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Investigation of J-shaped dose-responses induced by exposure to the alkylating agent *N*-methyl-*N*-nitrosourea

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Highlights

- A J-shaped dose-response was observed following *N*-methyl-*N*-nitrosourea treatment
- Mechanistic studies indicated a possible role of p53
- Hormesis is rare in Swansea University genotoxicity datasets generated since 2004
- Hormesis is, therefore, likely to be infrequent for genotoxic agents

Abstract

Hormesis is defined as a biphasic dose-response where biological effects of low doses of a stressor demonstrate the opposite effect to high-dose effects of the same stressor. Hormetic, or J-shaped, dose-response relationships are relatively rarely observed in toxicology, resulting in a limited understanding and even some skepticism of the concept. Low dose-response studies for genotoxicity endpoints have been performed at Swansea University for over a decade. However, no statistically significant decreases below control genotoxicity levels have been detected until recently. A hormetic-style dose-response following a 24h exposure to the alkylating agent *N*-methyl-*N*-nitrosourea (MNU) was observed in a previous study for HPRT mutagenesis in the human lymphoblastoid cell line AHH-1. A second recent study demonstrated a J-shaped dose-response for the induction of micronuclei by MNU in a 24h treatment in a similar test system. Following mechanistic investigations, it was hypothesized that p53 may be responsible for the observed hormetic phenomenon. As genotoxic carcinogens are a major causative factor of many cancers, consideration of hormesis in carcinogenesis could be important in safety assessment. The data examined here offer possible insights into hormesis, including its estimated prevalence, underlying mechanisms and lack of generalizability.

Keywords: Hormesis, adaptive response, genotoxicology, alkylating agents, low-dose, carcinogens

1. Introduction

1.1 Hormesis and genetic toxicology

In biological systems, it is sometimes observed that the frequency of adverse events decreases below the background frequency at low doses before it starts to increase [1, 2]. As the dose-response contains both sub-background damage and toxic phases, it conforms to a "biphasic" trend, producing a J- or U-shaped, or inverted J- or U-shaped, dose-response curve [3][4]. This form of dose-response is frequently referred to as the biological phenomenon "hormesis" if this follows a single exposure to a toxin, toxicant or radiation (**Figure 1**). If at least one exposure to an agent induces a protective effect against a subsequent larger exposure, this would be described as an "adaptive response". Although hormesis and adaptive responses demonstrate some overlap, it is important to recognize these as distinct phenomena. Such responses of a cell or organism to a low dose of toxic agent is considered to be an adaptive compensatory process following an initial disruption in homeostasis [4].

Hormesis and adaptive responses are concepts shrouded in controversy and confusion[5]. There is an ongoing debate on the probability of hormesis existing. Some proponents claim that hormesis has been ignored by the scientific community, due to bias towards a linear no threshold (LNT) dose-response model [6], which has been favoured in genotoxicology, or to a threshold model in other areas of toxicology. As well as debates over whether hormesis and adaptive responses exist, there is the issue of whether these are generalisable phenomena, partly owing to their relative infrequency in genotoxicological datasets. To be generalisable, such phenomena should occur at frequencies approaching universality, being observed with all or most organisms, agents, endpoints and genotypes [7]. Therefore, a key aspect of determining whether hormesis is generalisable in genetic toxicology is whether it occurs in all or in the vast majority of dose-responses, or whether its occurrence is more limited [7]. However, based on current dose-responses, hormetic-style responses are in fact in the minority [8][9-11] and it also appears that no universal mechanism exists for hormesis, perhaps preventing it from being adopted in toxicological risk assessment [12]. However, the mechanisms underlying hormesis have previously been hypothesized and speculated upon [13]. The fact that previous studies were not aimed at the low-dose region means that it is unsurprising that hormetic effects were rarely observed. Whether current study designs are not optimized to capture a transient hormetic effect, whether few studies use low doses, or whether hormesis is truly not generalisable is unclear.

1.2 Genotoxicity dose-response data generated at Swansea University

J-shaped dose-responses have been observed relatively recently in our laboratory at Swansea University. Thomas *et al.* reported the first of these in 2013 for HPRT mutant frequency in the human lymphoblastoid cell line AHH-1 (**Table 1**). In their study, a

statistically significant decrease in mutant frequency below the vehicle control level was observed for two low concentrations of MNU, 0.005 and 0.0075 μg/ml. The magnitude of the reduction relative to vehicle control was 45%. The second of these J-shaped dose-responses will be discussed in **Results**. Prior to these findings, no such J-shaped dose-responses had been identified. For over a decade, the effects of low concentrations of chemical agents on *in vitro* cell-based test systems have been studied within our laboratory (**Table 1**), which has extensive datasets from genetic toxicology testing conducted with a high standard of quality control. The use of low concentrations minimizes possible artefactual reductions in genotoxicity due to confounding cytotoxicity, and these conditions are therefore likely to facilitate the observation of hormesis should it occur. Both linear and non-linear (threshold) dose-responses have been observed thus far at Swansea University, yet only one previously published study to date has produced a J-shaped dose-response [14]. The data contain a large number of experiments performed using the same facilities, equipment and laboratory protocols, facilitating direct comparisons between different chemical dose-responses. Furthermore, similar chemicals, exposure times, assays and cell types were often used across the different studies. For example, the majority of studies used MCL-5, AHH-1 or TK6, which are all human lymphoblastoid cell lines, with MCL-5 even being derived from AHH-1. The collection of datasets therefore offers novel insights into the prevalence of hormesis in genetic toxicology and the conditions required for its occurrence. This paper explores hormesis further by presenting new data for another J-shaped dose-response and considering this finding in relation to previous datasets for the low-dose region. The study could also provide further information on whether hormesis is likely to be a generalizable phenomenon.

