Environmental Science & Technology

Article

pubs.acs.org/est

Toxicity of Drinking Water Disinfection Byproducts: Cell Cycle Alterations Induced by the Monohaloacetonitriles

Yukako Komaki,*[†,‡] Benito J. Mariñas,[†,‡] and Michael J. Plewa*[‡,§]

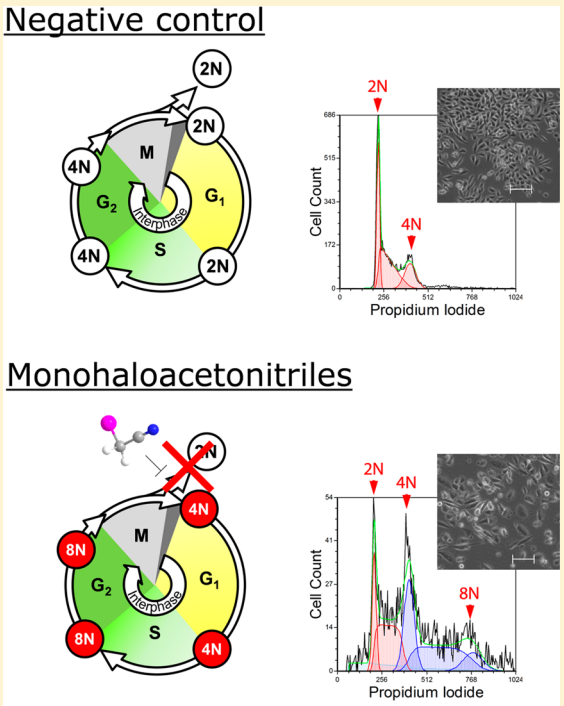
[†]Department of Civil and Environmental Engineering, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, United States

[‡]Center of Advanced Materials for the Purification of Water with Systems, Safe Global Water Institute, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, United States

[§]Department of Crop Sciences, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, United States

Supporting Information

ABSTRACT: Haloacetonitriles (HANs) are a chemical class of drinking water disinfection byproducts (DBPs) that form from reactions between disinfectants and nitrogen-containing precursors, the latter more prevalent in water sources impacted by algae bloom and municipal wastewater effluent discharge. HANs, previously demonstrated to be genotoxic, were investigated for their effects on the mammalian cell cycle. Treating Chinese hamster ovary (CHO) cells with monoHANs followed by the release from the chemical treatment resulted in the accumulation of abnormally high DNA content in cells over time (hyperploid). The potency for the cell cycle alteration followed the order: iodoacetonitrile (IAN) > bromoacetonitrile (BAN) ≫ chloroacetonitrile (CAN). Exposure to 6 μM IAN, 12 μM BAN and 900 μM CAN after 26 h post-treatment incubation resulted in DNA repair; however, subsequent cell cycle alteration effects were observed. Cell proliferation of HAN-treated cells was suppressed for as long as 43 to 52 h. Enlarged cell size was observed after 52 h post-treatment incubation without the induction of cytotoxicity. The HAN-mediated cell cycle alteration was mitosis- and proliferation-dependent, which suggests that HAN treatment induced mitosis override, and that HAN-treated cells proceeded into S phase and directly into the next cell cycle. Cells with multiples genomes would result in aneuploidy (state of abnormal chromosome number and DNA content) at the next mitosis since extra centrosomes could compromise the assembly of bipolar spindles. There is accumulating evidence of a transient tetraploid state proceeding to aneuploidy in cancer progression. Biological self-defense systems to ensure genomic stability and to eliminate tetraploid cells exist in eukaryotic cells. A key tumor suppressor gene, p53, is oftentimes mutated in various types of human cancer. It is possible that HAN disruption of the normal cell cycle and the generation of aberrant cells with an abnormal number of chromosomes may contribute to cancer induction and perhaps be involved in the induction of adverse pregnancy outcomes associated with long-term consumption of disinfected water. Here we present the first observation of the induction of hyperploidy by a class of DBPs.



INTRODUCTION

The disinfection of water protects public health against waterborne pathogens, however, water disinfection also results in the formation of toxic disinfection byproducts (DBPs).[1] Exposure to DBPs is associated with the induction of bladder and colon cancers and adverse pregnancy outcomes.[2−4] The haloacetonitriles (HANs) form from reactions between disinfectants (e.g., chloramines) and dissolved nitrogen precursors (e.g., amino acids) (Figure S1 in Supporting Information (SI)).[5−9] Interest in nitrogenous DBPs (N-DBPs) has increased because recent quantitative in vitro mammalian cell assays demonstrated that the HANs, haloacetamide and halonitromethanes were more cytotoxic and genotoxic than regulated trihalomethanes (THMs) and haloacetic acids (HAAs).[10−13] Population growth and increased water supply demand is putting stress on the availability of pristine water sources. As a result, water providers are

Received: July 3, 2014
Revised: September 2, 2014
Accepted: September 3, 2014
Published: September 3, 2014

 © 2014 American Chemical Society

increasingly using source waters impacted by algae blooms and municipal wastewater effluent discharge, which promotes N-DBP formation due to elevated levels of organic nitrogen content.[8,9,14−16] Utilities exploit disinfectant combinations to reduce the formation of regulated DBPs but some of these disinfection schemes results in enhanced N-DBP formation.[8] HANs measured in the nationwide occurrence studies of 12 U.S. water treatment plants were at concentrations of up to 14 μg/L (median: 3 μg/L).[17] HANs were also measured in chlorinated municipal wastewater treatment plant effluents (median: 16 μg/L) as well as in chloraminated wastewater effluents (median: 0.3 μg/L).[18] Recently HANs were shown to form as electrochemical oxidation byproducts of landfill leachate.[19] HANs can also form in biological systems in vivo from residual hypochlorite in drinking water.[20] HANs formed in swimming pool water can be absorbed through dermal exposure.[21,22]

A review of the metabolism and toxicity of HANs[23] revealed that several HANs directly interact with DNA,[24] and are mutagenic in *Salmonella typhimurium*,[25−27] genotoxic in mammalian cells,[11,26,27] clastogenic,[28] and developmentally toxic.[29,30] Dichloroacetonitrile (DCAN), but not dibromoacetonitrile (DBAN), was shown to be an effective inducer of aneuploidy in oocysts of *Drosophila melanogaster*.[31] DBAN was recently shown to be carcinogenic[32] and the contribution of HANs to lifetime risk of cancer was calculated to be approximately 3- to 10-fold more potent than that of trihalomethanes (THMs).[33] Chloroacetonitrile (CAN) and/or its metabolites were shown to cross the placenta and accumulate in fetal brain tissues in mice, and induced oxidative stress and neural apoptosis.[34,35] A systematic quantitative toxicity study on HANs demonstrated that in vitro mammalian cell toxicity expressed a descending rank order of DBAN > bromoacetonitrile (BAN) ≈ iodoacetonitrile (IAN) > bromochloroacetonitrile (BCAN) > DCAN > CAN > trichloroacetonitrile (TCAN) for chronic cytotoxicity, and IAN > BAN ≈ DBAN > BCAN > CAN > TCAN > DCAN for acute genotoxicity.[11]

