Chromosome Breakage Induced by the Genotoxic Agents Mitomycin C and Cytosine arabinoside is Concentration and p53 Dependent

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The p53 tumor suppressor protein plays an essential role in cellular integrity and inactivation of the TP53 gene by mutation is the most frequent alteration in human cancer. As loss of p53 function is associated with increased genetic instability, it is important in genotoxicity testing to explore the role of p53 competency. In vitro model systems for genotoxicity testing are sometimes prone to misleading positive results; some of this loss of predictivity may be caused by p53 inactivation in some cell models. To explore whether impaired p53 function plays a role in mutation sensitivity, TK6 cells (p53 competent) and NH32 cells (p53 deficient) were treated with two known genotoxicants, mitomycin C (MMC) and cytosine arabinoside (araC). Chromosomal damage was assessed in the low dose region by an automated micronucleus system and p53 activity was investigated by gene and protein expression analysis. Cell cycle progression studies were also assessed. Low levels of micronucleus and p53 induction were observed in TK6 cells treated with MMC. On the other hand, higher levels of micronucleus and p53 induction were shown in TK6 cells treated with araC and a G1/S arrest was observed after araC treatment. p53 deficient NH32 cells showed an increased sensitivity of micronucleus (MN) induction after araC treatment compared with TK6 cells and less of an active G1/S phase checkpoint. Thus, impaired p53 function sensitizes cells to genotoxicants and plays a central role in the DNA damage response. This data has clear importance for safety assessment of genotoxicity and shows how crucial p53 competence is.  

Key words: p53; genomic instability; cell cycle; micronucleus; threshold  

Genotoxic substances can potentially induce multiple types of DNA lesions simultaneously. For example, simple alkylating agents inducing specific DNA lesions (adducts) readily induce point mutations through misreplication of the adducted DNA base. However, these simple lesions can also result in chromosome breakage (and thereby amplifications, deletions, and translocations) through processing of the damaged base either spontaneously or by various DNA repair pathways (Doak et al., 2007; Jenkins et al., 2010). As a consequence of the key role of mutagenesis, the ability to detect substances which cause DNA damage is essential in the risk assessment for human populations. In addition to the identification of genotoxicants, investigating if these DNA reactive compounds follow linear or nonlinear (threshold) dose response relationships after exposure to low concentrations can further improve health risk assessments. Genotoxicants with thresholded dose responses pose very different risks to humans as compared with genotoxicants with linear dose responses. DNA reactive genotoxins are largely believed to induce nonthresholded, linear responses, because they directly induce DNA lesions that have the potential to be fixed as point mutations or chromosomal aberrations (Henderson et al., 2000). However, mammalian cells have a number of homeostatic mechanisms, e.g., DNA repair or detoxification processes, that provide protection to a certain extent, resulting in a no observed effect level (NOEL) (Doak et al., 2008; Seager et al., 2012; Thomas et al., 2013).  

The tumor suppressor gene TP53 plays an essential role in cellular integrity as a transcriptional activator that regulates the expression of various genes involved in cell cycle arrest and apoptosis in response to genotoxic and cellular stress (Arrowsmith, 1999). Cells deficient in functional p53 protein fail in cell cycle arrest and elimination of damaged cells via apoptosis after a genotoxic insult (Honma and Hayashi, 2011). Further, it was shown that loss of p53 function increases genetic instability causing aneuploidy, gene amplification, and point mutation (Shao et al., 2000). It is therefore essential to understand the role of p53 in model systems used in genotoxicity testing to as-
Assess the potential carcinogenicity of genotoxic agents. In recent publications, it was demonstrated that cells with impaired p53 function are more susceptible to cytotoxicity and micronucleus induction than p53 competent cells (Fowler et al., 2012; Pfuhler et al., 2011). Oversensitivity of in vitro test systems has been blamed for misleading positive responses in vitro, i.e., in vitro positives not confirmed in further in vivo studies.