2. Materials and methods

2.1 Test chemical. *N*-methyl-*N*-nitrosourea (MNU) (CAS Number: 684-93-5; molecular weight: 103.08; purity: 66%) was purchased from Sigma-Aldrich (Dorset, UK) and stored according to the manufacturer's instructions (4ºC, sealed). The relatively low purity of 66% was associated with the inherent instability of MNU, which required the "impurities" acetic acid (2.3%) and H_2O for its stabilisation. The presence of these compounds was not deemed to have any effects on genotoxicity or hormesis, due to their extremely low concentrations following subsequent dilutions of the test chemical stock. MNU, of which 1g (not including impurities) was provided by the manufacturer, was diluted in DMSO (Fisher Scientific), as per the methods followed in a previous study using MNU {Thomas, 2013 #15}. Dilutions from a master stock were made immediately before use and stored in the dark at 4ºC for approximately 5 min until use. It was ensured that the solid was fully dissolved prior to further dilution of the stock solution, to prevent underestimation of toxic effects. When handling MNU, safety precautions, including arm length gloves and protective clothing, were implemented at all times.

2.2 Cell culture. The human lymphoblastoid cell lines TK6 and NH32 were cultured in RPMI 1640 medium (Life Technologies) supplemented with 1% L-glutamine (Life Technologies) and 10% donor horse serum (BDGentest, Oxford). The cells were maintained in culture at densities between $1x10^5$ and $1x10^6$ cells/ml. TK6 cells were obtained from the Health Protection Agency Culture Collections, UK. NH32 cells were a kind gift from Professor Gerald Wogan, Massachusetts Institute of Technology, USA.

2.3 *In Vitro* Micronucleus Assay. Please refer to Chapman *et al.*[31] (2015) for the method followed.

2.4 RNA isolation and quantitative real time-PCR. Real-time PCR was used to investigate relative mRNA expression levels for methylpurine-DNA glycosylase (MPG) in response to 24h exposure to MNU in TK6 cells. Changes might indicate whether MPG, a DNA repair glycosylase targeting methylation at N^7-guanine sites, could have a notable impact on the observed dose-response. RNA was extracted from treated cell samples using RNeasy Mini Kit (Qiagen) and RNase-free DNase I Set (Qiagen) using the recommended protocols and for various treatment time points and concentrations. Synthesis of cDNA from RNA was completed using Quantitect Reverse Transcription Kit (Qiagen). qRT-PCR was performed using these samples, using Quantifast SYBR Green I (Qiagen) and appropriately designed and optimised primers. **Primer nucleotide sequences:** *MPG* forward: 5' GGTCCGAGTCCCACGAAGCC 3'; MPG reverse: 5' CTGCATGACCTGGGCCCCG 3'; ß-actin forward: 5' GATGGCCACGGCTGCTTC 3'; ß-actin (*ACTB*) reverse: 5' TGCCTCAGGGCAGCGGAA 3'. A BioRad iCycler was used to perform the real-time PCR and analysis using a standard curve was completed using BioRad iQ5 software.

2.5 Protein isolation and immunoblotting. To investigate MPG protein levels in TK6 cells, protein isolation and subsequent immunoblotting was used. This was to observe whether MPG expression was altered at the protein level without being altered at the transcription (in this case, mRNA) level. TK6 cell suspensions treated for 24h with MNU within the hormetic dose range were centrifuged at 250x*g* for 7min and washed twice in 4ºC phosphate-buffered saline (PBS) (Gibco). Cells were lysed at 4ºC using 1x radioimmuno-precipitation lysis buffer (RIPA) (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and kept on ice for 5 min prior to vortexing, followed by centrifugation at 9,300 x*g* for 10 min at 4°C. Protein concentration was determined using the DC quantification assay (Biorad). Proteins (40µg) were mixed at a 1:1 ratio with 1x Laemmli buffer (Sigma-Aldrich) and resolved on a 12% SDS polyacrylamide gel. Proteins were then electroblotted onto Immun-Blot PVDF membranes (Biorad) and blocked for 1h with 1x Tris-buffered saline-Tween 20 containing bovine serum albumin (BSA) (5% solution) (Sigma-Aldrich). Membranes were separated and probed at 4°C overnight with MPG antibody (1:1000 dilution; M6195; Sigma-Aldrich) diluted in 5% BSA. After four washes with 1x Tris-buffered saline-Tween 20 containing 5% BSA, the membrane was incubated for 1h in rabbit anti-mouse secondary antibody (1:10,000 dilution; ab6728-1, Abcam). Following this, a further three washes with 1x TBS-Tween 20 containing 5% BSA were performed. To correct for protein loading differences, blots were probed with mouse antibody to ß-actin (ab8226-100, abcam), followed by rabbit anti-mouse secondary antibody. Immun-Star WesternC Chemiluminescence Kit (Biorad) was used for the immunodetection of proteins. Band densitometry was determined using the Quantity One software (Biorad).