DNA damage in eukaryotic cells is highly associated with cell cycle modulation. A damage-response pathway triggered by genetic insult regulates cell cycle arrest and apoptosis, which controls the activation of DNA repair pathways.[36] The eukaryotic cell cycle consists of interphase (G_0/G_1, S, and G_2) and mitotic (M) phase (SI Figure S2). Each chromosome is duplicated during S phase and cell division occurs in M phase, in which chromosome condensation, alignment, and chromosome separation, and daughter cell separation (cytokinesis) occur. The cell cycle is controlled in a directional and sequential pattern by cell cycle regulators, complexes of proteins called cyclins, and cyclin-dependent kinases (Cdks). These cyclin/Cdk complexes are responsible for promoting cell survival by protecting the cell from replicating damaged DNA or from mutations that may lead to aberrant and uncontrolled cell growth (neoplasia). Each cyclin/Cdk complex in the cell cycle acts as a checkpoint and can halt progression through the cell cycle and either arrest progression or induce apoptosis.[37−40] Since HANs are mutagenic, genotoxic, and clastogenic,[11,25−28] we hypothesized that the mammalian cell cycle is affected by HAN treatment. The objective of this study was to determine if monoHANs affected the cell cycle profile of Chinese hamster ovary (CHO) cells, and determine if the cell cycle alteration was associated with genomic damage induction. Flow cytometry was used to measure the DNA content distribution of monoHAN-treated cells, combined with plating efficiency measurements and DNA strand break analyses. This is the first study that identified cell cycle alterations by a class of DBPs, with all three halogens included (I, Br, and Cl).

■ MATERIALS AND METHODS

Chemicals and Reagent. General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma-Aldrich Co. (St. Louis, MO). IAN (98%, CAS# 624-75-9), BAN (97%, CAS# 590-17-0) and CAN (99%, CAS# 107-14-2) were purchased from Sigma-Aldrich, diluted in dimethyl sulfoxide (DMSO) at a concentration of 1 M, and stored at −22 °C. Ethyl methanesulfonate (EMS, CAS# 62-50-0) was diluted in DMSO at 2.5 M and stored at −22 °C. Cytosine-β-D-arabinofuranoside hydrochloride (AraC, CAS# 64-74-9) was dissolved in Ham's F12 medium at 100 mM and stored at −22 °C. Propidium iodide (95%, CAS# 25535-16-4, Acros Organics, Morris Plains, NJ) was dissolved in sterile distilled−deionized water at 1.0 mg/mL and stored at 4 °C.

Cell Culture. Chinese hamster ovary (CHO) cell line AS52 clone 11−4−8[41,42] was maintained in modified Ham's F12 medium (Mediatech, Inc., Manassas, VA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO_2. The cells exhibit normal morphology, express cell contact inhibition, and grow as a monolayer without expressing neoplastic foci.

Cell Treatment for Flow Cytometric Analysis of Cell Cycle. Unsynchronized CHO cells were plated in sterile flat-bottom 6-well tissue culture plates at 2×10^5 cells/(2 mL F12 + 5% FBS)/well or 5×10^4 cells /(2 mL of F12 + 5% FBS)/well. After 16−20 h of incubation, cells were rinsed twice with 1 mL of divalent cation-free Hank's balanced salt solution (HBSS), and treated with each chemical in 1 mL of serum-free F12 for 4 h at 37 °C, 5% CO_2. Multiple wells were used per treatment group as needed. A sheet of sterile AlumnaSeal (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before covering the plate with a lid to prevent crossover contamination between wells due to volatilization. After the 4-h treatment, the treatment solution was aspirated, and the cells were washed twice with 1 mL HBSS. The cells were harvested immediately (0 h sample) with 0.025% trypsin +0.1 g/L EDTA (Hyclone Laboratories, South Logan, UT). Alternatively, 2 mL of drug-free F12 + 5% FBS were added and incubated for designated post-treatment times. An aliquot of the harvested cells was used for cell number count with Beckman Coulter Z1 Particle Counter (Beckman Coulter, Inc., Brea, CA) and for determination of acute cytotoxicity using the trypan blue dye exclusion assay.[10,43] The rest of the harvested cells were fixed in 70% ice cold ethanol and stored at −22 °C for up to 1 week. The fixed cells were rinsed twice with cold phosphate buffered saline (PBS) and incubated for 30 min at 37 °C with 50 μg/mL of the DNA fluorochrome, propidium iodide, in a solution containing 200 μg/mL of RNase A (Life Technologies, Grand Island, NY). Cell fluorescence was measured with a Biosciences LSR II flow cytometry analyzer (San Jose, CA) equipped with BD FACSDiva software. The sample flow rate was set at 12 μL/min or increased to allow for a good cell counting. Propidium iodide was excited by a 488 nm laser line (laser diode) and fluorescence emission was collected through a 695/40 nm band-pass filter. For each sample, approximately 10 000 events were collected, and aggregated cells were gated out. The collected data were analyzed by multicycle DNA analysis using

FCS Express Software version 4 (De Novo Software, Los Angeles, CA) and the best fit model was chosen to determine the fraction of subpopulations.

Cell Visualization. CHO cells were treated as described above and adherent cells at 0, 26, and 52 h of post-treatment incubation were imaged using phase contrast (100×) in an inverted microscope (Olympus, Center Valley, PA). The resulting images were saved as 8-bit grayscale files in TIFF format.

Plating Efficiency. Cells after 4-h exposure to HANs were transferred to a new 96-well microplate at the density of 3000 cells/(200 μL F12+FBS)/well. The resulting cell density after 72 h was compared to the concurrent negative control. The detailed procedure is presented in the SI. For each HAN concentration, eight replicates were conducted with three independent repeated experiments.

