

Accepted Manuscript

Title: Genotoxicity testing: Comparison of the γ H2AX focus assay with the alkaline and neutral comet assays

Authors: Teodora Nikolova, Federico Marini, Bernd Kaina

PII: S1383-5718(17)30092-X
DOI: <http://dx.doi.org/doi:10.1016/j.mrgentox.2017.07.004>
Reference: MUTGEN 402837

To appear in: *Mutation Research*

Received date: 10-4-2017
Revised date: 14-7-2017
Accepted date: 17-7-2017

Please cite this article as: Teodora Nikolova, Federico Marini, Bernd Kaina, Genotoxicity testing: Comparison of the γ H2AX focus assay with the alkaline and neutral comet assays, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* <http://dx.doi.org/10.1016/j.mrgentox.2017.07.004>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



**Genotoxicity testing: Comparison of the γ H2AX focus assay
with the alkaline and neutral comet assays**

Teodora Nikolova¹, Federico Marini² and Bernd Kaina^{1*}

¹Institute of Toxicology and ²Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI), University Medical Center, Mainz, Germany

*Corresponding author:

Institute of Toxicology

University Medical Center

Obere Zahlbacher Str. 67

D-55131 Mainz

Germany

kaina@uni-mainz.de

Tel: 0049-6131-17-9217

Fax: 0049-6131- 17- 8499

Highlights

- All genotoxicants were positive in the γ H2AX foci assay.
- In the alkaline and neutral comet assay, mitomycin C was found to be negative.
- γ H2AX and comet are highly correlated for methylating and oxidizing agents.
- There was no correlation between γ H2AX and comet for crosslinkers like mitomycinC.
- The γ H2AX foci assay is reliable, sensitive and robust in detecting DNA damage.

Abstract

Genotoxicity testing relies on the quantitative measurement of adverse effects, such as chromosome aberrations, micronuclei, and mutations, resulting from primary DNA damage. Ideally, assays will detect DNA damage and cellular responses with high sensitivity, reliability, and throughput. Several novel genotoxicity assays may fulfill these requirements, including the comet assay and the more recently developed γ H2AX assay. Although they are thought to be specific for genotoxicants, a systematic comparison of the assays has not yet been undertaken. In the present study, we compare the γ H2AX focus assay with the alkaline and neutral versions of the comet assay, as to their sensitivities and limitations for detection of genetic damage. We investigated the dose-response relationships of γ H2AX foci and comet tail intensities at various times following treatment with four prototypical genotoxicants, methyl methanesulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), mitomycin C, and hydrogen peroxide (H_2O_2) and we tested whether there is a correlation between the endpoints, i.e., alkali-labile sites and DNA strand breaks on the one hand and the cell's response to DNA double-strand breaks and blocked replication forks on the other. Induction of γ H2AX foci gave a linear dose response and all agents tested were positive in the assay. The increase in comet tail intensity was also a function of dose; however, mitomycin C was almost completely ineffective in the comet assay, and the doses needed to achieve a significant effect were somewhat higher for some treatments in the comet assay than in the γ H2AX foci assay, which was confirmed by threshold analysis. There was high correlation between tail intensity and γ H2AX foci for MMS and H_2O_2 , less for MNNG, and none for mitomycin C. From this we infer that the γ H2AX foci assay is more reliable, sensitive, and robust than the comet assay for detecting genotoxicant-induced DNA damage.

Keywords: genotoxicity; γ H2AX foci assay; alkaline comet assay; neutral comet assay; DNA damage

INTRODUCTION

The safety assessment of pharmaceutical products, cosmetics, food ingredients, and environmental pollutants as to their effects on human genetic material requires a combination of tests, i.e., a battery of genotoxicity assays [1]. Numerous studies have shown that, due to non-uniform modes of action of genotoxicants, no single approach can detect all genotoxic agents [2]. The test battery uses various endpoints which reflect types of DNA damage, from initial DNA lesions (base adducts, crosslinks, single-strand (SSB) and double-strand breaks (DSB), etc.) to late downstream events such as mutations, chromosome aberrations, and micronuclei.

Routine genotoxicity tests evaluate the frequencies of gene mutations in bacteria (Ames assay) or mutations, chromosome aberrations, or micronuclei in mammalian cells and tissues. The single cell gel electrophoresis (“comet”) assay measures the migration of DNA from agarose-embedded cells in an electric field; it detects primary SSB and DSB, DNA breaks resulting from spontaneous hydrolysis of adducts, and repair intermediates associated with abasic sites and DNA incisions [3]. Modifications of the initial protocol can specifically detect DSB [4] or crosslinks [5]. Although the assay was already used for many years for experimental purposes and genotoxicity testing, an OECD guideline for *in vivo* comet assay was issued only in 2014 [6] after a pending period [7]. The specificity and sensitivity of the assay can be enhanced by treatment of the lysed cells with lesion-specific enzymes such as formamidopyrimidine glycosylase (FPG) or 8-oxoguanine-DNA glycosylase (OGG1) [8].

The γ H2AX foci assay, on the other hand, depends on the cellular response to damage, specifically the recruitment of the phosphorylated (activated) form of histone 2AX (referred to as γ H2AX) to sites of DSB [9-12]. Histone 2AX proteins, phosphorylated over large stretches around the DSB or blocked replication fork, and acting through the PI3-like kinases ATM, ATR, and DNA-PK, form discrete intra-nuclear foci which can be visualized microscopically by indirect immunofluorescence, using specific primary antibodies and secondary antibodies coupled with fluorescent dyes [13]. Since the histone phosphorylation is a consequence of specific DNA lesions, the γ H2AX assay can be considered as a typical DNA damage indicator assay. The γ H2AX assay is highly sensitive compared to other assays, in terms of DSB that can be detected. The assay even allows the detection of a single DSB per nucleus [14].

In a previous study, we used the γ H2AX focus assay to compare well-defined genotoxicants with non-genotoxic agents. We showed that, in the toxic dose range, the genotoxicants induce, dose-dependently, γ H2AX foci while non-genotoxic compounds are toxic without inducing γ H2AX foci. Thus, the assay clearly distinguishes between genotoxic and non-genotoxic substances at sub-toxic and toxic (measured in the MTT viability assay)

concentrations [11]. In agreement with other reports, we concluded that the γ H2AX assay is a useful screening tool and additional test in the battery of genotoxicity assays [10,11,15].