2.6 O^6-benzylguanine as an inhibitor of O^6-methylguanine DNA-methyltransferase (MGMT). To investigate whether the DNA repair enzyme MGMT is responsible for the hormetic dose-response, O^6-benzylguanine (O^6BG) (Sigma-Aldrich) was used as a MGMT inhibitor. O^6BG in powder form was dissolved in methanol, and cells were treated with 10µM O^6BG for 1h prior to dosing. Appropriate controls were included, i.e., methanol control, DMSO control, untreated control and O^6BG only control.

2.7 Statistical analysis. At least three biological replicates were completed for all experiments (except where indicated): replicates were performed on separate days with separate vials of cells and test chemicals. “n” refers to the number of these individual biological replicates. The mean values of these replicates are presented on the graphs, with error bars referring to standard deviation between replicates. Data were log-transformed and assessed using the Levene statistic as to whether these conformed to a normal distribution. A one-way ANOVA with Dunnett’s 2-sided post hoc analysis was then performed to identify the first statistically significant increase or decrease relative to background MN levels (i.e., lowest observed effect level (LOEL)). The Dunnett’s test was selected because it permits multiple comparisons between treatments. Using the 2-sided Dunnett’s test is appropriate for the detection of both increases in frequency for genotoxicity relative to the vehicle control, and possible hormesis at low doses.

3. Results

3.1 *MNU induced a J-shaped dose-response for two genotoxicity endpoints*

J-shaped dose-responses are relatively infrequent in genetic toxicology, and it was noteworthy, therefore, that our laboratory has identified two hormetic dose-response curves on separate occasions for two different endpoints following a 24h treatment in human lymphoblastoid cell lines with the same chemical: *N*-methyl-*N*-nitrosourea (MNU).

The second J-shaped dose-response observed for MNU is presented here for the first time (**Figure 2A**), with the raw data in tabular form (**Appendix A**). The full dose-response for the higher dose region is included in a previous publication, with a LOEL at 0.46 µg/ml and a clear dose-dependent mutagenic effect at higher doses [31]. The full dose-response up to 1.1 µg/ml is also included here in a different form (**Appendix B**) for the purpose of illustrating that the hormetic dose belongs to a broader “J-shaped” dose-response. Rather than a frequency of gene mutations (HPRT), as in the study of Thomas *et al.*[14], this study measured the frequency of micronuclei, indicative of a clastogenic effect in mononucleated cells. The statistically significant decrease in DNA damage occurred at (0.009 µg/ml), similar to the study by Thomas *et al.* (2013). The magnitude of the reduction in micronucleus frequency relative to the vehicle control was 55%.

Further investigations were conducted to determine the mechanistic basis of the reduction below control levels for micronucleus frequency (**Figure 2A**). The induction of micronuclei in the p53-deficient cell line, NH32, was assessed at the same dose range as in TK6 to investigate whether functional p53 might be involved in the J-shaped dose-response. Unlike TK6, NH32 cells produced no significant decrease below control damage levels and therefore no “hormetic” dose-response (**Figure 2A**). NH32 cells have an approximately 1.8x greater background micronucleus frequency than TK6, these being 1.23% and 0.67%, respectively in **Figure 2A**.

Two DNA repair proteins were studied to determine their involvement in the J-shaped dose-response. *N*-methylpurine DNA glycosylase (MPG) is involved in identifying alkylated bases, such as N^7-methylguanine[34], and subsequent initiation of the base

excision repair (BER) pathway due to abasic site formation[35]. MPG has previously been identified as being responsible for the threshold dose-response induced by alkylating agent ethyl methanesulfonate [24]. qRT-PCR and Western blotting were employed to investigate relative MPG mRNA and protein abundance, respectively, to study gene expression. MPG mRNA concentrations did not produce a statistically significant change; however, there was an increase of 1.5-fold observed at 0.009 μg/ml, prior to a 0.5-fold reduction below control levels at 0.114 μg/ml (**Figure 2D**). Similarly, MPG protein abundance (**Figure 2C**) remained unchanged across the dose range, including the 0.009 μg/ml treatment.

As Thomas *et al.* identified the involvement of MGMT in the J-shaped dose-response, the MGMT-inhibitor O^6-benzylguanine (O^6BG) was used in TK6 cells to determine whether DNA repair via MGMT might contribute to the hormetic curve presented in **Figure 2A**. MGMT performs direct repair of O^6-methylguanine DNA adducts [36], which are typically induced by alkylating agents that target oxygen sites. No statistically significant difference was observed between cells with normal and inhibited MGMT (**Figure 2D**). Interestingly, while a decrease relative to control was observed at 0.009 μg/ml in cells not treated with the inhibitor, this was not statistically significant, in contrast to the dose-response in **Figure 2A**.

