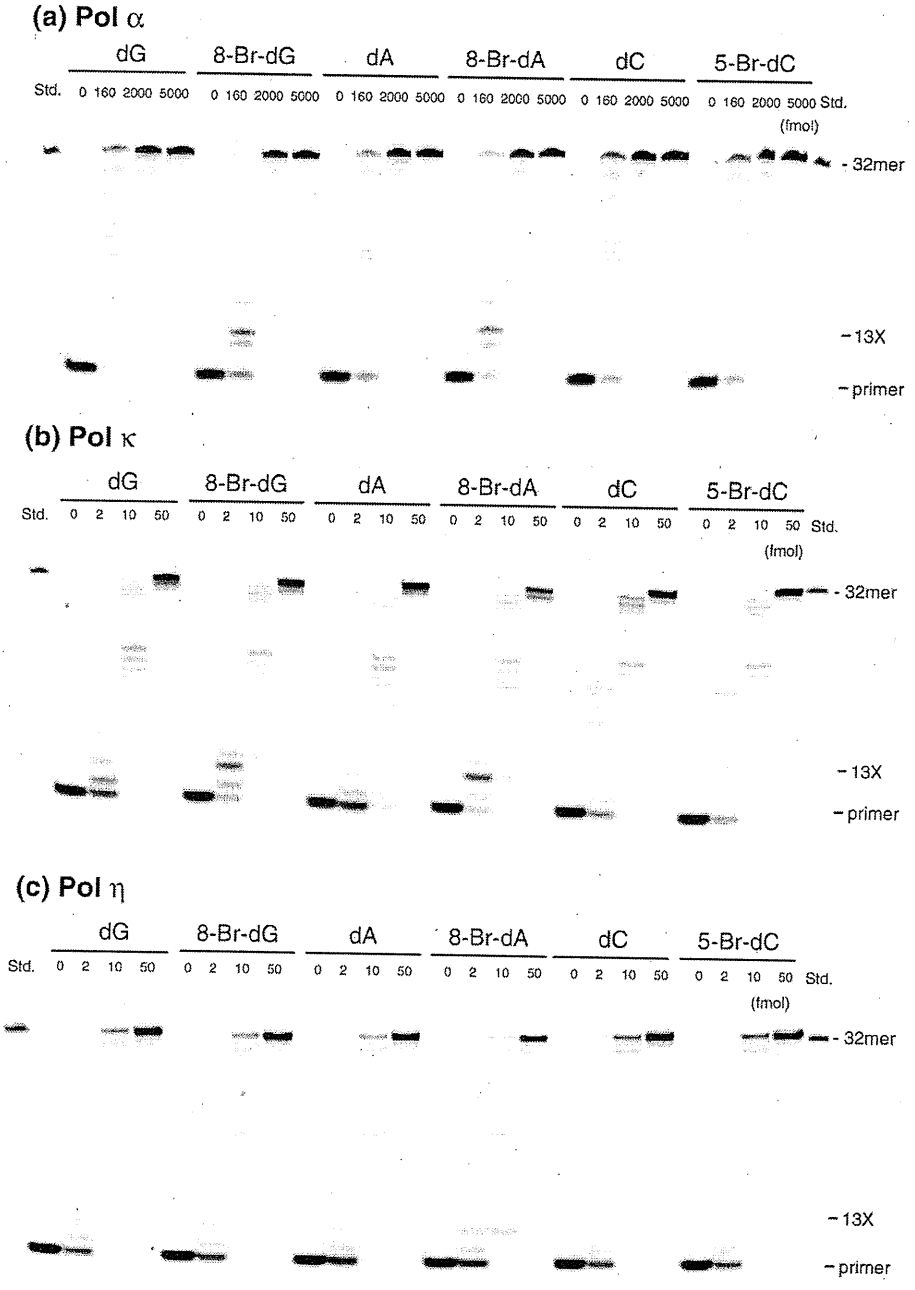

Fig. 3. Primer extension reactions catalyzed by DNA pols on 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified DNA templates. Unmodified and modified 38-mer templates were annealed to an Alexa546-labeled 10-mer primer. Primer extension reactions catalyzed by variable amounts (0–5000 fmol) of pols α, κ, and η were conducted at 25 °C for 30 min in the presence of four dNTPs using variable amounts of enzymes. The whole amount of the reaction mixture was subjected to 20% denaturing PAGE. 13X marks the location opposite the DNA adducts.

Discussion

8-Br-dG, 8-Br-dA, and 5-Br-dC can be produced by HOBr *in vitro*,[6,16] and 8-Br-dG has been detected in the genomic DNA of rat liver.[5] If the adducts are not rapidly repaired, DNA pols are more likely to be associated with mutagenic events generated by brominated DNA damage. We demonstrated that primer extension reactions catalyzed by pols α, κ, and η were carried out on site-specifically modified DNA templates containing the 8-Br-dG, 8-Br-dA, or 5-Br-dC lesion. Pols α and κ were slightly retarded

Table 1. Kinetic parameters for nucleotide insertion and chain extension reactions catalyzed by human DNA pols α and κ

		Insertion			Extension			
		dNTP ↓GAAGAAAGGAGA 5′-CCTTCXCTTCTTTCCTCTCCCTTT			dGTP ↓NGAAGAAAGGAGA 5′-CCTTCXCTTCTTTCCTCTCCCTTT			
	N:X	K_m (μM)[a]	V_{max} (% min-1)[a]	F_{ins}	K_m (μM)[a]	V_{max} (% min^{-1})[a]	F_{ext}	$F_{ins} \times F_{ext}$
Pol α	C:G	0.85±0.18	0.053±0.0029	1	1.68±0.31	0.27±0.013	1	1
	C:X	24.4±9.73	0.014±0.0024	9.20×10^{-2}	287.8±68.8	0.036±0.0042	7.78×10^{-4}	7.16×10^{-5}
	A:X	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	G:X	0.28±0.17	0.00038±0.000017	0.22	400.5±117.7	0.0016±0.00026	2.49×10^{-5}	5.48×10^{-6}
	T:X	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Pol κ	C:G	5.06±1.76	9.16±1.40	1	0.37±0.08	5.15±0.45	1	1
	C:X	13.0±4.98	2.31±0.31	9.82×10^{-2}	16.1±4.88	6.84±1.09	3.05×10^{-2}	3.00×10^{-3}
	A:X	215±68.0	2.85±0.40	7.32×10^{-3}	149.5±34.3	2.43±0.29	1.17×10^{-3}	8.55×10^{-6}
	G:X	4.02±1.04	1.03±0.08	0.14	90.0±25.5	3.19±0.40	2.54×10^{-3}	3.56×10^{-4}
	T:X	257±66.1	3.01±0.37	6.46×10^{-3}	65.5±12.0	1.99±0.14	2.18×10^{-3}	1.41×10^{-5}

Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the following equation: $F=(V_{max}/K_m)$[wrong pair]$/(V_{max}/K_m)$[correct pair=dC:dG]. X=8-Br-dG lesion.

N.D., not detectable.

[a] Data were expressed as mean±SD obtained from three independent experiments.

by the 8-Br-dG and 8-Br-dA sites on their templates. As shown in Fig. 3a and b, the primer extension profiles of 8-Br-dG and 8-Br-dA were very similar and thus expected to show similar miscoding specificities between 8-Br-dG and 8-Br-dA. We determined both miscoding specificities by using two-phase PAGE (Fig. 2), and only 8-Br-dG had a miscoding potential (Fig. 4), and not 8-Br-dA (Supplementary Fig. S1). Therefore, the miscoding specificities of 8-Br-dG and 8-Br-dA are quite different even though their primer extension profiles are similar.

Little is known about the mutation spectrum induced by reactive brominating species, except for the miscoding spectrum of 5-Br-dU lesion, which has been reported in detail.[15] In this work, the miscoding spectra of 8-Br-dG, 8-Br-dA, and 5-Br-dC lesions were quantitatively determined in primer extension reactions in the presence of all four dNTPs. Among the DNA adducts, we found that only 8-Br-dG was a mutagenic lesion. When pol α was used, one-base deletion was detected opposite the lesion. In reactions catalyzed by pol κ, one-base deletion was observed, accompanied by lower

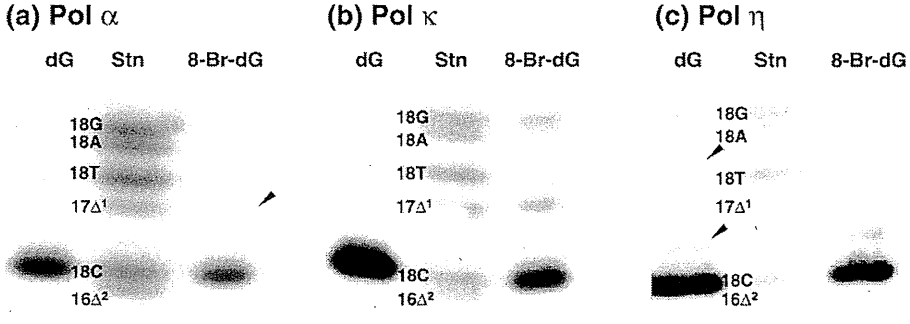

Fig. 4. Miscoding specificities of 8-Br-dG lesion in reactions catalyzed by pols α, κ, and η. Using unmodified and 8-Br-dG-modified 38-mer templates primed with an Alexa546-labeled 10-mer, we conducted primer extension reactions at 25 °C for 30 min in a buffer containing four dNTPs (100 μM each) and pol α (2000 fmol for unmodified and 8-Br-dG-modified templates), pol κ (10 fmol for unmodified and 8-Br-dG-modified templates), or pol η (50 fmol for unmodified and 8-Br-dG-modified templates), as described in Materials and Methods. The extended reaction products (>26 bases long) produced on the unmodified and modified templates were extracted following 20% denaturing PAGE. The recovered oligodeoxynucleotides were annealed to the complementary unmodified 38-mer and cleaved with EcoRI restriction enzyme. The entire product from the unmodified and 8-Br-dG-modified templates was subjected to two-phase PAGE (20×65×0.05 cm). The mobilities of the reaction products were compared with those of 18-mer standards (Fig. 2) containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions.

amounts of dGMP, dAMP, and dTMP misincorporation (Fig. 4b). Thus, 8-Br-dG can pair with all the wrong bases at low frequency. This indicates that the broad mutation spectrum of 8-Br-dG may be generated in cells at the inflamed site. The spectrum was also supported by the steady-state kinetic studies (Table 1).

