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Paper:
Chapman, K., Doak, S. & Jenkins, G. (2015). Acute Dosing and p53-Deficiency Promote Cellular Sensitivity to DNA Methylating Agents. *Toxicological Sciences, 144*, 357-365.
http://dx.doi.org/10.1093/toxsci/kfv004

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Acute dosing and p53-deficiency promote cellular sensitivity to DNA methylating agents

Katherine E. Chapman, Shareen H. Doak and Gareth J. S. Jenkins

In Vitro Toxicology Group, Institute of Life Science, Swansea University, SA2 8PP

Keywords: genotoxicity, chronic, dose-fractionation, micronucleus, dose-response, DNA repair, p53

Abstract

A current challenge of *in vitro* genotoxicity testing is the issue of misleading positive results (low specificity). This has in the past led to unnecessary testing on laboratory animals, as results for agents testing positive for genotoxicity *in vitro* are often checked *in vivo*. Such misleading *in vitro* positives have predominantly been attributed to *in vitro* tests not accurately representing *in vivo* test systems, nor typical human exposure. Human exposure scenarios almost invariably involve low-level, chronic exposure, whereas *in vitro* testing uses predominantly high dose and acute exposures. Here, the issue of misleading positives was addressed using comparisons between traditional acute dosing and alternative, chronic dosing regimes, uniquely using low dose experiments. The *In Vitro* Micronucleus Assay was used to measure chromosome aberrations induced by methylating agents methyl methanesulphonate (MMS) and methyl nitrosourea (MNU) in human lymphoblastoid cell line, TK6. Quantitative RT-PCR was used to measure mRNA level induction of DNA repair enzymes. Lowest observed genotoxic effect levels (LOGELs) for MMS were obtained at 0.7µg/ml for the acute study and 1.0µg/ml for the chronic study. For acute MNU dosing, a LOGEL was observed at 0.46µg/ml, yet genotoxicity was completely removed following the chronic study. Interestingly, a statistically significant decrease was observed at 0.009µg/ml following acute MNU dosing. Levels of selected DNA repair enzymes did not change significantly at the dose range tested. However, p53-deficiency (using the TK6 isogen cell line, NH32) increased sensitivity to MMS during chronic dosing, causing the LOGEL to equate to the LOGEL for acute dosing. Chronic dosing may prove an important *in vitro* alternative to the oversensitivity associated with acute dosing, leading to reductions in misleading positives and consequently, unnecessary *in vivo* tests.

Introduction

It is widely accepted that environmental mutagens that induce DNA damage are a potential cause of human cancers. Indeed, exposure to exogenous genotoxins occurs through a variety of sources, including food products, pharmaceuticals, cosmetics and pollutants. Currently, genotoxic effects of chemicals are assessed using a battery of regulatory-accepted *in vitro* assays, where a positive result for genotoxicity *in vitro* is often followed-up with *in vivo* tests (Pfuhler *et al.*, 2010). However, a potential limitation of current *in vitro* assays is their relatively low specificity, meaning a propensity for generation of "misleading" or "false" positive results (Kirkland *et al.*, 2007, Pfuhler *et al.*, 2010). Such irrelevant *in vitro* positives are those that are misleading when compared to their *in vivo* genotoxicity or rodent carcinogenicity counterpart data (Fowler *et al.*, 2012). Consequently, misleading positives are unlikely to be relevant to humans *in situ* and lead to unnecessary animal tests.

Another driver behind the requirement for the improvement of *in vitro* genotoxicity tests is the increasing pressure to limit the use of laboratory animals in toxicity testing. For example, the 7th Amendment to the Cosmetics Directive in 2009 has meant that testing of cosmetics on animals is now banned in the European Union. Further, the European Centre for the Validation of Alternative Methods (ECVAM) workshops and government-funded National Centre for the 3Rs (NC3Rs) have encouraged the implementation of the 3Rs principle (replacement, reduction and refinement of animals in research) to support animal welfare in toxicity testing (Holmes *et al.*, 2010). Indeed, Toxicity Testing in the 21st Century (TT21C) suggests that solely in vitro tests should be used. Considering such drivers, it is important to remember that current *in vitro* genotoxicity tests cannot satisfy the desire to rely more heavily on *in vitro* testing, as high rates of misleading positives will mean that some unnecessary *in vivo* follow up tests will be required. Therefore, it is crucial that dosing regime (Blakey *et al.*, 2008) and cell type (Fowler *et al.*, 2012) are carefully selected to maximize the representation of human exposure *in vitro* and, as a result, avoid false positives, improve *in vitro* testing *per se*, and reduce unnecessary animal testing.

The high rate of unnecessary animal tests combined with the low specificity of *in vitro* tests is a reflection that current *in vitro* tests are failing to accurately recapitulate human exposure to genotoxins. This is partly due to high, acute dosing dominating *in vitro* testing (Blakey *et al.*, 2008), while the majority of human exposures, and indeed exposures in *in vivo* tests involve longer-term, chronic dosing (Swenberg *et al.*, 1987). This highlights the fact that current *in vitro* tests are unlikely to accurately portray the kinetics of DNA adduct formation and repair that operate during chronic dosing at subtoxic doses of chemical (Swenberg *et al.*, 1987). It is, therefore, important to study subtoxic doses *in vitro* alongside toxic doses (Martin and Barrett, 2002). As well as being important for representation of human exposure *in vitro*, chronic dosing is also a valuable approach in cancer chemotherapy. For example, metronomic chemotherapy involves administration of regular and chronic doses of cytotoxic agent below the maximum tolerated dose (MTD) to optimally kill cancer cells, yet limit toxic side effects (Gasparini, 2001, Kerbel and Kamen, 2004). The cumulative dose with metronomic therapy might be significantly less than with MTD-based chemotherapy (Kerbel and Kamen, 2004), and is therefore likely to induce very different effects compared to the MTD. Therefore, characterization of

effects of chronic dosing in cells may further current understanding of metronomic chemotherapy.