The relative sensitivities of the γ H2AX and comet assays have not been determined comparatively. Therefore, in the present study, we have compared the γ H2AX assay with the alkaline and neutral comet assays in dose-response experiments *in vitro*. We investigated DNA damage induced by four typical model mutagens, the S_N2 methylating agent methyl methanesulfonate (MMS), the S_N1 methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), the cross-linker mitomycin C, and the strong oxidative agent hydrogen peroxide (H_2O_2), which is widely used as a positive control in studies on oxidative stress. We compared the data on the basis of best-fit correlation (hockey-stick model). Further, we assessed whether there is a correlation between the data obtained in the comet assay, which monitors DNA lesions indirectly, with the γ H2AX assay, which monitors the cell's response to DNA damage.

MATERIALS AND METHODS

Cell lines, culture conditions, and treatment

We used, as a cell model, the Chinese hamster cell line CHO-9 [16], which is well characterized as to DNA repair and DNA damage response. Cells were cultured in DMEM Ham's F-12 containing 5% fetal calf serum (FCS) in a humidified atmosphere with 7% CO_2 at 37°C. Cells were tested for mycoplasma contamination. Cells were pulse-treated for 1 h with increasing concentrations of the tested genotoxic agents added to the medium of exponentially growing cells. Thereafter, the medium was changed and cells were incubated at 37°C until further processed for the γ H2AX or comet assay.

Test compounds

The following genotoxic compounds were tested in the γ H2AX, alkaline and neutral comet assays: methyl methanesulfonate (MMS), hydrogen peroxide (H_2O_2) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were from Sigma-Aldrich (Taufkirchen, Germany) and mitomycin C (MMC) from Tocris Bioscience (Bristol, UK). MNNG was diluted in a small volume of DMSO, then to stock concentration (10 mM) in water, and further to working concentrations in culture medium; MMS was diluted in water to stock concentration 200 mM; H_2O_2 in water (0.1 mM stock concentration), and mitomycin C in DMSO to 1 mg/ml, or additionally in medium to 100 μ g/ml for treatments with low concentrations. For studies with the comet assay, it was necessary to perform the pulse treatment with H_2O_2 in PBS instead of culture medium; otherwise very little primary DNA damage was detected.

Quantitation of γ H2AX foci by microscopy

Cells (n = 20,000 per well) were seeded onto precleaned sterile cover slips in six-well culture plates. Two days later, they were treated for 60 min with the test chemical. After treatment, the medium was changed and cells were incubated further (6 or 24 h). Finally, the cells grown on cover slips were washed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were incubated with 1–2 ml ice-cold methanol for 10 min at -20°C . After three washing steps with PBS, the blocking reagent (100 l PBS + 0.25% Triton X-100 + 10% NGS) was added for 1 h. The primary antibody (mouse anti-phospho-H2AX Ser139, Millipore, cat. no. 05-636) was diluted to 1:1000 in PBS + 0.25% Triton X-100 buffer, added to the cells and left overnight. The samples were stored at 4°C . The next day, the cells on cover slips were washed three times in PBS and Alexa488-coupled F(ab)₂ anti-mouse antibody was added, diluted to 1:500 in the same buffer. The samples were stored in the dark at room temperature for 1 h. After washing, 10 μl antifade medium (Vectashield) with DAPI was dropped onto clean slides and the cover slips were transferred onto the slides and fixed with nail polish. For each treatment level, 100-500 cells were analyzed in each experiment. All experiments were repeated three times.

Microscopic images were screened and captured using a Zeiss Axio Imager M1 (Carl Zeiss) supplied with the Metafer4 Software (MetaSystems, Altlussheim, Germany). The slide scanning platform “Metafer” and the accompanying software “Metafer4” were used [11]. At brief intervals, the system automatically scans a preselected area and captures the images according to the settings defined by the user and saved as a classifier. The system scans and focuses using the DAPI signal and, after recognizing the presence of a cell, captures red (TRITC, Cy3) or green (FITC, Alexa Fluor 488) fluorescent signals according to the classifier settings. The signals are acquired as a z-stack with a total of five planes. The same classifier is used to acquire the images for each sample. The stored galleries of images can be reloaded on a review screen, where every captured cell can be relocated and the automatic analyses can be verified. In addition, we compared the automatic analyses of the saved images by the Metafer4 Software with visual analysis using ImageJ (Fiji) with suitable batch-macro.

Alkaline and neutral comet assays

Exponentially growing cells were exposed to the genotoxicants, post-incubated for the indicated time periods, trypsinized, washed and re-suspended in ice-cold PBS. Cell suspension (15l) was embedded in 120 μl low-melting-point agarose (0.5% in dH₂O at 37°C) onto agarose-coated (1.5% in PBS) and dried slides that were submersed for 1h in

precooled lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and 1% Na-laurylsarcosine, pH=10 for alkaline comet assay, pH=7.5 for neutral comet assay); before cooling, 1% Triton was added to the lysis buffer. Slides were denatured and equilibrated for 20 min in precooled running buffer (300 mM NaOH, 1 mM EDTA, pH>13 for alkaline comet assay; 90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH=7.5 for neutral comet assay). Following the denaturation step, slides were electrophoresed at 0.8 V/cm (300 mA for the alkaline version) for 22 min at 4°C. In the case of the alkaline version, the slides were neutralized three times and further (both for the neutral and alkaline comet assays) slides were rinsed in water, fixed in 100% ethanol, dried, and stained with propidium iodide (50 µg/ml). Stained slides were evaluated using a fluorescence microscope and the Comet IV software (Perceptive Imaging, Liverpool, UK). Data were expressed as tail intensity or Olive Tail Moment (OTM), which represents the percentage of DNA in the tail multiplied by the length between the center of the head and tail [17]. The experiments were repeated three times.

Statistical analyses

Mean values and standard deviations were calculated by Microsoft Office Excel. Comparison of foci values induced by the same dose at different time-points were performed by t-test in Excel and using the software GraphPad Prism 6. Tail intensity and the number of γ H2AX foci per cell were presented as a function of the concentration of the chemicals. Spearman's correlation coefficients, r , were calculated in GraphPad Prism 6. The r value is a measure of the monotonicity of the association between two continuous variables (e.g., number of γ H2AX foci per cell vs. tail intensity in the alkaline comet assay).