We revealed that both 8-Br-dA and 5-Br-dC adducts showed no miscoding events during *in vitro* DNA synthesis catalyzed by pols α, κ, and η. With respect to 8-Br-dA, all pols exclusively inserted dTMP, the correct base, opposite the lesion (Fig. S1). As the representative example of C8-dA DNA adducts, 8-oxo-7,8-dihydro-2′-deoxyadenosine also promoted the incorporation of dTMP, the correct base, in a similar two-phase PAGE system.[24] In contrast, 8-Br-dG and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-Oxo-dG), as C8-dG DNA adducts, promoted primary one-base deletion (Fig. 4) and misincorporation of dAMP,[25,26] respectively. Thus, we suggest that the miscoding potential of C8-dG adducts may be stronger than that of C8-dA adducts.

Moreover, we found that 5-Br-dC was also a nonmutagenic lesion. Pols α, κ, and η exclusively incorporated dGMP opposite the adduct (Fig. S1). However, 5-Br-dC may be converted to the thymidine analogue 5-Br-dU by deamination in cells. 5-Br-dU in the genomic DNA is paired with dA in the keto form and/or with dG in the enol form, which causes the mutations.[14,15] Thus, 5-Br-dC may cause the mutation via conversion to 5-Br-dU. On the basis of the current study, we suggest that 8-Br-dA and 5-Br-dC themselves are nonmutagenic DNA adducts.

Asahi *et al.* recently reported that the formation of 8-Oxo-dG and 8-Br-dG in LPS-treated rats was assessed by liquid chromatography–tandem mass spectrometry.[5] The study showed that the basal level of 8-Br-dG in the hepatic DNA and urine of the rats was at least 3 orders of magnitude lower than that of 8-Oxo-dG. Furthermore, the excretion of 8-Br-dG into the urine in the LPS-treated rats occurred earlier than for 8-Oxo-dG, implying that DNA repair of 8-Br-dG is more efficient than that of 8-Oxo-dG. In the present study, as shown in Table 2, the $F_{ins} \times F_{ext}$ ratio for the dG:8-Br-dG/dC:8-Br-dG pairs was 0.08. This number was remarkably lower than that for the dA:8-Oxo-dG/dC:8-Oxo-dG pairs (ratio = 1453). Similar results were observed with pol κ. The ratio of $F_{ins} \times F_{ext}$ past 8-Br-dG was at least 2 orders of magnitude lower than that of 8-Oxo-dG, indicating that the miscoding potential of 8-Br-dG was weaker than that of 8-Oxo-dG.[25,26] Overall, the miscoding potential, formation level, and accumulativeness in DNA of the 8-Br-dG adduct are lower than those of 8-Oxo-dG. Therefore, the contribution to mutagenesis at the inflamed site of 8-Br-dG may be limited.

Table 2. $F_{ins} \times F_{ext}$ past DNA adducts by mammalian DNA pols α and κ

	X=	8-Br-dG[a]	8-Oxo-dG[b,c]
Pol α	C:X	7.16×10^{-5}	1.70×10^{-5}
	A:X	N.D.	**2.47×10^{-2}**
	G:X	**5.48×10^{-6}**	N.D.
	T:X	N.D.	N.D.
Pol κ	C:X	3.00×10^{-3}	6.21×10^{-4}
	A:X	8.55×10^{-6}	**2.00×10^{-2}**
	G:X	**3.56×10^{-4}**	7.65×10^{-8}
	T:X	1.41×10^{-5}	5.98×10^{-5}

Values in boldface indicate a primarily misincorporated base opposite the DNA adduct.
N.D., not detectable.
[a] Data were taken from Table 1.
[b] Data for pol α were taken from Ref. 25.
[c] Data for pol κ were taken from Ref. 29.

In conclusion, two-phase PAGE analysis and steady-state kinetic studies were performed to determine the miscoding specificities of the brominated DNA lesions 8-Br-dG, 8-Br-dA, and 5-Br-dC. Among them, only 8-Br-dG, and not 5-Br-dC and 8-Br-dA, is a mutagenic lesion; the miscoding frequency and specificity vary depending on the DNA pol used. Thus, HOBr-induced 8-Br-dG adducts may contribute to mutagenic events at the site of inflammation.

Materials and Methods

Materials

Ultrapure dNTPs were from GE Healthcare. EcoRI restriction endonuclease was purchased from New England BioLabs. Blue Dextran was obtained from Sigma-Aldrich. Human pol α (1200 units/mg of protein) was purchased from CHIMERx (Milwaukee, WI). Human pol η was kindly provided by Drs. Chikahide Masutani and Fumio Hanaoka. Human pol κ was prepared as C-terminal truncations with 10 His-tags as previously described.[27]

Preparation of oligodeoxynucleotides

All oligodeoxynucleotides, Alexa546-labeled primers, standard markers, and site-specifically 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified template were obtained from Japan Bio Service Co. (Saitama, Japan). Alexa546 was conjugated at the 5′-terminus of primers and standard markers. A single brominated DNA adduct was located at the 20th position from the 5′-termini in the modified 38-mer template (5′-CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT, where X represents 8-Br-dG, 8-Br-dA, or 5-Br-dC). The oligomers were purified by 20% denaturing PAGE before use. We demonstrated that the 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified oligomers were stable at 25 °C for at least 6 h.

Primer extension reactions

Primer extension reactions catalyzed by pols α, κ, and η were conducted at 25 °C for 30 min in a buffer (10 μL) containing all four dNTPs (100 μM each) using 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified and unmodified 38-mer templates (750 fmol) primed with an Alexa546-labeled 10-mer (500 fmol, 5′-AGAGGAAAGA) (Fig. 2). The reaction buffer for pol α contains 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 60 mM KCl, 10 mM dithiothreitol, 250 μg/mL bovine serum albumin, and 2.5% glycerol. The reaction buffer for pol η and pol κ contains 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM dithiothreitol, 250 μg/mL bovine serum albumin, 60 mM KCl, and 2.5% glycerol. The reaction was stopped by the addition of 2 μL of formamide dye containing Blue Dextran (100 mg/mL) and ethylenediaminetetraacetic acid (50 mM) and incubation at 95 °C for 3 min. The whole amount of the reaction sample was subjected to 20% denaturing PAGE (30 × 40 × 0.05 cm). The separated products were visualized by using Molecular Imager FX Pro and Quantity One software (Bio-Rad Laboratories). We demonstrated that the linear range to quantitatively detect fluorescent-labeled oligomers was from 5 to 1500 fmol.[21]

Quantitation of miscoding specificities and frequencies

Using 8-Br-dG-modified and unmodified 38-mer oligodeoxynucleotides (750 fmol) primed with an Alexa546-labeled 10-mer (500 fmol, 5′-AGAGGAAAGA), we conducted primer extension reactions catalyzed by pol α (2000 fmol), pol κ (10 fmol), or pol η (50 fmol) at 25 °C for 30 min in a buffer (10 μL) containing all four dNTPs (100 μM each) and subjected them to 20% denaturing PAGE (30 × 40 × 0.05 cm). Extended reaction products (>26 bases long) were extracted from the gel. The recovered oligodeoxynucleotides were annealed with the unmodified 38-mer, cleaved with EcoRI, and subjected to two-phase PAGE (20 × 65 × 0.05) containing 7 M urea in the upper phase and no urea in the middle and bottom phases (each phase containing 18%, 20%, and 24% polyacrylamide, respectively). The phase width is approximately 10, 37, and 18 cm from the upper phase. To quantify base substitutions and deletions, we compared the mobility of the reaction products with those of Alexa546-labeled 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions (Fig. 2).[19–21]

Steady-state kinetic studies of nucleotide insertion and extension

Kinetic parameters associated with nucleotide insertion opposite the 8-Br-dG lesion and chain extension from the 3′ primer terminus were determined at 25 °C, using varying amounts of single dNTPs. For insertion kinetics, reaction mixtures containing dNTP (0–500 μM) and either pol α (50–1000 fmol) or pol κ (10 fmol) were incubated at 25 °C for 0.5–5 min in 10 μL of Tris–HCl buffer (pH 8.0) using a 38-mer template (750 fmol) primed with an Alexa546-labeled 12-mer (500 fmol; 5′-AGAGGAAAGAAG). To measure chain extension, reaction mixtures containing a 38-mer template (750 fmol) primed with an Alexa546-labeled 13-mer (500 fmol; 5′-AGAGGAAAGAAGN, where N is C, A, G, or T), with varying amounts of dGTP (0–500 μM) and either pol α (25–1000 fmol) or pol κ (10 fmol), were used. The reaction samples were subjected to 20% denaturing PAGE (30 × 40 × 0.05). The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were determined with Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software (SPSS Inc.). The frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dC:dG base pair according to the following equation:[28,29]

$$F = (V_{max}/K_m)_{[\text{wrong pair}]} / (V_{max}/K_m)_{[\text{correct pair = dC:dG}]}$$

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.01.005

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Induction of *TK* mutations in human lymphoblastoid TK6 cells by the rat carcinogen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX)

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ABSTRACT

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), a chlorine disinfection by-product in drinking water, is carcinogenic in rats and genotoxic in mammalian cells *in vitro*. In the current study, the mechanism of genotoxicity of MX in human lymphoblastoid TK6 cells was investigated by use of the Comet assay, the micronucleus test, and the thymidine kinase (*TK*) gene-mutation assay. MX induced a concentration-dependent increase in micronuclei and *TK* mutations. The lowest effective concentrations in the MN test and the *TK* gene-mutation assay were 37.5 μM and 25 μM, respectively. In the Comet assay, a slight although not statistically significant increase was observed in the level of DNA damage induced by MX in the concentration range of 25–62.5 μM. Molecular analysis of the *TK* mutants revealed that MX induced primarily point mutations or other small intragenic mutations (61%), while most of the remaining *TK* mutants (32%) were large deletions at the *TK* locus, leading to the hemizygous-type loss-of-heterozygosity (LOH) mutations. These findings show that aside from inducing point mutations, MX also generates LOH at the *TK* locus in human cells and may thus cause the inactivation of tumour-suppressor genes by LOH.