Mitomycin C (MMC) and cytosine arabinoside (araC) are known genotoxicants. MMC, a cross-linking agent, is known for its wide range of specific adverse biological effects in mammalian cells. Examples are selective inhibition of DNA synthesis, mutagenesis, induction of DNA repair in bacteria (SOS response), and induction of apoptosis (Mao et al., 1999). MMC causes damage to DNA by mono- and bifunctional alkylatation of guanine residues that lead to mitomycin C-guanine mono- or bis-adducts as well as DNA interstrand and intrastrand cross-links (Paz et al., 1999). These cross-links are formed specifically at CpG and GpC sequences. The main cause of MMC cytotoxicity is the interstrand MMC-DNA cross-links (Boamah et al., 2007). araC is a nucleoside analogue of both cytidine and deoxycytidine and is known as a “phase-specific” anticancer agent, because of its ability to inhibit the de novo DNA synthesis effectively only when cells are in S-phase of the cell cycle and its rapid in vivo detoxification (Henderson, 1982). Cellular effects of araC include: inhibition of cell growth, inhibition of DNA synthesis, and proliferation-dependent cytotoxicity (Vale-riote, 1982). AraC is phosphorylated in the cell with ATP as the phosphate donor and the triphosphate, araCTP, inhibits DNA replication (Kufe and Major, 1982).

The current study aimed to investigate the dose response curves at very low concentration levels of the genotoxic agents MMC and araC in p53 competent TK6 and p53-null NH32 cells to examine whether p53 abrogation affects DNA damage induction. Chromosomal damage was assessed using the in vitro cytokinesis blocked micronucleus (CBMN) assay, whereas p53 activation was investigated using gene and protein expression analysis. Furthermore, cell cycle changes were investigated using flow cytometry.

**MATERIALS AND METHODS**

**Test Agents**

MMC and araC were purchased from Sigma-Aldrich (Dorset, UK) and dissolved in water at a stock concentration of 1 mg/ml. Chemical dilutions were freshly prepared from stock solutions with water.

**Cell Lines and Culture Conditions**

In this study, the human lymphoblastoid cell lines TK6 and NH32 were used.

**TK6.** The human lymphoblastoid cell line TK6 is a derivative of the WIL-2 cell line. The cells are heterozygous at the thymidine kinase (TK) locus and contain the wild-type TP53 gene. TK6 cells were acquired from the European Collection of Cell Cultures (ECACC), Salisbury, UK (Cat. no. 95111735).

**NH32.** The NH32 cell line was a kind gift from Prof. Dr Gerald N. Wogan (MIT, Cambridge, MA). Like TK6, the human lymphoblastoid cell line NH32 is a derivative of the WIL-2 cell line. The cells contain a double p53 knockout mutation through a promoterless gene targeting approach (Chuang et al., 1999; Hashimoto et al., 2011).

All cell lines were cultured in RPMI 1640 (Gibco Invitrogen, Paisley, UK) supplemented with 10% donor horse serum (BD Gentest, Oxford, UK) and 1% l-glutamine (Gibco Invitrogen). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in air.

**Treatment with Test Agents**

For the CBMN assay, TK6 (p53 competent) and NH32 (p53 deficient) cell cultures were seeded at 1 × 105 cells/ml for 24 h and then treated with either MMC (4 h) or araC (24 h) followed by one cell cycle of cytotoxicity. Chromosomal damage was assessed using the CBMN assay, whereas p53 activation was investigated using gene and protein expression analysis. Furthermore, cell cycle changes were investigated using flow cytometry.

**The CBMN Assay**

All cell suspensions were seeded at 1 × 105 cells/ml for 24 h at 37°C, 5% CO2. Each cell suspension was treated with the appropriately diluted test chemical and 4.5 μg/ml cytochalasin B (Merck, Darmstadt, Germany) for one cell cycle. After the incubation times, the suspensions were removed from each flask and transferred into appropriately labeled tubes. The cells were spun down (10 min, 200 × g), washed with PBS and resuspended in 10 ml fresh medium.

