:3904-3911; Johnson et al., 2009. \textit{Mutat Res} 678:95-100)

\textbf{Multi-step Data Analyses} - to determine NOGEL (one-way Dunnett's), Td and Td-L (via Lutz and Lutz in R), BMD10 and BMDL10 (using exponential family of models in PROAST)
ToxStrategies Analyses - Thompson et al. (2012-14)

(1) Develop “toolbox” of analytical methods for PoD determination; apply to MNU and ENU datasets in G4.

(2) Unite tools in R-based package; SOP for PoD determination - including smoothing spline regression and break-point analyses.

RECOMMENDED WORKFLOW

1. Initial data screening and transformation.
2. Statistical evaluation and PoD determination
   i. NOGEL (Dunnett’s, Dunnet’s T3 or Dunn’s 1-sided )
   ii. Td via Lutz and Lutz method (optional)
   iii. Td (now called BPD) via segmented in R
   iv. STD via mgcv in R
   v. BMD_{10} (PROAST)
   vi. BMD_{1SD} (using BMDS v.2.2)
Flow-Chart Showing the Workflow for Determination of NOGELs, BPDs and STDs

1 - Normal Distribution
   (Shapiro-Wilk test)
2 - Homogeneous Variance
   (Bartlett test)
   With or without Transformation

1 - Yes
2 - Yes

1 - Yes
2 - No

1 - No
2 - No

NOGEL
Dunnett’s

BPD/STD
1. Segmented
2. MGCV
3. L&L
Breakpoint Dose Modelling -

- Discontinuity or “Breakpoint” below which function is linear, and slope not significantly >0.
- L&L - piecewise linear regression & assumes errors normally distributed & equal response variance across range of observations.
- Segmented - open source algorithm, does not require censoring at top doses. Similar assumptions, but can uses weighted modelling.
- When BPDL ≤0, single linear segment provides better fit.

Smoothing Regression Splines -

- Examines “continuous functional relationship” without need to assume linearity or non-linearity across any dose ranges.
- “Semi-parametric” - no distributional or constant variance assumptions.
- STD or “Slope Transition Dose” is the lowest dose where the first derivative >0.
- When STDL ≤0 - hypothesis that slope is increasing above dose=0 cannot be rejected.
Step-wise Process for PoD Determination

1. Data screening, assumptions and data transformations - outlier analysis, distribution of residual error, and homogeneity of response variance across doses. Log and Sqrt transformation often equalize variance and meet assumptions.

2. Determine NOGEL using multiple comparison methods such as Dunnett’s, Dunnett’s T3 or Dunn’s (non-parametric).

3. Dose-response shape testing - multi-step process to determine if we can reject $H_0$ that response is linear across entire dose-range; linear or non-linear below a cut-off dose.


5. Determine BPD using segmented in R. Does not require homogeneous variance or truncation due to sub-linear responses at high doses.

6. Determine STD using mgcv in R. Semi-parametric method that does not require normality or variance homogeneity assumptions.

7. Determine BMD$_{10}$ using PROAST (38.9) and/or BMD$_{1SD}$ using BMDS 2.5. Nested family of functions, model selection using AIC (Akaike’s Information Criterion) or Log-likelihood ratio to determine “parsimonious” model.

*Mutat Res 678:118-122
§ Toxicol Lett 190:298-302
Benzo[a]pyrene Extended Dose Experiment – Muta™Mouse, 28-day Repeat Dose Oral, 10 doses plus control, Bone Marrow

LacZ Mutant Frequency
DNA Adduct Frequency

LacZ Mutant Frequency x 10^-5
DNA Adduct Frequency (RAL per 10^9 Nucleotides)

Dose (mg/kg/day)
drsmooth (Dose-Response Modeling with Smoothing Splines). Available from CRAN (Comprehensive R Archive Network)
Screen for outliers and evaluate transformation options.

