Acute Dosing and p53-Deficiency Promote Cellular Sensitivity to DNA Methylating Agents

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ABSTRACT

Risk assessment of human exposure to chemicals is crucial for understanding whether such agents can cause cancer. The current emphasis on avoidance of animal testing has placed greater importance on in vitro tests for the identification of genotoxicants. Selection of an appropriate in vitro dosing regime is imperative in determining the genotoxic effects of test chemicals. Here, the issue of dosing approaches was addressed by comparing acute and chronic dosing, uniquely using low-dose experiments. Acute 24 h exposures were compared with equivalent dosing every 24 h over 5-day, fractionated treatment periods. The in vitro micronucleus assay was used to measure clastogenicity induced by methyl methanesulfonate (MMS) and N-methyl-N-nitrosourea (MNU) in human lymphoblastoid cell line, TK6. Quantitative real-time (qRT) PCR was used to measure mRNA level induction of DNA repair enzymes. Lowest observed genotoxic effect levels (LOGELs) for MMS were obtained at 0.7 µg/ml for the acute study and 1.0 µg/ml for the chronic study. For acute MNU dosing, a LOGEL was observed at 0.46 µg/ml, yet genotoxicity was completely removed following the chronic study. Interestingly, acute MNU dosing demonstrated a statistically significant decrease at 0.009 µg/ml. Levels of selected DNA repair enzymes did not change significantly following doses tested. However, p53 deficiency (using the TK6-isogenic cell line, NH32) increased sensitivity to MMS during chronic dosing, causing this LOGEL to equate to the acute treatment LOGEL. In the context of the present data for 2 alkylating agents, chronic dosing could be a valuable in vitro supplement to acute dosing and could contribute to reduction of unnecessary in vivo follow-up tests.

Key words: genotoxicity; p53; DNA repair; clastogen; toxicity; chronic; alkylating agents

It is widely accepted that environmental agents that induce genotoxicity are a potential cause of human cancers. Currently, genotoxic effects of chemicals are assessed using a battery of regulatory-accepted in vitro assays, where a positive result for genotoxicity is often followed up with in vivo tests (Pfuhler et al., 2010). High, acute dosing regimes currently dominate in vitro testing (Blakey et al., 2008), yet the majority of human exposures involve longer-term, chronic dosing (Swenberg et al., 1987). Chronic dosing and dose fractionation of low doses of genotoxins have 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 the importance of cytoprotective mechanisms at low doses (Brisehafer et al., 2014; Chapman et al., 2014; Doak et al., 2007; Seager et al., 2012; Thomas et al., 2013; Zair et al., 2011). Indeed, low doses of genotoxins may even invoke hormetic dose-responses (Gocke et al., 2009; Thomas et al., 2013; Touil et al., 2002), meaning that the default assumption of a linear dose-response is a gross oversimplification, causing chemicals to be excluded from products and treatments that are in fact safe, or even beneficial, at low doses.

A further potential limitation of current in vitro assays is their relatively low specificity, meaning a propensity for generation of “misleading” positive results (Kirkland et al., 2007; Pfuhler et al.,...
are prevalent in the environment, with sources of contact nucleophilic sites, such as the N7 site of guanine, which can these 2 potent genotoxins, MMS favors reaction with highly DNA repair mechanisms operating for different adducts (Doak et al., 2012). Further, most normal human cells are p53 competent; therefore, the use of p53-competent cells in vitro is likely to better represent human exposure. Here, we further explore the role of p53 deficiency on genotoxic dose-responses.

While the selection of in vitro dosing regime is imperative, the selection of appropriate cell type is also important (Fowler et al., 2012). Traditionally, rodent cell lines have been used in genotoxicity testing, which are known to often be p53 deficient (Fowler et al., 2012). However, 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 normal human cells are p53 competent; therefore, the use of p53-competent cells in vitro is likely to better represent human exposure. Here, we further explore the role of p53 deficiency on genotoxic dose-responses.