In order to fit "hockey-stick" models to the data, the statistical software R was used (see legend of Supplementary Fig. S1). In detail, we adapted a function provided in a work aimed at estimating threshold doses [18], to determine the parameters and the confidence intervals. As in the original implementation, the full line shows the best fit of the hockey stick, based on the highest limit of a confidence interval for the threshold dose. R was calculated as described in the legend of Supplement, Fig. S1.

RESULTS

For most chemical mutagens, the conversion of initial DNA lesions into DSB depends on the progression of cells through S-phase [19,20]. Therefore, similarly to our previous study [11], we analyzed cells that have passed through S-phase by using harvest times 6 and 24 h following a 1 h treatment with the agents. We also determined the effects immediately after mutagen treatment (measure points designated as 0 h), because some of the primary DNA lesions (e.g., SSB induced directly or as a result of BER [9]) are repaired

very rapidly [21]. For γ H2AX foci analyses, an expression time of 45 min after 1 h mutagen treatment was chosen. This time is required for achieving maximum histone phosphorylation, as confirmed by a time-course of γ H2AX formation following ionizing radiation in this [22] and other cell systems [23,24], demonstrating that phosphorylation at serine 139 of histone 2AX starts within minutes and reaches a maximum 45-60 min after DSB induction. The dose range for all mutagens used in this study was established by the MTT cell viability assay as described previously [11]. Representative images for migrated DNA measured in the alkaline and neutral comet assays and γ H2AX foci following H₂O₂ treatment are shown in Fig. 1.

γ H2AX foci formation as a function of dose

The genotoxicants MMS, MNNG, MMC and H₂O₂ are well-known reference compounds frequently used in genotoxicity studies [25,26]. They represent specific groups of genotoxicants. Thus, MMS is a representative of N-alkylators, while MNNG represents agents of high O-alkylation potency. MMS is a crosslinker and H₂O₂ a powerful producer of ROS, inducing DNA breaks and oxidative base damage. As shown in Figure 2A, the agents induce γ H2AX foci immediately (0 h), 6, and 24 h after a 60 min pulse of treatment, and the foci number increases with dose up to a saturation level. For MMS, saturation was obviously not achieved, while for MNNG, 24 h after treatment saturation was obvious at 5 μ M. For MMC, the foci numbers were only slightly enhanced above the control even at high dose levels immediately after pulse-treatment, while 6 and 24 h after treatment the foci numbers were significantly enhanced. This is likely a result of processing and repair of monoadducts and crosslinks formed by MMC in the post-exposure period [27]. The dose-response of H₂O₂ revealed no clear differences between the time point of assessment. A significant increase in the foci level was observed with a dose of 50 μ M.

Neutral and alkaline comet assays as a function of dose

Data on tail intensity obtained by the alkaline and the neutral comet assays are presented in Fig. 2B and 2C, respectively. In the alkaline comet assay, which primarily detects SSB directly induced or formed as a result of BER and alkali labilization of abasic sites, the response is linearly dependent on the dose of MMS, with the strongest effect immediately after treatment (0 h). This was also seen in the neutral comet assay, which was markedly different from the γ H2AX foci assay, in which the 0 h values were lower than 6 and 24 h values. Differences were also observed for MNNG for which the 24 h dose-response was not above the 0 and 6 h response. For MMC, the neutral and alkaline comet assay did not yield positive effects even at a high dose level of 20 μ g/ml exposure, which was clearly effective in the γ H2AX assay. For H₂O₂, the alkaline and the neutral comet

assay were clearly positive if cells were harvested immediately after treatment (0 h). At times 6 and 24 h post-treatment, the alkaline assay was negative while the neutral assay and the γ H2AX assay yielded positive results. Overall, the dose-response determined immediately and 6 and 24 h after treatment was different in the γ H2AX, alkaline and neutral comet assay.

Correlation analysis of γ H2AX foci and data obtained with the comet assay

Next, we investigated the correlation between data obtained by the γ H2AX and the alkaline comet assay. As shown in Fig. 3, there is a high correlation ($r > 0.8$) between number of foci and tail intensity for MMS and H_2O_2 when cells were harvested immediately after treatment. For MNNG, the correlation was generally low. For MMC, the correlation coefficient was high 6 and 24 h after treatment, which is, however, not relevant, since the tail intensity was generally low. Interestingly, if we extrapolate the curve to the abscissa, it becomes obvious that the lowest dose of MMS, MNNG and H_2O_2 already induces γ H2AX without a concomitant increase in tail intensity.

The correlation between γ H2AX and tail intensity determined by the neutral comet assay following treatment with the agents is shown in Fig. 4. Again, for MMS and H_2O_2 , a significant correlation was found if cells were harvested immediately after treatment. The correlation was also significant for H_2O_2 , 6 and 24 h after treatment. For MNNG, there was a rough correlation between both end points. For MMC, the correlation coefficients were meaningless, since tail intensity was not significantly above the background. An extrapolation of the curves to the abscissa at 0 h revealed, for MMS, MNNG and H_2O_2 , a nearly linear correlation, indicating that γ H2AX foci correlate with DSB measured by the neutral comet assay. For MMS, the γ H2AX assay appears to be more sensitive while for MNNG and H_2O_2 the neutral comet assay might be slightly more sensitive. For MMC, the γ H2AX assay was clearly positive, but the neutral comet assay was almost negative.

Data analysis via hockey stick model

In order to substantiate the conclusions, we decided to fit a hockey-stick model [18] to the dose-response data. The results are presented in Fig. 5 for MMS and in Supplement Fig. S1, S2 and S3 for the other genotoxicants used in this study. The best fit of the data shows, for MMS and the endpoint γ H2AX, no threshold at any measurement time, while for the alkaline comet assay, a no-effect threshold was obvious at 0 and 6 h post-incubation, and for the neutral comet assay at 6 and 24 h post-incubation. This data shows that for MMS, which induces DNA alkylation lesions linearly with dose [28], the DNA damage response is a linear function of dose, irrespective of the post-incubation time. This was not the case in the comet assays. The only threshold we observed in the γ H2AX assay was for

MNNG measured 6 h after treatment (Supplement Fig. S1), which is likely due to the fact that in a low dose range, *O*⁶-methylguanine is the major response-triggering lesion. Two replication cycles are needed for it to be converted into DSBs [20] that trigger H2AX phosphorylation, which explains the late (24 h) linear response. For H₂O₂, thresholds were observed in the alkaline comet assay, but in neither the γ H2AX foci nor the neutral comet assay (Supplement Fig. S3).