A semiautomated scoring protocol was performed with the Metafer-System (MetaSystems, Altlussheim, Germany). For semiautomated scoring, cells were prepared as described by Varga et al. (2004). Shortly, after washing the cells with PBS, the cells were treated with 0.56% KCl solution and centrifuged (10 min, 200 × g) immediately. After resuspending the cells were fixed with a methanol/acetic acid/0.09% NaCl (5:1:6) solution for 10 min. The first fixative was removed by centrifugation and a second fixation step was then performed with a methanol/acetic acid (5:1) solution for 10 min, followed by four
wash steps with fixative 2. The cells were maintained in the final fixative 2 wash at 4°C overnight. The fixed cells were then dropped across the length of polished and hydrated slides. Subsequently, the cells were stained for 10 min in the dark with a DAPI (4′,6-diamidino-2-phenylindole) + vectashield (Vector Lab., Inc., UK) solution and scanned with the Metafer4 (version 3.8.5) software. A minimum of 2000 binucleated cells per replicate (experiments were conducted in triplicates) were scored.

**Cytotoxicity**

Relative population doubling (RPD) was used to measure cytotoxicity in the absence of cytochalasin B. Therefore, satellite cultures were seeded as above for the CBMN assay. Cells were counted with the Beckman coulter counter (Z1 Coulter Particle Counter, Beckman, UK) 1 h before treatment (control cultures) and at the cell harvest stage (treated cultures).

RPD was then determined as:

\[
\text{RPD} = \frac{\text{number of population doublings in treated cultures}}{\text{number of population doublings in control cultures}} \times 100
\]

where

\[
\text{population doubling} = \frac{\log_{2}(\text{posttreatment cell number}/\text{initial cell number})}{\log_{2}2}
\]

**Gene Expression Analysis**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Sussex, UK) according to the manufacturer’s instructions. The RNA content was measured and the purity was assessed (260:280 ratio) with a NanoDrop ND-1000 Spectrophotometer (Labtech International, Uckfield, UK).

Real-time reverse transcription-PCR (RT-PCR) was used to quantify the expression of \( p21 \). The RNA was reverse transcribed using the QuantiTect Reverse Transcript Kit (Qiagen). The manufacturer’s instructions were followed. Real-time PCR was then performed on an iCycler iQ5 Thermal Cycler (Bio-Rad, Hertfordshire, UK) using the QuantiFast SYBR Green PCR Kit (Qiagen). Appropriate primer sets for the test gene were designed: \( \beta\)-actin forward 5′-GATGCCAGGCTGGCTTC-3′ and reverse 5′-TGCCCTAGGCAAGGGAA-3′; \( p21 \) forward 5′-GACCTCTAGGGGTCGAAAACG-3′ and reverse 5′-GGATTAGGGCTTCTCTTG-3′. The resultant data were analyzed by the standard curve method.

**Western Blotting**

Treated cell pellets were resuspended in 200 \( \mu l \) ice-cold 1 × radioimmuno-precipitation lysis (RIPA) buffer (Sigma-Aldrich) supplemented with 2 \( \mu l \) protease inhibitor cocktail (Sigma-Aldrich) and 2 \( \mu l \) phosphatase inhibitor cocktail 2 (Sigma-Aldrich) and the cell suspensions were transferred to prechilled microcentrifuge tubes. After 5 min incubation at 4°C, the cells were lysed by vortexing thoroughly and spun down for 10 min at 10,000 \( \times \) g in a centrifuge precooled to 4°C. Finally, the supernatant was transferred to a new microcentrifuge tube and after protein quantification (Bio-Rad DC Protein Assay; Bio-Rad) aliquots of the protein samples were stored at \(-80°C \) until further use.

Protein extracts (40 \( \mu g \)) were mixed with one volume of Laemmli buffer (Sigma-Aldrich) and then boiled for 5 min at 95°C. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were electroblotted onto an Immun-Blot PVDF membrane (Bio-Rad) using the Mini Trans-Blot Cell system (Bio-Rad). The membranes were incubated with 1:1000 dilutions (\( p53/\text{phospho}-p53 \)) or 1:2000 dilutions (\( \beta\)-actin) of primary antibodies specific to \( p53 \), phospho-\( p53 \) (Ser15) and \( \beta\)-actin (Cell Signaling, New England Biolabs, Herts, UK). After washing the membranes, a secondary horsereadish peroxidase (HRP) conjugated goat polyclonal antibody to rabbit IgG (1:1000 dilution; Abcam, Cambridge, UK) was applied.

