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Non-linear dose–response of DNA-reactive genotoxins: Recommendations for data analysis

George E. Johnson*, Shareen H. Doak, Sioned M. Griffiths, Emma L. Quick, David O.F. Skibinski, Zoulikha M. Zaïr, Gareth J. Jenkins

DNA Damage Group, School of Medicine, Swansea University, Wales, UK

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ABSTRACT

Until recently, there has only been a limited amount of data available on the kinetics of mutation induction in the low dose region of exposure. In our publication Doak et al. [S.H. Doak, G.J. Jenkins, G.E. Johnson, E. Quick, E.M. Parry, J.M. Parry, Mechanistic influences for mutation induction curves after exposure to DNA-reactive carcinogens, Cancer Res. 67 (2007) 3904–3911] we showed that the two alkylating agents methyl-methanesulfonate (MMS) and ethyl-methanesulfonate (EMS) possess non-linear dose–response curves with no observed effect levels (NOEL) for mutation or chromosomal damage *in vitro*. These experiments were carried out in the AHH-1 human lymphoblastoid cell line, using the hypoxanthine phosphoribosyl transferase (*HPRT*) assay and the cytokinesis-block micronucleus (CBMN) assay, respectively. We have now carried out more advanced statistical analyses to define threshold values, which is critical as it has a dramatic impact on hazard and risk assessment. To do this, we re-analysed the data to see if the linear model or a more complex model (hockey stick or quadratic) gave a significant better fit of the data. For both EMS and MMS cytokinesis-block micronucleus data sets, the hockey stick model gave the most significant fit. The same was true for EMS, MMS and surprisingly ethylnitrosourea (ENU) in the *HPRT* assay in human AHH-1 cells. However, methylnitrosourea (MNU) was linear in both assays. These further analyses have shown that EMS and MMS have clear thresholds for both gene mutation and chromosome damage, as does ENU for gene mutation in AHH-1 cells. MNU was linear for gene and chromosome mutation and so was ENU for chromosome mutations at the concentrations tested. These findings correlate closely with those *in vivo* findings of Gocke et al. [E. Gocke, L. Müller, In vivo studies in the mouse to define a threshold for the genotoxicity of EMS and ENU, Mutat. Res. (this issue)] and together these data show a true threshold for EMS both *in vitro* and *in vivo*. In this report, we will discuss the approaches that were taken to investigate potential threshold dose–response curves for DNA-reactive genotoxic compounds, with recommendations for further studies.

1. Introduction

The dogma, that DNA-reactive genotoxins exhibit a linear relationship between exposure and mutagenic response was investigated and discussed extensively in the Mutation Research Special Issue 464 on thresholds in radiation and chemical carcinogenesis [1]. At the time of publication in 2000, non-DNA-reactive genotoxins were already accepted as having threshold dose–response curves. Spindle poisons were one such class of non-DNA-reactive compound, and the mechanism involved multiple target deactivations being required, before aneuploidy was induced. Therefore, low levels of the compounds were tolerated [2–4]. Threshold mechanisms were also predicted but not proven for DNA-reactive genotoxins and we have recently shown that the two alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) possess non-linear dose–response curves for mutation [5]. The predicted mechanisms for the non-linear dose responses produced by these compounds are likely to involve DNA repair, where a low level of DNA damage is efficiently repaired by the cells. This was supported by a recent study that showed that MMS induced N7-methyl guanine (N7-meG) adducts at concentrations below the no observed effect level (NOEL) for mutation (1 μg/ml) in AHH-1 cells [6]. These low concentrations of MMS, whilst causing DNA adducts, did not cause a significant increase in gene mutations or chromosome mutations, as they were presumably repaired preventing widespread point mutation and chromosome damage [5]. Therefore, DNA adducts are a biomarker for exposure and an excellent tool for investigating mode of action however they do not always

* Corresponding author at: DNA Damage Group, Institute of Life Science, School of Medicine, Swansea University, Swansea, Wales SA2 8PP, UK. Tel.: +44 1792 295158.
E-mail address: g.johnson@swansea.ac.uk (G.E. Johnson).

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equate to the induction of point mutations or chromosome mutations.