1. Introduction

Epidemiological evidence indicates an association between life-time exposure to disinfection by-products (DBPs) in chlorinated drinking-water and an increased risk for cancer [1]. Of all the DBPs tested for carcinogenicity (~20 DBPs), 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is the most potent rodent carcinogen, producing tumours in multiple organs in rats [2]. In rat-liver epithelial cells, the target cells of MX-induced tumorigenicity in rats, MX causes DNA damage and gene mutations [3] and it also has tumour-promoting properties [4,5]. These studies together with others suggest that MX may be a complete carcinogen in rats. In the overall evaluation conducted by the International Agency for Research on Cancer (IARC), MX was designated as a group 2B carcinogen (possibly carcinogenic to humans) [1].

In spite of the extensive literature showing that MX is a potent genotoxicant in a wide variety of cell lines and endpoints *in vitro* [for a review, see 6], the primary mechanism of genotoxicity and animal tumorigenicity has remained unclear. MX has shown both clastogenic (sister chromatid exchange, chromosomal aberrations, micronuclei) and mutagenic properties. *In vitro*, MX induces point mutations (mainly GC→TA transversions) in *Salmonella typhimurium* strains [7–9] and at the hypoxanthine-phosphoribosyl transferase (*Hprt*) locus in Chinese hamster ovary (CHO) cells [10]. On the other hand, MX was not found to be mutagenic *in vivo*, in transgenic *gpt* delta mice [5]. Furthermore, analyses of mutations in the MX-induced rat liver tumours revealed no point mutations in *Ras* genes and only few point mutations in the *p53* gene [11]. The mechanism that initiates the MX-induced point mutations *in vitro* is unknown. DNA adducts are formed *in vitro* in the reaction of MX with nucleosides and calf-thymus DNA in buffer solutions [12–15], but there are no reports indicating that MX-induced DNA adducts are formed in bacteria or mammalian cells in culture or *in vivo*.

The aim of the present work was to study the genotoxic properties of MX in human lymphoblastoid TK6 cells. TK6 cells have been used in numerous mutation studies, because the *TK* gene-mutation assay detects not only intragenic events – mainly point mutations – but also loss of heterozygosity (LOH), which can result from large-scale chromosomal deletions, recombinations, and aneuploidy [16,17]. This feature makes the assay useful for evaluating the ability of chemicals to induce various mutational events. A major part of the genetic damages detected in *TK* mutants occur in human tumours and thus have relevance to carcinogenicity. In the present study, we investigated the capability of MX to induce *TK* mutants in TK6 cells and determined the types of mutation at the molecular level. In addition, we evaluated the ability of MX to induce DNA damage, analyzed by the use of Comet assay, and chromosomal damage, measured as micronuclei in the TK6 cells.

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2. Materials and methods

2.1. Cell growth and treatment

The human lymphoblastoid TK6 cell line was isolated by Skopek et al. [18] as a non-tumour, p53-proficient cell line, immortalized by Epstein-Barr virus. The cell line is heterozygous at the *thymidine kinase* (*TK*) locus and thus the remaining wild-type allele serves as the target for a recessive mutation. The cells were grown in RPMI-1640 medium (Nacalai Tesque Inc., Kyoto, Japan) containing 10% (v/v) heat-inactivated horse serum (SAFC Biosciences, Lenexa, KS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 200 μg/ml sodium pyruvate at 37 °C in an atmosphere of 5% CO_2.

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX, CAS No. 77439-76-0) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). For the exposure of the cells to MX, 20 ml aliquots of the cell suspension at a concentration of 5.0×10^5 cells/ml were pipetted into 50-ml tubes (1 tube per concentration). MX (final concentrations: 6.25, 12.5, 25, 37.5, 50, and 62.5 μM) was added to the tubes in 200 μl of RPMI 1640 plus supplements. After 4 h of treatment at 37 °C with gentle shaking (26–27 rpm), 500 μl of the cell suspension was pipetted into a 1.5-ml microcentrifuge tube for the Comet assay. The remaining cells were centrifuged (1000 rpm, 5 min), washed, and re-suspended in 50 ml of fresh RPMI-1640 plus supplements. For the cell-viability study, the cells were diluted for plating into 96-well plates. For the micronucleus test and the *TK* gene-mutation assay, the cell suspensions were cultured in 75-cm² flasks. Thus, the assays were conducted with aliquots of the same batch of cells.

The assays were also performed in the presence of an exogenous metabolic activation system (rat liver S9).

2.2. Comet assay

The alkaline Comet assay (single-cell gel electrophoresis, SCGE) was carried out according to the method of Singh et al. [19]. Briefly, 500 μl of treated cell suspension was centrifuged, the cells were washed and suspended in 1 ml of Hank's balanced salt solution (HBSS) supplemented with 20 mM Na_2EDTA and 10% DMSO. A suspension of cells was mixed with 0.5% NuSieve® GTG® Agarose (Cambrex Bio Science Rockland Inc., Rockland, ME) and spread on a microscope slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. The agarose was allowed to solidify for 5 min at room temperature. The cells were lysed at 4 °C overnight in 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10. The following day, slides were transferred to an electrophoresis chamber filled with the ice-cold solution of 0.3 M NaOH, 1 mM EDTA at pH > 13 for 20 min to allow the DNA to unwind. Then electrophoresis was carried out for 15 min at 300 mA. Finally, the slides were neutralized for 5 min in 0.4 M Tris buffer (pH 7.5). Analysis of the Comets was done on SYBR® Gold stained slides (100 cells analyzed per concentration) using the Comet assay IV (Perceptive Instruments Ltd., UK) image-analysis system. The Comet response-parameter used in the statistical analysis was tail intensity (% of DNA migrating into the tail).

2.3. Micronucleus test

The micronucleus test was conducted 48 h after treatment. Approximately 10^6 cells were suspended in hypotonic solution (0.075 M KCl), and then fixed in two steps (2 × 5 min) in ice-cold fixative solution (methanol:acetic acid, 3:1). Finally, the cells were suspended in methanol containing 1% acetic acid. A drop of the cell suspension was spotted on a glass slide and air-dried. The slides were stained with 40 μg/ml acridine orange solution just before microscopic observation. A total of at least 1000 intact interphase cells per concentration were analyzed for the presence of micronuclei by use of a fluorescence microscope.

2.4. TK gene-mutation assay

For the measurement of plating efficiency (PE_0) on day 0 after exposure, the TK6 cells were plated into wells of a 96-well plate (1.6 cells/0.2 ml/well). After 14 days of incubation at 37 °C in an atmosphere of 5% CO_2, the colonies were scored. For the determination of mutation frequency (MF) ($TK^{-/-}$ mutants per 10^6 viable cells) after exposure, the TK6 cells were transferred into the 75-cm² flasks and maintained in logarithmic growth for three days to permit expression of the *TK*-deficient phenotype. The cell suspensions were diluted to below 3×10^5 cells/ml each day to prevent overgrowth. At the end of the expression period, the cell density was adjusted to 2×10^5 cells/ml in 50 ml of growth medium. For the *TK*-deficient mutant selection (containing the recessive mutation), the cells were cloned into 96-well plates at 40,000 cells/well in growth medium (0.2 ml) in the presence of the selective agent trifluorothymidine (TFT, final concentration 3 μg/ml). Cells were also seeded at a density of 1.6 cells/well into 96-well plates in the absence of TFT to determine plating efficiency (PE). The plates were incubated at 37 °C in an atmosphere of 5% CO_2 for 14 days. Then the colonies in the PE and TFT plates (normally growing (NG) *TK* mutants) were scored. The plates containing TFT were then re-fed with TFT at a final concentration of 30 μg/ml. After re-feeding, the plates were incubated for another 14 days, and again the colonies (slowly growing (SG) *TK* mutants) were scored. Cytotoxicity for the TK6 cells was evaluated as relative survival (RS) calculated from PE_0 and relative suspension growth (RSG), derived from cell growth-rate during a 3-day expression period.

2.5. Analysis of loss of heterozygosity

For the determination of loss of heterozygosity (LOH) an independent experiment was done with MX at 37.5 μM. After a 4-h exposure, the TK6 cells were washed and re-suspended in 20 ml of growth medium. Cells were transferred into 25-cm² flasks and incubated for 3 days (37 °C, 5% CO_2). For the *TK*-deficient mutant selection, the cells were then fed with TFT (final concentration 3 μg/ml) and cloned into 96-well plates. After 14 days of incubation (37 °C, 5% CO_2), the NG mutants were transferred from 96-well to 24-well plates in growth medium containing 3 μg/ml TFT, in order to expand the number of cells. The cells in the 96-well plates containing TFT were re-fed with TFT (final concentration 30 μg/ml) and incubated for an additional 14 days. After incubation, the SG mutants were also transferred from 96- to 24-well plates with 3 μg/ml TFT. The NG and SG mutant clones were also tested for growth in medium containing CHAT (deoxycytidine, hypoxanthine, aminopterin, and thymidine), which reduces the number of background mutants. Clones were classified as mutants if they were sensitive to CHAT but able to grow in the presence of TFT. The 24-well plates were incubated for 1–2 weeks (37 °C, 5% CO_2) followed by transfer of the cells into 25-cm² flasks. The cells were cultured for 1–2 weeks until the number of *TK* mutant cells was high enough ($1–2 \times 10^6$ cells) to extract genomic DNA, which was done with the Puregene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN). PCR was performed for those parts of exons 4 and 7 of the human *TK* gene that were heterozygous for frameshift mutations. β-Globin was used as the internal control. For the determination of LOH, a microsatellite locus on chromosome 17q was also subjected to PCR amplification. The PCR products were analyzed with an ABI PRISM® 3100-Avant™ Genetic Analyzer (PE Applied Biosystems, Chiba, Japan) and classified into three classes: non-LOH, hemizygous LOH (hemi-LOH), or homozygous LOH (homo-LOH) mutants. The results were processed by ABI PRISM® GeneMapper™ software v3.5 (PE Applied Biosystems) according to the manufacturer's guidelines. For the additional determination of the extent of LOH at the *TK* locus of TK6 cells, 10 microsatellite regions (D17S799, THRA, D17S1299, D17S855, D17S588, D17S807, D17S789, D17S785, D17S802, and D17S784) were analyzed on chromosome 17 by means of multiple PCR reactions and ABI PRISM® 3100-Avant™ Genetic Analyzer, and GeneMapper™ software v3.5.