Genomic DNA Damage. Genomic DNA damage of cells exposed to HAN was measured with the single cell gel electrophoresis (SCGE) assay. The detailed procedure is presented in the SI. Cells immediately after 4-h treatment and after 26 h post-treatment incubation were collected for genomic DNA damage analysis. The percentage of DNA in the tail (% tail DNA) was the primary measurement as the DNA genomic damage since it bears a linear relationship to DNA strand break frequency, and is relatively unaffected by threshold setting.[44,45]

■ RESULTS AND DISCUSSION

HANs are known to induce genomic DNA damage detectable by the SCGE assay[11] and thus assessing the association of genotoxic damage with cell cycle alteration was of interest. CHO cells were treated with CAN, BAN or IAN, and subsequent cell cycle profiles were observed using flow cytometry, which quantifies DNA content in individual cells by measuring the incorporated propidium iodide. Cell cycle distribution of HAN-treated cells was always compared to its concurrent negative control.

Careful attention was given to the initial cell loading because CHO cells grow as a monolayer and they express cell contact inhibition when they become confluent. Xenobiotics can exhibit growth phase-specific cell-cycle effects.[46] With an initial cell loading of 2×10^5 cells/well, negative control cells could grow (increase in number) exponentially up to 26 h of post-treatment incubation (Figure 1). When the post-treatment incubation exceeded 26 h, the initial cell loading for the negative control was reduced to 5×10^4 cells/well. The cell loading for each HAN treatment group was kept at 2×10^5 cells/well since the cell number did not reach confluency for 52 h.

The HAN concentrations for cell treatment were empirically optimized. All three HANs shifted the cell cycle distribution toward hyperploidy in a concentration-dependent manner. Concentrations of 900 μM CAN, 12 μM BAN, and 6 μM IAN were chosen because these concentrations induced cell cycle alteration without cytotoxicity. After 4-h treatment with each HAN, cells were rinsed and incubated in fresh F12 + 5% FBS for 0, 8, 17, 26, 34, 43, and 52 h. The post-treatment incubation allowed the time for cells to express altered cell cycle effects (Figure 2; SI Table S1). For all HAN-treated cells, DNA content distribution shifted toward high DNA content compared to the concurrent negative control. All HAN treatments induced 8N DNA containing cells while suppressed 2N DNA containing cell population. Concurrent negative

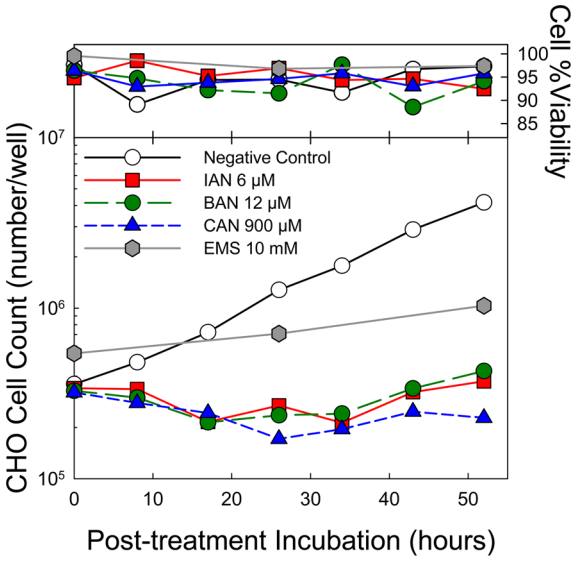


Figure 1. CHO cell viability (top) and growth (bottom) curves during the post-treatment incubation after treated without or with 900 μM CAN, 12 μM BAN, 6 μM IAN, and 10 mM EMS.

control cells did not show the accumulation of an 8N cell population. Over the 52 h post-treatment incubation, no cytotoxicity was observed in any HAN-treated cells, while cell growth was greatly suppressed (Figure 1). It is worth noting that a 4-h noncytotoxic treatment with the HANs induced such long-lasting suppression of cell division with massive impacts on cell ploidy.

Plating efficiency of HAN-treated cells was examined (SI Figure S3). Post-treated cells were seeded onto a new microplate. The resulting cell density after 72 h was compared to the concurrent negative control. The concurrent negative controls had their mean density set to 100%. At the HAN concentrations used for cell cycle analysis, the mean (±SE) cell densities were 14.0 ± 0.8%, 28.9 ± 1.8%, and 27.2 ± 1.8% for 900 μM CAN, 12 μM BAN, and 6 μM IAN, respectively (SI Figure S3).

HAN treatment changed the cell morphology to an elliptical shape (Figure 3). After 26 h of post-treatment incubation, HAN-treated cells still expressed this altered morphology. Interestingly, HAN-treated cells became prominently elongated (>100 μm) after an additional 26 h incubation period. Negative control cells exhibited consistent cell size and normal spindle-shape morphology at 0, 26, and 52 h post-treatment incubation. Treatment with the positive controls 10 mM EMS or 50 μM AraC did not change the morphology of CHO cells after the 4-h treatment, but these agents also increased cell size at 26 and 52 h post-treatment incubation.

We initially hypothesized that genomic DNA damage induced by HANs[11] would affect cell cycle. Genomic DNA damage induction was measured for HAN-treated cells with and without 26 h post-treatment incubation. Treatment with 900 μM CAN, 12 μM BAN and 6 μM IAN induced % tail DNA of 66.4 ± 4.2, 45.5 ± 5.9 and 25.3 ± 1.6, was reduced to 15.6 ± 0.5, 2.4 ± 0.2 and 1.6 ± 0.3 after 26 h post-treatment incubation, respectively (Figure 4). Since the altered cell cycle persisted longer than 26 h, our hypothesis that cells with HAN-damaged DNA delayed cell cycle progression was rejected.

HANs are known to form DNA adducts,[24] and it was hypothesized these adducts may contribute to cell cycle