Chronic dosing and dose-fractionation of low doses of genotoxin has not, to our knowledge, been extensively studied *in vitro*. However the effects of acute low doses have recently been investigated: a seminal publication from our laboratory (Doak *et al.*, 2007) challenged the assumption that dose of genotoxin is always directly proportional to genotoxic effect. While induction of DNA adducts is predicted to be linear (Osterman-Golkar *et al.*, 2003), the threshold dose-response for mutation obtained for some genotoxins confirms that cytoprotective mechanisms are crucial for limiting damage at low doses (Doak *et al.*, 2007, Zaïr *et al.*, 2011, Seager *et al.*, 2012, Thomas *et al.*, 2013, Brüsehafer *et al.*, 2014, Chapman *et al.*, 2014). Indeed, low doses of genotoxin may even invoke hormetic dose-responses (Touil *et al.*, 2002, Gocke *et al.*, 2009, Thomas *et al.*, 2013), meaning that the default assumption of a linear dose-response is a gross oversimplification, leading to the exclusion of chemicals from products and treatments that are in fact safe, or even beneficial, at low doses.

Not only is selection of *in vitro* dosing regime imperative, but also the selection of the cell type (Fowler *et al.*, 2012). Traditionally, rodent cell lines have been used in genotoxicity testing, and these are known to be often p53-deficient (Fowler *et al.*, 2012). p53-deficiency is associated with increased sensitivity to DNA damage, micronucleus induction and cytotoxicity when compared to p53-proficient cells, suggesting that a lack of p53 is contributing to misleading positive induction (Fowler *et al.*, 2012). As p53 is crucial in the DNA damage response, it is preferable that p53-competent mammalian cells, such as cell line TK6, are used for assessment of genotoxicity (Fowler *et al.*, 2012). Further, most healthy human cells in our bodies are p53 competent; therefore, the use of p53-competent cells *in vitro* is likely to better reflect human exposure. Here, we further explore the role of p53 deficiency on genotoxic dose responses.

The alkylating agents constitute a class of compounds commonly utilised in genotoxicity studies, due to their defined DNA adduct profiles and high DNA-reactivity under physiological conditions (Beranek, 1990, Jenkins *et al.*, 2005). Such alkylators are prevalent in the environment, with sources of contact including cigarette smoke, food compounds and occupational exposure (Beranek, 1990). Due to their toxicity, methylating agents such as methyl methanesulphonate (MMS) and *N*-methyl-*N*-nitrosourea (MNU) (Donelli *et al.*, 1967) have been used as chemotherapeutic agents. Of these two potent genotoxins, MMS favours reaction with highly nucleophilic sites, such as the N^7 site of guanine, which can induce clastogenicity and subsequent micronucleus formation (Beranek, 1990, Zaïr *et al.*, 2011). However, MNU is more mutagenic at the nucleotide level, inducing around 27-fold greater levels of the highly mutagenic lesion O^6-methylguanine than MMS (Beranek, 1990). Despite their ability to induce the same adducts, alkyl sulphonates and alkyl nitrosoureas have been shown to generate contrasting dose-responses for genotoxicity *in vitro* and *in vivo* (Doak *et al.*, 2007, Gocke and Müller, 2009). This is likely to be partly determined by the different dynamics of DNA repair mechanisms operating for different adducts (Doak *et al.*, 2008). For example, at doses below the no observed effect level (NOEL), O^6-methylguanine-DNA methyltransferase (MGMT) repairs O^6-methylguanine lesions (Doak *et al.*, 2008, Thomas *et al.*, 2013),

whereas base excision repair initiated by *N*-methylpurine-DNA glycosylase (MPG) is important in repair of N^7-methylguanine at such doses (Zaïr *et al.*, 2011).

The objective of the present study was to explore the effects of acute and chronic dosing of MMS and MNU within the low dose region, to further challenge the high-dose paradigm, help to refine *in vitro* testing design and to address the issue of false positives. A second aim was to investigate the mechanistic basis of the observed dose-responses, in terms of levels of DNA damaging agents and the influence of p53 on the observed dose-response.

Materials and Methods

Chemicals. MMS and MNU were purchased from Sigma-Aldrich (Dorset, UK) and stored according to the manufacturer's instructions. MMS was diluted in dH_2O and MNU was diluted in DMSO (Fisher Scientific). Dilutions from a master stock were made freshly each day for MNU and on the day of seeding for MMS, and stored in the dark at 4°C. Chemicals were tested to ensure that a statistically significant reduction in micronucleus frequency did not occur following stock storage over 10 days.

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 a density of between $1x10^5$ and $1x10^6$ cells/ml.

***In Vitro* Micronucleus Assay.** TK6 or NH32 suspensions (flasks of 10ml) with cells at $1x10^5$ cells/ml were seeded for 24h at 37°C and 5% CO_2. Each flask was dosed with appropriately diluted test chemical (100µl in total) and incubated for 24h, or 5x24h, as appropriate (**Table i**), under standard tissue culture conditions (37°C, 5% CO_2). Medium containing chemical was removed by centrifugation and replaced with fresh medium. Treated cells were then harvested after a further 24h. Pellets were centrifuged for 8min at 1000 revolutions per minute (rpm) with phosphate-buffered saline (PBS, Invitrogen), centrifuged with 0.56% KCl for 10min at 800rpm, then fixed using methanol and acetic acid mixtures. Slides were prepared for analysis using the automated Metafer Slide Scanning Platform. The full protocol is outlined in Seager *et al.* (in press). Nuclei were then stained using DAPI and viewed under an Olympus BX50 Fluorescence Microscope. A total of 12,000 cells were scored per treatment for three replicates.

Cytotoxicity measurement. A haemocytometer was used to count 10µl samples of cells on each day of the assay. Relative population doubling (%) (Fellows *et al.*, 2008, Lorge *et al.*, 2008) was then calculated as a measure of observed cytotoxicity. RPD did not decrease below 50% (RPD of 67.6% was the minimum) for any of the doses tested (**Supplementary Figure 1**), in line with the Organisation for Economic Co-operation and Development (OECD) requirements for use of the *In Vitro* Micronucleus Assay.

Table i. Example of the dose fractions of chemical administered to cells for a total dose of 2µg/ml, to demonstrate the dose-fractionation approach. The overall concentration quoted hereafter, represents a cumulative dose for the chronic studies. The "+2" nomenclature refers to two additional, non-dosing days: 1x 24h's seeding and 1x 24h of recovery.

Study	Total number of doses	Total dose (µg/ml)	Dose/day (µg/ml)
Acute (1+2 day)	1	2	2
Chronic (5+2 day)	5	2	0.4
Chronic (10+2 day)	10	2	0.2

RNA isolation and quantitative real time-PCR. 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 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 SYBRGreen I (Qiagen) and appropriately designed and optimised primers, or for MGMT, TaqMan® probe PCR (Qiagen) was used. A BioRad iCycler was used to perform the real-time PCR and analysis was completed using BioRad iQ5 software.