4. Discussion

The aim of this study was to further explore the biphasic dose-responses observed in genotoxicity studies within our laboratory, including the underlying mechanistic basis of such phenomena. The primary data presented here focused on a single chemical, MNU, which was found to induce two separate biphasic dose-responses for two different genotoxicity endpoints.

4.1 J-shaped dose-responses are observed infrequently in our laboratory

Following this study, a total of two J-shaped dose-responses were identified among the 137 datasets considered here. In over a decade of the low-dose region being specifically studied in this laboratory, the fact that these were the first dose-responses to exhibit hormesis was intriguing, especially since they occurred for the same genotoxic agent for two different endpoints in studies of comparable design. In many cases, hormesis may be missed due to a lack of focus on the low-dose region and a lack of statistical power of the studies undertaken; this was not the case with our studies. It was noted that only one concentration (**Figure 2A, B**) of 0.009 μg/ml produced a significant decrease corresponding with hormesis. However, due to the high level of statistical significance ($p < 0.01$) and reproducibility of this result, we can be confident that this is a true biological effect. These reasons also imply that the performance of three replicates is sufficient. Indeed, it is possible that other doses within the region of 0.009 μg/ml may have been hormetic; for example, a small decrease was observed at 0.011 μg/ml (**Appendix A**). However, as this was not statistically significant, this dose cannot be concluded to be hormetic.

The HPRT assay study of Thomas *et al.*[14] produced the statistically significant reduction in mutation levels below vehicle control at concentrations in the region of 0.009 μg/ml, as was observed in the present study (**Figure 2**). The fact that similar hormetic dose ranges were identified makes a case for hormesis more convincing in both instances. However, previous studies that used a similar dose range of MNU did not produce a J-shaped dose-response. A study by Doak *et al.* in 2007 included a linear dose-response for MNU in AHH-1 cells for micronucleus induction in binucleated cells and HPRT mutant frequency [17]. In contrast to the data presented in **Figure 2**, Doak *et al.* used a different cell line, AHH-1, and also the cytokinesis-blocked version of the assay involving cytochalasin B as a cytokinesis inhibitor. Prior to Thomas *et al.*[14], Doak *et al.* [17] also performed the HPRT experiment with MNU using the same protocol and a similar dose-range, yet 0.0075 μg/ml was found to be the LOEL, rather than a "hormetic" dose as Thomas *et al.* observed. It is unclear why the two studies produced different outcomes when the same concentrations, endpoints and cell lines were used. It is possible that minor technical differences could be responsible. For example, the Doak[17] and Thomas[14] studies were performed several years apart and undoubtedly used different batches or ages of test chemical.

Only the acute, 24h dosing revealed hormetic effects for MNU, whereas the chronic dosing for both MNU and MMS did not give evidence for hormesis [31]. Originally, it was hypothesized that if hormesis were to be identified it would more likely be observed following the chronic dosing. It is possible that this relates to a narrow dose window and specific temporal conditions being required for observation of a hormetic effect.

Due to the abundance of published data that centered on the low-dose region (**Table 1**), this study presents an opportunity to estimate the prevalence of J-shaped (i.e., biphasic) dose-responses for experiments performed in the same laboratory using similar protocols. Based on this literature, J-shaped dose-response curves were found to occur infrequently (2 cases in 137 dose-responses, a 1.4% frequency). This very low frequency of biphasic dose-responses suggests that hormesis itself is not a generalisable phenomenon, as it does not occur for all endpoints, cell types and chemicals tested in systems that have been shown to identify J-shaped curves. Chemical specificity also appears to be an important factor, as only MNU thus far has exhibited a hormetic-like effect. Hormesis could be assay-, endpoint- or cell-specific, and different conditions may be required for hormesis in the different cases. If so, this also argues that it is not broadly generalisable.

While there are similarities between hormesis occurring after exposure to an array of individual doses and adaptive responses occurring after sequential treatments, the two should not be conflated. **Appendix C** presents data relating to the possibility of an adaptive response resulting from a pre-treatment with genotoxic agent prior to a subsequent larger concentration. To extend the present study, the "hormetic" dose of MNU, 0.009μg/ml, was employed as a priming dose. A reduction in the genotoxic effect of a high dose of MNU was not observed (**Appendix C**) and therefore provided no evidence of an adaptive response when a dose that had been shown to induce hormesis (**Figure 2**) was used as a priming dose prior to a larger "challenge" dose.

Statistical power is a factor in the ability to detect hormesis and adaptive responses. Statistical power will increase with a greater magnitude of biological effects. In priming dose experiments, therefore, the reduction from the more potent treatment level is relatively easily measured. However, a chronic study is likely to involve comparisons between smaller exposures, meaning a reduction effect is more difficult to detect within a dataset of the same size. That hormesis and adaptive responses should be regarded as distinct processes, rather than manifestations of a single process, is also reflected in studies in yeast, where a dose of hydrogen peroxide that induces a clear adaptive response to a subsequent high-dose challenge does not induce hormesis in the original sense of a biphasic curve from single exposures to each dose[37]. Thus, one may see hormetic treatments that do not induce an adaptive response, and one may see an adaptive response with no concomitant hormesis.