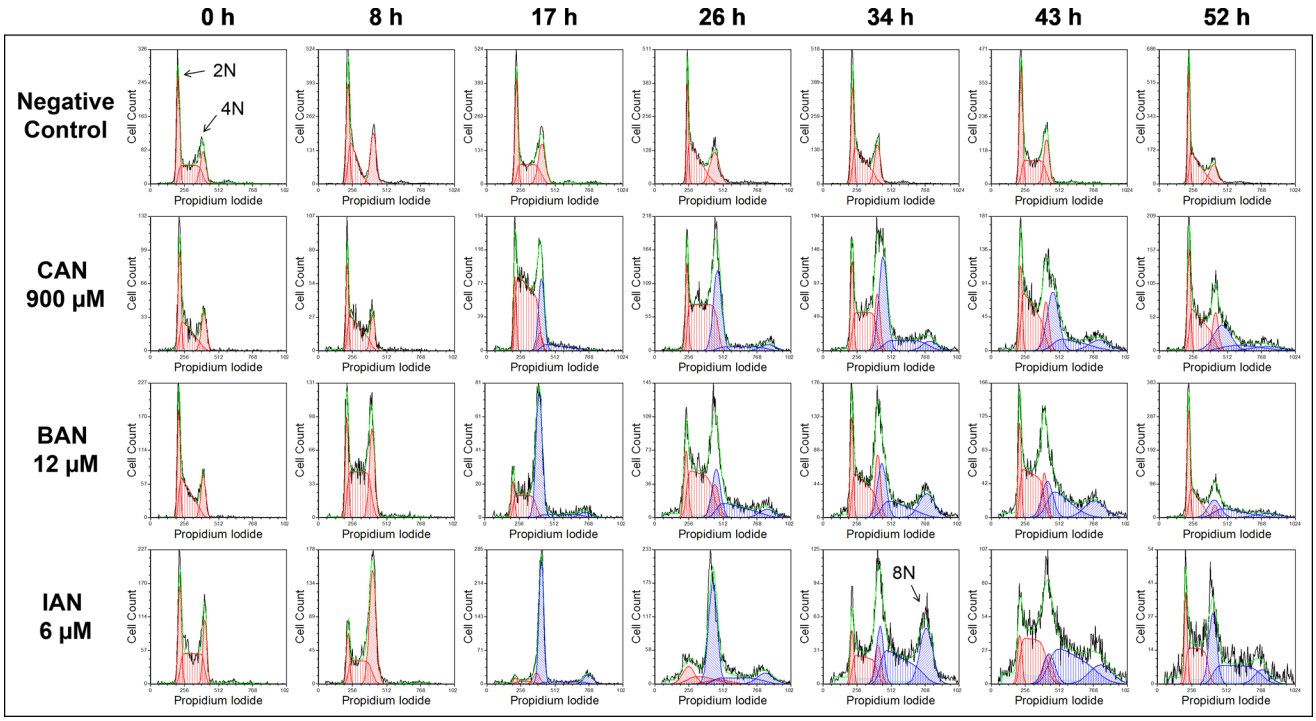


Figure 2. CHO cell cycle distributions at 0, 8, 17, 26, 34, 43, and 52 h post-treatment incubation. Flow cytometric diagram obtained with LSR II flow cytometer.

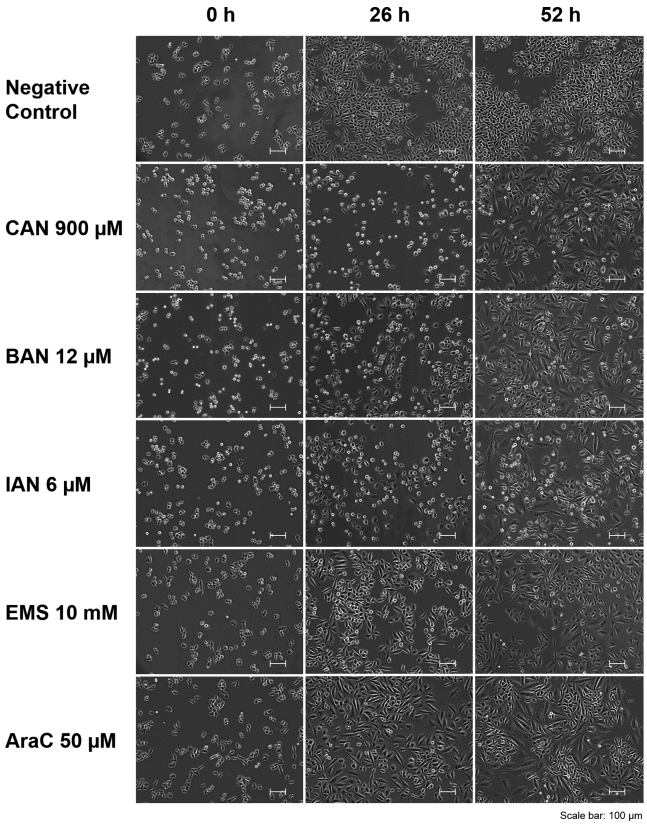


Figure 3. Microscopic images of adherent cells with phase contrast (100×).

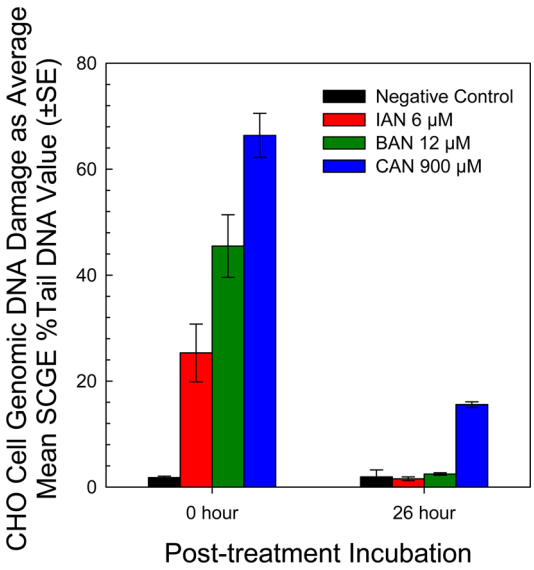


Figure 4. DNA strand breaks measured by single cell gel electrophoresis assay. Treated cells were harvested immediately after the treatment (0 h) and after 26 h post-treatment incubation. In each microgel, 25 or 50 randomly chosen nuclei were analyzed. The average and the standard error of the mean (SE) was calculated based on the mean % tail DNA from each microgel (n = 2−11).

alterations by HANs. The rank order of cell cycle alteration and the rank order of DNA damage induction[11] follow the same order (IAN > BAN > CAN), which indicates a mechanism that involves displacement of a HAN halogen atom at the α-carbon by a S_{N2} reaction.[47] HAN treatment was compared to EMS, a well-known monofunctional alkylating agent that forms adducts at the N- and O-atoms in DNA bases.[48,49] Cells treated with noncytotoxic but a genotoxic concentration of EMS[50,51] expressed G_2 blockage at 26 and 52 h post-treatment incubation times (Figure 5; SI Table S2). However, EMS-treated cells did not express a large percentage of 8N cell as seen with HAN-treated cells. Treatment of CHO cells with 10 mM EMS suppressed the cell growth (Figure 1 and SI Figure