The Bio-Rad Immun-Star Western Chemiluminescent Kit (Bio-Rad) was used for protein detection after washing off the excess and nonspecifically bound antibodies. The membranes were visualized using the Chemidoc XRS system (Bio-Rad).

**Cell Cycle Analysis**

The cell cycle status was analyzed by quantitation of DNA content using flow cytometry. All cell suspensions (10 ml) were seeded at 1 \( \times \) 10\(^5\) cells/ml for 24 h at 37°C, 5% CO\(_2\) and treated with test chemicals. An appropriate amount of cells (1 \( \times \) 10\(^6\)) were transferred to each tube and spun down at 4°C for 10 min at 200 \( \times \) g. After removing the supernatant, the cells were resuspended in 300 \( \mu l \) ice-cold PBS. To fix the cells, 700 \( \mu l \) cold ethanol (70%) were added dropwise to the tube containing 300 \( \mu l \) of cell suspension in PBS, while vortexing gently. The cells were left for 1 h on ice or up to a few days at 4°C. Subsequently, the cells were spun down, washed once with cold PBS, before recentrifugation and removal of the supernatant. Afterward, the cell pellet was resuspended in 250 \( \mu l \) PBS, 5 \( \mu l \) of 10 mg/ml RNase A were added at a final concentration of 0.2–0.5 mg/ml and the cells were incubated at 37°C for 1 h.

Finally, 10 \( \mu l \) of 1 mg/ml propidium iodide (PI) solution (Sigma-Aldrich) at a final concentration of 10 \( \mu g/ml \) was added and the cells were kept in the dark at 4°C until analyzed.

The DNA content was quantified using the BD FACS Aria flow cytometer (BD Biosciences, Oxford, UK) and the BD FACSDiva software (BD Biosciences) was used for analysis. The forward scatter (FSC) and side scatter (SSC) were measured to identify single cells and a suitable filter (PE-A: 585/42) was chosen to measure PI (excitation = 536 nm; emission = 617 nm). Cell count was plotted on the y-axis, while PI was plot-
response modeling software package PROAST (Gollapudi et al., 2013). 

For qRT-PCR experiments and the cell cycle type of work a one-way ANOVA approach, followed by a two-sided Dunnett’s post hoc test was used to determine if any of the treatment concentrations were significantly different from the control concentration for the MN data. BMD values were derived using the dose response modeling software package PROAST (Gollapudi et al., 2013). 

For qRT-PCR experiments and the cell cycle type of work a one-way ANOVA approach, followed by a two-sided Dunnett’s post hoc test was used to determine if any of the treatment concentrations were significantly different from the control concentrations.

RESULTS

NH32 cells are human lymphoblastoid cells and like TK6 cells derived from the WIL-2 cell line with a double p53 knock-out (Chuang et al., 1999; Hashimoto et al., 2011). In order to assess the role of p53 in the genotoxic dose responses for two genotoxicants (MMC and araC), NH32 and TK6 cells were compared in parallel. Firstly, however, p53 expression was investigated in TK6 and NH32 cells after treatment with araC for 24 h (0, 0.01, and 0.1 µg/ml) to confirm p53 status. As expected, no p53 protein expression was observed in NH32 cells, whereas total p53 was clearly evident in TK6 cells and was also clearly induced by araC exposure (Fig. 1).

Chromosome damage induction was subsequently investigated in TK6 and NH32 cells after exposure to low concentrations of MMC and araC. The low range of concentrations of MMC and araC were chosen based on the cytotoxicity profile following a dose range finding assay (data not shown).