Conclusions

In summary, a comparison of dose-responses determined by the γ H2AX foci and the alkaline and neutral comet assay revealed both similarities and differences. The most striking difference was the inability of the alkaline and the neutral comet assay to identify mitomycin C as a genotoxicant, while in the γ H2AX assay, mitomycin C induced a genotoxic response linearly with dose at 0, 6 and 24 h post-incubation time. MMC induces both monoadducts and DNA interstrand crosslinks (ICL) and both seem not to be able to enhance DNA migration in the comet assay. The opposite is true: ICL enhance the molecular weight of DNA fragments following denaturation and, therefore, the commonly used alkaline assay is not suitable for detecting ICL [5]. The modified alkaline comet assay detecting ICL is laborious, requires internal controls, and is hardly useful as a routine assay [29,30]. During crosslink repair SSBs and DSBs are formed as repair intermediates [27,31], but the amount is obviously below the detection level in the alkaline and neutral comet assays. The lesions can, however, activate the DNA damage response, giving rise to significant numbers of γ H2AX foci. Interestingly, the focus level following mitomycin C was higher 24 h compared to 6 and 0 h after treatment. This might be explained by replication and transcription blockage at ICL, which are long-lived lesions compared to DNA alkylation and oxidative damage. ICL repair, which is a slow process compared to BER, gives rise to DSBs as repair intermediates even a day after damage induction, which was previously shown in cells treated with cisplatin [30] or chloroethylnitrosoureas [32-34].

For MMS, the γ H2AX dose response revealed the highest DNA damage level immediately after mutagen treatment (60 min) and lowest determined 24 h later. This is explained by BER activity, which corresponds with the level of DNA adducts such as N7-methylguanine, N3-methyladenine, and N3-methylguanine, giving rise to SSB by N-methylpurine-DNA glycosylase and apurinic endonuclease activities. With increasing recovery time, the amount of N-methylpurines declines, which reduces the response in the comet assay.

It is of interest to note that for the genotoxicants tested, a no-effect threshold was not observed in the γ H2AX assay, except for MNNG, 6 h post-incubation (Fig. 2A, Fig. 5 and data shown in Supplement). Obviously, even very low amounts of a variety of critical DNA lesions

(different primary lesions that give rise to DSB, directly formed DSB and arrested replication forks) can trigger the DNA damage response, indicating that the kinases ATM, ATR, and DNA-PK represent highly sensitive DNA damage indicators. For MNNG, almost all genotoxic effects in the low-dose range result from O^6 -methylguanine. This critical lesion blocks replication and induces DSBs in the 2nd cell cycle after treatment [35], which needs to be taken into account in the experimental set up. Interestingly, downstream in the response are decision-makers regulating cell cycle, proliferation, and death pathways. The link between histone H2AX phosphorylation and downstream DNA damage response activation [36] explains why the γ H2AX foci level strongly correlates with the viability (determined in the MTT assay) of cells treated with genotoxic agents [11].

The γ H2AX foci assay and the comet assays can be performed in large scale by means of automatic scoring systems [11,29,37-39]. The comet assay needs viable cells that are lysed in agarose before electrophoresis, while the γ H2AX foci assay can be performed in a simplified way with cells immobilized on a cover slip and air dried [40]. This is of advantage if samples are collected without having the opportunity to carry out the assay immediately. This and the low amount of cells required seem to be another advantage of the γ H2AX focus assay. Thus, the γ H2AX assay appears to be useful for *in vitro* genotoxicity testing as a highly sensitive, robust, and reliable tool focusing on critical DNA damage [36], deserving further validation [10,11,15,41].

Acknowledgments

We thank Andrea Pi e-Staffa for technical support. Dr. Teodora Nikolova was financed by a grant of the DFG (NI 1319/1-2). Work was also supported by DFG KA724 to BK and University Medical Center Mainz.

References

1. Mahadevan B, Snyder RD, Waters MD, Benz RD, Kemper RA, et al. (2011) Genetic toxicology in the 21st century: reflections and future directions. *Environ Mol Mutagen* 52: 339-354.
2. Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, et al. (2009) Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. *Mutagenesis* 24: 341-349.
3. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175: 184-191.
4. Olive PL, Wlodek D, Banath JP (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res* 51: 4671-4676.
5. Merk O, Speit G (1999) Detection of crosslinks with the comet assay in relationship to genotoxicity and cytotoxicity. *Environ Mol Mutagen* 33: 167-172.
6. OECD (2014) Test No. 489: In Vivo Mammalian Alkaline Comet Assay. OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects.
7. Moller P (2006) The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic Clin Pharmacol Toxicol* 98: 336-345.