The potential merit in identifying the hormetic dose range relative to the toxic dose range extends to the possible use of hormesis in risk assessment. However, this would require full dose-responses with multiple doses both in the "hormetic" and "toxic" regions. Given the lack of generalisability of hormesis, as was reflected in the datasets analysed in this study (**Table 1**) and other sources [7,9,38,60], hormesis-based risk-assessment remains an unrealistic aim.

4.2 p53 status may contribute to the J-shaped dose-response

The mechanisms underlying the J-shaped dose-response observed in **Figure 2** were investigated using several different experimental approaches. The first of these involved a comparison of p53-null NH32 cells to isogenic, p53-competent TK6 cells. The results suggested that functional p53 might be responsible for the J-shaped dose-response for micronucleus frequency observed in the latter cell line. Previous publications have also identified the involvement of p53 in genotoxicity dose-responses for TK6 cells [28, 31]. It is unclear whether p53 was responsible for Thomas *et al.*'s dose-response; unlike TK6 cells, AHH-1 cells are heterozygous for p53 function due to a base pair substitution at codon 282, with consequences including loss of the G1 checkpoint and delayed apoptotic cell death[37]. Indeed, more efficient repair of apurinic/apyrimidic sites by MPG in wild-type p53 cells than mutant p53 cells[38] may explain why a different mechanism may be responsible for Thomas *et al.*'s J-shaped dose-response. In relation to this, MPG protein expression (**Figure 2C**) remained unchanged at the present study's hormetic dose, although a minor, non-significant increase in mRNA, which corresponded with the hormetic dose, was observed (**Figure 2B**). The fact that MPG expression was essentially unchanged does not rule out a role in the hormetic dose-response, as MPG is known to be a selective regulator of p53[38]. In unstressed cells, MPG binds to p53 and represses its activity [38]. Perhaps the 0.009 μg/ml concentration of MNU was sufficient to dissociate p53 and MPG without being sufficient to up-regulate MPG expression.

In a similar mechanistic analysis, MGMT activity was not observed to change in the hormetic dose range, suggesting that it was not responsible for the TK6 J-shaped dose-response curve in **Figure 2A**. Heterogeneity in efficiency of inhibition by O^6BG across a cell population has been suggested as a source of high statistical variability in MGMT- samples[14], and this may mask any effect of MGMT. This may also be due to the cell line, TK6, expressing MGMT at non-detectable levels[39]. As there is limited evidence linking MGMT and p53 in human cells [19], this perhaps indicates that MGMT operates independently of p53, as Thomas *et al.* found that MGMT appeared to be responsible for the J-shaped dose-response in AHH-1 cells.

DNA repair mechanisms have been implicated in genotoxin-induced hormesis in a variety of systems [14, 40-43]. Indeed, MGMT has been hypothesized to be responsible for hormetic dose-responses following exposure to alkylating agents [44, 45]. As MGMT is an inducible enzyme and repairs the highly mutagenic O^6-methylguanine lesion, induction of MGMT would readily explain the reduction in mutant frequency observed by Thomas *et al*. It is acknowledged that there may be additional underlying mechanisms for hormesis, which include but are not limited to alterations in apoptosis [46, 47], [48], [49], [50] cell proliferation [42, 51, 52] and antioxidant capacity [53].[54, 55],[56],[57],[25].

4.3 Conclusions

The mechanisms responsible for J-shaped curves for low-dose regions and genotoxicity endpoints were further explored. New MNU data were presented, with p53 predicted to partly explain the dose-response. J-shaped curves were found to occur at very low frequency in the literature generated at Swansea University, suggesting that hormesis is unlikely to be generalisable across different endpoints, cell types and test chemicals. This fact argues that hormesis cannot serve as a default assumption in risk assessment. While hormesis-based risk assessment is not feasible, an awareness of hormesis contributes to the broad-based understanding of dose-response relationships.

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Conflicts of interest

There are no conflicts of interest to declare.

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Appendix A. Raw data for the micronucleus dose-response presented in **Figure 2A**. Only 0.009 µg/ml produced a statistically significant decrease compared to the DMSO-treated vehicle control, denoted by ** (i.e., p=0.003).