2.6. Statistics

Three independent experiments were conducted in each assay. Statistical analyses were done on the combined data of the experiments. Levene's test for equality of variances was used for all the responses and, consequently, a parametric or a non-parametric test was chosen. The analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was used for the analysis of data from the *TK* gene-mutation assay and the cytotoxicity test. The results of the micronucleus test and the Comet assay were analyzed with the non-parametric Kruskall–Wallis test. *P*-values ≤ 0.05 were considered statistically significant. The data were analyzed with the PASW statistics version 18.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Cytotoxicity and genotoxicity of MX

The TK6 cells were treated with various concentrations of MX for 4 h. The results of cytotoxicity and genotoxicity assays are presented in Fig. 1. Two parameters were used to assess the cytotoxicity of MX: relative survival (RS) and relative suspension growth (RSG). RS is the plating efficiency immediately after exposure, whereas RSG is the relative cell growth during the three days following treatment. For the *TK* gene-mutation assay, the highest concentration of the test chemical should cause 10–20% RS or RSG [20]. Over the concentration range used, MX evoked a concentration-dependent reduction in cell viability, permitting 9.5% RS and 14.9% RSG at the maximum concentration of 62.5 μM.

The Comet assay was conducted to assess the induction of DNA damage in TK6 cells. The test concentrations used covered the range from maximum acceptable toxicity to little or no toxicity in the assay [21]. A concentration-related positive trend was observed for the DNA-damaging effect of MX in TK6 cells, but the effect was not statistically significant (Kruskall–Wallis test).

Genotoxic effects of MX were also evaluated with the micronucleus test and the *TK* gene-mutation assay. Exposure to MX increased the number of micronuclei (MN) and *TK* mutants in TK6 cells in a concentration-related fashion (Fig. 1). The induction of

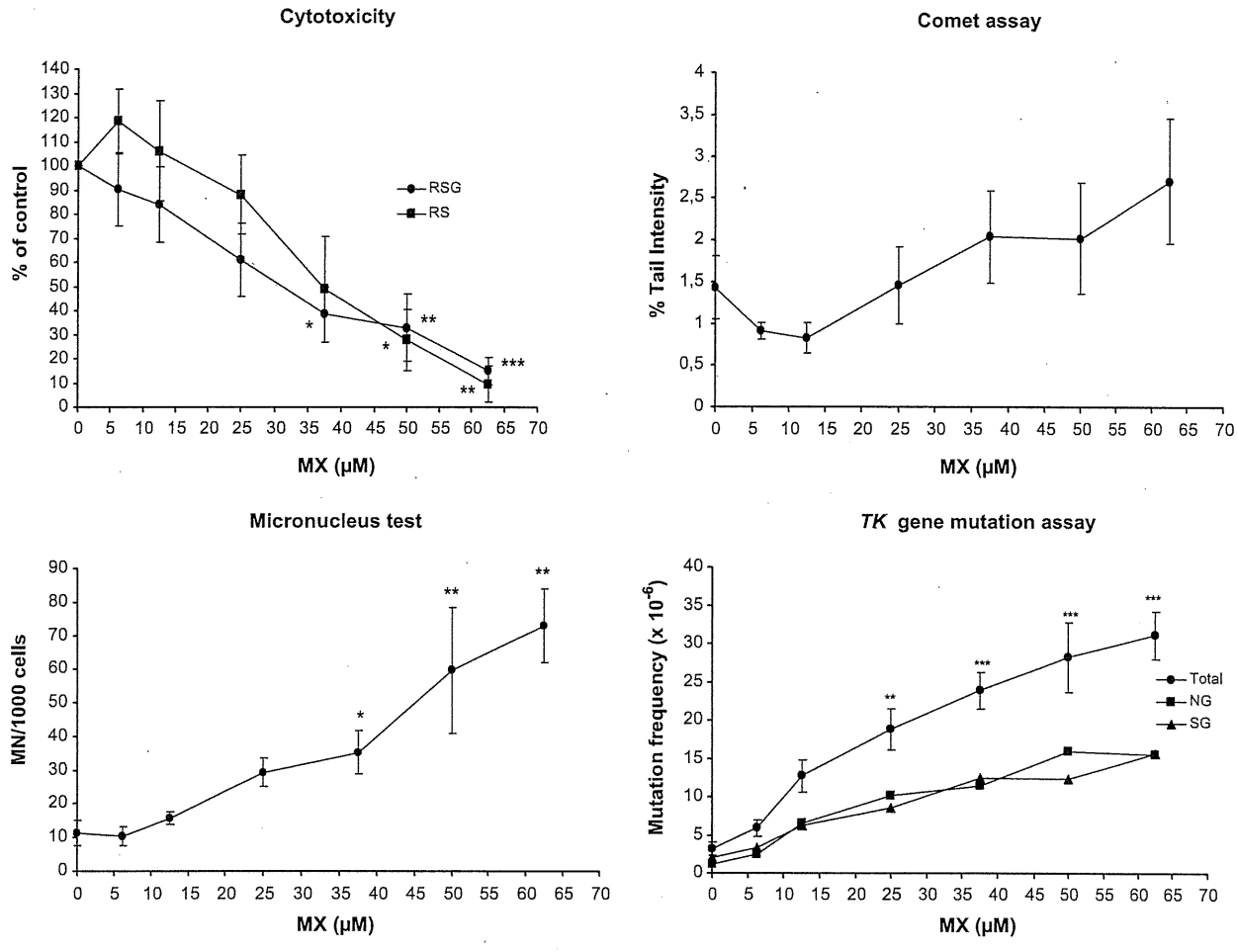

Fig. 1. Cytotoxic (relative survival, RS; relative suspension growth, RSG) and genotoxic responses (Comet assay, micronucleus test, and *TK* gene-mutation assay) in TK6 cells exposed to MX for 4 h (three experiments, mean values ± SEM). Asterisks indicate statistically significant differences with the negative control $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ (Kruskall–Wallis test for Comet assay and micronucleus test, and ANOVA followed by Dunnett's *post hoc* test for *TK* gene-mutation assay and cytotoxicity test).

MN and *TK* mutants was statistically significant from 37.5 μM and 25 μM, respectively. Even concentrations that were not strongly cytotoxic induced both MN and *TK* mutants. At the highest concentration (62.5 μM), the potencies of MX to induce MN and *TK* mutations were 6.5 and 9.7 times the control level, respectively.

Two distinct phenotypic classes of *TK* mutants were generated in the assay. Normally growing (NG) mutants proliferate with a growth rate similar to that of the wild-type cells (doubling time, 13–17 h), and slowly growing (SG) mutants grow with doubling time of >21 h. NG mutants are generally the result of small intragenic mutations (point mutations, small deletions and insertions), while SG mutants result from gross structural changes involving a putative growth-regulating gene near the *TK* gene [17]. The NG and SG mutants induced by MX were almost equal in number (Fig. 1 and Table 1).

Methyl methanesulfonate (MMS) was used as a positive control chemical in the assays (30 μg/ml for the Comet assay and 5 μg/ml for the micronucleus test and the *TK* gene-mutation assay). MMS produced a positive response in each assay: Comet assay; % tail intensity: 42.2; micronucleus test: 74.7 MN/1000 cells; *TK* mutation frequency ($TK^{-/-}$ mutants per 10^6 viable cells): 47.9

The assays were also performed in the presence of a rat-liver exogenous metabolic activation system (S9 mix). Fig. 2 shows the data obtained from one independent experiment. In the presence of S9 mix no increases in the induction of DNA damage, frequency of MN, or *TK* mutants were detected at any MX concentration tested. S9 mix enhanced the activity of cyclophosphamide (5 μg/ml), the positive control chemical: cytotoxicity: 22.3% RS and 21.6% RSG; micronucleus test: 85 MN/1000 cells; *TK* mutation frequency: 28×10^{-6}.

3.2. Molecular analyses of TK mutants

The *TK* mutants were independently isolated from the cells treated with 37.5 μM MX for 4 h. The *TK* mutation frequency for

Table 1
LOH analysis of normally growing (NG) and slowly growing (SG) *TK* mutants induced by MX.

	Number of *TK* mutants	Non-LOH	Hemi-LOH	Homo-LOH
Spontaneous[a]				
NG mutants	19	14 (74%)	3 (16%)	2 (11%)
SG mutants	37	0 (0%)	9 (24%)	28 (76%)
Total	56	14 (25%)	12 (21%)	30 (54%)
37.5 μM MX				
NG mutants	70	68 (97%)	2 (3%)	0 (0%)
SG mutants	60	11 (18%)	40 (67%)	9 (15%)
Total	130	79 (61%)	42 (32%)	9 (7%)

[a] Data from Zhan et al. [22].