The alkylating agents constitute a class of compounds commonly utilized 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 and foods (Beranek, 1990). Due to their toxicity, methylating agents such as methyl methanesulfonate (MMS) and N-methyl-N-nitrosourea (MNU) (Donelli et al., 1967) have been employed as chemotherapeutic agents. Of these 2 potent genotoxins, MMS favors reaction with highly nucleophilic sites, such as the N7 site of guanine, which can induce clastogenicity and subsequent micronuclear formation (Beranek, 1990; Zaïr et al., 2011). However, MNU is more mutagenic at the nucleotide level (Beranek, 1990). Despite their ability to induce the same adducts, alkylsulfonates and alkylnitrosoureas have generated 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 different DNA repair mechanisms operating for different adducts (Doak et al., 2008; Thomas et al., 2013; 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 and help to refine in vitro testing design. The in vitro micronuclear assay was selected as the test system as this is a currently recommended in vitro genotoxicity test that allows full dose-responses to be obtained. The additional information on the effects of chemicals that chronic dosing provides might allow human exposure levels to be modeled more accurately in an in vitro test system. Therefore, chronic dosing approaches could be used to supplement current in vitro acute dosing in genotoxicity testing.

**MATERIALS AND METHODS**

**Chemicals.** MMS (CAS Number: 66-27-3; molecular weight: 110.13; and purity: 99%) and MNU (CAS Number: 684-93-5; molecular weight: 103.08; and purity: 66%) were purchased from Sigma-Aldrich (Dorset, UK) and stored according to the manufacturer’s instructions. MMS was diluted in dH2O 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. Final H2O and DMSO concentrations in test cultures were the same for dosing in both acute and chronic approaches. When handling MMS and MNU, safety precautions, including arm length gloves and protective clothing, were implemented at all times.

**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 1 × 10^4 and 1 × 10^5 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.

In vitro micronucleus assay. TK6 or NH32 suspensions (10 ml) with cells at 1 × 10^5 cells/ml were seeded for 24 h at 37 °C and 5% CO2. Each flask was dosed with appropriately diluted test chemical (100 μl in total) and incubated for 24 h, or 5 × 24 h, as appropriate (Table 1), under standard tissue culture conditions (37 °C, 5% CO2). Table 1 indicates the dosing regime adhered to during the study, including the number of individual doses administered to give a cumulative dose of 2 μg/ml as an example. The resulting individual doses tested are also indicated. Concentrations of chemical were selected based on those used in previous studies in AHH-1 human lymphoblastoid cells (Doak et al., 2007). The mononucleate version of the assay was used, rather than the cytokinesis block micronucleus assay, due to the study duration being several days. Medium containing chemical was removed by centrifugation and replaced with fresh medium. Treated cells were then harvested after a further 24 h recovery period, during which no further test chemical was added. Pellets were centrifuged for 8 min at 170 × g with phosphate-buffered saline (PBS; Invitrogen), centrifuged with 0.56% KCl for 10 min at 110 × g, 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 by Seager et al. (2014). Nuclei were then stained using 4′,6-diamidino-2-phenylindole (DAPI) and viewed under an Olympus BX50 Fluorescence Microscope. A total of 12 000 cells were scored per treatment for 3 replicates.

**Cytotoxicity measurement.** A hemocytometer was used to count 10μl samples of cells on each day of the assay. Relative population doubling (RPD) (%) (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 Fig. 1), in line with the Organization for Economic Co-operation and Development requirements for use of the in vitro micronuclear assay.

**RNA isolation and quantitative real-time PCR.** Real-time PCR (RT-PCR) was completed to investigate relative mRNA expression levels for relevant DNA repair genes in response to chronic 5 + 2 day treatment with MMS in TK6 cells. Changes might indicate whether DNA repair could have a notable impact on the
Table 1. Example of the Dose Fractions of Chemical Administered to Cells for a Total Dose of 1 μg/ml, to Demonstrate the Dose-Fractionation Approach

<table>
<thead>
<tr>
<th>Study</th>
<th>Total Number of Doses</th>
<th>Dose/Day (μg/ml)</th>
<th>Dose (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute (1 + 2 day)</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Chronic (5 + 2 day)</td>
<td>5</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Chronic (10 + 2 day)</td>
<td>10</td>
<td>2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Notes: The overall concentration quoted hereafter represents a cumulative dose for the chronic studies. The "1-2" nomenclature refers to 2 additional, non-dosing days: 1 × 24 h seeding and 1 × 24 h of recovery.