In order to assess the dose–response of genotoxins, it is imperative that sensitive tests are employed and that the experiments are carried out with large numbers of replicates to achieve sufficient power to calculate the NOEL, LOEL and the inflection point. A NO(A)EL (no observed (adverse) effect level) is the highest dose that does not cause an adverse effect [7] and the lowest observed (adverse) effect level (LO(A)EL) is the lowest dose that causes an adverse effect. In our recent paper [5] we assessed the NOEL and LOEL doses for four alkylating agents MMS, MNU, EMS and ENU using simple statistical methods based on comparisons to the untreated controls. However, in this present publication we will analyse the data using more advanced statistical methods to assess the linear or threshold dose responses. For the purposes of this report a threshold is defined as the point below which there was no dose–response. A threshold has a characteristic called the inflection point which is the point where the change in gradient is at its maximum. This can be calculated by using the hockey stick model incorporated in the statistical analysis package described by Lutz and Lutz [8] in this special issue journal. If fitting of the more complex hockey stick model explains a greater proportion of the variation than the linear model, then it is a threshold dose–response with a defined inflection point. It is interesting to note that although the nitrosourea compounds ethyl nitrosourea (ENU) and methyl nitrosourea (MNU) were suspected to have linear dose responses [5] unexpected observations were found.

Detailed knowledge of the dose–response relationship of a given compound is paramount in the area of genetic toxicology. If a compound has a threshold, such as a mitotic spindle poison, then safe levels can be calculated [1–3,9–11]. If a compound does not have a threshold and gives a linear dose–response, then there are no safe exposure levels. This can have economic implications, as these compounds are heavily regulated and principles such as the threshold of toxicological concern (TTC) are used to minimise human exposure [12]. In mechanistic terms, the importance of mutation thresholds involves the key changes in biological effect, occurring in the cells (or tissues) when the threshold dose is reached. At concentrations below the inflection point the effect is within the endogenous level of mutation and the exposure is presumably tolerated through homeostatic mechanisms (e.g. DNA repair, metabolic deactivation, redundant targets) [13–15]. Obviously, in this part of the dose range it is important to first confirm that cellular exposure is occurring (usually through the measurement of protein or DNA adduct formation). However, above the inflection point the homeostatic mechanisms are 'saturated' and there is a change in biological response to the genotoxic compound. The mode of action is of great importance alongside the dose–response and we have been investigating both of these aspects for the genotoxic effects of MMS and EMS in mammalian cells (particularly the role of DNA repair). In this article we will analyse the Doak et al. [5] data further to see if the data fit the linear model or if fitting a more complex model (hockey stick or quadratic) gives a significantly better fit to the data.

2. Materials and methods

In this report, the raw data that were published in [5] were re-analysed using more advanced statistical approaches to assess whether the dose responses were truly thresholded or not. In brief, the hypoxanthine phosphoribosyl transferase (*HPRT*) and cytokinesis-block micronucleus (CBMN) assays were previously used to test for gene mutation and chromosomal damage in AHH-1 human lymphoblastoid cells after treatment with MMS, MNU, EMS and ENU. In that study we found there to be a NOEL and LOEL for both endpoints after treatment with MMS and EMS. However, ENU and MNU produced more linear responses.

2.1. Statistical methods

The dose–response curves were analysed using several statistical models and approaches. This involved fitting hockey stick, linear and quadratic non-linear models (Fig. 1). When non-linearity, suggestive of an inflection point, was established, further statistical analysis was used to determine the first statistically significant dose (LOEL).

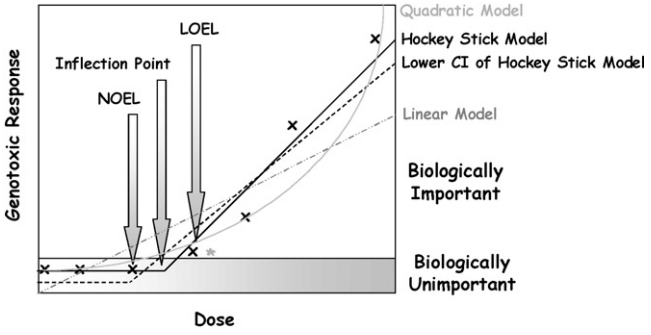

Fig. 1. Diagram of the different terminology used for statistical analysis and statistical modelling. Inflection point (IP) is the point at which the change in gradient is at its maximum; NOEL = no observed effect level; LOEL = lowest observable effect level; * = first statistically significant point.