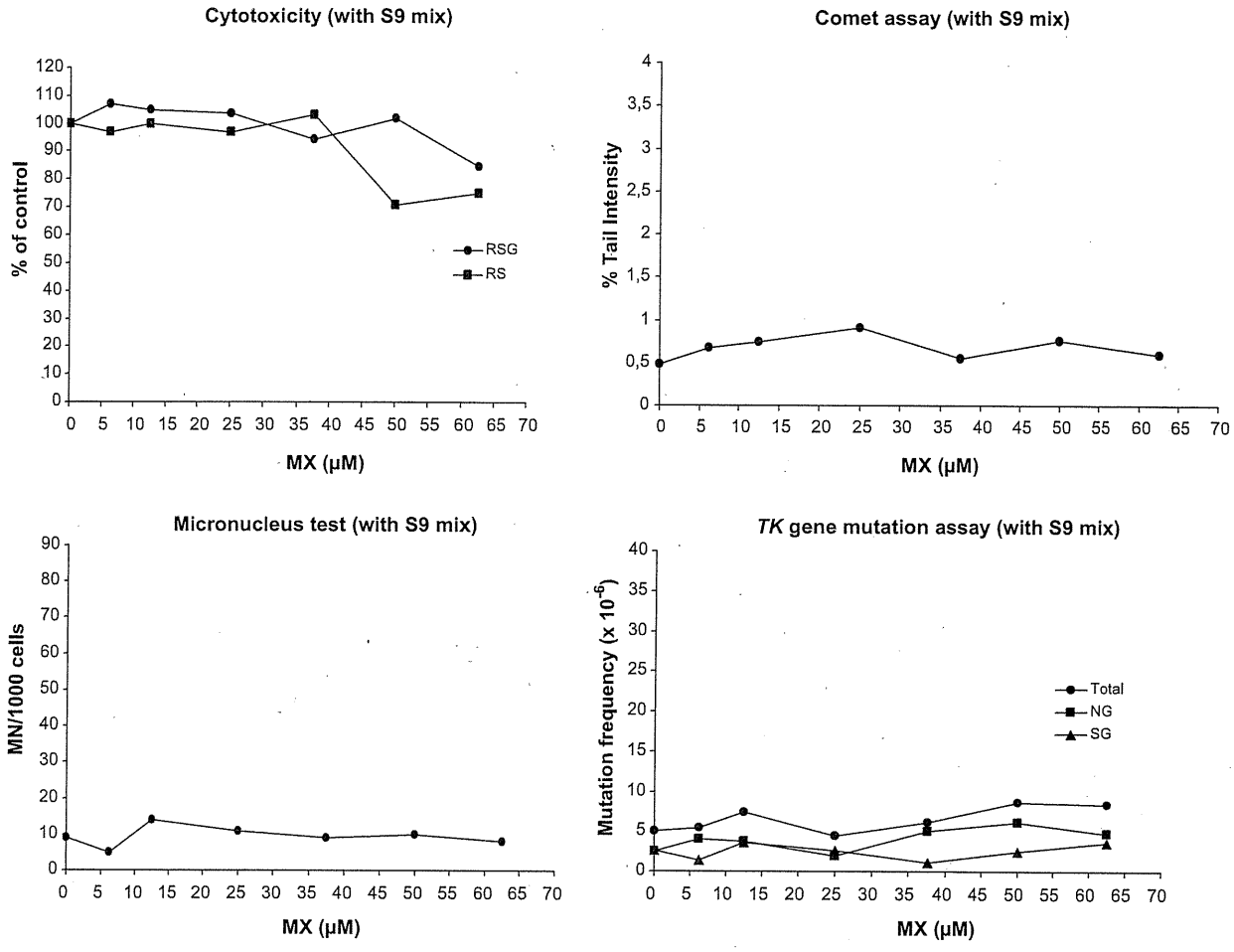

Fig. 2. Cytotoxic (relative survival, RS; relative suspension growth, RSG) and genotoxic responses (Comet assay, micronucleus test, and *TK* gene-mutation assay) in TK6 cells exposed to MX for 4 h with metabolic activation (S9 mix) (one experiment).

37.5 μM MX was about 5-fold higher than the concurrent control. The cytotoxic responses were 82% RS and 66.8% RSG. A total of 130 MX-induced *TK* mutants (70 NG and 60 SG mutants) were compared to those of spontaneously occurring *TK* mutants described previously [22] (Table 1). The numbers of NG and SG mutants analyzed depended on the ratio of NG and SG mutants in the assay. In this independent experiment, the proportion of SG mutants among the total number of *TK* mutants was lower compared with the combined data of the three experiments (Table 2). PCR-based loss of heterozygosity (LOH) analysis of genomic DNA from *TK* mutants was used to classify the mutants into non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, non-LOH results from a small intragenic mutation (e.g., point mutation) in the *TK* gene, hemi-LOH from a chromosomal deletion (the functional *TK* allele is lost), and homo-LOH from inter-allelic homologous recombination (the functional *TK* allele is replaced by a non-functional *TK* allele) [17]. Most (61%) of the MX-induced *TK* mutants (NG and SG mutants) were of the non-LOH type (Table 1), indicating that MX predominantly induced point or other small intragenic mutations in TK6 cells. Three percent of NG and 82% of SG mutants resulted from LOH. Of those, all the NG and most of the SG mutants were hemi-LOH. Thus, deletion was a major event in the induction of LOH mutations in TK6 cells by MX. This is in contrast to spontaneous *TK* mutants, which were mainly homo-LOH (Table 1, reported previously [22]).

For a better illustration of the extent of LOH responsible for deletion and recombination at 10 microsatellite loci of chromosome 17, hemi-LOH and homo-LOH mutants are shown as bars in Fig. 3. Molecular analyses of MX-induced LOH mutants showed an extensive loss of functional *TK* sequences. In all of the MX-induced LOH events, the deleted or exchanged chromosome segment extended to the telomere.

Table 2
Total mutation frequencies (MF) and the proportion of slowly growing (SG) *TK* mutants induced by MX in the *TK* gene-mutation assay.

MX treatment (μM)	Mutational response	
	MF[a]	% SG
Combined data of the three experiments		
0	3.2	65.6
6.25	5.9	57.6
12.5	12.7	49.6
25	18.8	45.7
37.5	23.9	51.9
50	28.2	43.6
62.5	31.1	50.2
An independent experiment for the molecular analysis of *TK* mutants		
0	6.9	46.4
37.5	31.6	39.0

[a] $TK^{-/-}$ mutants per 10^6 viable cells.

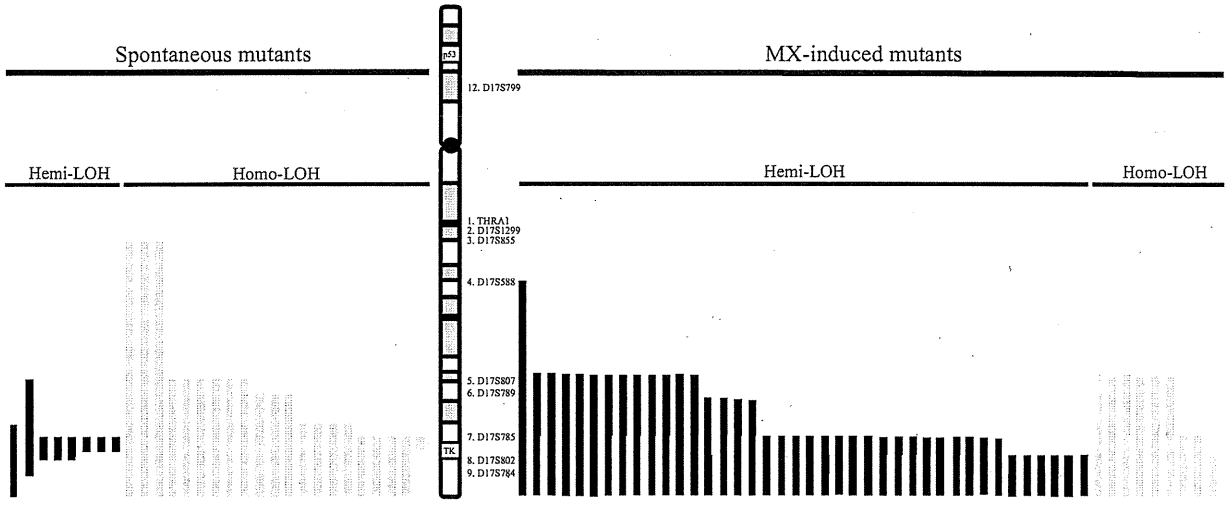

Fig. 3. The extent of loss of heterozygosity (LOH) in spontaneous and MX-induced LOH mutants from TK6 cells. Ten microsatellite loci of chromosome 17 that are heterozygous in TK6 cells were examined. Black and grey bars represent hemi-LOH and homo-LOH, respectively. The length of the bars indicates the extent of LOH. Twenty-nine spontaneous mutants (10 NG and 19 SG mutants) and 49 MX-induced mutants (1 NG and 48 mutants) were analyzed. The data on spontaneous mutants were taken from the previous paper [17].

4. Discussion

MX, a carcinogen in rats, exhibits both tumour-initiating and -promoting properties *in vitro* and *in vivo* suggesting that this compound may be a complete carcinogen. A large number of *in vitro* studies on the genotoxicity of MX indicate a clearly positive response for MX in various bacterial strains and mammalian cell lines [for a review, see 6]. However, the mechanisms of this genotoxicity are still obscure. In the present study, the genotoxicity of MX and its mechanisms were investigated in the human lymphoblastoid TK6 cells through analysis of three endpoints: DNA damage (alkaline Comet assay), clastogenesis (micronucleus test), and gene mutation (thymidine kinase (*TK*) assay). One of the goals of the current study was to gain insight into the mechanism behind MX tumorigenicity and obtain further information for assessment of MX's role in cancer risk.

The *TK* gene-mutation assay has an advantage over the bacterial and mammalian *hprt* and *Hprt* gene-mutation assays, because it can detect not only point mutations, but also small- and large-scale chromosomal changes, recombination and aneuploidy, which can lead to loss of the functional allele (loss of heterozygosity, LOH). This in turn may result in the inactivation of tumour-suppressor genes. Thus, LOH is considered to be an important event in carcinogenesis [16,17,23].

The present results reveal that MX induced *TK* mutants in TK6 cells in a concentration-dependent manner. The effect was statistically significant at the concentrations of 25–62.5 μM (~5.4–13.6 μg/ml). Our results agree with the previous study of Woodruff et al. [24], which indicated that MX was mutagenic at the *TK* locus in three human B-lymphoblastoid cell lines (MCL-5, AHH-1 $TK^{+/-}$, and h1A1v2 cells) at the same concentration range (3–10 μg/ml). However, the ratio of normally (NG) and slowly growing (SG) colonies and the distribution of mutant types (non-LOH, hemi-LOH, homo-LOH) in that study are not known.

Our findings are also in line with earlier reports that demonstrated the induction of mutations by MX in non-human mammalian cells. It has been shown that MX induces mutations at the hypoxanthine phosphoribosyl transferase (*Hprt*) and Na/K ATPase gene loci in Chinese hamster ovary (CHO) cells [10,25,26], at the *Hprt* gene locus in Chinese hamster V79 [27] and rat liver epithelial WB-F344 cells [3], and at the *Tk* gene locus in L5178Y mouse lymphoma cells [28]. However, the ratio of NG (large-colony) and SG (small-colony) mutants was not similar between TK6 cells and mouse lymphoma cells. The majority of the MX-induced *Tk* mutants in mouse lymphoma cells were small-colony mutants (60–90%, depending on the MX concentration) [28] while our findings with TK6 cells revealed that frequencies of NG and SG mutants were similar. In the study of Harrington-Brock et al. [28], molecular analysis of the *Tk* mutants was not performed, but MX also induced a significant number of chromatid breaks and rearrangements in mouse lymphoma cells and the authors stated that the production of small-colony *Tk* mutants – which imply large genetic alterations such as deletions, rearrangements, and recombinations – was in accordance with the positive clastogenic response. In fact, regardless of the type of mutagen, molecular analysis of *Tk* mutants has demonstrated that most of the large-colony mutants in the mouse lymphoma assay (MLA) also result from large chromosomal alterations. In the MLA, the large-colony/small-colony ratio has been used for evaluating the clastogenicity rather than for evaluating the potential to induce point mutations [29]. In contrast to the *in vitro* gene-mutation studies, no induction of mutations by MX in transgenic *gpt* delta mice was detected [5].