8. Collins AR (2011) The use of bacterial repair endonucleases in the comet assay. *Methods Mol Biol* 691: 137-147.
9. Ensminger M, Iloff L, Ebel C, Nikolova T, Kaina B, et al. (2014) DNA breaks and chromosomal aberrations arise when replication meets base excision repair. *J Cell Biol* 206: 29-43.
10. Garcia-Canton C, Anadon A, Meredith C (2012) gammaH2AX as a novel endpoint to detect DNA damage: applications for the assessment of the in vitro genotoxicity of cigarette smoke. *Toxicol In Vitro* 26: 1075-1086.
11. Nikolova T, Dvorak M, Jung F, Adam I, Kramer E, et al. (2014) The gammaH2AX assay for genotoxic and nongenotoxic agents: comparison of H2AX phosphorylation with cell death response. *Toxicol Sci* 140: 103-117.
12. Nikolova T, Ensminger M, Lobrich M, Kaina B (2010) Homologous recombination protects mammalian cells from replication-associated DNA double-strand breaks arising in response to methyl methanesulfonate. *DNA Repair (Amst)* 9: 1050-1063.
13. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 273: 5858-5868.
14. Rothkamm K, Lobrich M (2003) Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A* 100: 5057-5062.
15. Watters GP, Smart DJ, Harvey JS, Austin CA (2009) H2AX phosphorylation as a genotoxicity endpoint. *Mutat Res* 679: 50-58.
16. Tjio JH, Puck TT (1958) Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. *J Exp Med* 108: 259-268.
17. Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat Res* 122: 86-94.
18. Lutz WK, Lutz RW (2009) Statistical model to estimate a threshold dose and its confidence limits for the analysis of sublinear dose-response relationships, exemplified for mutagenicity data. *Mutat Res* 678: 118-122.
19. Bender MA, Griggs HG, Bedford JS (1974) Mechanisms of chromosomal aberration production. 3. Chemicals and ionizing radiation. *Mutat Res* 23: 197-212.
20. Kaina B (2004) Mechanisms and consequences of methylating agent-induced SCEs and chromosomal aberrations: a long road traveled and still a far way to go. *Cytogenet Genome Res* 104: 77-86.
21. Ward JF, Blakely WF, Moberly JB (1983) A Comparison of the Enzymatic Repair Kinetics of Ionizing-Radiation and of Hydrogen-Peroxide Induced DNA Strand Breaks in V79 Cells. *Radiation Research* 94: 629-630.
22. Staszewski O, Nikolova T, Kaina B (2008) Kinetics of gamma-H2AX focus formation upon treatment of cells with UV light and alkylating agents. *Environ Mol Mutagen* 49: 734-740.
23. Berdelle N, Nikolova T, Quiros S, Efferth T, Kaina B (2011) Artesunate induces oxidative DNA damage, sustained DNA double-strand breaks, and the ATM/ATR damage response in cancer cells. *Mol Cancer Ther* 10: 2224-2233.
24. Redon CE, Dickey JS, Bonner WM, Sedelnikova OA (2009) gamma-H2AX as a biomarker of DNA damage induced by ionizing radiation in human peripheral blood lymphocytes and artificial skin. *Adv Space Res* 43: 1171-1178.
25. Brambilla G, Martelli A (2009) Update on genotoxicity and carcinogenicity testing of 472 marketed pharmaceuticals. *Mutat Res* 681: 209-229.
26. Kirkland D, Kasper P, Muller L, Corvi R, Speit G (2008) Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: a follow-up to an ECVAM workshop. *Mutat Res* 653: 99-108.
27. Nikolova T, Roos WP, Kramer OH, Strik HM, Kaina B (2017) Chloroethylating nitrosoureas in cancer therapy: DNA damage, repair and cell death signaling. *Biochim Biophys Acta* 1868: 29-39.
28. Thomas AD, Jenkins GJ, Kaina B, Bodger OG, Tomaszowski KH, et al. (2013) Influence of DNA repair on nonlinear dose-responses for mutation. *Toxicol Sci* 132: 87-95.

29. Berte N, Piee-Staffa A, Piecha N, Wang M, Borgmann K, et al. (2016) Targeting Homologous Recombination by Pharmacological Inhibitors Enhances the Killing Response of Glioblastoma Cells Treated with Alkylating Drugs. *Mol Cancer Ther* 15: 2665-2678.
30. Usanova S, Piee-Staffa A, Sied U, Thomale J, Schneider A, et al. (2010) Cisplatin sensitivity of testis tumour cells is due to deficiency in interstrand-crosslink repair and low ERCC1-XPF expression. *Mol Cancer* 9: 248.
31. Muniandy PA, Liu J, Majumdar A, Liu ST, Seidman MM (2010) DNA interstrand crosslink repair in mammalian cells: step by step. *Crit Rev Biochem Mol Biol* 45: 23-49.
32. Batista LF, Roos WP, Christmann M, Menck CF, Kaina B (2007) Differential sensitivity of malignant glioma cells to methylating and chloroethylating anticancer drugs: p53 determines the switch by regulating xpc, ddb2, and DNA double-strand breaks. *Cancer Res* 67: 11886-11895.
33. Naumann SC, Roos WP, Jost E, Belohlavek C, Lennerz V, et al. (2009) Temozolomide- and fotemustine-induced apoptosis in human malignant melanoma cells: response related to MGMT, MMR, DSBs, and p53. *Br J Cancer* 100: 322-333.
34. Nikolova T, Hennekes F, Bhatti A, Kaina B (2012) Chloroethylnitrosourea-induced cell death and genotoxicity: cell cycle dependence and the role of DNA double-strand breaks, HR and NHEJ. *Cell Cycle* 11: 2606-2619.
35. Quiros S, Roos WP, Kaina B (2010) Processing of O6-methylguanine into DNA double-strand breaks requires two rounds of replication whereas apoptosis is also induced in subsequent cell cycles. *Cell Cycle* 9: 168-178.
36. Roos WP, Kaina B (2013) DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett* 332: 237-248.
37. Dehon G, Catoire L, Duez P, Bogaerts P, Dubois J (2008) Validation of an automatic comet assay analysis system integrating the curve fitting of combined comet intensity profiles. *Mutat Res* 650: 87-95.
38. Faust D, Nikolova T, Watjen W, Kaina B, Dietrich C (2017) The Brassica-derived phytochemical indolo[3,2-b]carbazole protects against oxidative DNA damage by aryl hydrocarbon receptor activation. *Arch Toxicol* 91: 967-982.
39. Frieauff W, Hartmann A, Suter W (2001) Automatic analysis of slides processed in the Comet assay. *Mutagenesis* 16: 133-137.
40. Heylmann D, Kaina B (2016) The gammaH2AX DNA damage assay from a drop of blood. *Sci Rep* 6: 22682.
41. Smart DJ, Ahmed KP, Harvey JS, Lynch AM (2011) Genotoxicity screening via the gammaH2AX by flow assay. *Mutat Res* 715: 25-31.

Figure legends

Figure 1. Representative images of ‘comets’ and γ H2AX foci in CHO-9 cells. (A) Alkaline comet assay. Cells were either untreated or treated for 1 h with 50 or 100 μ M H_2O_2 and harvested immediately (0 h) and 6 h after mutagen treatment. (B) Neutral comet assay. Cells were either untreated or treated for 1 h with 50 or 100 μ M H_2O_2 and harvested immediately (0 h) and 6 h after mutagen treatment. (C) Representative images of nuclei from CHO-9 cells not treated (control) or treated with H_2O_2 using the same concentrations as in the comet assay. Cells grown on cover slips were fixed and stained immediately (0 h) and 6 h after treatment.