MNU (µg/ml)	Data points for individual replicates		
0	0.679	0.861	0.485
	0.593	0.670	0.683
	0.928	1.025	0.977
	0.620	0.572	0.364
0.002	0.544	0.590	0.492
0.005	0.772	0.660	0.585
	0.475	0.502	0.244
0.009 **	0.357	0.310	0.220
0.011	0.455	0.389	-
0.018	0.878	0.658	0.390
0.023	0.744	0.750	0.561
0.034	0.609	0.455	0.562
0.045	1.206	0.319	0.440

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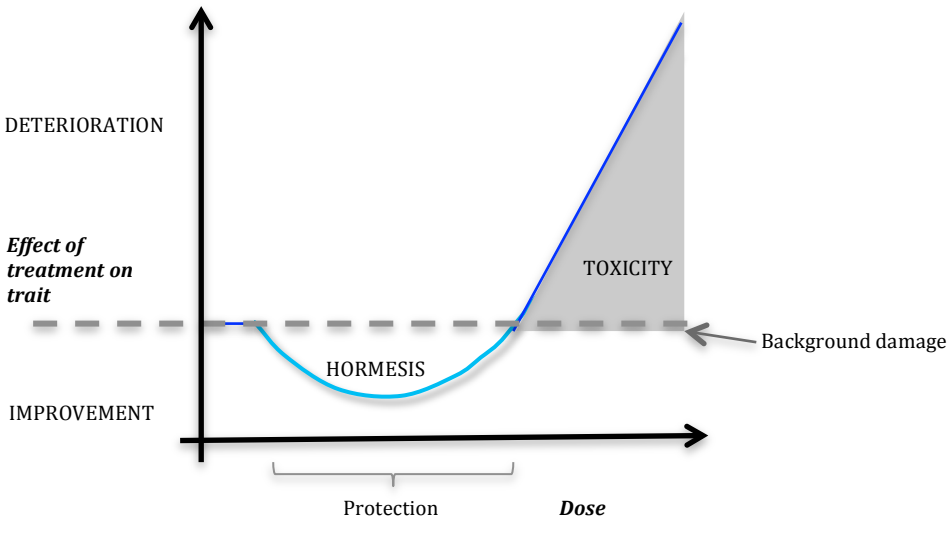
Legends for figures

Figure 1. Schematic diagram of a J-shaped (biphasic) dose-response curve characteristic of hormesis. The hormetic region represents protective overcompensation by cellular processes to reverse damage, resulting in a proportion of endogenous damage also being repaired and leading to reduction of damage below background damage levels. This leads to an apparent improvement in cellular fitness. At doses lower than those within the hormetic zone, levels of agent are too low to stimulate a measurable effect. At high doses, toxic effects are observed. Adapted from: [58] and [59]

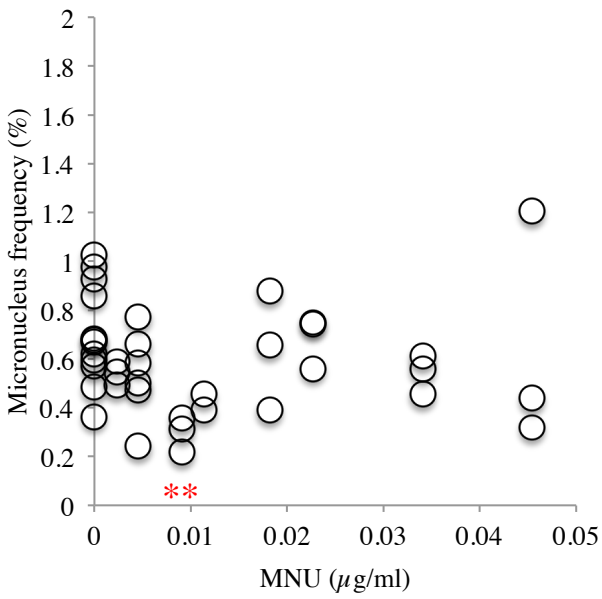
Figure 2A. MNU 24h treatment dose-response for the micronucleus assay in TK6 cells (n= or >2), centering on the low-dose, “hormetic” region. Data points for individual replicates are represented by circular symbols. A statistically significant decrease relative to the 0µg/ml control was observed at 0.009µg/ml (p<0.01, denoted by **). **B.** Average values for MNU 24h treatment dose-response for the micronucleus assay in TK6 cells (n=3, black line) and NH32 cells (n=2, grey line). No statistically significant decrease was observed in NH32 (p>0.05). **C.** Western blotting for DNA repair glycosylase, MPG, demonstrated no dose-dependent changes across MNU doses tested (n=3). One representative replicate is shown in **C**. **D.** No statistically significant changes in MPG mRNA expression levels (n=3, line) were observed. DNA repair enzyme MGMT’s (n=4, bars) effects upon micronucleus frequency (%), studied via use of a MGMT inhibitor (O^6BG), also remained unchanged (p>0.05) at the hormetic dose range.

Appendix B. Illustration of the approximately “J-shaped” nature of the dose-response curve. A full dose-response for MNU MN frequency (%), including significantly genotoxic doses not included in Figure 2A and 2B, as well the hormetic dose ($p < 0.05$ denoted by *, $p < 0.01$ denoted by **, $p < 0.001$ denoted by ***). The $\log_{10}$ of the values for MNU dose (x axis) was plotted in order to account for the differences in magnitude across the tested dose-range.

Appendix C. Micronucleus frequency (%) following a priming dose study (n=2). TK6 cells were pre-treated with the dose of MNU observed previously to induce a statistically significant decrease in micronucleus frequency (0.009 µg/ml, **Figure 2A**), for 24h. A dose of 0.2 µg/ml was then administered to cells and cells were incubated for 24h. No statistically significant difference was observed between cells treated with the priming dose and those with no priming dose.