In this study, the distribution of mutant types (non-LOH, hemi-LOH, homo-LOH) of MX was not similar to that observed in spontaneous mutants (Table 1), indicating that the increase in mutation frequency by MX is not associated with a global enhancement of the genomic instability. Instead, the LOH pattern of MX resembled that induced by ethyl methanesulfonate (EMS) [17,30]. The present data show that the predominant mutation type in TK6 cells induced by MX was non-LOH (point mutations and small deletions and insertions), but also a considerable proportion of *TK* mutants exhibited LOH events (large deletions and recombinations) indicating MX's clastogenic capacity. A higher proportion of deletions (32% *vs* 21%) were observed in the *TK* mutants induced by MX compared with the spontaneous *TK* mutants. It has been demonstrated that EMS also induces mainly non-LOH mutations (65.2%). The percentages of hemi-LOH and homo-LOH were found to be 31.6% and 3.3%, respectively [30]. Thus, MX and EMS produce similar mutation spectra of *TK* mutants. EMS is an alkylating agent reacting readily with DNA producing alkylated nucleotides. *In vitro*, EMS induces predominantly GC to AT base substitutions [31] while MX induces mainly GC → TA transversions [7–10], but to a lesser

extent also GC→AT transitions [7–9]. In contrast to MX and EMS, the majority of spontaneously arising *TK* mutants were of the LOH type and of those, the predominant mutation type was homo-LOH (Table 1, reported previously [22]).

LOH is the result of inaccurate repair of DNA double-strand breaks (DSBs). There are two major mechanisms for generating LOH. DSBs can be repaired in a non-conservative manner through non-homologous end-joining, resulting in hemi-LOH mutation (deletion). Homo-LOH mutation, on the other hand, is considered to be the result of mitotic recombination between homologous alleles [32]. Molecular analysis of LOH mutants reveals the mechanisms leading to LOH. In the present study, the LOH-mapping analysis showed that the LOH events (deleted or substituted portions of the chromosome) extended to the telomere of chromosome 17q and encompassed exons 4 and 7 of the *TK* locus (Fig. 3). Thus, all the homo-LOH and hemi-LOH mutations induced by MX were due to homologous recombination with crossing-over and large terminal deletions, respectively. Since TK6 cells have high levels of telomerase activity, DSBs can be repaired by the addition of new telomere to the broken chromosome end [17]. In contrast to MX, the spontaneously arising hemi-LOH mutations are generally interstitial deletions, that is, the altered chromosome segments do not extend to the telomere (Fig. 3).

MX has been shown to cause DNA and chromosomal damage in a number of human and mammalian cell lines *in vitro* and positive results have also been observed in some *in vivo* studies [for a review, see 6]. In the current study, MX induced a statistically significant concentration-related increase in the frequency of micronuclei (MN), whereas in the DNA damage study by means of the Comet assay only a slight, statistically insignificant increase of DNA damage was found. In this study, the DNA strand breaks (leading to hemi-LOH and homo-LOH) may not be caused by DNA damage directly. Instead, MX's clastogenic activity (MN formation) may involve DSBs supporting a clastogenic mechanism of MX-induced LOH mutation formation. There is, however, some discrepancy between the results of the Comet assay and the gene-mutation assay. The alkaline (pH > 13) version of the Comet assay, which was used in the present study, is capable of detecting diverse types of genetic damage such as DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA–DNA/DNA–protein cross-links, and SSB associated with incomplete excision-repair sites [21]. The assay, on the other hand, is not a mutagenicity test, and the DNA alterations detected with it are not necessarily related to mutagenesis [33]. But, it is not clear why the DNA lesions leading to mutations (e.g., a high number of small intragenic mutations) observed in the gene-mutation assay in this study did not produce a positive response in the Comet assay. In the MLA, 3 μg/ml (0.01 mM) cyclophosphamide (CPA) is used as a positive control [29]. Some studies have shown that concentrations of CPA higher than 3 μg/ml will be needed to induce a positive response in the Comet assay [34,35]. Potent cytotoxicity was found at the two highest MX concentrations (Fig. 1). Thus, the genetic damage induced at these concentrations may be a consequence of cytotoxicity-related mechanisms. One possible explanation for the contrasting findings between the micronucleus test and the Comet assay may indeed be the influence of cytotoxicity, because it can induce elevated MN frequencies but may not lead to positive effects in the Comet assay [36].

The assays used in this study were also performed in the presence of a rat-liver exogenous metabolic activation system (S9 mix). The finding that S9 mix did not enhance the activity of MX in TK6 cells was not unexpected. In fact, the addition of S9 has been found to decrease the bacterial mutagenicity of MX [37–41]. MX is a direct-acting mutagen inducing point mutations in *S. typhimurium* strains [7–9] and also at the *Hprt* locus in CHO cells [10]. Thus, our observation that most of the MX-induced *TK* mutants were the non-LOH type is supported by the positive results in bacterial and mammalian gene-mutation assays. However, the molecular mechanisms by which MX induces point mutations are unknown. There is as yet no evidence that DNA adducts induced by MX are formed in bacteria or mammalian cells in culture. Thus, it is possible that stable DNA adducts do not play a role in the mutagenic activity of MX. Instead, King et al. [42] have found some evidence for the ability of MX to induce a-basic sites in DNA from MX-treated *Salmonella* TA100 cells.

One common mechanism for chemicals to induce DNA damage is stimulation of the production of reactive oxygen species (ROS) [43]. The report of Díaz-Llera et al. [44] indicated that ROS induced small-scale DNA damages, such as point mutations, small insertions and deletions at the *HPRT* locus in primary human T-lymphocytes. MX has been shown to induce ROS production in murine L929 fibrosarcoma cells, but only at high concentrations (100–1000 μM) [45,46]. The ROS production was slightly stimulated also in murine NIH 3T3 fibroblasts [45]. It is thus possible that ROS production is one mechanism involved in the induction of gene mutations by MX in TK6 cells, but it is not known whether MX can induce oxidative stress in these cells. On the other hand, the results obtained in the current study with the Comet assay, which has been used to measure oxidative DNA damage [43], do not support the role of ROS production in the induction of mutations by MX in TK6 cells.

The tumour promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and microcystin-LR (MCLR), which is also a compound with tumour-promoting activity, preferably induce homo-LOH through homologous recombination [22,30]. Furthermore, the tumour promoter okadaic acid induces minisatellite mutation in NIH3T3 cells, probably through recombinational events [47]. It has been demonstrated that MX also has tumour-promoting activity both *in vitro* and *in vivo* [for a review, see 6]. However, the findings of this study are not comparable with other studies showing that homo-LOH is the predominant mutation type induced by tumour promoters. According to the results of the present study, recombinational events have only a minor contribution among the MX-induced *TK* mutants.

In conclusion, we have shown that MX induces primarily small mutations (e.g., point mutations), but it has also clastogenic properties causing LOH in human cells. Point mutations were generally not found at two key genes (*ras*, *p53*) in MX-induced liver tumours in rats [11]. Thus, other specific genes involved in tumorigenesis may be affected by treatment with MX. Indeed, the results of this study give some indication that MX is capable of inactivating tumour-suppressor genes in human cells as a result of LOH which may contribute to cancer development.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Genotoxicity of Colloidal Fullerene C_{60}

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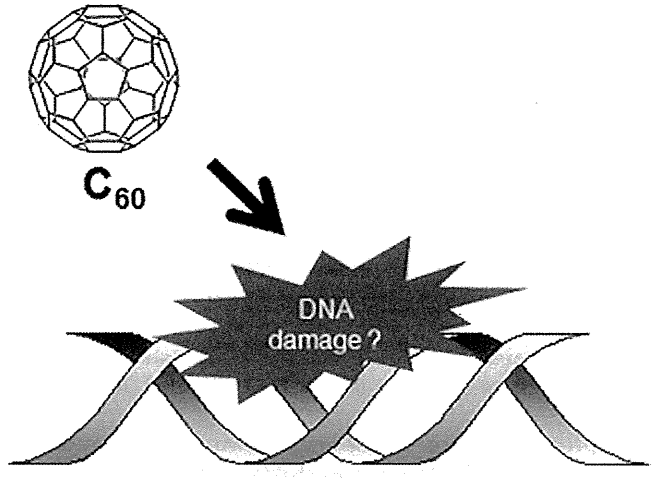
Supporting Information

ABSTRACT: Previous genotoxicity tests of aqueous fullerene C_{60} suspension (aqu-C_{60}) yielded both positive and negative results. In the present study, aqu-C_{60} elicited positive responses in two bacterial genotoxicity tests, the *Bacillus subtilis* Rec-assay and the *umu* test at concentrations as low as 0.048 mg/L and 0.43 mg/L, respectively. In mammalian cell experiments, aqu-C_{60} showed a significant growth inhibitory effect on human hepatocarcinoma HepG2 cells at 0.46 mg/L. The level of the oxidative DNA lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine, measured by liquid chromatography tandem mass spectrometry, was slightly but not significantly increased in HepG2 cells treated with 0.46 mg/L for 24 h, whereas the level of the lipid peroxidation-related DNA lesion α-methyl-γ-hydroxy-1, N^2-propano-2′-deoxyguanosine was not changed. Under the same conditions, we did not detect any bulky DNA adducts, as measured by ^{32}P-postlabeling/polyacrylamide gel electrophoresis analysis. Our data suggest that aqu-C_{60} has DNA-damaging potential and that the DNA damage is not due to covalent DNA adduct formation by C_{60} itself.



1. INTRODUCTION

Fullerene C_{60} (C_{60}) is one of the most attractive nanoparticles because of its unique physical and chemical properties and its burgeoning application to electronics, cosmetics, medicine, and so on. Greater use of C_{60} will lead to its increased emission into the environment and greater exposure opportunity for living organisms. Therefore, many researchers are concerned not only with the convenience of C_{60} but also its potential hazardous effects. Although C_{60} is insoluble in water, it can be stably dispersed in aqueous solution by simply stirring in water for a long time.[1−3] This raises the possibility that C_{60} could remain a stable aquatic pollutant, prompting toxicological evaluation of aqueous C_{60} suspension (aqu-C_{60}).