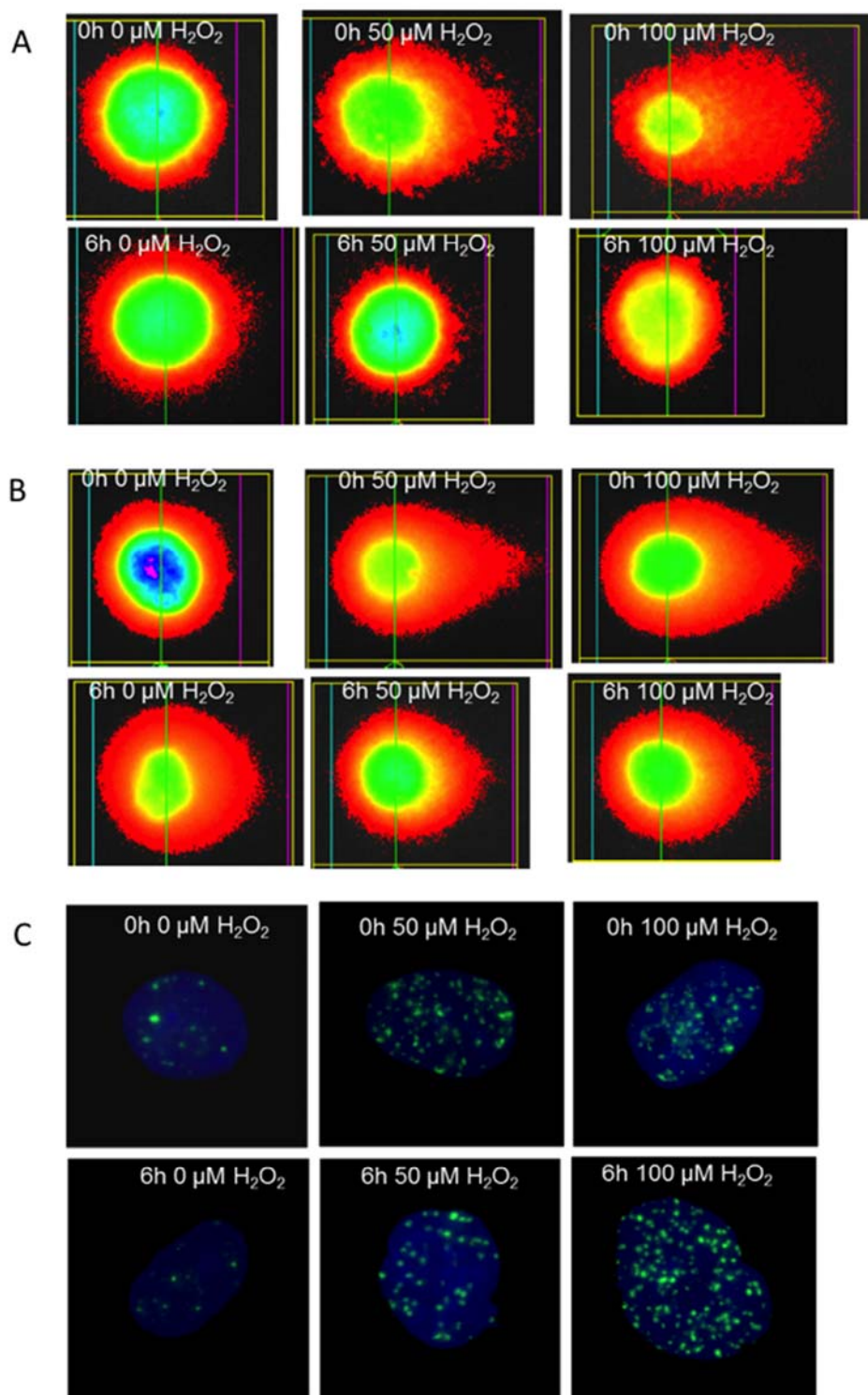


Figure 1

Figure 2. γ H2AX foci and tail intensity determined in the alkaline and neutral comet assay as a function of dose of the mutagens. (A) CHO-9 cells were pulse-treated with increasing

concentrations of MMS, MNNG, MMC and H₂O₂. Cells were fixed and stained 0 h (i.e. 45 min after removing the agents), 6h and 24 h after treatment. The number of foci was determined using ImageJ Fiji. Mean values and SD were calculated in Microsoft Excel. (B) Tail intensity in the alkaline comet assay after treatment with the genotoxicants. CHO-9 cells were treated with increasing concentrations of MMS, MNNG, MMC and H₂O₂ for 60 min, harvested immediately (0 h), 6 h and 24 h after treatment. Cells were embedded in agarose, lysed and subjected to electrophoresis as described in Material and Methods. Image analysis and calculation of tail intensity were performed with Comet IV software. (C) Tail intensity in the neutral comet assay. Cell culture, treatments, harvesting, preparation for electrophoresis, image analyses and calculation of tail intensity were performed exactly as in the alkaline comet assay. In all assays, each measure point represents the mean of

three

independent

experiments.