A.



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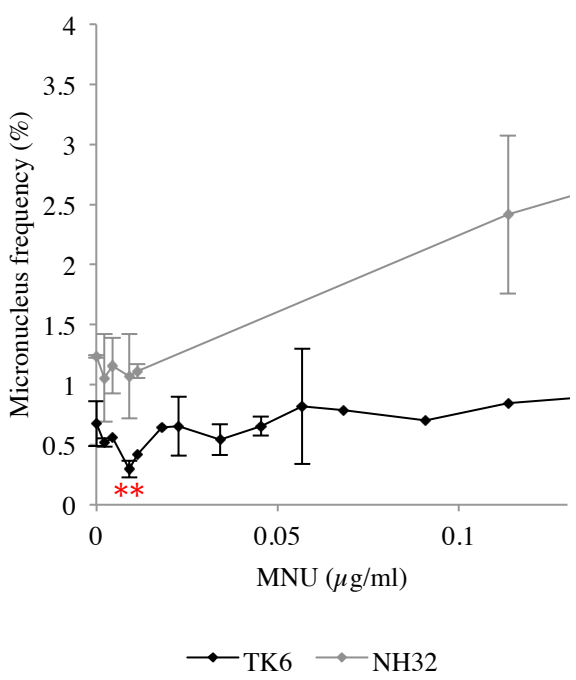

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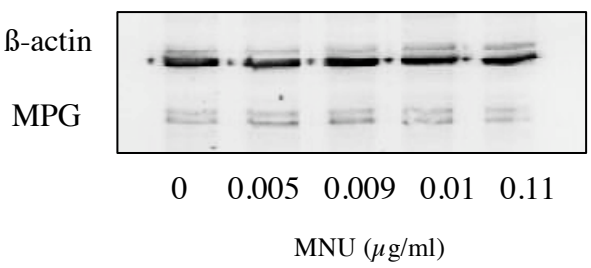
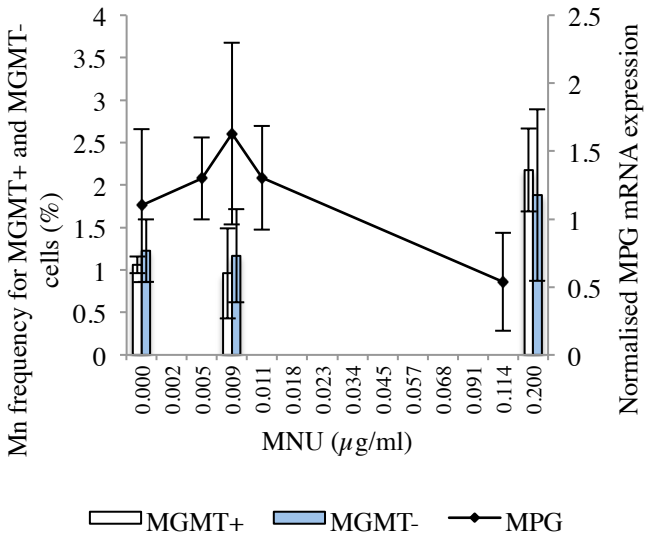
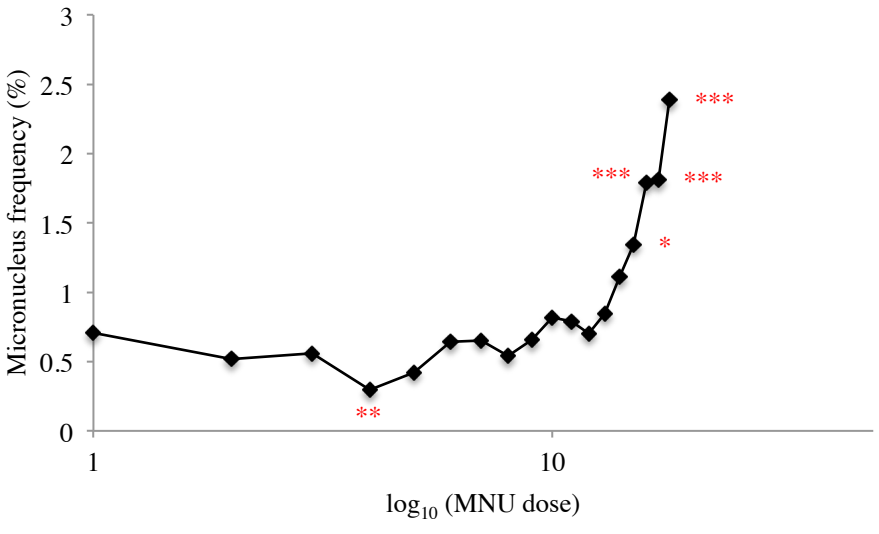


D.