O^6-benzylguanine (O^6BG). Cells were treated with the MGMT inhibitor, O^6BG (Sigma-Aldrich) dissolved in methanol (Fisher Scientific). Dosing was performed for 1h at 37°C, 5% CO_2, with a final concentration of 10µM, prior to dosing with MNU.

Protein isolation and immunoblotting. Cell suspensions were centrifuged at 1200rpm and washed twice in 4°C phosphate-buffered saline (PBS) (Gibco). Cells were lysed at 4°C 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 10,000 revolutions per minute 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% (MPG) or 10% (p53) 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 5% bovine serum albumin (BSA) (Sigma-Aldrich). Membranes were separated and probed with MPG antibody (1:1000 dilution; M6195; Sigma-Aldrich) or p53 antibody (1:1000 dilution; p53: 9282, phospho^{ser15}-p53: 9284. Cell Signaling) diluted in 5% BSA at 4°C overnight. After four washes with 1x Tris-buffered saline-Tween 20 containing 5% BSA, rabbit anti-mouse secondary antibody was used at 1:10,000 dilution (ab6728-1, abcam) for MPG and goat anti-rabbit secondary antibody (ab6721, abcam). The membrane incubated for a further hour prior to three washes with 1x TBS-Tween 20 containing 5% BSA. 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).

Statistical analysis. Data was square root (√) transformed prior to the performance of a Dunnett's 2-sided *post hoc* analysis to identify the first statistically significant increase above control levels (i.e., lowest observed effect level (LOEL)).

Results

Chronic dosing reduces micronucleus frequency relative to acute dosing

Dose-responses for matched chronic 5+2 day exposure and acute 1+2 day exposure to MMS and MNU, were generated in TK6 cells using the *In Vitro* Micronucleus Assay, coupled to high-powered acquisition of data through use of a semi-automated image analysis system (Metafer, Zeiss). Concentrations of chemical were selected based on those used in previous studies (Doak *et al.*, 2007) in AHH-1 human lymphoblastoid cells. The mononucleate assay was used, rather than the cytokinesis block micronucleus (CBMN) assay, due to the study duration being over several days. Following a positive response in the acute 1+2 day study with MMS and MNU, the same dose range, fractionated, was employed for the 5+2 day study **(Figure 1)**.

To explore alternative dosing regimes, chronic dosing of TK6 cells was performed over a five-day period with MMS or MNU (**Figure 1 C, D**). RPD values, indicating cytotoxicity levels, are shown in **Supplementary Figure 1**. The LOGEL, or first statistically significant increase in micronucleus frequency above control levels, increased from 0.7µg/ml for the acute MMS study to 1.0µg/ml for the chronic 5+2 day MMS study. The LOGEL for the acute MNU study was 0.46µg/ml, whereas no LOGEL was observed for the chronic study in the dose range tested. Therefore, chronic dosing caused the LOGEL to shift to the right along the x-axis for both chemicals with respect to acute dosing. A 10+2 day study was also performed with MMS (**Supplementary Figure 2**), where genotoxicity was removed completely for the dose range of MMS tested. This supports the notion that acute dosing is overly sensitive.

Statistical analysis was performed on the data that produced a LOGEL, i.e., acute 1+2 day MMS and MNU, and chronic 5+2 day MMS. The results (**Supplementary figure 3**) implied that threshold dose-responses were a possibility for MMS, whereas the dose-response was determined to be linear for the acute MNU study. Further investigation suggested that micronuclei accumulated over time during the chronic 5+2 day MMS study for 2.0µg/ml, a dose above the LOGEL (**Supplementary figure 4**).