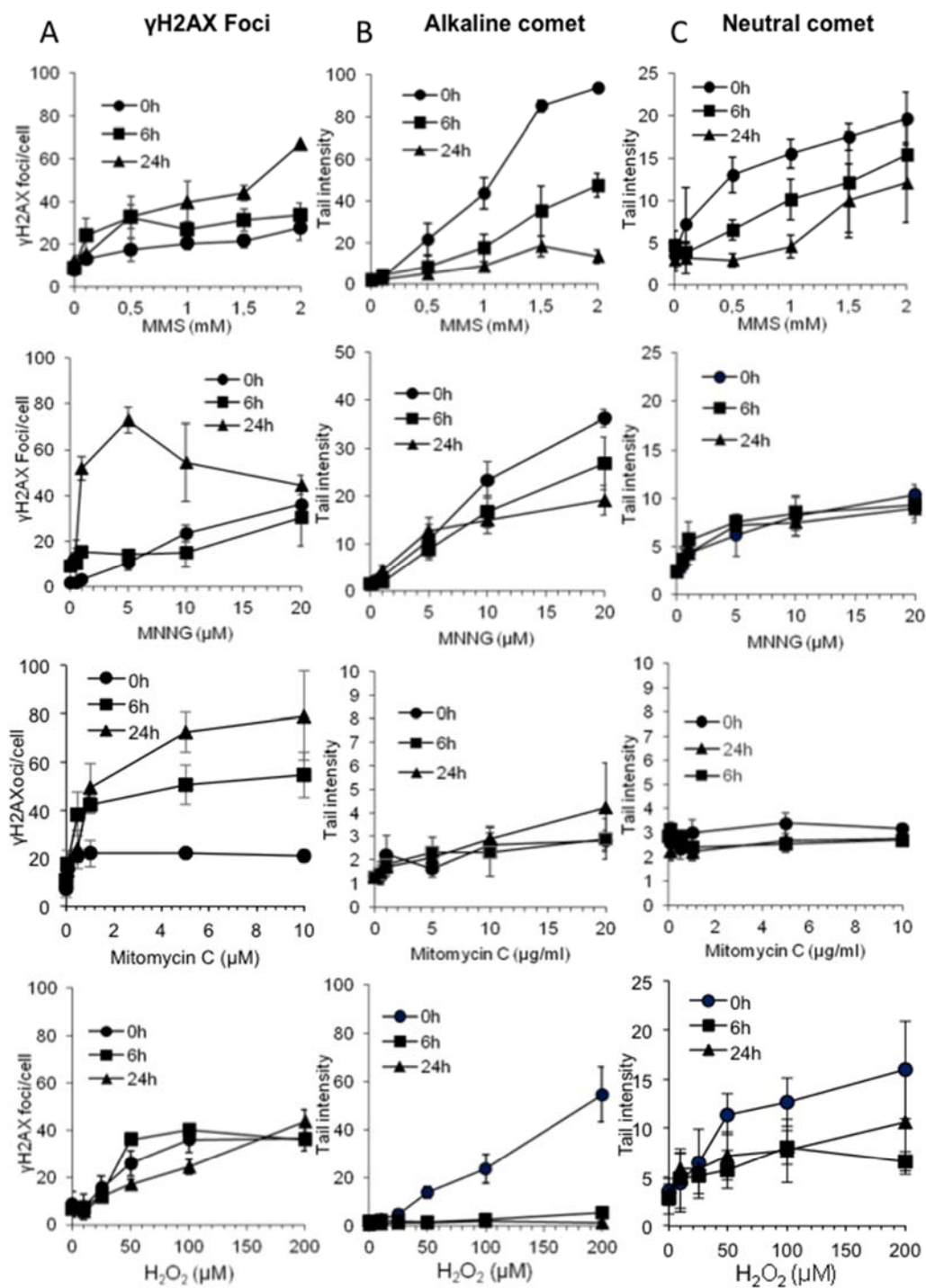


Figure 2

Figure 3. Correlation analysis between tail intensity determined by the alkaline comet assay and the number of γ H2AX foci following treatment with increasing concentrations of the genotoxicants. Data are from Fig. 1A and Fig. 1B. Each measure point represents the mean of three independent experiments. The correlation coefficient r was calculated using

the Spearman method (GraphPad Prism 6).

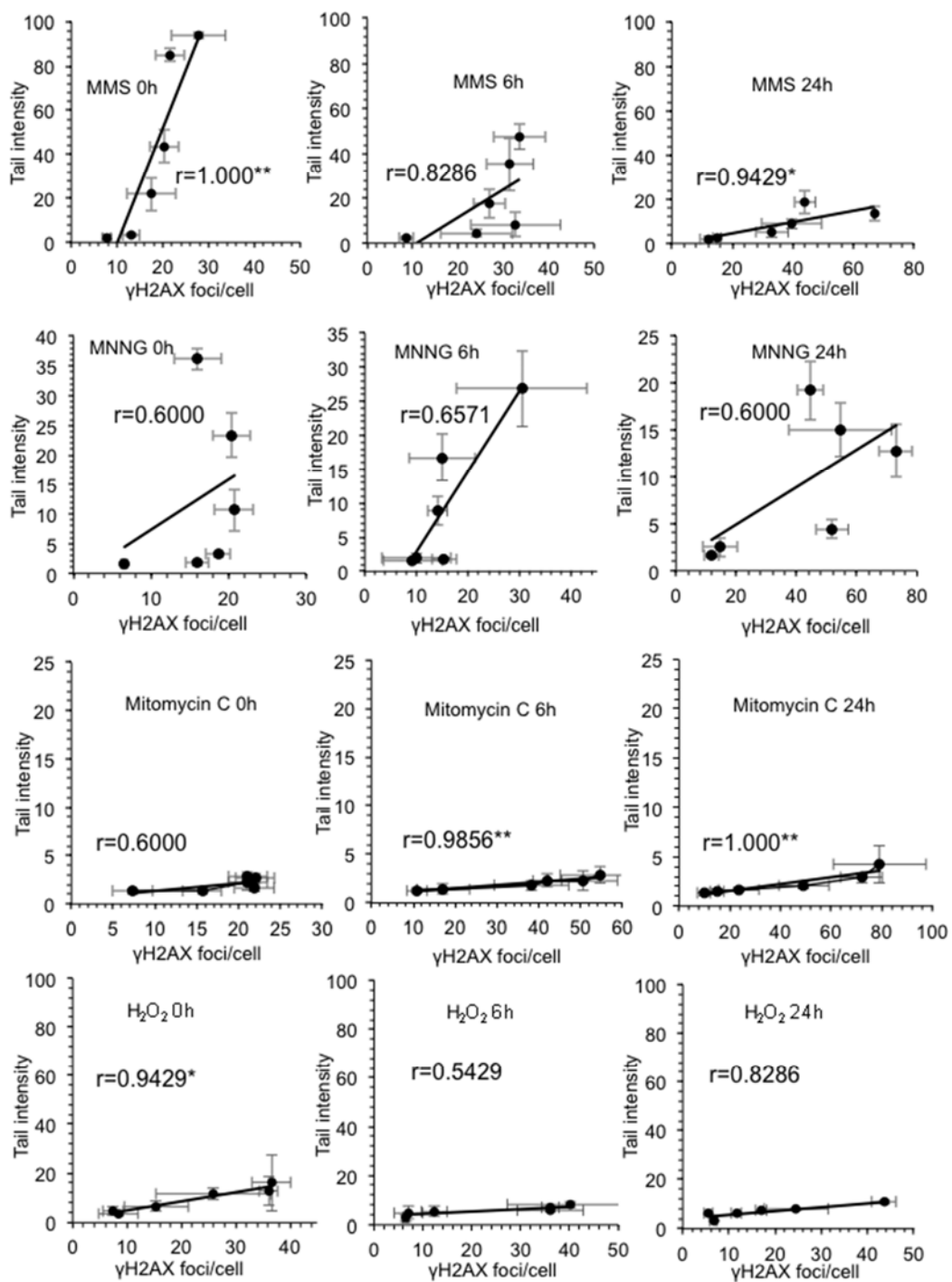


Figure 3

Figure 4. Correlation analysis between tail intensity determined by the alkaline comet assay and the number of γ H2AX foci following treatment with increasing concentrations of the genotoxicants. Data are from Fig. 1A vs. Fig.1C. The correlation coefficient r was calculated using the Spearman method (GraphPad Prism 6). Asterisks indicate the