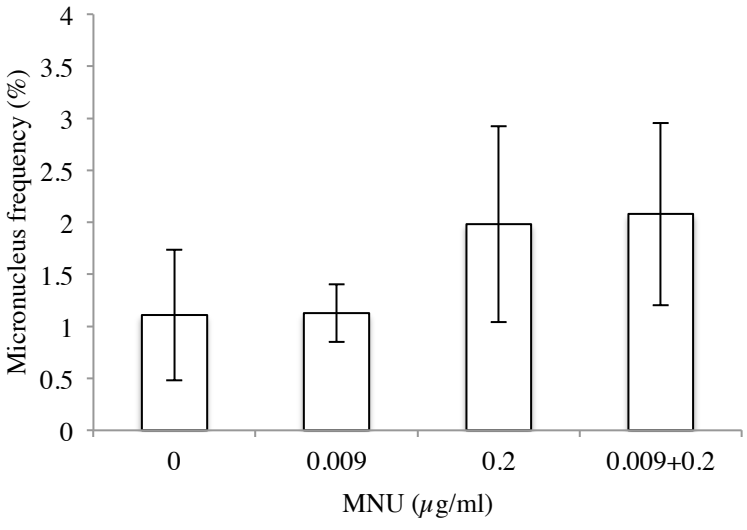


Table 1. Summary of the dose-responses from positive genotoxicity assays for diverse endpoints generated at Swansea University. Only one of 136 published datasets produced a J-shaped dose-response (in bold). The current study increases the number to two of 137 datasets. Criteria for inclusion included a dose-response (i.e., > or = 3 treatment concentrations) for a genotoxicity endpoint. CBMN = Cytokinesis-blocked micronucleus assay. EMS = ethyl methanesulfonate. ENU = *N*-ethyl-*N*-nitrosourea. HPRT = Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) Gene Mutation Assay. MMS = methyl methanesulfonate. MN = Micronucleus Assay. MNU = *N*-methyl-*N*-nitrosourea. RSMN = Reconstructed Skin Micronucleus Assay.

Publication	**Chemical**	**Number of dose-responses**	**Genotoxicity Assay/Endpoint**	**Exposure time**	**Cell line/type**
Parry *et al.*, 2004 [15]	8-Hydroxyquinoline, MMS, 4-nitroquinoline 1-oxide	3	CBMN	1 CC	AHH-1
Jenkins *et al.*, 2006 [16]	Deoxycholic acid (pH 5.5 or 7.4)	2	CBMN	24 h	OE33
Doak *et al.*, 2007 [17]	MMS, MNU, EMS, ENU	12	CBMN, HPRT	24 h	AHH-1, MT-1
Jenkins *et al.*, 2008 [18]	Deoxycholic acid	1	CBMN	21 h	OE33
Doak *et al.*, 2008 [19]	MMS	2	HPRT, N^7-meG adducts	24 h	AHH-1
Johnson and Parry, 2008[20]	Bisphenol A, rotenone	2	CBMN	24 h	AHH-1, MCL-5, V79
Johnson *et al.*, 2010a [21]	Vinblastine, diethylstilboestrol	8	CBMN, MN (no cytochalasin B)	3h, 24h	CHO
Johnson *et al.*, 2010b [22]	Sudan-1, Para Red	7	CBMN, HPRT	24 h	AHH-1, MCL-5
Kayani and Parry, 2010 [23]	Ethanol, acetaldehyde	2	CBMN	22 h	MCL-5
Zair *et al.*, 2011 [24]	EMS, ENU	12	CBMN, HPRT	24 h	AHH-1
Seager *et al.*, 2012 [25]	H_2O_2, $KBrO_3$, menadione	6	CBMN, HPRT	4 h	AHH-1
Singh *et al.*, 2012 [26]	Fe_2O_3, Fe_3O_4 (dextran-coated and uncoated ultrafine superparamagnetic iron oxide nanoparticles)	4	CBMN	24 h	MCL-5
Thomas *et al.*, 2013 [14]	**MNU**	**1**	**HPRT**	**24 h**	**AHH-1**

Manshian *et al.*, 2013 [27]	Single walled carbon nanotubes	11	CBMN, HPRT	24 h, 48 h	BEAS-2B, MCL-5
Brüsehafer *et al.*, 2014 [28]	Mitomycin-C (4h), cytarabine (24h)	4	CBMN	4 h, 24 h	TK6/NH32
Chapman *et al.*, 2014 [29]	MMS, Mitomycin-C, H_2O_2, Methyl carbamate	5	RSMN (CBMN) in EpiDerm	48 h	Primary epidermis
Brüsehafer *et al.,* 2015 [30]	4-Nitroquinoline 1-oxide	11	CBMN, MN (no cytochalasin B), HPRT, Comet assay	4 h	MCL-5, AHH-1 L5178Y
Chapman *et al.*, 2015 [31]	MMS, MNU	6	MN (no cytochalasin B)	24h, 5 day, 10 day	TK6, NH32
Manshian *et al.*, 2015 [32]	CdSe/ZnS nanoparticles	24	CBMN	1 and 3 cell cycles	HFF-1, BEAS-2B, TK6
Shah *et al*., 2016 [33]	Benzo[*a*]pyrene	11	CBMN, HPRT	4h, 23h	TK6
		Total = 136			