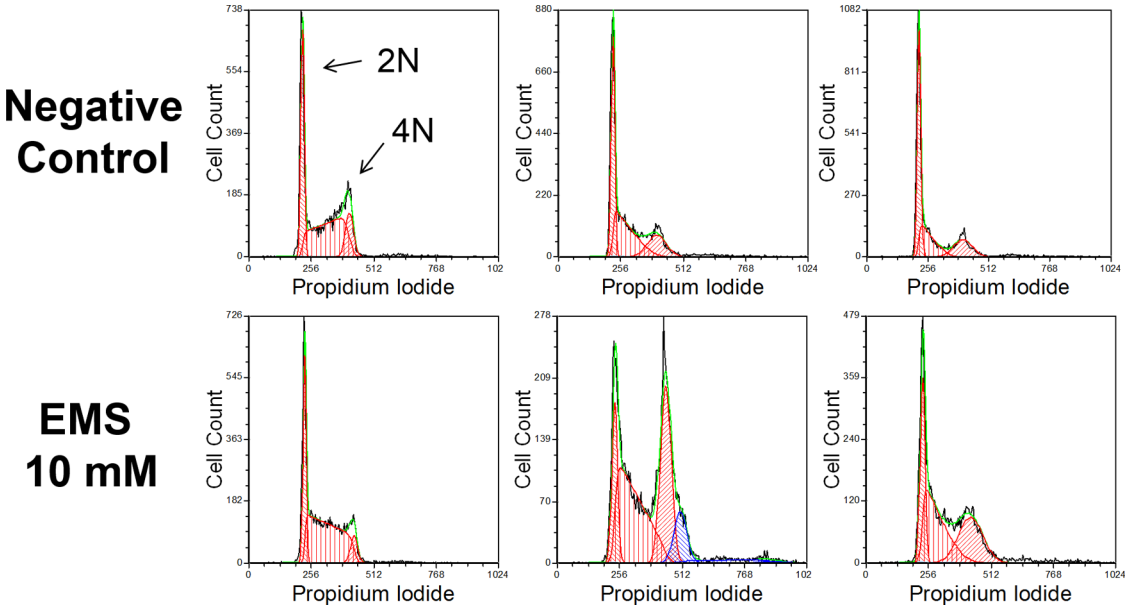


Figure 5. Cell cycle distributions of CHO cells without or with 10 mM EMS at 0, 26, and 52 h post-treatment incubation. Flow cytometric diagram obtained with LSR II flow cytometer.

Table 1. DNA Content Distribution (Percentage) in HAN-Treated CHO Cells with Different Initial Loadings at 26 H Post-Treatment Incubation[a]

	2×10^5 cells/well				4×10^5 cells/well			
Treatment group	2N	2N to 4N	4N	>4N	2N	2N to 4N	4N	>4N
Negative control	34.58	53.27	12.15	0	65.72	26.09	8.18	0
CAN 900 μM	18.22	17.73	64.05	0	20.85	21.12	58.03	0
BAN 12 μM	6.22	35.11	40.33	18.34	21.65	43.66	17.75	16.94
IAN 6 μM	1.12	8.39	46.30	44.19	32.34	28.06	18.49	21.11

[a]DNA content distribution was obtained with LSR II flow cytometer. The cell cycle distributions were calculated using the FCS Express software.

S3) and resulted in larger cell size compared to the concurrent negative controls after 26 and 52 h of post-treatment incubation (Figure 3). This lack of accumulation of an 8N cell population in EMS-treated cells indicated that direct alkylation of DNA cannot account for the cytogenetic toxicity of the HANs.

Initial cell loading was doubled to 4×10^5 cells/well and the HAN effect on cell cycle was examined. CHO cells are not neoplastic and express cell contact inhibition of cell division. Concurrent negative control cells after 26 h post-treatment incubation exhibited a suppressed S phase population frequency (53.3% from 2×10^5 cells/well as opposed to 26.1% for 4×10^5 cells/well), indicating cell contact inhibition was exerted with the higher initial cell loading (Table 1). BAN- and IAN-treated cells showed distinctly different cell cycle profiles when initial cell loading was doubled. When the initial cell loading was 4×10^5 cells/well, IAN- and BAN-treated cells did not show the accumulation of hyperploidy cells as opposed to when 2×10^5 cells/well was initially plated. These results demonstrated a density-dependence on HAN-mediated cell cycle alternation, although it is somewhat less stringent in CAN-treated cells than observed in BAN- and IAN-treated cells. Also, when CHO cells were treated at confluency and subsequently incubated in fresh serum-free F12 medium for 24 h, cells were able to endure as high as 60 μM IAN, 60 μM BAN, and 1200 μM CAN, which was too cytotoxic when treated at low cell density and incubated in complete medium afterward (unpublished data). This demonstrated that the suppression of cell proliferation by cell contact inhibition and serum starvation increased the resistance of CHO cells to HANs. Importantly, these results indicated that cell cycle alteration was neither DNA damage-dependent nor alkylation-dependent, but cell mitosis- and proliferation-dependent.

Andreassen and colleagues treated mitotic CHO cells with chemical agents that inhibit different stages of mitosis, and the mitostatic chemicals forced CHO cells out of mitosis without undergoing cell division.[52] Moreover, after two additional cell cycles, tetraploid cells remained viable and rapidly became aneuploid.[52] The study by Sakaue-Sawano et al. demonstrated that the inhibition of DNA topoisomerase II halted normal murine mammary gland (NMuMG) cells at the G_2/M checkpoint. However, the NMuMG cells then overrode the checkpoint and progressed from G_2/M to G_1 without undergoing cell division, which increased the cellular genome DNA content.[53] Animal cells have self-defense systems to eliminate tetraploid cells that arises from abortive cell cycle.[54] The tetraploid state itself acts as a checkpoint to arrest cells in G_1 regardless of the induction mechanism of tetraploidy.[55] This tetraploid checkpoint enhances the fidelity of cell proliferation by preventing aneuploidization by inducing the tetraploid cells to withdraw from the cell cycle before DNA replication.[55,56] G_1 arrest involved inactive Cdk2 kinase (required for G_1-S progression), hypophosphorylated retinoblastoma protein (pRB) (a gatekeeper of the pathway of activation of S phase and entry into a new cell cycle), and elevated levels of cell cycle progression regulating protein $p21^{WAF1}$ and cyclin E.[55] Arrest in

the G_1 phase of the cell cycle is mainly mediated by the p53 tumor suppressor, a transcription factor that induces cell cycle arrest, damage repair, and apoptosis.[57,58] The p53-dependent induction of p21 in response to DNA damage is the major mechanism enforcing arrest in G_1 in response to DNA damage.[59] Thus, the blockage of cell cycle progression upon abortive mitosis requires intact p53.[56] Andreassen et al. observed indefinite tetraploid G_1 arrest in nontransformed REF-52 (rat embryo fibroblast), mouse NIH3T3, and human IMR-90 cells after mitosis was inhibited by drug treatment.[55] A transformed derivative of REF-52 cells with inactivated p53 (p53DD cell) did not arrest but accumulated with 8N DNA content with mitotic activity. After release from the drug treatment, p53DD cells proceeded through a full cell cycle and became highly aneuploid with DNA content ranging from <2N to 4N.[55] CHO cells also became tetraploid after being treated with protein kinase inhibitor, microtubule assembly inhibitor, topoisomerase II-dependent DNA decatenation inhibitor, or actin assembly inhibitor, and all became aneuploid within 30 h after release from treatment. Another mechanism for the generation of aneuploid cells is by the action of spindle poisons.[60]