The hockey stick modelling of apparent thresholds was carried out using software, kindly provided by Lutz and Lutz [8] and implemented in the R package. This method attempts to fit two straight line segments to the data. A line with zero slope at low dose is forced and then joined to a line with positive slope at higher dose. The junction between these two line segments is a point of inflection at which increasing dose begins to cause a measurable response. The change in gradient is at its maximum at this point of inflection. Confidence intervals are also calculated and the lower confidence interval for the inflection point is presented graphically as a dotted line in the output. Further parameters are also calculated, including the gradient of the line above the inflection point, the *Y*-intercept and the probability value for fitting the linear model to a greater degree than the hockey stick.

The fitting of linear and quadratic models was carried out using SPSS v. 13.0. A straight line (with two parameters, intercept and slope) was first fitted to the data. This was always significant, but not necessarily a good visual fit to the data. Then a quadratic (with additional parameter) was fitted. In both cases the variation in response explained statistically by dose was determined by the coefficient of determination (R^2). If the R^2 change value on fitting the quadratic was significant then the quadratic was deemed a significantly better fit to the data than the linear model. The inflection point of the quadratic can then be regarded as a threshold.

Further statistical analyses to determine LOEL was based on several methods. The first approach, Dunnett's test, is a widely used and well regarded analysis method for the *HPRT* and CBMN assay data. Dunnett's test was already carried out on *HPRT* and CBMN data for the four alkylating agents [5]. Dunnett's test is a multiple comparison post hoc method, performed after a one-way ANOVA by comparing all treatment data versus the control.

The second approach uses the ANOVA + *t*-test method designed and kindly provided by the BioStatistics Department at Covance Laboratories UK in 2008, for the analysis of linear vs. threshold dose responses. It takes the response below the inflection point into consideration and unlike the Dunnett's it is not affected when there is increased error at high concentrations. This method is carried out as follows. (1) The controls (Group 0) are compared with the lowest concentration (Group 1) using a one-sided two-sample *t*-test. (2) Groups 0 and 1 pooled are compared with the next highest concentration (Group 2) using a one-sided two-sample *t*-test. (3) The process from this point forward is to compare each concentration against the previous three pooled, after first testing for homogeneity of the means. For example, Groups 0, 1 and 2 are compared for homogeneity the means using one-way ANOVA. Group 3 is then compared with Groups 0, 1 and 2 combined together, using a one-sided two-sample *t*-test. The testing continues to higher doses, each time testing the high dose with the three preceding lower doses, when these do not show heterogeneity of means. If statistically significant differences ($p < 0.05$) are found in step 1 or 2, or in the homogeneity of means test, these differences are taken into account when later steps are interpreted. The process, however, moves onto the next step regardless of the statistical significance. A potential threshold value is defined as a concentration that produces a statistically significant difference from the preceding three concentrations, with all subsequent concentrations having the same or higher response. When such a potential threshold concentration is identified, the process of *t*-testing stops.

When these methods of analysis have been carried out the dose–response can categorised as being linear or as having a threshold.

3. Results and discussion

It is essential to have an objective manner to analyse dose–response data for genotoxic activity. Without such a systematic approach, we are left with subjective analysis of shapes of