Chromosomal Damage Induction

The CBMN assay was performed following OECD guidelines (OECD 487, 2010) utilizing for MMC a short (4 h) exposure plus 18 h recovery time. The chosen exposure/recovery time were optimized using empirical experiments bearing in mind the need to keep toxicity levels below 55 ± 5% as dying cells can confound the micronucleus assay. TK6 cells showed significant increases in MN induction only at higher concentrations after treatment with MMC (0–0.1 µg/ml), whereas at the lower range of concentrations, no significant increases in MN were observed (Fig. 2). A NOEL for TK6 cells of 0.04 µg/ml was identified. Statistically significant increases in MN frequency were observed thereafter, resulting in a lowest observable effect level (LOEL) of 0.06 µg/ml MMC. Furthermore, 45.8% cell death and cytostasis was observed in TK6 cells at 0.1 µg/ml MMC (Fig. 2).

In comparison, NH32 cells treated with MMC for 4 h, followed by one cell cycle of cytochalasin B (18 h), showed increases in MN induction at the relatively lower concentrations of 0.02 and 0.04 µg/ml (LOEL) MMC. Furthermore, higher levels of cell death and cytostasis (54.3%) were observed at the lower concentration of 0.06 µg/ml MMC, highlighting the greater sensitivity of this cell line to both chromosome damage and toxicity (Fig. 2).

Extended treatments were used for the investigation of the low dose response curves for araC in TK6 and NH32 cells. Due to the “phase-specific” mechanism of action of araC, more than one cell cycle is needed for chromosome damage to be expressed. As for MMC, dose exposure time and recovery were optimized using empirical experiments. TK6 cells treated with araC for 24 h over a range of concentrations between 0 and 0.07 µg/ml, followed by a further cell cycle (18 h) in the presence of cytochalasin B showed significant increase in MN induction only at the highest concentrations tested (Fig. 3). A NOEL of 0.01 µg/ml was identified in TK6 cells and a LOEL concentration at 0.03 µg/ml araC was determined. Furthermore, ≥55 ± 5% cell death and cytostasis were observed in TK6 cells at ~0.07 µg/ml araC (62.5%) (Fig. 3). In comparison, NH32 cells treated with araC, showed significant increase in MN induction at concentrations as low as 0.001 µg/ml (LOEL) and above, whereas higher levels of toxicity (90.6%) were observed at the...
lower concentration of 0.03 μg/ml araC (Fig. 3). Similarly to MMC, this highlights the increased sensitivity of the NH32 cells to both toxicity and chromosome damage.

The dose responses after MMC and araC were further assessed by quantitative means using BMD modeling. For the TK6 data after treatment with MMC, a BMD was calculated at 0.008 μg/ml with its confidence interval between 0.007 μg/ml (BMDL10) and 0.01 μg/ml. The NH32 data set revealed a BMD at 0.018 μg/ml with its confidence interval between 0.01 μg/ml (BMDL10) and 0.02 μg/ml. The BMD for the dose response data in TK6 cells treated with araC was calculated at 0.001 μg/ml with its confidence interval between 0.0007 μg/ml (BMDL10) and 0.002 μg/ml, and for the NH32 data set, the BMD was calculated at 0.001 μg/ml with its confidence interval between 0.0004 μg/ml (BMDL10) and 0.002 μg/ml (Table 1).

In summary, NH32 cells treated with araC showed a much higher magnitude of MN induction than TK6 cells treated with the same concentration of the nucleoside analogue. However, NH32 cells treated with MMC showed a similar magnitude of MN induction compared with the parent TK6 cells line treated with the cross-linker. This suggests a difference in response between the two agents in p53 deficient versus proficient cells.

**p53 Activation After MMC and araC Treatment in TK6 Cells**

p53 activation assessment was only performed in the p53 competent TK6 cell line. In line with the chromosomal damage studies, TK6 cells were treated with MMC for 4 h over a range of concentrations (0, 0.004, 0.008, 0.02, 0.04, and 0.08 μg/ml) below and above the LOEL observed for chromosome damage induction. No significant increases in p21 gene expression were observed in TK6 cells (Fig. 4A). However, an overall increase in phospho-p53 (Ser15) in TK6 cells with increasing concentrations of MMC was noticed in all replicates. No increases in total p53 protein expression were observed however (Fig. 4B).