significance

level

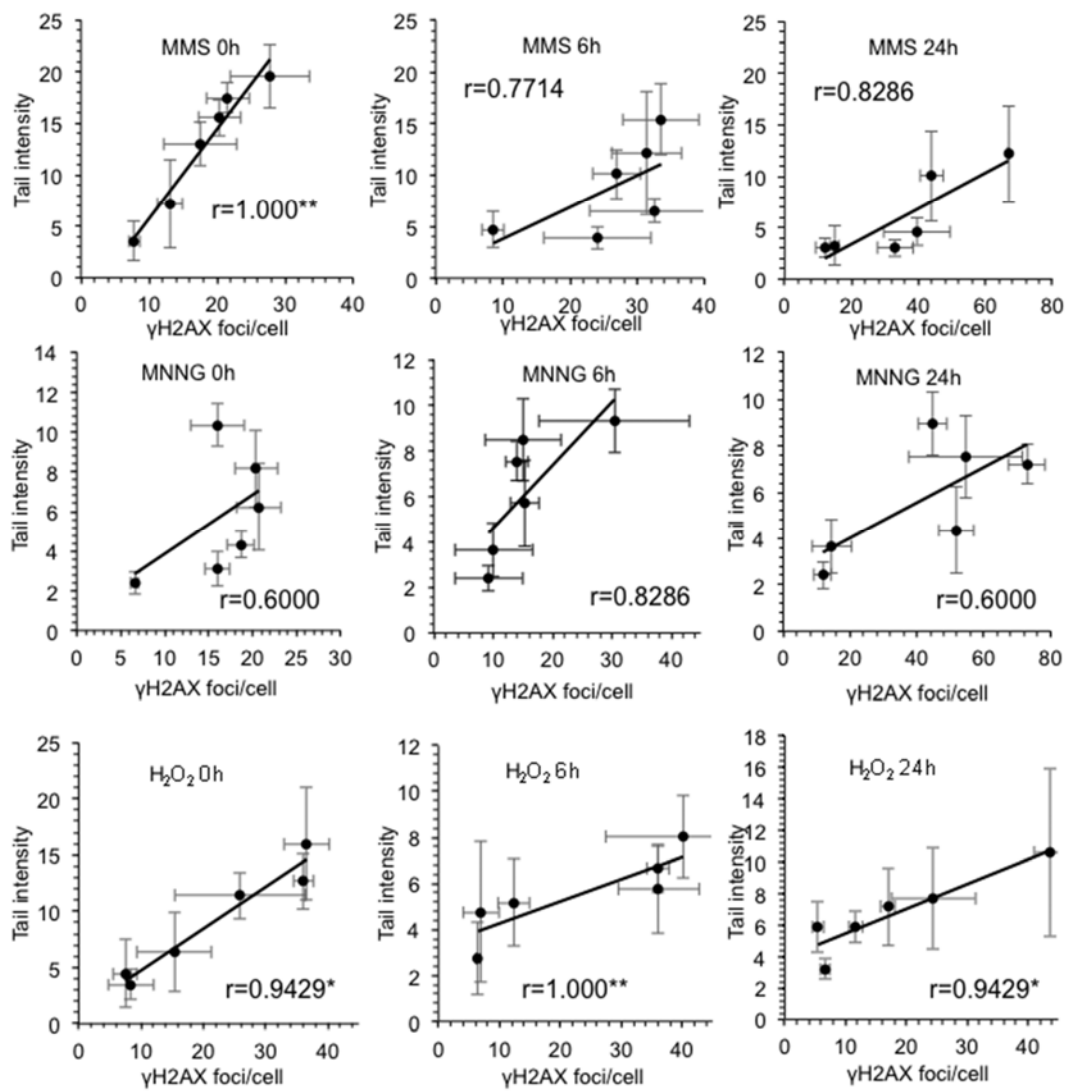
(* $p < 0.05$;** $p < 0.01$).

Figure 4

Figure 5. Dose-response curves for CHO-9 cells treated with MMS for 60 min, where the response is measured as (A) number of γ H2AX foci, and tail moment for (B) alkaline comet and (C) neutral comet. Data of three independent experiments are shown and used for calculation of the best fit according to the hockey-stick model (see Material and Methods).

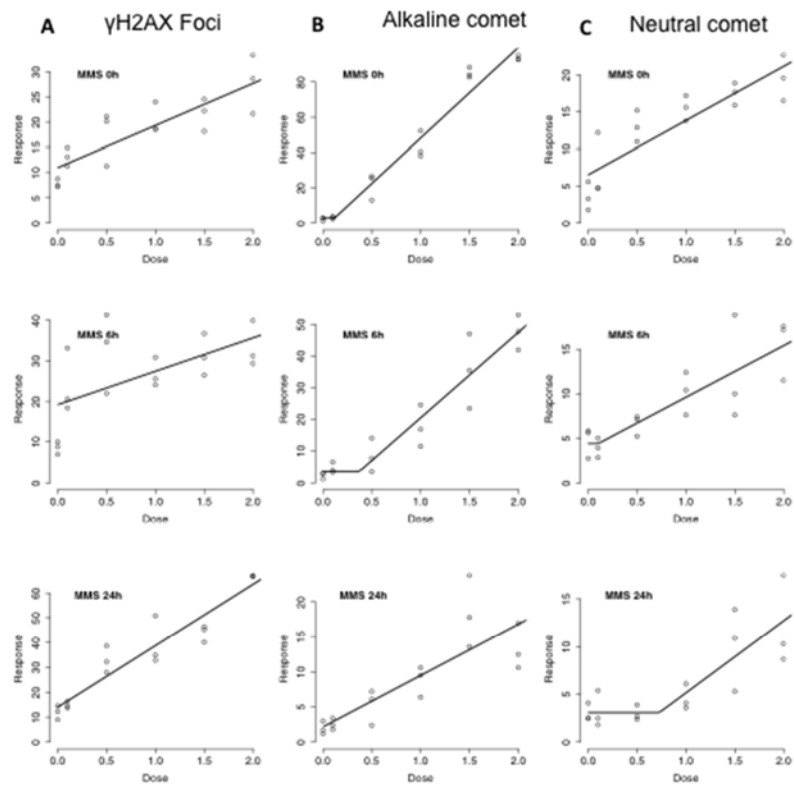


Figure 5