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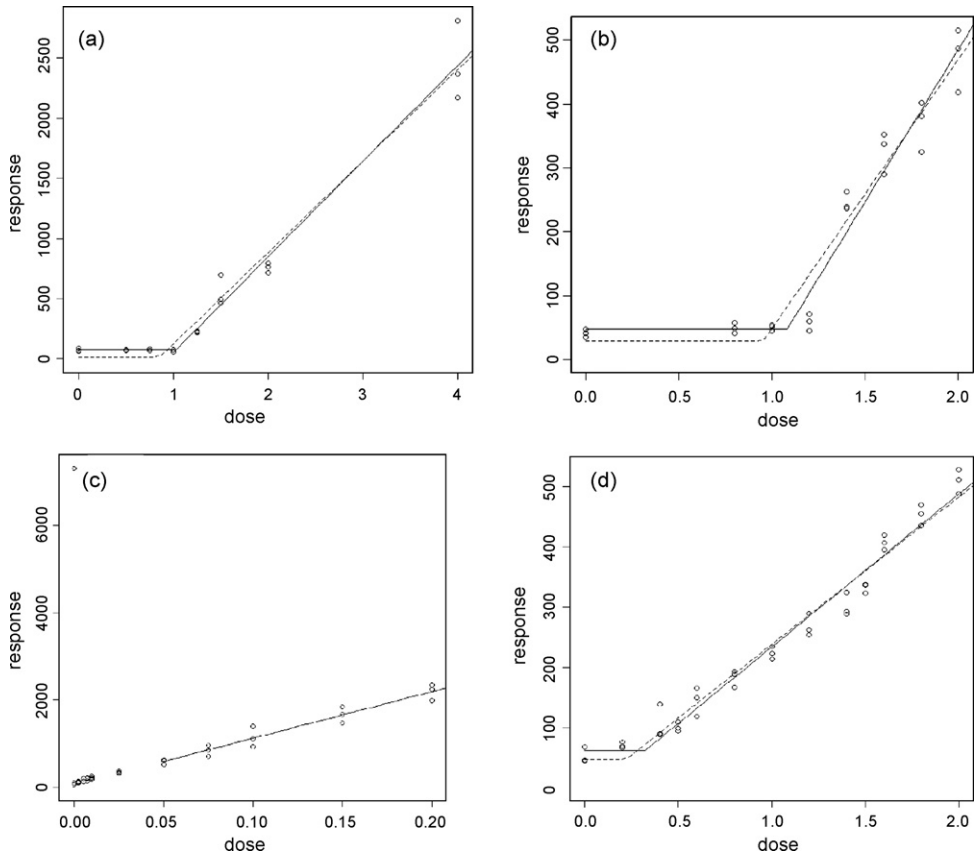

Fig. 2. HPRT forward mutation assay with hockey stick statistical modelling of MMS, EMS, MNU and ENU dose–response as presented in Doak et al. [5]. All values apart from the *p*-value are in μg/ml MMS. Inflection point (IP), probability for linearity (*p*) and *Y*-intercept were calculated using the hockey stick statistical modelling package kindly provided by Lutz and Lutz [8]. (a) MMS, threshold, IP = 1.03, lower IP/CI = 0.86, *Y* = 72.38, and *p* = 2.6e−08. (b) EMS, threshold, IP = 1.08, lower IP/CI = 0.95, *Y* = 46.78, and *p* = 6.0e−10. (c) MNU, linear, and *p* = 0.37. (d) ENU, threshold, IP = 0.32, lower IP/CI = 0.22, *Y* = 62.08, and *p* = 1.1e−04.

curves (sometimes by eye) which are not reliable. The objective approaches are also essential if we are to compare dose responses between experiments, between related chemicals and in mechanistic studies using cells differing in genetic backgrounds. Hence, we have compared several statistical approaches here to analyse the large data sets obtained by us previously for the alkylating agents ENU, MNU, EMS and MMS.

3.1. Linear regression and hockey stick model

In Fig. 2a for MMS in the *HPRT* assay, although there was a significant fit of the linear regression model to the data, the hockey stick model gave a significantly better fit ($p < 0.05$). Hockey stick modelling of the data also showed that MMS had an inflection point at 1.03 μg/ml with lower confidence interval (CI) of 0.86 μg/ml. This was also true for EMS in the *HPRT* assay (Fig. 2b) and the hockey stick modelling of the data showed EMS to have an inflection point at 1.08 μg/ml with lower CI of 0.95 μg/ml. A surprising finding was that the hockey stick gave a significantly better fit than the linear model ($p < 0.05$) for ENU in the *HPRT* assay data (Fig. 2d) whereas a linear response for ENU induced *HPRT* mutation had been previously assumed. ENU had an inflection point at 0.32 μg/ml with lower CI of 0.22 μg/ml. In Fig. 2c for MNU in the *HPRT* assay, the linear regression was significant ($p < 0.05$).