Althougth there are several studies of the genotoxicity of aqu-C_{60}, some test systems showed positive results[1,4,5] but others negative.[4,6,7] Therefore, further studies on the genotoxic potential of aqu-C_{60} are required.

In the present study, we carried out an array of genotoxicity tests: 1) we used the *Bacillus subtilis* Rec-assay and the *umu* test to model induction of a DNA repair response caused by aqu-C_{60}; and 2) we exposed mammalian cells to aqu-C_{60} to measure the levels of bulky DNA adducts by using ^{32}P-postlabeling/polyacrylamide gel electrophoresis analysis as well as the lipid peroxidation (LPO)-related lesion α-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (CdG) and the oxidative lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) by using liquid chromatography tandem mass spectrometry (LC/MS/MS).

2. MATERIALS AND METHODS

Materials. C_{60} was a kind gift from Dr. Hirohito Tsue (Kyoto University, Kyoto, Japan). Yeast extract and Bacto tryptone were purchased from BD Bioscience (Franklin Lakes, USA). S-9 mix was purchased from Wako. Alkaline phosphatase was purchased from SIGMA (St. Louis, USA). Micrococcal nuclease and spleen phosphodiesterase were purchased from Worthington (Lakewood, USA).

Bacterial Strains. *Salmonella typhimurium* TA1535/pSK1002 strain for the *umu* test was a kind gift from Dr. Yoshimithu Oda (Osaka Prefectural Institute of Public Health, Osaka, Japan). For the *Bacillus subtilis* Rec-assay, the *Bacillus subtilis* H17 (Rec+) and M45 (Rec-) strains were used.

Preparation of Aqueous C_{60} Suspension and Characterization. Before experiments, C_{60} was purified with HPLC. C_{60} was dissolved in toluene, and the solution was injected onto the Shimpack FC-ODS column (150 mm × 4.6 mm) (Shimadzu, Kyoto, Japan) and subsequently eluted in an isocratic mode with 60% toluene in acetonitrile at flow rate of 1.0 mL/min with monitoring the absorbance at 333 nm. The peak showing UV spectrum at 333 nm of C_{60} was collected and evaporated to dryness.

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Preparation of aqu-C_{60} followed Deguchi et al.[8] with slight modification. Ten mg of C_{60} was dispersed in 100 mL of THF, degassed with a nitrogen purge for five hours, and stirred overnight in the dark. After filtration (pore size: 0.45 μm), an equal amount of water was added to the solution. The solution was evaporated to 90 mL by using a rotary evaporator, following which 20 mL of water was added. The evaporation and water-addition steps were repeated twice. Finally the solution was evaporated to 100 mL and insoluble C_{60} in the solution was removed by filtration (pore size: 0.45 μm). UV−vis spectra of the aqu-C60 were scanned within the wavelength of 220−600 nm using Gene Spec V (Hitachi, Tokyo, Japan). Size distribution was determined by SALD-2100 laser diffraction particle size analyzer (Shimadzu, Kyoto, Japan). Concentration of the prepared aqu-C_{60} was determined by absorbance at 263 nm with molar absorbance coefficient reported by Mchedlov-Petrossyan et al. and Deguchi et al.[8,9] (ε, 1.1 × 10^5 M^{-1} cm^{-1}). Final concentration in toxicity tests was calculated from dilution magnification of the prepared aqu-C_{60}.

***Bacillus subtilis* Rec-Assay.** *Bacillus subtilis* M45 (Rec-) strain is a *recA* gene deficient strain lacking DNA recombination repair system and SOS response induction. Consequently, Rec- strain is much more sensitive than *Bacillus subtilis* H17 (Rec+) strain to wide spectra of DNA damage such as DNA strand breaks, pyrimidine dimers, alkylations, cross-links, and bulky DNA adducts.[10] Therefore, genotoxicity of the interested chemical can be judged to compare the survival curve of Rec- with that of Rec+.

Both Rec+ and Rec- strains were grown in Luria−Bertani (LB) broth at 37 °C with vigorous shaking until the turbidity of the cultures at 595 nm reached from 0.1 to 1.0 using a microplate reader. The cultures were then diluted with LB broth until the turbidity at 595 nm ≈ 0.02. Four microliters of water (negative control), the aqu-C_{60} sample were pipetted into the wells of a 96-well microplate. Fifty microliters of the diluted culture and 46 μL of LB broth were added to each well, and then the turbidity of the mixtures at 595 nm (A_{595} (before)) was measured. The microplate was sealed and incubated at 37 °C for 5 h with vigorous shaking. After incubation, the turbidity of the mixtures at 595 nm (A_{595} (after)) was measured. Survival of Rec+ and Rec- strains was calculated using the following equation: Survival (%) = $\{A_{595}\text{ (after)} - A_{595}\text{ (before)}\}_{sample}/\{A_{595}\text{ (after)} - A_{595}\text{ (before)}\}_{control}$.

***Umu* Test.** *Salmonella typhimurium* TA1535/pSK1002 strain used for *umu* test is introduced a plasmid pSK1002 carrying a fused gene *umuC-lacZ* and the expression of *umuC* is inducible by these DNA-damaging agents. The strain enables us to judge genotoxicity of the interested chemical by measuring the β-galactosidase activity in the cells produced by the fusion gene.

The detailed protocol of the *umu* test was described elsewhere.[11] Four microliters of water (negative control) or the aqu-C_{60} sample and 96 μL of an exponentially growing culture of TA1535/pSK1002 for the S9-absent experiment or the mixture of the bacterial culture and the S9 mix at a ratio of 1.7:0.3 for the S9-present experiment were added to the wells of a 96-well microplate. 4-Nitroquinoline 1-oxide (4-NQO) and 2-aminoanthracene (2-AA) as positive controls for the S9-absent and the S9-present experiment, respectively, and dimethylsulfoxide (DMSO) as their negative control were used. After incubation, bacterial growth was measured as turbidity at 595 nm with a microplate reader. For chlorophenol red-β-D-galactopyranoside (CPRG), the absorbance at 540 nm was measured. The relative β-galactosidase activity (RGA) was calculated using the following equation: RGA (units) = A_{540} (CPRG)/A_{595} (growth turbidity). The values in this equation were corrected by subtracting the value of the absorbance blank. The experiment was performed independently three times.

Cell Culture. Human hepatocarcinoma cell line HepG2 was obtained through the courtesy of the Cell Resource Center for Biomedical Research of Tohoku University, Sendai, Japan and was maintained in Dulbecco's modified Eagle's medium (DMEM) (IWAKI, Funabashi, Japan) or phenol red-free DMEM (for MTS assay) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

MTS Assay. HepG2 cells seeded in a 24-well plate were treated with 450 μL of culture medium plus 50 μL of aqu-C_{60} (final concentration, 0.46 mg/L) for 24 and 72 h. Cell viability after treatment was assessed by using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay according to the manufacturer's instructions (Promega, WI, USA).

Cell Treatment with aqu-C_{60} and DNA Extraction. HepG2 cells grown to 70% confluence in 100-mm dishes were incubated for 24 h with either 1 mL of water (negative control) or aqu-C_{60} (the final concentration, 0.46 mg/L) plus 9 mL of the culture medium. After the cells were harvested, DNA was extracted from the cellular pellet according to Ravanat et al.[12] using NaI for DNA precipitation. The DNA pellet was dissolved in 200 μL of 0.1 mM desferrioxamine. The DNA concentration of the samples was calculated by measuring the absorbance at 260 nm.

Enzymatic DNA Digestion. For LC/MS/MS experiments, each DNA sample (25 μg) was mixed with 15 μL of digestion buffer (17 mM sodium succinate, 8 mM $CaCl_2$, pH 6.0) containing 22.5 units of micrococcal nuclease and 0.075 units of spleen phosphodiesterase. Together, three stable isotope-labeled DNA adduct internal standards, [$^{15}N_5$]-8-oxodG, and [$^{15}N_5$]-α-S-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (CdG_1), and [$^{15}N_5$]-α-R-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (CdG_2), were also added to the solution. After incubation at 37 °C for 3 h, 1.5 units of alkaline phosphatase (SIGMA, St. Louis, USA), 5 μL of 20 mM $ZnSO_4$, 10 μL of 0.5 M Tris-HCl, pH 8.5, and 67 μL of water were added. The mixture was then incubated for another 3 h at 37 °C. The digested DNA was concentrated to approximately 20 μL by speed-vac concentrator, and 100 μL of methanol was added to precipitate enzymes and excess salt. The supernatant was recovered, and the precipitate was washed by 100 μL of methanol. The supernatant and the methanol fraction were combined and evaporated to dryness.

DNA Adducts Quantification. LC/MS/MS experiments were performed on a Shimadzu LC system (Shimadzu, Kyoto, Japan) and a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters-Micromass, Milford, MA). The digested DNA samples were resuspended in 50 μL of 30% dimethyl sulfoxide (DMSO). 50 μL aliquots of sample were injected onto a Shim-pack FC-ODS column (150 mm × 4.6 mm) (Shimadzu, Kyoto, Japan) and subsequently eluted in an isocratic mode with 2% methanol in water initially for 0.1 min, a linear gradient of 2% to 40% methanol in water from 2 to 40 min, and a subsequent linear gradient of 40% to 80% methanol in water from 40 to 45 min at flow rate of 0.4 mL/min. The mass spectrometric conditions were performed with the following parameters: ion mode, positive; capillary voltage, 3.5 kV; ion source temperature, 130 °C; desolvation gas flow rate, 700 L/h; cone gas flow rate, 35 L/h. The characteristic parameters for each DNA adduct

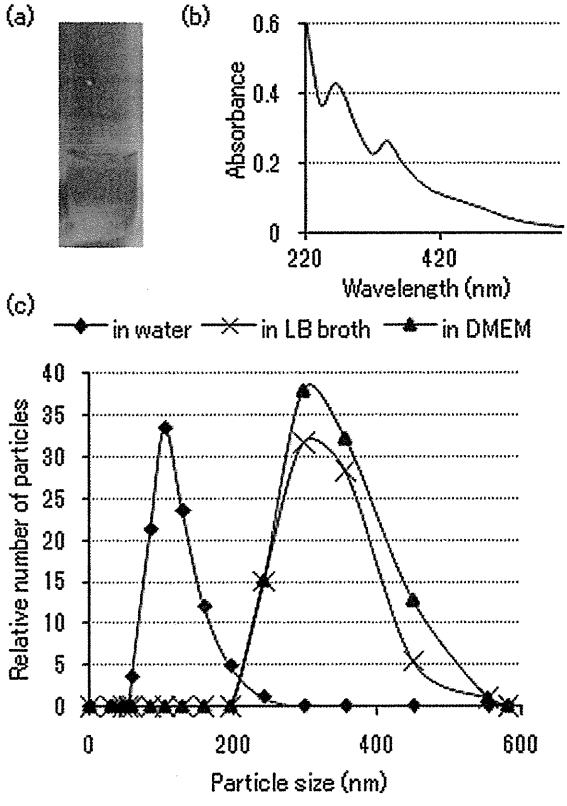

Figure 1. Characterization of aqu-C60. (a) The color images of aqu-C60. (b) UV–vis absorbance spectra of aqu-C60. (c) Particle size distribution of 2.3 mg/L aqu-C60, aqu-C60 in LB broth (2.3 mg/L) and aqu-C60 in DMEM (2.3 mg/L). The average particle size of C60 was determined to be 122 nm in water, 320 nm in LB broth, and 330 nm in DMEM.