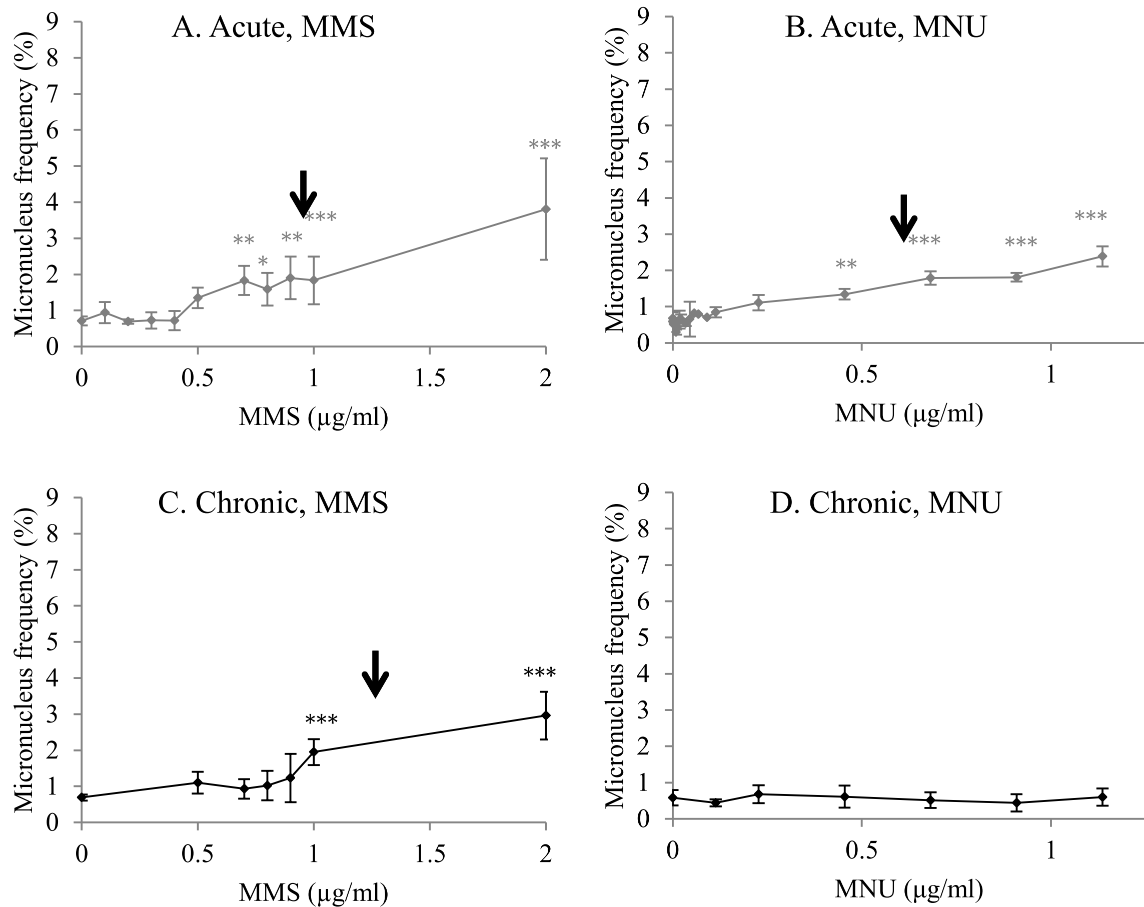


Figure 1. Influence of MMS (**A**, **C**) and MNU (**B**, **D**) dose on micronucleus frequency for acute 1+2 day (**A**, **B**) and chronic 5+2 day (**C**, **D**) treatments in TK6 cells (n=3). *Points*, mean of treatments in triplicate; *bars*, SD. The x axes represent cumulative dose for the chronic studies. For MMS, the first statistically significant increases in micronucleus frequency above solvent control (LOGELs) were at 0.7µg/ml (p=0.007) for acute exposure and 1.0µg/ml (p<0.001) for chronic exposure. The first statistically significant increase in micronucleus frequency for MNU acute was observed at 0.46µg/ml (p=0.007), whereas no significant differences were observed for the 5+2 day treatment (p≥0.89). Micronucleus frequency (%) represents the percentage of mononucleated cells containing one or more micronuclei. * p≤0.05; ** p≤0.01; *** p≤0.001. LOGELs are indicated by arrows.

p53 deficiency increases the sensitivity to MMS during chronic dosing

p53 is an important mediator in the DNA damage response and is mutated in many human cancers. p53 has previously been implicated in the BER pathway (Seo *et al.*, 2002), which is strongly associated with repair of lesions induced by alkyl sulphonates (Kaina, 1993). Therefore, the chronic 5+2 day study with MMS was repeated using the p53-deficient cell line, NH32, which is isogenic to TK6 (WT p53), to observe how p53 influences micronucleus induction. MMS was selected due to a LOGEL having been identified in the chronic 5+2 day study, which enabled a quantitative comparison between the different cell lines for the same chemical, unlike with MNU.

The LOGEL for MMS in NH32 cells was reduced relative to TK6, from 1.0µg/ml in TK6, to 0.7µg/ml in NH32 (**Figure 2A**). Interestingly, the NH32 chronic LOGEL occurred at the same dose as for the acute MMS study in TK6, which was also 0.7µg/ml. With increasing dose of MMS in NH32 cells, micronucleus frequency increased more rapidly compared to TK6, with NH32 reaching a maximum of 8.07% micronucleated cells at 2µg/ml, whereas for the same MMS dose, TK6 cells demonstrated an almost threefold lower induction of 2.96%. The p53 deficient cells were also more sensitive to cytotoxicity. At day 7, the RPD value for TK6 at 2µg/ml was 84.3% of control, whereas greater cytotoxicity was observed for NH32, where the RPD value was 60.3% (**Supplementary Figure 5A**). Interestingly, the untreated control micronucleus frequency for NH32 following the chronic regime, 1.7%, was around 2.5-fold greater than that of TK6, 0.69%. Interestingly, the basal micronucleus level for acute studies in NH32 was less than 1% (Brüsehafer *et al.*, 2014), which was lower than the 1.7% obtained with the chronic regime.

To further investigate the role of p53 in chronic dosing with MMS, Western blotting for detection of total p53 and phospho^{ser15}-p53 was performed in TK6 (**Supplementary Figure 5B**). The blots confirmed the presence of p53 in TK6, although produced no evidence of high p53 activation. A double-band was observed at 53kDa for phospho-p53, perhaps owing to multiple phosphorylated forms of p53 present in the sample.

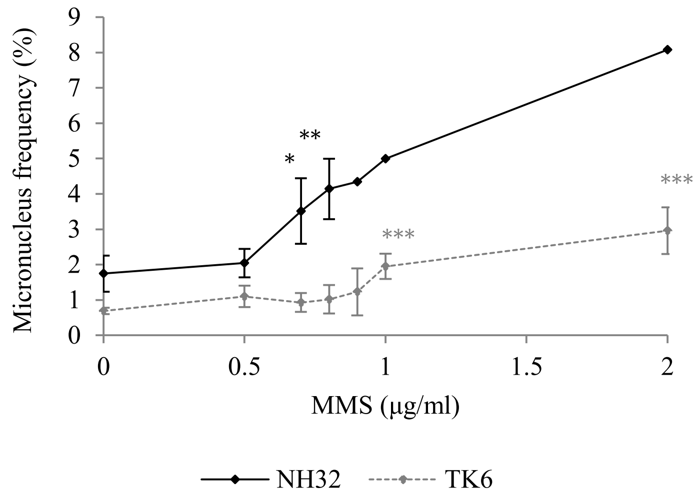


Figure 2. p53-deficiency increased sensitivity to MMS during the chronic 5+2 day study, although p53 protein levels remained unchanged in p53-proficient TK6 **(Supplementary Figure 5B)**. Micronucleus frequency (%) following chronic 5+2 day dosing with MMS in NH32 cells, as compared to the chronic 5+2 day study in TK6 cells (as presented in **Figure 1C**). For NH32, the first statistically significant increase was observed at 0.7µg/ml (p=0.026). Doses 0.9, 1.0 and 2.0µg/ml for NH32 are in duplicate and error bars and asterisks are therefore excluded.

Chronic dosing with MMS did not increase sensitivity to DNA damage

In order to determine whether the chronic dosing regime led to adaptation of the TK6 cells through enhanced DNA repair enzyme expression, we analysed the mRNA expression of four DNA repair genes during the chronic dosing period (**Figure 3**). MMS was selected as this agent generated both a LOGEL and NOGEL for the chronic exposure.

No significant changes in gene expression were observed, although DMC1, MPG and XRCC3 demonstrated similar trends in terms of DNA repair gene expression changes. For example, expression of all three genes increased at day 6, following a cumulative dose of 1µg/ml MMS. Further, expression peaked for these genes at day 4, following 0.5µg/ml treatment. Real-time DNA repair PCR arrays were used for initial assessment of differences between mRNA levels for 84 different DNA repair genes (data not shown). The arrays suggested a slight decrease in RAD51L1 expression with chronic 5+2 day MMS treatment, and RAD51L1 was therefore selected for subsequent real-time PCR analysis. Although this produced no statistically significant changes in RAD51L1 expression, 0.5 and 1.0µg/ml levels were less than untreated control levels (**Figure 3C**). Similarly, the remaining three genes produced no statistically significant changes in mRNA expression, although a general increasing trend was observed with increasing number of days for 1.0µg/ml for all genes, whereas expression at 0.5µg/ml peaks at day 4 prior to decreasing at day 6.