The situation was similar for both MMS (Fig. 3a) and EMS (Fig. 3b) in the CBMN assay, and although there was a significant fit of the linear regression model to the data, the hockey stick model gave a significantly better fit ($p < 0.05$) for %Mn/Bn. There were inflection points at 0.38 μg/ml (lower CI of 0.14 μg/ml) for MMS and 1.06 μg/ml (lower CI of 0.87 μg/ml) for EMS. As predicted, MNU (Fig. 3c) and ENU (Fig. 3d) were both significant ($p < 0.05$) for the linear regression for %Mn/Bn.

3.2. Linear regression and quadratic model

Table 1 shows the linear vs. quadratic probability (*p*) values calculated for EMS and MMS along with the other statistical tests. For EMS in both the *HPRT* and CBMN assays the quadratic had a significantly better fit than the linear model ($p < 0.05$). For MMS only the *HPRT* assay showed the quadratic model to have a significantly better fit when compared to the linear model ($p < 0.05$). However, for MMS in the *HPRT* assay the quadratic model did not explain a significant additional proportion of the variation in response above that explained statistically by the linear model. Nevertheless, as previously stated for MMS in the *HPRT* assay, the hockey stick model gave a significantly better fit. Therefore, for EMS and MMS in the CBMN and *HPRT* assays, fitting of the more complicated models (hockey stick or quadratic) explained a greater proportion of the variation than the linear model.

Table 2 shows the linear vs. quadratic *p*-values calculated for ENU and MNU along with the other statistical tests. For MNU in the *HPRT* and CBMN assays, the linear had a significantly better fit than the quadratic model. For ENU in the CBMN assay the linear also had a significantly better fit than the quadratic model. However, for ENU in the *HPRT* assay the quadratic had a significantly better fit than the linear model, as did the previous hockey stick model. Therefore, the main point of interest was that for ENU the response was significant for the linear model but fitting a more complex model (hockey stick or quadratic) explained a significantly greater proportion of the

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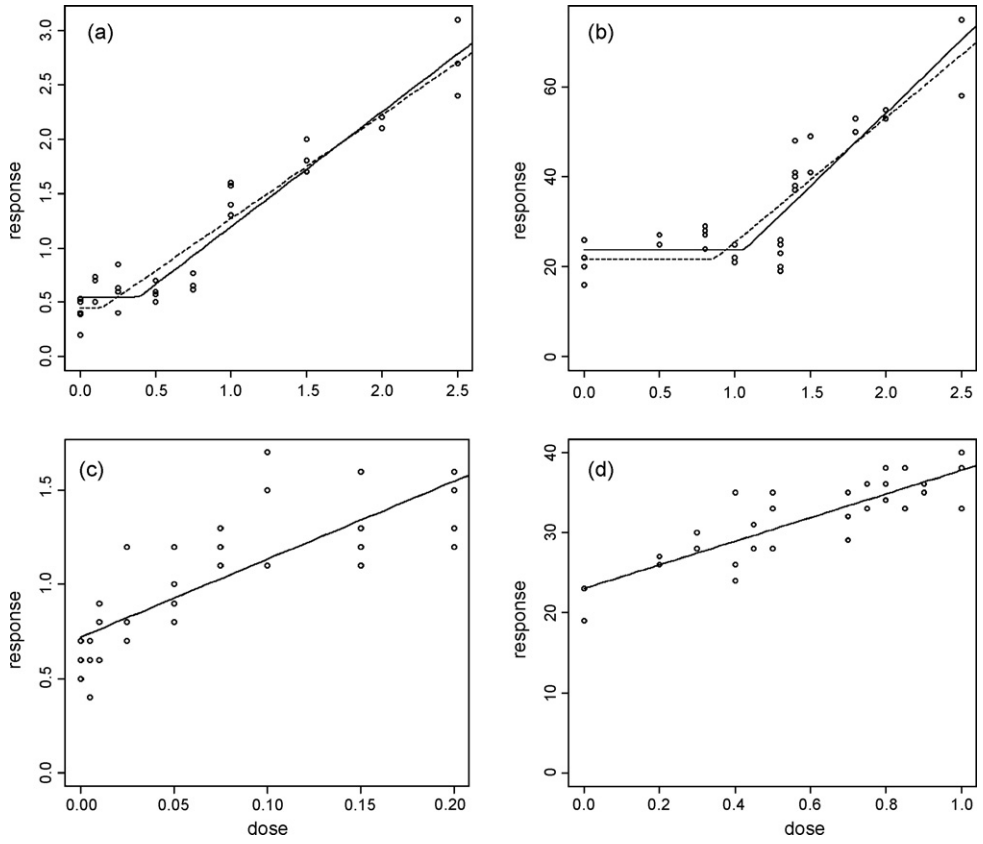