Log transformation appropriate for this dataset.
Choose type of test

1. NOGEL
   - Response
   - Response sq. root
   - Response Log

2. Shape Testing
   - Response
   - Response sq. root
   - Response Log

3. Breakpoint Dose
   - Response
   - Response sq. root
   - Response Log

4. Slope Transition Dose
   - Response
   - Response sq. root
   - Response Log

Depending on dataset this could take few seconds

NOEL Derivation (No-Observed Genotoxic Effect Level)

NOGEL is defined as the highest dose in a genotoxicity assay in which no statistically significant effect can be detected. If the best data transformation had homogeneous variance and normal distribution then use the 1 sided Dunnett's test. If either tests failed then use the 1 sided Dunn's test. The Dunnett's T3 can also be used for data that have failed the Bartlett's test.

<table>
<thead>
<tr>
<th>Dose</th>
<th>vs</th>
<th>Zero</th>
<th>Estimate</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>-0.007</td>
<td>0.0895</td>
<td>-0.082</td>
<td>0.9626512206</td>
</tr>
<tr>
<td>0.2</td>
<td>-0.074</td>
<td>0.0895</td>
<td>-0.835</td>
<td>0.9975946423</td>
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<tr>
<td>0.39</td>
<td>0.0750</td>
<td>0.0895</td>
<td>0.8381</td>
<td>0.6996577549</td>
</tr>
<tr>
<td>0.78</td>
<td>0.1931</td>
<td>0.0895</td>
<td>2.1557</td>
<td>0.1222758691</td>
</tr>
<tr>
<td>1.56</td>
<td>0.0995</td>
<td>0.0895</td>
<td>1.1107</td>
<td>0.5637317105</td>
</tr>
<tr>
<td>3.13</td>
<td>0.4541</td>
<td>0.0895</td>
<td>5.0693</td>
<td>1.35305e-05</td>
</tr>
<tr>
<td>6.25</td>
<td>1.0880</td>
<td>0.0895</td>
<td>12.143</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>1.5856</td>
<td>0.0895</td>
<td>17.697</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>1.9624</td>
<td>0.0895</td>
<td>21.902</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>2.1034</td>
<td>0.0895</td>
<td>23.476</td>
<td>0</td>
</tr>
</tbody>
</table>
a. Non Linear Across All Doses

It tests whether a linear or nonlinear model better fit the dose-response data as follows. A significant result (e.g. $p \leq 0.05$) indicates that a nonlinear model fits the data better than a linear model.

<p>| Analysis of Variance Table |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Res.Df</th>
<th>RSS</th>
<th>Df</th>
<th>Sum of Sq</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81.000</td>
<td>15.1042</td>
<td>1.9851</td>
<td>12.083</td>
<td>161.17</td>
<td>&lt; 2.2e-16***</td>
</tr>
</tbody>
</table>

Signif. codes: 0 "****" 0.001 "***" 0.01 "**" 0.05 "*" 0.1 "." 1

✓ Determine whether we can reject that null hypothesis that a linear model provides the “correct” fit across the entire range. YES

b. Linear Below Cutoff Dose

LBDC assesses whether the slope of the initial portion of a dose-response curve differs significantly from zero. A significant result (e.g. $p \leq 0.05$) indicates that the slope differs significantly from zero between zero/background dose and the NOGEL. The presence of a negative sign (-) indicates a negative slope.

| Residuals: |
|------------|-------------|-------------|-------------|
| Min       | -0.6641     | -0.2957     | -0.1030     |
| 1Q         | 0.0956      | 0.05468     | 0.03126     |
| Median     | 0.0619498   | 0.05468     | 0.03126     |
| 3Q         | 0.1755      | 0.1138      | 0.0751      |
| Max        | 0.9596      | 0.5556      | 0.3268      |

| Coefficients: |
|-----------------|-------------|-------------|-------------|
| Estimate        | 0.619498    | 0.045488    | 11.38       |
| Std. Error      | 0.05468     | 0.03126     | 15.52       |
| t value         | < 2e-16***  | < 2e-16     | < 2e-16     |
| Pr(>|t|)         |             |             |             |

Residual Standard Err.: 0.4124 on 74 degrees of freedom

Multiple R-squared: 0.7651
Adjust R-squared: 0.7619
F-statistics: 241 on 1 and 74 DF, p-value: <

Signif. codes: 0 "****" 0.001 "***" 0.01 "**" 0.05 "*" 0.1 "." 1

✓ Determine whether the “initial” slope below NOGEL is significantly $>0$. YES

✓ Determine whether we can reject the null hypothesis that the function is linear below the NOGEL. YES

c. Non Linear Below Cutoff Dose

A significant result (e.g. $p \leq 0.05$) indicates that the nonlinear model better fits the dose-response data than a linear model from zero/background dose to the NOGEL.