The cell line used for this study, CHO AS52 is derived from CHO-K1.[42] A single missense mutation at position 633 (codon 211) in CHO-K1 corresponds to an amino acid change from threonine (ACA) to lysine (AAA) in the DNA-binding domain, which compromises the ability to induce G_1 checkpoint.[61−63] Since CHO-K1 is the parental cell line of CHO AS52, it is most likely that the gene encoding p53 in AS52 carries the same mutation as in K1 cell line. HAN-treated CHO AS52 cells in this study showed accumulation of cells with >4N DNA content after 17 h of post-treatment incubation. The hyperploid cells accumulated with time but the proportion of hyperploid cells went down after 34 h of post-treatment incubation. It is not likely that 8N cells could segregate normally to become diploid. Polyploid cells cannot overcome the issue of multipolar mitosis, which leads to aneuploidy.[54] The accumulation of 4N and 8N DNA containing cell population, combined with the increased cell size and proliferation-dependency, indicates the inhibitory effect of HANs into M phase of the cell cycle, and that the cell cycle progression was not halted due to the lack of p53-dependent G_1 arrest in CHO AS52 cells.

Mutations of the p53 tumor suppressor gene are common in various types of human cancer.[58] Polymorphism in wild-type p53 exists at codon 72 that presents the arginine (CGC) or proline (CCC) genotype, which has been associated with increased risk or urothelial cancer in smokers.[64] Aneuploidy is a hallmark of tumorigenesis,[39] and there is accumulating evidence of transient 4N state proceeding aneuploidy in cancer progression.[39,65] It is possible that HAN-mediated cell cycle alteration may impose an elevated threat to vulnerable subpopulation with compromised p53 function.

Epidemiological studies associated DBPs with urinary bladder cancer but rodent single-chemical bioassays have not been able to demonstrate tumor induction at the same organ sites.[2,66] This discrepancy may be an artifact due to the use of regulated DBPs as an indicator of DBP exposure in epidemiology. Induction of aneuploidy in HAN-treated cells suggests that HANs play a role in cancer induction and adverse pregnancy outcomes associated with long-term exposure to disinfected drinking water. Further research to elucidate the molecular bases of the toxic action of HAN is needed since compromised source waters for use in the generation of drinking water will enhance HAN formation.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, including additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: 217-333-6967; fax (217) 333-0687; e-mail: ykumada@illinois.edu.

*Phone: 217-333-3614; e-mail: mplewa@illinois.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Authors thank Dr. Barbara Pilas and Mr. William Hanafin at the University of Illinois Roy J. Carver Biotechnology Center Flow Cytometry Facility. This research has been supported by NSF STC WaterCAMPWS (Award CTS-0120978) and the U.S. EPA STAR Grant R834867. Although the research described in the article has been funded in part by the U.S. Environmental Protection Agency's STAR program, it has not been subjected to any EPA review, and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

■ REFERENCES

(1) Krasner, S. W. The formation and control of emerging disinfection by-products of health concern. *Philos. Trans. R. Soc. A: Math., Phys. Eng. Sci.* **2009**, *367* (1904), 4077−4095.

(2) Hrudey, S. E. Chlorination disinfection by-products, public health risk tradeoffs and me. *Water Res.* **2009**, *43* (8), 2057−2092.

(3) Bove, F.; Shim, Y.; Zeitz, P. Drinking water contaminants and adverse pregnancy outcomes: A review. *Environ. Health Perspect.* **2002**, *110* (Suppl1), 61−74.

(4) Villanueva, C. M.; Cantor, K. P.; Cordier, S.; Jaakkola, J. J.; King, W. D.; Lynch, C. F.; Porru, S.; Kogevinas, M. Disinfection byproducts and bladder cancer: A pooled analysis. *Epidemiology* **2004**, *15* (3), 357−367.

(5) Trehy, M. L.; Yost, R. A.; Miles, C. J. Chlorination byproducts of amino acids in natural waters. *Environ. Sci. Technol.* **1986**, *20* (11), 1117−1122.

(6) Bond, T.; Henriet, O.; Goslan, E. H.; Parsons, S. A.; Jefferson, B. Disinfection byproduct formation and fractionation behavior of natural organic matter surrogates. *Environ. Sci. Technol.* **2009**, *43* (15), 5982−5989.

(7) Oliver, B. G. Dihaloacetonitriles in drinking water: Algae and fulvic acid as precursors. *Environ. Sci. Technol.* **1983**, *17* (2), 80−83.

(8) Shah, A. D.; Mitch, W. A. Halonitroalkanes, halonitriles, haloamides, and *N*-nitrosamines: A critical review of nitrogenous disinfection byproduct formation pathways. *Environ. Sci. Technol.* **2011**, *46* (1), 119−131.

(9) Dotson, A.; Westerhoff, P.; Krasner, S. W. Nitrogen enriched dissolved organic matter (DOM) isolates and their affinity to form emerging disinfection by-products. *Water Sci. Technol.* **2009**, *60* (1), 135−143.

(10) Plewa, M. J.; Wagner, E. D. *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*; Water Research Foundation: Denver, CO, 2009.

(11) Muellner, M. G.; Wagner, E. D.; McCalla, K.; Richardson, S. D.; Woo, Y. T.; Plewa, M. J. Haloacetonitriles vs. regulated haloacetic

acids: Are nitrogen-containing DBPs more toxic? *Environ. Sci. Technol.* **2007**, *41* (2), 645−651.

(12) Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D., Jr.; Woo, Y. T.; McKague, A. B. Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004**, *38* (18), 4713−4722.