Fig. 3. CBMN assay of hockey stick statistical modelling of MMS, EMS, MNU and ENU as presented in Doak et al. [5]. Inflection point (IP), probability for linearity (p) and Y-intercept were calculated using the hockey stick statistical modelling package kindly provided Lutz and Lutz [8]. (a) MMS, threshold, IP = 0.38, lower IP/CI = 0.14, Y = 0.54, and p = 0.02. (b) EMS, threshold, IP = 1.06, lower IP/CI = 0.87, Y = 23.79, and p = 2.0e−05. (c) MNU, linear, and p = 1. (d) ENU, linear, and p = 1.

Table 1
EMS and MMS treatment with CBMN and HPRT assays—Dunnett's and ANOVA + t-test and statistical modelling comparing hockey stick versus linear model and comparing quadratic versus linear model. Dunnett's test and ANOVA + t-test used to determine the NOEL and LOEL. THRESHOLD, threshold model fits significantly better than linear model. LINEAR, linear best fit model. Quadratic, quadratic model fits significantly better than linear model.

	Dunnett's		ANOVA + t-test		Linear model	Hockey stick (threshold) or linear		Quadratic or linear	
	NOEL (μg/ml)	LOEL (μg/ml)	NOEL (μg/ml)	LOEL (μg/ml)	p	Inflection point (μg/ml)	p		p
EMS									
CBMN	1.3	1.4	1.3	1.4	1.93E−10	1.06 THRESHOLD	2.00E−05	Quadratic	1.80E−04
HPRT	1.2	1.4	1	1.2	2.08E−07	1.08 THRESHOLD	6.00E−10	Quadratic	4.30E−08
MMS									
CBMN	0.8	0.85	0.8	0.85	1.83E−15	0.38 THRESHOLD	0.02	LINEAR	
HPRT	1	1.25	1	1.25	1.51E−12	1.03 THRESHOLD	2.00E−08	Quadratic	7.50E−07

variation. Further experiments and statistical modelling may show that there is a threshold dose–response for ENU for *HPRT* mutation. Examining lower doses of ENU and MNU may yield interesting data in terms of dose–response thresholds. Given the argument that DNA repair can impose a genotoxic threshold, it is plausible that, at low enough doses, there may be genotoxic thresholds for both nitrosoureas. However, if ENU is thresholded and MNU is not, then this may be due to the lower reactivity of ENU and less O^6-alkylG lesions produced, and therefore more chance of O^6-methyl guanine methyl transferase (MGMT/AGT) repairing them.

Table 2
ENU and MNU treatment with CBMN and HPRT assays—Dunnett's and ANOVA + t-test and statistical modelling comparing hockey stick versus linear model and comparing quadratic versus linear model. Dunnett's test and ANOVA + t-test used to determine the NOEL and LOEL. THRESHOLD, threshold model fits significantly better than linear model. LINEAR, linear best fit model. Quadratic, quadratic model fits significantly better than linear model.

	Dunnett's		ANOVA + t-test		Linear model	Hockey stick (threshold) or linear		Quadratic or linear	
	NOEL (μg/ml)	LOEL (μg/ml)	NOEL (μg/ml)	LOEL (μg/ml)	p	Inflection point (μg/ml)	p		p
ENU									
CBMN	0.45	0.5	LINEAR		1.09E−08	LINEAR		LINEAR	
HPRT	0.2	0.4	LINEAR		5.52E−28	0.32 THRESHOLD	1.10E−04	Quadratic	3.5E−08
MNU									
CBMN	0.1	0.15	LINEAR		5.53E−13	LINEAR		LINEAR	
HPRT	0.005	0.0075	LINEAR		5.61E−28	LINEAR		LINEAR	