In contrast, when TK6 cells were treated with araC for 24 h over a range of concentrations (0, 0.0007, 0.005, 0.01, and 0.07 μg/ml) below and above the chromosomal damage LOEL, an overall increase, but not to a significant level, in p21 gene expression with increasing concentrations of araC was observed (Fig. 4C). Furthermore, both total p53 and phospho-p53 (Ser15) protein expression was substantially increased over the range of concentrations tested in all replicates (Fig. 4D).

In conclusion, p53 activation was observed in TK6 cells at concentrations that caused significant increases in MN induction for chromosomal damage, but this was particularly striking following araC exposure.

**Cell Cycle Analysis in TK6 and NH32 Cells**

Due to the link between p53 and chromosome damage induction identified above, coupled to the role of p53 in controlling cell cycle checkpoints, the cell cycle status of treated and untreated TK6 and NH32 cells after exposure to MMC and araC were assessed using flow cytometry. Cell cycle analysis in TK6 cells treated with MMC for 4 h, followed by a recovery period of 18 h, revealed a significant decrease in the percentages of cells in the G1/G0 phase at the top concentration of 0.1 μg/ml (42.2% in untreated cells, 30.8% at 0.1 μg/ml MMC), with more cells being arrested in G2/M phase (25.9% in untreated, 39.4% at 0.1 μg/ml MMC), when compared with the control (Table 2). Similar results were observed in NH32 cells treated with MMC. At 0.04 μg/ml, a significant decrease of cells in

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**FIG. 3.** Effect of araC on TK6 and NH32 cells using the CBMN assay. TK6 and NH32 cells were treated with araC for 24 h, followed by 18 h of cytochalasin B. Bars: percentage micronucleated binucleated cells. Line: relative population doubling (RPD). Values represent mean ± SD. **p < 0.01, ***p < 0.001.

**FIG. 4.** p53 activation in TK6 cells following MMC and araC treatment. (A) Relative fold change in p21 gene expression in TK6 cells, following exposure to increasing concentrations of MMC. Levels of p21 mRNA were assessed by real-time RT-PCR. (B) Representative Western blots for p53 protein, phospho-p53 (Ser15) protein, and β-actin as loading control following treatment in TK6 cells with 0–0.07 μg/ml araC for 24 h. Values represent mean ± SD. **p < 0.01, ***p < 0.001.
the G1/G0 phase was demonstrated (although the reduction of 7.1% was less than in TK6 cells—11.4%) (Table 2).

In contrast, TK6 cells treated with araC for 24 h, followed by a recovery period of 18 h showed an arrest of cells in the G1/G0 phase at the higher range of concentration, where DNA damage induction was also observed, whereas cells in G2/M phase decreased (Table 2). NH32 cells treated with araC showed no arrest of cells in G1/G0 phase and/or a decrease of cells in G2/M phase over the range of concentrations.

In conclusion, NH32 cells were found to be less prone to cell cycle arrest in comparison to TK6 cells in response to treatment with both chemicals. This is a particularly interesting observation as cell cycle arrest induced by DNA damage at the G1/S checkpoint is known to be p53 dependent, whereas cell cycle arrest at the G2/M checkpoint can be initiated in the presence or absence of p53.

**DISCUSSION**

p53 abrogation affect on DNA damage induction after treatment with two genotoxicants was investigated to better understand the role of p53 status in well validated model systems. The chromosomal damage studies revealed that p53 competent TK6 cells treated with MMC and araC demonstrate increases in MN frequency only at higher exposure concentrations, whereas NH32 (p53 deficient) cells were more sensitive to MN induction compared with the TK6 cell line after identical MMC and araC treatment. In addition, NH32 cells were more sensitive to cytotoxicity after MMC and araC treatment than the p53-proficient TK6 cells. Hence, in vitro test systems with defective p53 function will be overly sensitive to genotoxicants, potentially leading to misleading positive responses. Our data also indicate that p53 deficient cells have higher levels of genomic instability and thus p53 mutated cancer cells may mutate and evolve faster than p53 proficient cells. Further work to optimize this killing effect is warranted.