(13) Plewa, M. J.; Muellner, M. G.; Richardson, S. D.; Fasano, F.; Buettner, K. M.; Woo, Y. T.; McKague, A. B.; Wagner, E. D. Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: An emerging class of nitrogenous drinking water disinfection byproducts. *Environ. Sci. Technol.* **2008**, *42* (3), 955−961.

(14) Bond, T.; Templeton, M. R.; Graham, N. Precursors of nitrogenous disinfection by-products in drinking water − A critical review and analysis. *J. Hazard. Mater.* **2012**, *235−236* (0), 1−16.

(15) Bond, T.; Huang, J.; Templeton, M. R.; Graham, N. Occurrence and control of nitrogenous disinfection by-products in drinking water − A review. *Water Res.* **2011**, *45* (15), 4341−4354.

(16) Huang, H.; Wu, Q.-Y.; Hu, H.-Y.; Mitch, W. A. Dichloroacetonitrile and dichloroacetamide can form independently during chlorination and chloramination of drinking waters, model organic matters, and wastewater effluents. *Environ. Sci. Technol.* **2012**, *46* (19), 10624−10631.

(17) Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D. Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol.* **2006**, *40* (23), 7175−7185.

(18) Krasner, S. W.; Westerhoff, P.; Chen, B.; Rittmann, B. E.; Amy, G. Occurrence of disinfection byproducts in United States wastewater treatment plant effluents. *Environ. Sci. Technol.* **2009**, *43* (21), 8320−8325.

(19) Anglada, Á.; Urtiaga, A.; Ortiz, I.; Mantzavinos, D.; Diamadopoulos, E. Boron-doped diamond anodic treatment of landfill leachate: Evaluation of operating variables and formation of oxidation by-products. *Water Res.* **2011**, *45* (2), 828−838.

(20) Mink, F. L.; Coleman, W. E.; Munch, J. W.; Kaylor, W. H.; Ringhand, H. P. In vivo formation of halogenated reaction products following peroral sodium hypochlorite. *Bull. Environ. Contam. Toxicol.* **1983**, *30* (1), 394−399.

(21) Trabaris, M.; Laskin, J. D.; Weisel, C. P. Effects of temperature, surfactants and skin location on the dermal penetration of haloacetonitriles and chloral hydrate. *J. Expos. Sci. Environ. Epidemiol.* **2012**, *22* (4), 393−397.

(22) Trabaris, M.; Laskin, J. D.; Weisel, C. P. Percutaneous absorption of haloacetonitriles and chloral hydrate and simulated human exposures. *J. Appl. Toxicol.* **2012**, *32* (6), 387−394.

(23) Lipscomb, J. C.; El-Demerdash, E.; Ahmed, A. E. Haloacetonitriles: Metabolism and Toxicity. In *Reviews of Environmental Contamination and Toxicology*; Whitacre, D. M., Ed.; Springer: New York, 2009; Vol. *198*, pp 169−200.

(24) Nouraldeen, A. M.; Ahmed, A. E. Studies on the mechanisms of haloacentronitrile-induced genotoxicity IV: In vitro interaction of haloacetonitriles with DNA. *Toxicol. In Vitro* **1996**, *10* (1), 17−26.

(25) Simmon, V. F.; Kauhanen, K.; Tardiff, R. G. Mutagenic Activity of Chemicals Identified in Drinking Water. In *Progress in Genetic Toxicology*, Scott, D., Bridges, B. A., Sobels, F. H., Eds.; Elsevier/North-Holland Biomedical Press: New York, 1977; pp 249−258.

(26) Bull, R. J.; Meier, J. R.; Robinson, M.; Ringhand, H. P.; Laurie, R. D.; Stober, J. A. Evaluation of mutagenic and carcinogenic properties of brominated and chlorinated acetonitriles: By-products of chlorination. *Fundam. Appl. Toxicol.* **1985**, *5* (6, Part 1), 1065−1074.

(27) Muller-Pillet, V.; Joyeux, M.; Ambroise, D.; Hartemann, P. Genotoxic activity of five haloacetonitriles: Comparative investigations in the single cell gel electrophoresis (comet) assay and the Ames-fluctuation test. *Environ. Mol. Mutag.* **2000**, *36* (1), 52−58.

(28) Le Curieux, F.; Giller, S.; Gauthier, L.; Erb, F.; Marzin, D. Study of the genotoxic activity of six halogenated acetonitriles, using the SOS chromotest, the Ames-fluctuation test and the newt micronucleus test. *Mutat. Res./Gen. Toxicol.* **1995**, *341* (4), 289−302.

(29) Smith, M. K.; George, E. L.; Zenick, H.; Manson, J. M.; Stober, J. A. Developmental toxicity of halogenated acetonitriles: Drinking water by-products of chlorine disinfection. *Toxicology* **1987**, *46* (1), 83−93.

(30) Smith, M. K.; Randall, J. L.; Stober, J. A.; Read, E. J. Developmental toxicity of dichloroacetonitrile: A by-product of drinking water disinfection. *Fundam. Appl. Toxicol.* **1989**, *12* (4), 765−772.

(31) Osgood, C.; Sterling, D. Dichloroacetonitrile, a by-product of water chlorination, induces aneuploidy in *Drosophila*. *Mutat. Res., Genet. Toxicol.* **1991**, *261* (2), 85−91.

(32) National Toxicity Program *Toxicology and carcinogenesis studies of dibromoacetonitrile (CAS No.3252−43−5) in F344/N rats and B6C3F1 mice (drinking water studies). National Toxicology Program Technical Report TR-544*; NIEHS; National Toxicology Program, Research Triangle Park, N.C., 2010.

(33) Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E. Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011**, *286* (1−3), 1−19.

(34) Ahmed, A. E.; Jacob, S.; Campbell, G. A.; Harirah, H. M.; Perez-Polo, J. R.; M. Johnson, K. Fetal origin of adverse pregnancy outcome: The water disinfectant by-product chloroacetonitrile induces oxidative stress and apoptosis in mouse fetal brain. *Dev. Brain Res.* **2005**, *159* (1), 1−11.

(35) Ahmed, A. E.; Campbell, G. A.; Jacob, S. Neurological impairment in fetal mouse brain by drinking water disinfectant byproducts. *Neurotoxicology* **2005**, *26* (4), 633−640.

(36) Zhou, B.-B. S.; Elledge, S. J. The DNA damage response: Putting checkpoints in perspective. *Nature* **2000**, *408* (6811), 433−439.

(37) Uhlmann, F.; Bouchoux, C.; López-Avilés, S. A quantitative model for cyclin-dependent kinase control of the cell cycle: Revisited. *Philos. Trans. R. Soc. B: Biol. Sci.* **2011**, *366* (1584), 3572−3583.