MGMT (O^6-methylguanine-DNA methyltransferase) levels were also analysed (data not shown), as MGMT is involved in the direct reversal of methylation of O^6-methylguanine lesions (Kaina *et al.*, 2007). However, accurate assessment of MGMT expression was not possible, as TK6 cells express MGMT at barely detectable levels (Hickman and Samson, 2004). This has also been observed previously in our laboratory.

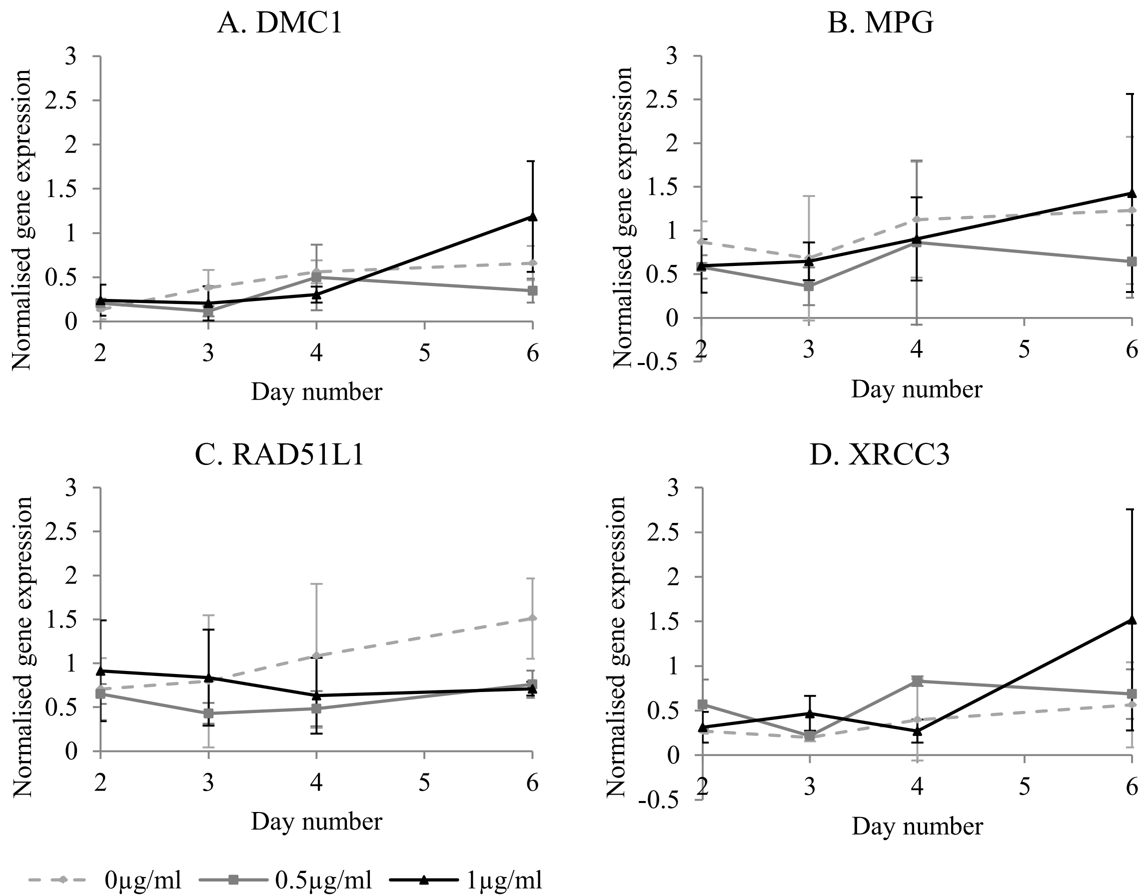


Figure 3. mRNA levels remained unchanged during the chronic 5+2 day study with MMS. Expression of mRNA of four DNA repair genes, normalized against housekeeping gene, ß-actin, as determined by qRT-PCR. The time points and doses selected during the chronic 5+2 day study with MMS did not produce any statistically significant differences. Gene products of DMC1 (Disrupted meiotic recombinase 1), RAD51L1 (RAD51 (*S. cerevisiae*)-like 1, synonymous with RAD51 homolog B) and XRCC3 (X-ray repair complementing defective repair in Chinese hamster cells 3) are involved in DNA double strand break repair. MPG (*N*-methylpurine-DNA glycosylase) is involved in base excision repair (BER).

Discussion

Exploration of chronic dosing regimes *in vitro* in the low dose region may be helpful for a comprehensive understanding of the effects of chemicals without bias towards high, acute doses. Such dosing regimes are important for safety assessment of chemicals on the basis that this type of dosing better represents human exposure levels than standard *in vitro* assays. This is particularly important if the oversensitivity of current *in vitro* tests is to be addressed. Chronic dosing may also provide further information on the effects of metronomic chemotherapy, the direct effects of which are difficult to determine *in vivo*.

In this study, we have demonstrated that dose-fractionation of two methylating agents led to substantial reductions in micronucleus frequency, relative to comparative acute dosing. Dose-fractionation was associated with the eventual removal of genotoxicity for both MMS and MNU within the tested dose range (**Figure 1, Supplementary Figure 2**). Previously, dose-fractionation of a mutagenic dose to smaller doses has produced similar effects *in vivo* for both MMS (Tang *et al.*, in preparation) and its ethyl form, ethyl methanesulphonate (EMS) (Gocke *et al.*, 2009, Gocke and Müller, 2009). In these studies, dose-fractionation abolished mutagenic effects of these chemicals *in vivo*. Interestingly, Gocke *et al.* (2009) observed *in vivo* that dose-fractionation produced an additive effect with *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation, whereas doses of EMS, which gave a threshold dose-response, did not lead to an accumulation of mutation (Gocke and Müller, 2009). This supports the trends observed here for MMS, where the probable explanation for a lack of accumulation lies in the combination of cellular DNA repair activity (Doak *et al.*, 2007) and intervals between doses enabling time for repair (Tang *et al.*, in preparation). However, for MNU, accumulation of damage was not observed for the 5+2 day study relative to acute. Rather, damage induction was lost following chronic exposure, suggesting that under certain conditions, MNU's genotoxic potency is removed. This is in part supported by *in vitro* data demonstrating a mutagenic threshold for MNU (Pottenger *et al.*, 2009) and even possible hormetic effects (Thomas *et al.*, 2013) at low doses of MNU. Such findings support a possible safe exposure at low levels of MNU, probably based on efficient DNA repair. The effect of MNU perhaps depends on the nature of the biological system, including repair capacity and MNU efficacy and metabolism, although this would require further investigation. However, in the context of this study, it would be expected that time for repair between doses would reduce genotoxicity regardless of chemical type. Equally, the longer recovery period in this assay, due to no cytokinesis block stage, may allow continued repair of DNA adducts.