Overall, p53 induction in TK6 cells treated with MMC for 4 h was low, suggesting that p53’s impact might be less pronounced under the chosen short treatment condition (and because low concentrations were deliberately targeted here). Natural defense mechanisms, like DNA repair might be responsible for the low DNA damage induction of MMC in TK6 and NH32 cells. A nonlinear dose response of araC was observed after an extended treatment in TK6 cells. Significant increases in MN induction were only seen at the higher range of concentrations. A study by Asano et al. (2006) provided evidence for the existence of a practical threshold that is defined as a concentration below which any effect is biologically unimportant for araC and MMC *in vivo*, supporting the findings in this study. In addition, BMD modeling estimated a concentration that produces predetermined biologically relevant increases in response over the control. The lower limit on the BMD is termed BMDL and is often considerate an adequate point-of-departure (POD) extrapolation for dose response data (Gollapudi et al., 2013), providing further evidence for nonlinear dose response relationships in the human lymphoblastoid cell lines in particular after araC treatment.

p53 predominantly acts at the DNA damage checkpoint in the cell cycle, influencing cell proliferation (Pucci et al., 2000). DNA damage induction triggers p53 activation, which leads to downstream signaling to target genes such as p21. Consequently p53 activation was investigated by p21 gene expression and p53/phospho-p53 (Ser15) protein expression analysis. No increases in p21 gene expression were observed in TK6 cells after MMC exposure, whereas TK6 cells treated with araC showed increases in p21 gene expression over background with increasing concentrations. Chromosomal damage was more strongly induced in araC exposed TK6 cells, when compared with the MMC treatment, which correlates with the elevated levels of p21 in TK6 cells treated with araC. Loss of p53 function is associated with increased genetic instability. p53 activation was investigated by assessing p53 and phospho-p53 (Ser15) protein expression analysis, as DNA damage induces phosphorylation of p53 at serine residues (Lakin and Jackson, 1999). Increases in phospho-p53 (Ser15) levels were observed in TK6 cells treated with MMC for 4 h, but the overall p53 induction after MMC treatment was low. It has been suggested that DNA strand breaks might be critical to trigger p53 induction and thus agents capable of directly causing strand breaks are more effective in activating p53 than agents that cause DNA cross-links (such as MMC), base modifications, intercalate into the DNA, or cause interference with the cell cycle or cellular metabolism (Nelson and Kastan, 1994).

TK6 cells treated with araC showed high increases in p53 and phospho-p53 (Ser15) protein expression over the range of concentrations. These results suggest a direct relationship between p53 levels and the induction of MN. High increases in p53 expression were observed at concentrations that were found to be

<table>
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<tr>
<th>Genotoxicant</th>
<th>Treatment</th>
<th>Cell line</th>
<th>Units</th>
<th>NOEL</th>
<th>BMDL10</th>
</tr>
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<tr>
<td>MMC</td>
<td>4 + 18 h</td>
<td>TK6</td>
<td>µg/ml</td>
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<td>0.007</td>
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<td>MMC</td>
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<td>araC</td>
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<td>0.0007</td>
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<tr>
<td>araC</td>
<td>24 + 18 h</td>
<td>NH32</td>
<td>µg/ml</td>
<td>0.009</td>
<td>0.0004</td>
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</table>
showed a direct relationship between p53 levels and the induction of MN in RKO cells treated with genotoxic chemicals, indicating that the level of p53 might be associated with chromosomal damage (Salazar et al., 2009). Further, p53 binds directly to sites of DNA damage, such as mismatches and single-stranded DNA, showing the potential of p53 as a direct DNA damage detector (Amundson et al., 2009).