<p>| Analysis of Variance Table |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Res.Df</th>
<th>RSS</th>
<th>Df</th>
<th>Sum of Sq</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>74.000</td>
<td>12.5871</td>
<td>1.943</td>
<td>9.6266</td>
<td>132.91</td>
<td>&lt; 2.2e-16***</td>
</tr>
</tbody>
</table>

Signif. codes: 0 "****" 0.001 "***" 0.01 "**" 0.05 "*" 0.1 "." 1

✓ Determine whether we can reject the null hypothesis that a linear model provides the “correct” fit across the entire range. YES

✓ Determine whether the “initial” slope below NOGEL is significantly $>0$. YES

✓ Determine whether we can reject the null hypothesis that the function is linear below the NOGEL. YES
If the lower confidence limit on the breakpoint dose (BPDL) is ≤0, then ignore the breakpoint dose and report the BPD and BPDL values as "no BPD".

<table>
<thead>
<tr>
<th>Breakpoint Dose</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.56</td>
<td>-2.7936</td>
<td>5.9136</td>
<td>2.6468</td>
</tr>
</tbody>
</table>

- Lower confidence limit on BPD<0, therefore BPD could not be determined.
- No detectable "Breakpoint" below which function is linear and slope not significantly >0.
If the lower confidence limit on the slope transition dose (STDL) is 0, then ignore the STD dose and report the STD and STDL values as "no STD."

### Table

<table>
<thead>
<tr>
<th>STD</th>
<th>STD_l</th>
<th>STD_u</th>
<th>STD_bias</th>
<th>iLOGEL</th>
<th>iLOGEL_l</th>
<th>iLOGEL_u</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.30</td>
<td>0.05</td>
<td>0.60</td>
</tr>
</tbody>
</table>

- Lower confidence limit on STD<0.
- Could not detect a dose>0 where the first derivative >0 (i.e., lowest dose associated with slope transition to positive).
Two Excellent Tools Available for Determination of Benchmark Dose (e.g., BMD$_{10}$, BMD$_{1SD}$)

Wide range of functional forms for a wide range of data structures, experimental designs, endpoints, and covariates.
PROAST (38.9) Output for BaP-induced LacZ Mutants in Muta™Mouse
(28-day repeat-dose oral, 10 doses plus control, bone marrow)
Benchmark Dose Determination (i.e., BMD$_{10}$) Using PROAST in R (Log-transformed)

- CES (Critical Effect Size or BMR) = 0.1
- CED (Critical Effect Dose) or BMD$_{10}$ = 0.80
- CEDL (Lower Confidence Limit of CED) or BMDL$_{10}$ = 0.55
BMDS (v2.5) Output for BaP-induced *LacZ* Mutants in *Muta™*Mouse
(28-day repeat-dose oral, 10 doses plus control, bone marrow)

Exponential Model. (Version: 1.9; Date: 01/23/2013)
Input Data File: C:/Program Files/BMDS250/Data/exp_Dax_Setting.d
Output Plotting File:  
Tue Jul 01 10:04:41 2014

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Benchmark Dose Computations:
Specified Effect = 1.000000
Risk Type = Estimated standard deviations from control
Confidence Level = 0.950000

BMD and BMDL by Model

<table>
<thead>
<tr>
<th>Model</th>
<th>BMD</th>
<th>BMDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.329741</td>
<td>0.279295</td>
</tr>
<tr>
<td>3</td>
<td>0.329741</td>
<td>0.279295</td>
</tr>
<tr>
<td>4</td>
<td>0.183518</td>
<td>0.147415</td>
</tr>
<tr>
<td>5</td>
<td>0.449273</td>
<td>0.393341</td>
</tr>
</tbody>
</table>

Exponential Model 5, with BMR of 1 Std. Dev. for the BMD and 0.95 Lower Confidence Level for BMDL

BMDL<sub>1SD</sub> = 1.31 mg/kg
BMDL<sub>10</sub> = 0.55 mg/kg

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The form of the response function by Model:
Model 2: $Y(dose) = a \times \exp(sign \times b \times dose)$
Model 3: $Y(dose) = a \times \exp(sign \times (b \times dose)^d)$
Model 4: $Y(dose) = a \times [c-(c-1) \times \exp(-b \times dose)]$
Model 5: $Y(dose) = a \times [c-(c-1) \times \exp(-(b \times dose)^d)]$

Note: $Y(dose)$ is the median response for exposure - dose;
$sign = +1$ for increasing trend in data;
$sign = -1$ for decreasing trend.