As mentioned previously, it is likely that differences observed in genotoxicity between chronic and acute exposure regimes can be attributed to the extent of DNA repair pathway saturation (Doak *et al.*, 2007) and the number of adducts that, therefore, remain unrepaired. Here, we did not observe significant changes in DNA repair mRNA levels during the chronic 5+2 day MMS study. This interesting result suggests that cells were as repair competent at assay commencement as at the end of the assay. This perhaps owes to the low doses tested, with adducts generated being repaired by basal levels of DNA repair enzymes (Doak *et al.*, 2007), particularly below the NOGEL. It is important to note that mRNA analysis, as used here, does not inform us of levels of protein activity and phosphorylation state, for example. This

aspect would be important to consider in follow up studies. We also hypothesise that the time between doses allows DNA repair to occur, limiting pathway saturation. Therefore, the cell might not be required to invest resources in up-regulation of DNA repair activity. Alternatively, different pathways to those investigated could be responsible in this case, or timing of extraction may be important in determining the observed effect (Doak *et al.*, 2008). Interestingly, at the chronic 5+2 day MMS LOGEL of 1.0µg/ml, no significant changes in repair enzyme expression were observed. However, this is supported by previous studies, which suggested that different repair mechanisms operate at high doses and low doses for the same chemical (Doak *et al.*, 2008, Zaïr *et al.*, 2011).

Secondly, the unchanged levels of DNA repair enzymes suggest that repair mechanisms did not increase sensitivity to alkylating agents. This is important because at low doses, up-regulation of repair pathways may be more damaging than the chemical itself. For example, over-expression of MPG has been associated with increased sensitivity to chromosome damage and increased cell death, due to the error-prone nature of the base excision repair pathway (Ibeanu *et al.*, 1992, Coquerelle *et al.*, 1995, Fishel *et al.*, 2003, Rinne *et al.*, 2004, Rinne *et al.*, 2005, Fishel *et al.*, 2007). However, as MPG recognizes N^7methylguanine adducts (O'Connor and Laval, 1991), it was unexpected that MPG expression would remain unchanged throughout the chronic MMS 5+2 day treatment. However, Doak *et al.* (2008) similarly observed no change in MPG expression in response to MMS in AHH-1 cells, which was suggested to relate to MPG being tightly regulated to avoid unnecessary DNA damage.

An alternative explanation for lack of change observed in repair gene expression is the timing of mRNA or protein extraction, or technique used for analysis. For example, p53 expression is predicted to occur in "waves" in response to genotoxic stimuli (Loewer *et al.*, 2010), which would not necessarily be detectable when analyzing total protein expression as an average in a cell population. Further MGMT expression appears to occur as a single increase at around 4h post-dosing with genotoxin (Doak *et al.*, 2008). Therefore, a continuous analysis method may be useful in future to ascertain precisely how DNA repair responds to chronic exposure and whether cells adapt to chemical. It is worth noting that only a small percentage of cells exhibit micronuclei, so it is possible that only these cells are responding to the treatment.

The role of p53 in the chronic study was also investigated, as p53 is a crucial signaling node in the DNA damage response (Kastan *et al.*, 1991), for example, in upregulating some DNA repair enzymes, as well as the *TP53* gene encoding p53 being mutated in a high proportion of human cancers (Greenblatt *et al.*, 1994). We found p53-deficiency to confer heightened sensitivity to MMS, as adjudged by micronucleus induction and cytotoxicity **(Figure 2, Supplementary figure 5A)**. This was unsurprising, as p53-deficiency has previously been associated with reduced growth arrest and DNA repair, and therefore a higher incidence of micronuclei (Masunaga *et al.*, 2002, Driessens *et al.*, 2003, Brüsehafer *et al.*, 2014). Indeed, studies with DNA repair-proficient and -deficient Ames strains emphasised the importance of efficient DNA repair in genotoxic tolerance (Tang *et al.*, 2014). Interestingly, protein levels of p53 and phospho^{ser15}-p53 in p53-competent cell line TK6 remained unchanged during the chronic 5+2 day study. Doak *et al.* (2008)

observed a similar effect, with levels of p53 remaining unchanged in response to low-dose, acute MMS treatments. This would suggest that basal levels of p53 were sufficient under these dosing conditions to promote repair of DNA damage. Indeed, p53 protein levels are kept low during growth of normal cells through rapid protein turnover (Michael, 2003), to avoid both the down-regulation of the base excision repair pathway and excessive induction of apoptosis in response to increased p53 levels (Offer *et al.*, 2002).

Conclusions

We have observed that the use of dosing regimes more pertinent to human exposure than acute exposures may dramatically reduce the damage induced. Furthermore, low-level, chronic dosing appears to minimally influence DNA repair mechanisms. Therefore, chronic dosing appears to limit cellular sensitivity to methylating agents, further supporting the theory of low dose tolerance in genetic toxicology. These data, therefore, suggest that dose-fractionation could be a valuable additional approach in *in vitro* testing for assessing the relevance of *in vitro* positive results. If chronic dosing can be incorporated into initial *in vitro* tests, this may lead to reductions in follow-up animal tests.