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Once the dose–response was determined using the linear regression and model fitting, the next step was the statistical analysis using the two different methods (Dunnett's and ANOVA + *t*-test) to determine the LOEL and the NOEL for EMS and MMS. They both gave similar values (Table 1). However, there was a difference between the NOEL and LOEL values for EMS in the *HPRT* assay when using the ANOVA + *t*-test compared to the Dunnett's. This could be because the ANOVA + *t*-test is not affected by events in the data set at higher concentrations such as increased error, as it only includes the concentrations below and including the one being tested, and does not take the whole dose–response into consideration. It should be noted from Table 1 that the inflection point as calculated from the hockey-stick software falls outside the LOEL to NOEL range, as determined from the two statistical approaches. One might expect that the inflection point would sit within the NOEL and LOEL values however they are different analyses and they may produce different results. Both the ANOVA + *t*-test and the Dunnett's approaches proved to be good approaches but it is useful to have this extra validation for the linear regression results, and the ANOVA + *t*-test provides this. However, when using the ANOVA + *t*-test to analyse ENU in the *HPRT* assay, this test indicates a linear dose–response which is not in line with the linear regression where both the hockey stick and quadratic models gave a better fit for the data. Consequently, there is not a clearly defined threshold for ENU in the *HPRT* assay at the concentrations tested. This highlights the fact that linear regression should be carried out before further statistical analysis is carried out. We also suggest that ANOVA + *t*-test might give a non-linear dose–response if at least two more concentrations were tested below 0.2 μg/ml ENU in the *HPRT* assay. Non-linear dose responses with clear NOEL, LOEL and inflection points were defined for EMS and MMS in both the *HPRT* and CBMN assays using these statistical analyses. Moreover, the inflection points are now considered as the threshold values for EMS and MMS, as a measure of gene mutation and chromosome mutation, respectively, *in vitro*.

At the current time *in vitro* data can only be used to determine a yes or no answer when talking about thresholds and *in vivo* data is required to produce concentrations that can be used for hazard and risk assessment as they are more relevant to the human system [16–18]. Until this special issue journal was published, there was not any suitable data to show that any given DNA-reactive genotoxic compound had a threshold both *in vitro* and *in vivo*. However, Gocke and Müller will present the findings of their work in this special issue along with a further publication [19–22]. In this extensive project they investigated EMS by using the MutaMouse™ model and the bone marrow micronucleus assay (in Crl:CD-1 mice), and found a threshold at both the gene and chromosome mutation endpoints, respectively, along with much other supporting data.

Since we now know that genotoxic thresholds can exist for some genotoxins, determining the lowest doses that do not affect background mutation levels (the NOEL) are essential and represent an important area in the field of genotoxicology. However, in order to ascertain if thresholds exist for specific genotoxins it is recommended that the experiments make note of the following:

- Sensitive and robust genotoxicity tests are required that allow large numbers of individual cells to be analysed.
- A sufficient number of replicates are required to give a data set with enough power.
- Doses need to be close enough together to produce a clear distinguished dose–response.
- Suitable statistical modelling followed by statistical analysis carried out on the data.
- The background level in each test is within the historical background for each laboratory.
- A sufficient level of genotyping is required for each cell line that is used, especially with studies into specific mechanisms of action.

From the statistical testing that was carried out on these data, we think that the best statistical approach is to test each dose–response for the best fit using linear vs. non-linear model (hockey stick and quadratic). Once the response has been shown to be linear or non-linear then the statistical analysis can be used and the ANOVA + *t*-test would appear to be most suitable for a wider range of data sets, including ones where there is large variation at the higher dose levels, compared to the Dunnett's test. All of the current data analyses show that the methylsulfonates MMS and EMS, have thresholds for chromosome mutation and gene mutations *in vitro* in human AHH-1 cells, which is in line with our previous statements [5]. However, the linear regression analysis also showed that ENU had a threshold for gene mutations, which was not in line with our previous statements. This could mean that if lower concentrations of the nitrosoureas ENU and MNU were tested then we may find that they also have thresholds for gene and point mutations *in vitro*. Our current view is that thresholds of DNA-reactive genotoxic activity should be proven for each agent, on an individual (case-by-case) basis. This may change when we have data for compounds with similar modes of action (MOA).

Conflict of interest

The authors declare that there are no conflicts of interest.

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