(38) Camins, A.; Pizarro, J. G.; Folch, J., Cyclin-dependent kinases. In *Brenner's Encyclopedia of Genetics*, 2nd ed.; Stanley, M., Kelly, H., Eds.; Academic Press: San Diego, 2013; pp 260−266.

(39) Davoli, T.; de Lange, T. The causes and consequences of polyploidy in normal development and cancer. *Annu. Rev. Cell. Dev. Biol.* **2011**, *27* (1), 585−610.

(40) Depamphilis, M. L.; de Renty, C. M.; Ullah, Z.; Lee, C. Y. "The octet": Eight protein kinases that control mammalian DNA replication. *Front. Physiol.* **2012**, *3*.

(41) Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J. Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998**, *32* (4), 360−368.

(42) Tindall, K. R.; Stankowski, L. F., Jr.; Machanoff, R.; Hsie, A. W. Analyses of mutation in pSV2gpt-transformed CHO cells. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1986**, *160* (2), 121−131.

(43) Phillips, H. J. Dye exclusion tests for cell viability. In *Tissue Culture: Methods and Applications*, Kruse, P. F.; Patterson, M. J., Eds. Academic Press: New York, 1973; p 406.

(44) Collins, A. R. The comet assay for DNA damage and repair: Principles, applications, and limitations. *Mol. Biotechnol.* **2004**, *26* (3), 249−261.

(45) Kumaravel, T. S.; Vilhar, B.; Faux, S. P.; Jha, A. N. Comet Assay measurements: A perspective. *Cell Biol. Toxicol.* **2009**, *25* (1), 53−64.

(46) Zhang, P.; Zhou, Y.; Tao, D.; Zhou, J.; Gong, J. Cytarabine and paclitaxel exhibit different cell-cycle specificities in different cell growing status. *Chinese-German Journal of Clinical Oncology* **2006**, *5* (6), 416−419.

(47) Loudon, G. M. *Organic Chemistry*. 3rd ed.; Benjamin/Cummings Publ. Co.: Redwood, CA, 1995.

(48) Beranek, D. T. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1990**, *231* (1), 11−30.

(49) Gocke, E.; Bürgin, H.; Müller, L.; Pfister, T. Literature review on the genotoxicity, reproductive toxicity, and carcinogenicity of ethyl methanesulfonate. *Toxicol. Lett.* **2009**, *190* (3), 254−265.
(50) Rundell, M. S.; Wagner, E. D.; Plewa, M. J. The comet assay: Genotoxic damage or nuclear fragmentation? *Environ. Mol. Mutag.* **2003**, *42* (2), 61−7.
(51) Wagner, E. D.; Anderson, D.; Dhawan, A.; Rayburn, A. L.; Plewa, M. J. Evaluation of EMS-induced DNA damage in the single cell gel electrophoresis (Comet) assay and with flow cytometric analysis of micronuclei. *Teratog. Carcinog. Mutagen.* **2003**, *23* (S2), 1−11.
(52) Andreassen, P. R.; Martineau, S. N.; Margolis, R. L. Chemical induction of mitotic checkpoint override in mammalian cells results in aneuploidy following a transient tetraploid state. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1996**, *372* (2), 181−194.
(53) Sakaue-Sawano, A.; Kobayashi, T.; Ohtawa, K.; Miyawaki, A. Drug-induced cell cycle modulation leading to cell-cycle arrest, nuclear mis-segregation, or endoreplication. *BMC Cell Biol.* **2011**, *12* (1), 2.
(54) Storchova, Z.; Pellman, D. From polyploidy to aneuploidy, genome instability and cancer. *Nat. Rev. Mol. Cell Biol.* **2004**, *5* (1), 45−54.
(55) Andreassen, P. R.; Lohez, O. D.; Lacroix, F. B.; Margolis, R. L. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell* **2001**, *12* (5), 1315−1328.
(56) Margolis, R. L.; Lohez, O. D.; Andreassen, P. R. G1 tetraploidy checkpoint and the suppression of tumorigenesis. *J. Cell. Biochem.* **2003**, *88* (4), 673−683.
(57) Hofseth, L. J.; Hussain, S. P.; Harris, C. C. p53:25 years after its discovery. *Trends Pharmacol. Sci.* **2004**, *25* (4), 177−181.
(58) Hermeking, H. The miR-34 family in cancer and apoptosis. *Cell Death Differ.* **2010**, *17* (2), 193−199.
(59) Scorah, J.; McGowan, C. H., Regulation of cell cycle progression. In *Handbook of Cell Signaling*, 2nd ed.; Ralph, A. B., Edward, A. D., Eds.; Academic Press: San Diego, 2010; pp 2545−2553.
(60) Matson, D. R.; Stukenberg, P. T. Spindle poisons and cell fate: A tale of two pathways. *Mol. Interv.* **2011**, *11* (2), 141−150.
(61) Hu, T.; Miller, C. M.; Ridder, G. M.; Aardema, M. J. Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL, and CHL: Implications for genotoxicity testing. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1999**, *426* (1), 51−62.
(62) Tzang, B.-S.; Lai, Y.-C.; Hsu, M.; Chang, H.-W.; Chang, C.-C.; Huang, P. C.; Liu, Y.-C. Function and sequence analyses of tumor suppressor gene p53 of CHO.K1 cells. *DNA Cell Biol.* **1999**, *18* (4), 315−321.
(63) Lee, H.; Larner, J. M.; Hamlin, J. L. Cloning and characterization of Chinese hamster p53 cDNA. *Gene* **1997**, *184* (2), 177−183.
(64) Kuroda, Y.; Tsukino, H.; Nakao, H.; Imai, H.; Katoh, T. p53 codon 72 polymorphism and urothelial cancer risk. *Cancer Lett.* **2003**, *189* (1), 77−83.
(65) Galipeau, P. C.; Cowan, D. S.; Sanchez, C. A.; Barrett, M. T.; Emond, M. J.; Levine, D. S.; Rabinovitch, P. S.; Reid, B. J. 17p (p53) allelic losses, 4N (G_2/tetraploid) populations, and progression to aneuploidy in Barrett's esophagus. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93* (14), 7081−7084.
(66) Melnick, R. L.; Hooth, M. J. Carcinogenicity of Disinfection Byproducts in Laboratory Animals. In *Encyclopedia of Environmental Health*; Jerome, O. N., Ed.; Elsevier: Burlington, 2011; pp 516−523.