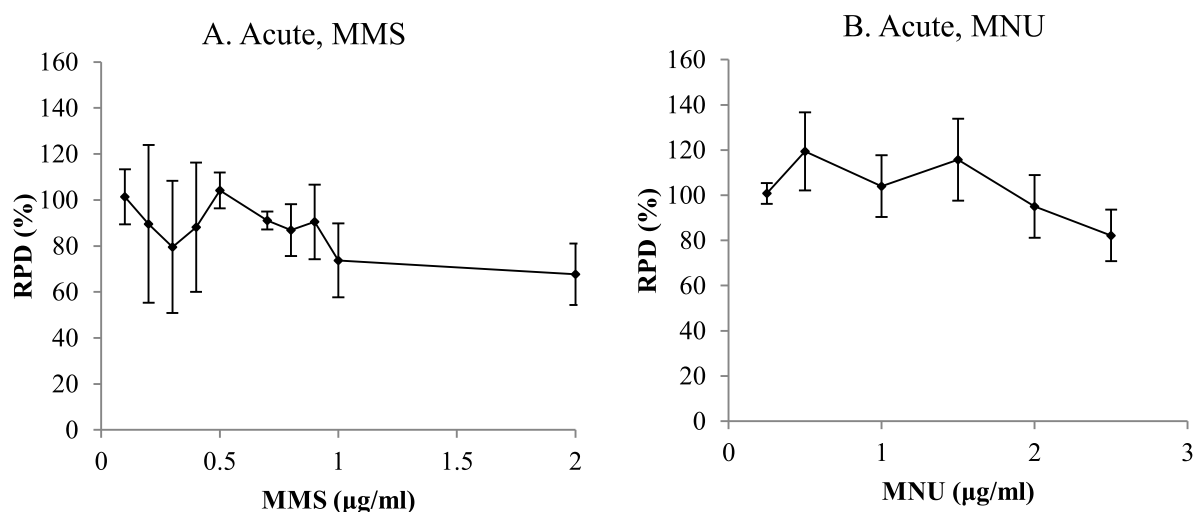
Acknowledgements

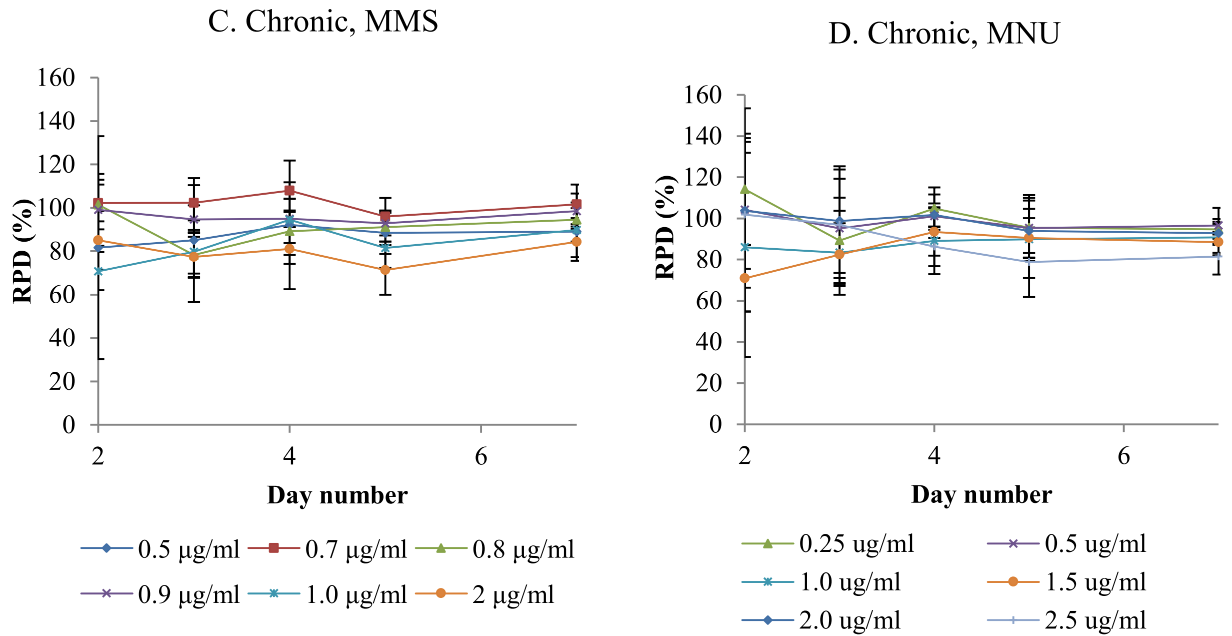
The authors would like to thank Margaret Clatworthy for her technical assistance. The authors also thank Professor Gerald Wogan for kindly providing the NH32 cells.

Funding

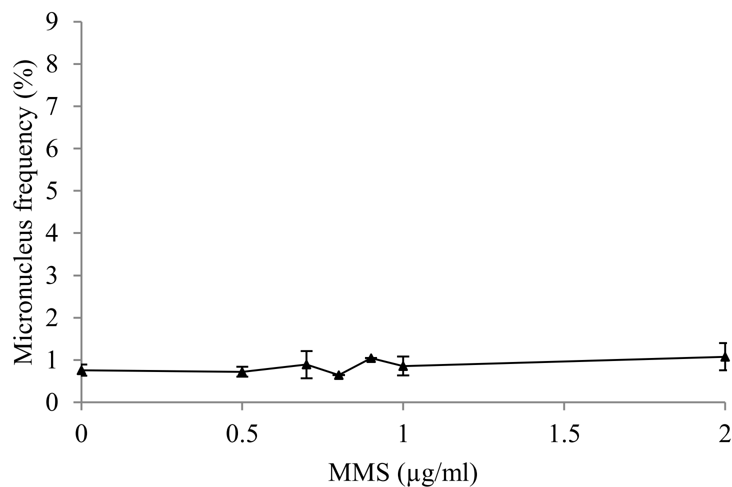
The work was supported by a PhD studentship to KEC from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