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 timepoints and concentrations. Synthesis of cDNA from RNA was completed using Quantitect Reverse Transcript Kit (Qiagen). Quantitative RT (qRT) PCR was performed using these samples, using Quantifast SYBRGreen I (Qiagen) and appropriately designed and optimized primers, or for O6-methylguanine-DNA methyltransferase (MGMT), TaqMan probe PCR (Qiagen) was used. Primer sequences are presented in Table 2. A BioRad iCycler was used to perform the RT-PCR and analysis was completed using BioRad iQ5 software.

Endpoint PCR and DNA polyacrylamide gel electrophoresis. To investigate whether MGMT was expressed in TK6 and NH32 cells, endpoint PCR and DNA polyacrylamide gel electrophoresis (PAGE) were performed. DNA extraction and cDNA synthesis were performed as outlined in the previous section. Endpoint PCR was then performed on a BioRad T100 Thermal Cycler for each sample, using the GoTaq Flexi DNA Polymerase kit (Promega). MGMT primer sequences (Doak et al., 2008) are presented in Table 2. Polyacrylamide gels (5%) were cast and DNA PAGE performed at 170 V for 30 min for PCR products. DNA was stained by washing the gel for 7 min in 1 g/l silver nitrate solution (Promega), followed by a 3-min wash in sodium hydroxide/formaldehyde solution (Sigma-Aldrich) until the bands were visible, followed by submersion in ddH2O.

To explore alternative dosing regimes, chronic dosing of TK6 cells was performed over a 5-day period with MMS or MNU, the same dose range, fractionated, was employed for the 5 + 2 day study (Fig. 1). To investigate p53 and phospho-p53 levels in TK6 cells, protein isolation and subsequent immunoblotting were used. This is due to p53 being an important node in the cellular DNA damage response. TK6 cell suspensions treated using the chronic 5 + 2 day dosing regime at concentrations 0, 0.5, and 2.0 μg/ml were centrifuged at 250 × g for 7 min and washed twice in 4°C PBS (Gibco). Cells were lysed at 4°C using 1× radioimuno-precipitation lysis buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and then 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 (40 μg) were mixed at a 1:1 ratio with 1× Laemmli buffer (Sigma-Aldrich) and resolved on a 12% (N-methylpyrrole-DNA glycosylase) or 10% (p53) SDS polyacrylamide gel. Proteins were then electroblotted onto Immun-Blot PVDF membranes (BioRad) and blocked for 1 h with 1× 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-ser53; p53: 9284. Cell Signaling) diluted in 5% BSA at 4°C overnight. After 4 hours, washes with 1× Tris-buffered saline-Tween 20 containing 5% BSA, rabbit anti-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 3 washes with 1× TBS-Tween 20 containing 5% BSA. To correct for protein-loading differences, blots were probed with mouse antibody to β-actin (ab8226-100, Abcam), followed by rabbit anti-mouse secondary antibody. Immun-Star WesternChemiluminescence Kit (BioRad) was used for the immunodetection of proteins. Band densitometry was determined using the Quantity One software (BioRad).

RESULTS

Chronic Dosing Reduces Micronucleus Frequency Relative to Acute Dosing

Dose-responses for matched chronic 5 + 2 day exposure and acute 1 + 2 day exposures to MMS and MNU were generated in TK6 cells using the in vitro micronucleus assay. This was coupled to high-powered acquisition of data through use of a semi-automated image analysis system (Metafer, Zeiss). 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 (Fig. 1).

To investigate alternative dosing regimes, chronic dosing of TK6 cells was performed over a 5-day period with MMS or MNU (Figs. 1C and 1D). RPD values, indicating cytotoxicity levels, are presented in Table 2. The "Notes: The overall concentration quoted hereafter represents a cumulative dose for the chronic studies. The "1-2" nomenclature refers to 2 additional, non-dosing days: 1 × 24 h seeding and 1 × 24 h of recovery.