Model 2 is nested within Models 3 and 4.
Model 3 is nested within Model 5.
Model 4 is nested within Model 5.

Dependent variable = Log10_Response
Independent variable = Log10_Dose
Data are assumed to be distributed: normally
Variance Model: $\exp(\ln(alpha + \rho \times \ln(Y(dose)))$
The variance is to be modeled as $Var(1) = \exp(\ln(alpha + \log(\text{mean}(1)) \times \rho)$

The p-value for Test 7a is greater than .1. Model 5 seems to adequately describe the data.

The p-value for Test 7b is less than .05. Model 5 appears to fit the data better than Model 3.

The p-value for Test 7c is less than .05. Model 5 appears to fit the data better than Model 4.
**Comparisons of PoD Values for Benzo[a]pyrene In Vivo Endpoints 28-day Repeat Dose Oral (mg/kg/day), Muta™Mouse Bone Marrow**

<table>
<thead>
<tr>
<th></th>
<th>NOGEL</th>
<th>BMDL&lt;sub&gt;10&lt;/sub&gt;</th>
<th>BMDL&lt;sub&gt;15D&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Adduct Frequency</td>
<td>0.10</td>
<td>0.0009</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>LacZ</strong> Mutant Frequency</td>
<td>1.56</td>
<td>0.55</td>
<td>1.31</td>
</tr>
<tr>
<td>Micronucleus Frequency in RETs</td>
<td>1.56</td>
<td>2.46</td>
<td>4.73</td>
</tr>
<tr>
<td>Micronucleus Frequency in NCEs</td>
<td>1.56</td>
<td>2.70</td>
<td>4.44</td>
</tr>
<tr>
<td>Pig-a Mutant Frequency in RETs</td>
<td>6.25</td>
<td>1.76</td>
<td>5.05</td>
</tr>
<tr>
<td>Pig-a Mutant Frequency in RBCs</td>
<td>6.25</td>
<td>1.32</td>
<td>3.66</td>
</tr>
</tbody>
</table>
• Awarded $485,750 for April 2014 through March 31, 2017.

Quantitative Approaches for Improved Regulatory Evaluation & Risk Assessment of Genotoxic Substances

Paul White, George Johnson, Wout Slob, and Lya Soeteman-Hernández

• Collection and analysis of data to continue evaluation of quantitative methods for genotoxic substances (i.e., broader range of compounds); cross-endpoint empirical potency comparisons, extrapolation from PoDs for Risk Assessment/Management and regulatory decision-making.

• Aflatoxin B1, acrylamide, benzo[a]pyrene, 2-acetylaminoﬂuorene, PhIP, MeIQx, vinyl chloride, butadiene and doxorubicin.
Concluding Remarks

- Rapid progress towards establishing streamlined methodologies for quantitative analyses of genetic toxicity dose-response data.
- Numerous tools available for a vast array of data structures, endpoints, functional forms, covariates, etc – BMDS, PROAST, drsmooth, L&L. Evaluated using G4 data on EMS, MMS, ENU and MNU.
- BMD concept been around since mid-1980's (i.e., Crump, 1984; USEPA, 2000), is well established, well accepted for numerous endpoints, and methodologies somewhat “more forgiving” and flexible.

**MAJOR ISSUES:**

1. Establishment of “pragmatic” BMRs for genetic toxicity endpoints.
2. Establish framework for interpretation of BMDs in a human risk assessment/management context.
3. Can we use genetic toxicity BMDs to construct empirical models to predict regulatory endpoints (e.g., *in vivo* from *in vitro*, cancer from *in vivo* genetic toxicity)?

Acknowledgements & Funding

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Canada Canada

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IWGT Quantitative Sub-group members.