Supplementary Figures

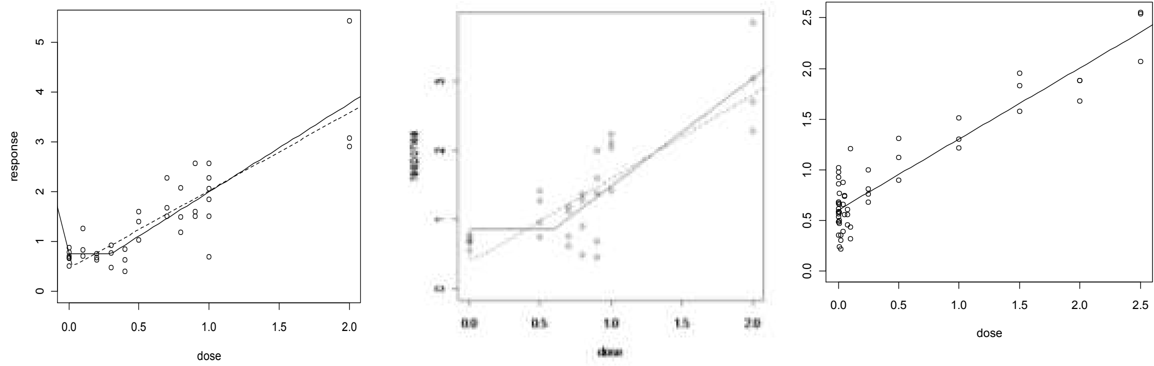




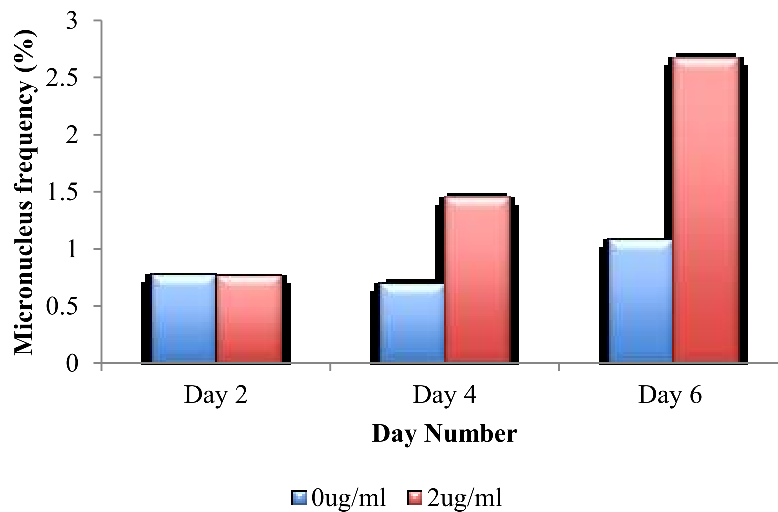
Supplementary figure 1. Relative population doubling (RPD (%)) values as a measure of cell survival relative to control levels for acute (1+2 day) (A, B) and chronic (5+2 day) (C, D) studies for MMS (A, C) and MNU (B, D), in TK6 cells.



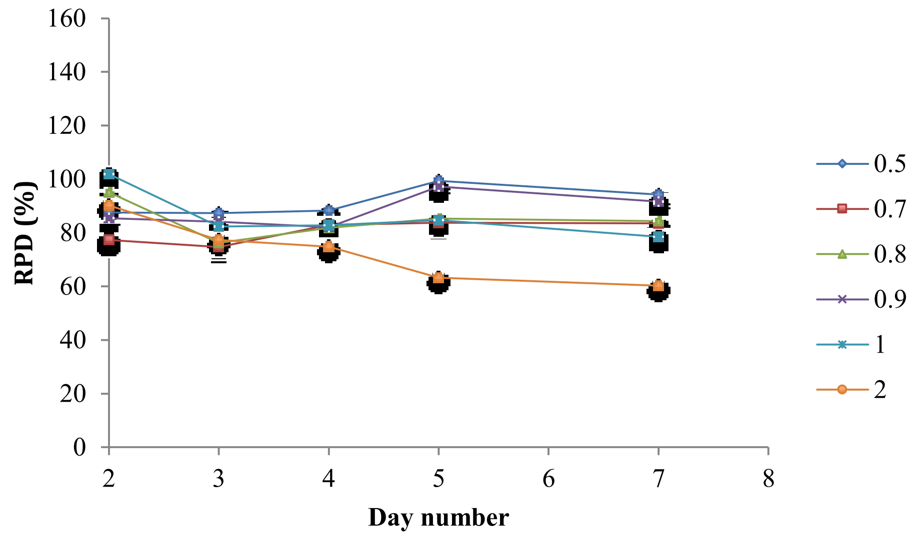
Supplementary figure 2. Micronucleus frequency (%) following a chronic 10+2 day treatment. Relative to the untreated control, there was no statistically significant increase in micronucleus frequency following treatment ($p \geq 0.61$).

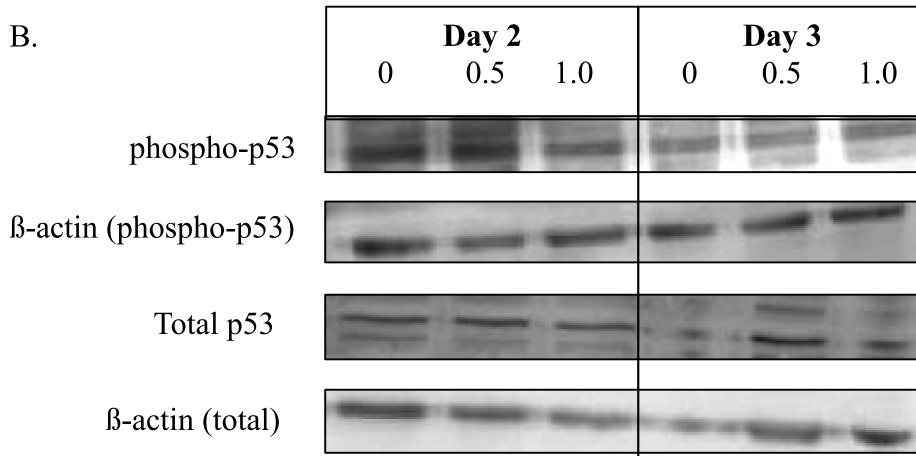


Supplementary figure 3. Broken Stick Dose-Response Model used for prediction of probability of threshold dose-responses demonstrating statistically significant increases above control levels of micronucleus frequency. Acute (1+2 day) MMS inflection point = 0.3 µg/ml (p=0.07); chronic (5+2 day) MMS inflection point = 0.6 µg/ml (p=0.07); acute (1+2 day) MNU inflection point = none (p=1), implying a linear dose-response, as determined by a *t*-test.



Supplementary figure 4. Micronucleus frequency measured at different time points (days 2, 4 and 6) throughout the chronic 5+2 day assay for 0µg/ml and 2µg/ml MMS treatments (n=1).





Supplementary figure 5. A. Relative population doubling (RPD (%)) values as a measure of cell survival relative to control levels for chronic (5+2 day) studies for MMS in NH32 cells. **B.** Western blots were performed for total p53 and phospho-p53 (p-p53, ser15) expression, although no dose-dependent differences were observed over different time points and doses below and at the LOGEL. Examples of blots for 0, 0.5 and 1.0µg/ml are shown for days 2 and 3, and similar results were obtained for the same doses at days 4 and 6.

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