Table 2. Primers Utilized for PCR Experiments Were Obtained from Sigma-Aldrich, UK

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence</th>
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<tr>
<td>β-Actin forward</td>
<td>5′-GATGGCCACGGCTGTTC-3′</td>
</tr>
<tr>
<td>β-Actin reverse</td>
<td>5′-TGCCTCAGGCGAGGAA-3′</td>
</tr>
<tr>
<td>DMC1 forward</td>
<td>5′-TGTCAGTCAGAGGCTGCGG-3′</td>
</tr>
<tr>
<td>DMC1 reverse</td>
<td>5′-AATCCTCCTCCCTGACCAC-3′</td>
</tr>
<tr>
<td>MGMT forward</td>
<td>5′-CTATCAGAATTCCCGTG-3′</td>
</tr>
<tr>
<td>MGMT reverse</td>
<td>5′-CGCCTTAAAGTTCCCGTAAGAATG-3′</td>
</tr>
<tr>
<td>MPG forward</td>
<td>5′-GGTCCGACTCCACAGAAGC-3′</td>
</tr>
<tr>
<td>MPG reverse</td>
<td>5′-CTGCGATGCCTGGCCCG-3′</td>
</tr>
<tr>
<td>RAD51L1 forward</td>
<td>5′-GCCCAAAACTGACACGCGTT-3′</td>
</tr>
<tr>
<td>RAD51L1 reverse</td>
<td>5′-AGCCACACCGATCGACGGG-3′</td>
</tr>
<tr>
<td>XRC23 forward</td>
<td>5′-GAACCCGCGGGAGATGTCGAC-3′</td>
</tr>
<tr>
<td>XRC23 reverse</td>
<td>5′-CCGGCTGTTTTGCTGACTGCAC-3′</td>
</tr>
</tbody>
</table>

Note: Primers were optimized using DNA PAGE.
performed with MMS (Supplementary Fig. 2), where genotoxicity was removed completely for the dose range tested.

Statistical analysis, specifically the Broken Stick Dose-Response Model, 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 (Fig. 2) implied that threshold dose-responses were a possibility for MMS, whereas the dose-response was predicted 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 2.0 μg/ml, a dose above the LOGEL (Fig. 3).

**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 base excision repair pathway (Seo et al., 2002), which is strongly associated with repair of lesions induced by alkyl sulfonates (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 1.0 μg/ml in TK6 to 0.7 μg/ml in NH32 (Fig. 4). Interestingly, the NH32 chronic LOGEL occurred at the same dose as for the acute MMS study in TK6, which was also 0.7 μg/ml. With increasing dose of MMS in NH32 cells, micronucleus frequency increased more rapidly compared with TK6, with NH32 reaching a maximum of 8.07% micronucleated cells at 2 μg/ml, whereas for the same MMS dose, TK6 cells demonstrated an almost 3-fold lower induction of 2.96%. The p53-deficient cells were also more sensitive to cytotoxicity. At day 7, the RPD value for TK6 at 2 μg/ml was 84.3% of control, whereas greater cytotoxicity was observed for NH32, where the RPD value was 60.3% (Supplementary Fig. 3A). 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%. 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

**FIG. 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 0.7 μg/ml (P < 0.001) for acute exposure and 1.0 μg/ml (P < 0.001) for chronic exposure. The first statistically significant increase in micronucleus frequency for MNU acute was observed at 0.46 μg/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.
phospho<sup>Ser15</sup>-p53 was performed in TK6 (Supplementary Fig. 3B). The blots confirmed the presence of p53 in TK6, although produced no evidence of high p53 activation. A double band was observed at 53 kDa for phospho-p53, perhaps owing to multiple phosphorylated forms of p53 present in the sample.

MGMT levels were also analyzed, as MGMT is involved in the direct reversal of methylation of O<sub>6</sub>-methylguanine lesions (Kaina et al., 2007). The aim was to assess whether TK6 and NH32 express MGMT at comparable levels. However, these results confirmed that MGMT could not be detected in either cell line. Therefore, this suggests that differences in rates of DNA repair of potentially clastogenic lesions by MGMT would not be likely to influence the results comparing the 2 cell lines.

**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 analyzed the mRNA expression of 4 DNA repair genes during the chronic dosing period (Fig. 5). qRT-PCR was used to assess mRNA levels extracted at selected timepoints (days 2, 4, and 6) throughout the chronic 5+2 day assay for 0 and 2 μg/ml MMS treatments (n = 1).