Activation of p53 can lead to cell cycle arrest to prevent the proliferation of damaged cells and to allow DNA repair before replication and mitosis or induce apoptosis to eliminate irreparable damaged cells (Amundson et al., 1998). TK6 cells treated with MMC for 4 h, followed by a recovery time of one cell cycle showed G2/M arrest in particular at 0.1 μg/ml MMC. Similar results were gained in NH32 cells treated with the cross-linker (less so though). A study by Islaih et al. (2005) analyzed the cell cycle in TK6 cells after treatment with different genotoxins, such as MMC. The cells were exposed for 4 h and cell cycle analysis was performed at 4 and 20 h following treatment. Cells collected at the 8 h time point showed significant increases in the percentage of cells in S phase, whereas cells collected at the 24 h time point showed a strong G2/M arrest (Islaih et al., 2005). Furthermore, a study by Lukamowicz et al. (2011) revealed a dose-dependent accumulation of cells in G2/M phase after MMC treatment. The G2/M checkpoint seems to be activated, when DNA synthesis is blocked to prevent segregation of damaged or incompletely synthesized DNA (Pucci et al., 2000).

The cyclinB1/Cdc2 complex is the main regulatory factor for the entry into M phase and phosphorylation of cdc2 or inhibition of cyclinB1 can cause G2 arrest (Amundson et al., 1998).

In addition, p53-regulated genes can regulate the G2 arrest to a certain extent. Further experiments are required to clarify the mechanism behind the G2/M arrest by MMC.

TK6 cells exposed to araC for 24 h with an extended recovery period of one cell cycle, showed concentration-dependent increases in the percentage of cells in G1/G0 phase. p53 predominately acts at the G1/S phase in the cell cycle, influencing cell proliferation. The sequence-specific transactivation function of p53 clearly mediates the G1/S phase arrest. Activation of p53 in this study was shown after araC treatment by increases in p21 gene expression as well as p53 and phospho-p53 (Ser15) protein expression. p53 was activated by DNA damage induced by araC and initiates the transcription of p21, a G1 cyclin-dependent kinase inhibitor (CK1) (Amundson et al., 1998; Pucci et al., 2000). Cell cycle progression is prevented by inhibition of phosphorylation of Rb/E2F complexes by p21 and inhibition of further downstream signaling (Amundson et al., 1998). Hence, the data support a general link between araC induced chromosome damage, p53 activation, and G1/S phase block in TK6 cells. NH32 cells treated with araC showed no cell cycle arrest in G1/G0 at concentrations that caused chromosomal damage. Without p53 expression damaged DNA was not prevented from being replicated, supporting the fact that p53 plays an essential role in cellular integrity and genomic instability. Genomic instability increases the risk of aneuploidy, point mutations, and homologous

### TABLE 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chemical</th>
<th>Dose (μg/ml)</th>
<th>G1/G0</th>
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<th>G2/M</th>
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<td>22.80 ± 2.95</td>
<td>25.93 ± 1.18</td>
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<td>4 h + 18 h</td>
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<td>32.03 ± 2.20</td>
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Note. Values represent mean ± SD.

* * * * * * p < 0.01.

** * * * p < 0.001.
recombination and therefore the development of tumorigenesis (Shao et al., 2000).

In conclusion, low levels of p53 induction were observed in TK6 cells treated with MMC. No significant increases in either p21 gene expression or p53/phospho-p53 (Ser15) protein expression were detected, suggesting that other defense mechanisms play a major role in the MMC dose response. This was further confirmed by the low DNA damage induction in NH32 cells. On the other hand, high p53 activity was shown in TK6 cells treated with araC. Increases in p21 expression as well as increases in total p53 and phospho-p53 (Ser53) were observed over the dose range, suggesting a G1/S arrest after araC treatment. In addition, NH32 cells showed a higher magnitude of MN induction and sensitivity after treatment (shift of LOEL to a lower concentration) than TK6 cells. The data presented in this study emphasize the central role of p53 in the DNA damage response and the usage of p53 competent cell lines in genotoxicity testing to reduce misleading positive results in in vitro testing systems. Further high increases in p53 expression were only observed at concentrations that were found to be statistically significant for MN induction, showing the potential of p53 as a direct DNA damage detector and as a potential endpoint for genotoxicity testing.

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