**FIG. 4.** p53 deficiency increased sensitivity to MMS during the chronic 5+2 day study (n = 3), although p53 protein levels remained unchanged in p53-proficient TK6 (n = 3) (Supplementary Fig. 3B). Micronucleus frequency (%) following chronic 5+2 day dosing with MMS in NH32 cells, when compared with the chronic 5+2 day study in TK6 cells (as presented in Fig. 1C). For NH32, the first statistically significant increase was observed at 0.7 μg/ml (P = 0.026). Doses 0.9, 1.0, and 2.0 μg/ml for NH32 are in duplicate and error bars and asterisks are therefore excluded.
timepoints. 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 3 genes increased at day 6, following a cumulative dose of 1 \( \mu g/ml \) MMS. Further, expression peaked for these genes at day 4, following the 0.5 \( \mu g/ml \) treatment. qRT-PCR human DNA repair 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 RT-PCR analysis. Although this produced no statistically significant changes in RAD51L1 expression, 0.5 and 1.0 \( \mu g/ml \) levels were less than untreated control levels (Fig. 5C). Similarly, the remaining 3 genes produced no statistically significant changes in mRNA expression, although a general increasing trend was observed with increasing number of days for 1.0 \( \mu g/ml \) for all genes, whereas expression at 0.5 \( \mu g/ml \) peaks at day 4 prior to decreasing at day 6. Levels of MGMT were also analyzed (data not shown), although as mentioned previously, accurate assessment of MGMT expression was not possible as TK6 cells express MGMT at barely detectable levels (Supplementary Fig. 4) (Hickman and Samson, 2004). This has also been observed previously in our laboratory.

DISCUSSION

Exploration of chronic dosing regimes in vitro in the low dose region may facilitate a comprehensive understanding of the effects of chemicals without bias toward high, acute doses. Such dosing regimes could be important for safety assessment of chemicals on the basis that this type of dosing is likely to better represent 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 2 methylation agents led to 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 (Fig. 1 and Supplementary Fig. 2). Previously, dose fractionation of a mutagenic dose to smaller doses has produced similar effects in vivo for both MMS (Leilei Tang, George Johnson, Stephen Dertinger, Thomas Singer, Andreas Zeller and Melanie Gue´rard, in preparation) and its ethyl form, ethyl methanesulfonate (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-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 reversal of damage by 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. Another interesting factor that may contribute to the differences between acute and chronic dosing regimes is the issue of test chemical persistence in the chronic test system. While test chemical may be removed from the cells following the acute exposure period, during chronic exposure test chemical added with the first dose, for example, could persist in the test system throughout the assay. Therefore, this might contribute to test chemical accumulation within the system. However, the half-lives of MMS and MNU are likely to be hours in solution, rather than days. Nonetheless, this might be important to consider for other agents.

Although these data might assist with avoidance of misleading positive results, it could equally be concluded that real-positive chemicals could produce “false negative” outcomes with the chronic dosing regime. This is highlighted by the observation that MNU produced a negative result in the 5 + 2 day study, whereas a positive result was produced following acute exposure (Fig. 1). However, this could simply owe to the fact that MNU is a relatively poor clastogen when compared with MMS (Beranek, 1990); this perhaps explains why MNU produced a negative result for the 5 + 2 day study, whereas MMS did not. Nonetheless, based on the present data, it is unclear whether the chronic regime is too insensitive, or whether acute dosing is oversensitive. It is worth noting 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 hypothesize 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 1.0 μg/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; Zair 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 (Coquerelle et al., 1995; Fishel et al., 2003, 2007; Ibeanu et al., 1992; Rinne et al., 2004, 2005). However, as MPG recognizes N7 methylguanine 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 4 h 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 up-regulating 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 (Fig. 4 and Supplementary Fig. 3A). This was unsurprising, as p53 deficiency has previously been associated with reduced growth arrest and DNA repair, and therefore a higher incidence of micronuclei (Brüsehafer et al., 2014; Driessens et al., 2003; Masunaga et al., 2002). Indeed, studies with DNA repair-proficient and -deficient Ames strains emphasized the importance of efficient DNA repair in genotoxic tolerance (Tang et al., 2014). Interestingly, protein levels of p53 and phosphoSer15-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 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 to in vitro testing for assessing the relevance of in vitro positive results. The results from this initial study indicate that the incorporation of chronic dosing into initial in vitro tests could lead to the reduction of unnecessary follow-up tests in animals. The validity of this approach needs to be verified with a larger and structurally more diverse set of compounds in the future.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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