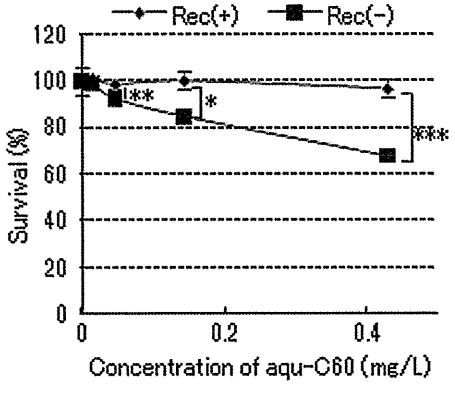

Figure 2. Survival curves for Rec+ and Rec- cells treated with aqu-C60 in the *Bacillus subtilis* Recassay. The values represent the mean of three independent experiments ± SD. Asterisks (*, **, and ***) denote $p <$ 0.05, 0.01, and 0.001 calculated using Student's t test between Rec+ and Rec-.

measurement were as follows (cone voltage (V), collision energy (eV), base ion → product ion): [$^{15}N_5$]-8-oxodG (40, 12, 288.8 → 172.8), [$^{15}N_5$]-CdG$_1$ and CdG$_2$ (35, 10, 343.0 → 227.0), 8-oxo-dG (40, 12, 283.8 → 167.8), and CdG$_1$ and CdG$_2$ (35, 10, 338.0 → 222.0). The amount of each DNA adduct was quantified by calculating the peak area ratio of the target DNA adduct and its specific internal standard. Calibration curves were obtained by

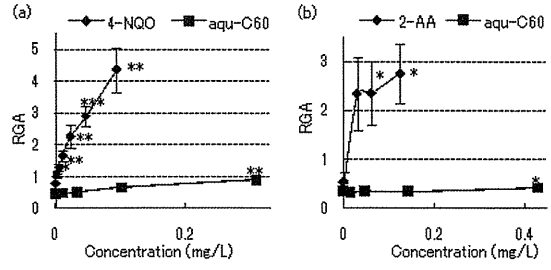

Figure 3. Aqu-C60 showed a positive genotoxic response in the umu test with S9- (a) or S9+ (b). 4- NQO and 2-AA were used as positive controls in the S9- and S9+ experiments, respectively. The values represent the mean of three independent experiments ± SD. Asterisks (*, **, and ***) denote $p <$ 0.05, 0.01, and 0.001 calculated using Student's t test versus the control.

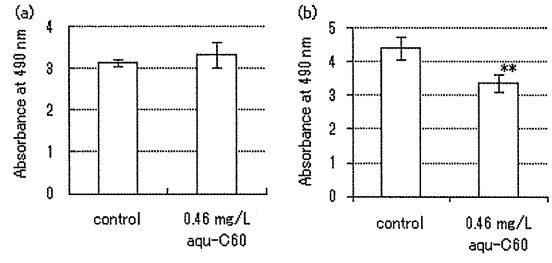

Figure 4. Cytotoxicity of aqu-C60 against HepG2 cells. After cells were treated with 0.46 mg/L aqu-C60 or water (control) for 24 h (a) and 72 h (b), MTS assay was performed. The values represent the mean of three independent experiments ± SD. Asterisks (**) denote $p <$ 0.01 calculated using Student's t test versus the control.

authentic standards applied with isotope internal standards. DNA adduct levels in each sample were calculated as described in a previous report.[13]

3. RESULTS

Aqu-C_{60} Characterization. The aqu-C_{60} exhibited yellow color (Figure 1(a)). The UV–visible absorption spectra of the aqu-C_{60} shown in Figure 1(b) were consistent with the spectra shown in a previous report.[8] Size distribution of the aqu-C_{60} ranged from 59 to 241 nm (Figure 1(c)). The average size was determined to be 117 nm. However, the distribution was shifted to larger (241–554 nm) when aqu-C_{60} was dispersed in LB broth or DMEM. The average size was determined to be 320 nm in LB broth and 330 nm in DMEM.

Bacterial Genotoxicity Test. The results of the *Bacillus subtilis* Rec-assay are shown in Figure 2. While aqu-C_{60} did not affect the survival of the Rec+ strain even at 0.43 mg/L, the survival of the Rec- strain decreased in a concentration-dependent manner (Figure 2). The surviving fraction (67.7 ± 1.98%) of the Rec- strain at the highest concentration (0.43 mg/L) was significantly lower than that (96.7 ± 3.67%) of the Rec+ strain ($p <$ 0.001).

The results of the *umu* test are shown in Figure 3. The RGA represents the relative genotoxic strength of the tested chemical. 4-NQO and 2-AA, which are positive controls for the S9-absent and S9-present experiments, respectively, produced dose-dependent increases in RGA. In the S9-absent experiment (Figure 3 (a)), the RGA of aqu-C_{60} was increased dose-dependently

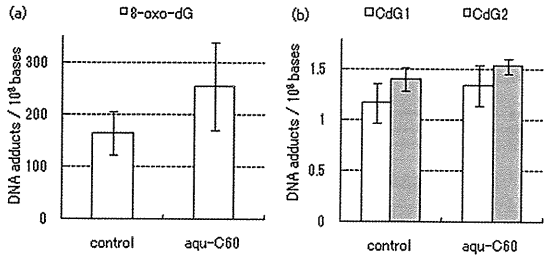

Figure 5. DNA adduct levels of the oxidative lesion 8-oxodG (a) and the LPO-related lesions CdG1 and CdG2 (b). 70% confluent HepG2 cells in 100-mm dishes were incubated for 24 h with either 0.46 mg/L aqu-C60 or water (negative control). The cells were then harvested, and DNA adduct levels were measured. The values represent the mean of three independent experiments ± SD.

and showed a significant increase at the highest concentration tested (0.43 mg/L) ($p < 0.01$). However, the increase in RGA was not evident in the S9-present experiment (Figure 3 (b)). The results of the *Bacillus subtilis* Rec-assay and the *umu* test indicate that aqu-C_{60} elicits a genotoxic response in bacterial cells.

Effect of aqu-C_{60} on Mammalian Cell Proliferation. Because it was reported that C_{60} tends to accumulate in the liver,[14] we used human hepatocarcinoma HepG2 cells for a cell viability assay. The cells were treated with 0.46 mg/L C_{60} for 24 and 72 h, and then the absorbance at 490 nm was measured using MTS assay. While no effect was observed in cell viability after the short time aqu-C_{60} exposure (24 h), aqu-C_{60} produced a significant inhibitory effect on cell proliferation after long time aqu-C_{60} exposure (72 h) (Figure 4).

Table 1. Summary of Aqueous C_{60} Suspension Genotoxicity Tests[a]

experimental system	cell line or tissue	dose	result	ref
bacterial reverse mutation assay	*Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2*uvr*A/pKM101	5 mg/plate	–	7
		1 mg/plate	–	6
	Chromosomal Damage in Mammalian Cells			
comet assay	human lympocytes	0.0022 mg/L	+	1
	FE1-Muta mouse lung epithelial cells	100 mg/L	+	16
	lung of male C57BL/6J mice	0.2 mg/mouse, intratracheal instillation	+	4
micronuclei test	A549 cells	0.02 mg/L	+	4
chromosomal aberration test	Chinese hamster CHI/IU cells	5000 mg/L	–	7
		200 mg/L	–	6
	Transgenic Mutagenesis Systems			
Gpt delta transgenic mouse				
Spi- mutation assay	primary embryo fibroblasts (*in vitro*)	14 μM	+	5
	lung (*in vivo*)	0.2 mg/mouse 4 times, intratracheal instillation	–	4
Gpt mutation assay	lung (*in vivo*)	0.2 mg/mouse, intratracheal instillation	+	4
FE1-MutaTM Mouse				
Cll mutation assay	lung epithelial cells (*in vitro*)	100 mg/L	–	16
8-oxodG level in DNA	liver (Li), lung (Lu), and colon (Co) of female Fisher 344 rats	0.064 (Li), 0.64 (Lu, Co) mg/kg body weight, oral gavage	Li +, Lu +, Co -	27
	Results from This Study			
Bacillus subtilis Rec-assay	*Bacillus subtilis* H17 and M45	0.048 mg/L	+	this study
Umu test	*Salmonella typhimurium* TA1535/pSK1002	0.43 mg/L	+	this study
^{32}P-postlabeling	HepG2	0.46 mg/L	–	this study
Oxidative DNA adduct formation				
8-oxodG	HepG2	0.46 mg/L	±	this study
CdG	HepG2	0.46 mg/L	–	this study

[a] +: positive/significantly increased: -: negative/not changed; ±: tended to increase but the increase is not significant.

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Quantification of Oxidative DNA Adducts and Bulky DNA Adducts. C_{60} is known as an ROS generator[15−17] and an inducer of LPO.[18−23] ROS and LPO products can modify nucleic acid bases to form DNA adducts, such as 8-oxodG, implicated in genotoxicity.[24] Therefore, we elucidated whether aqu-C_{60} can increase the levels of oxidative DNA adducts in human hepatocarcinoma HepG2 cells. As candidate oxidative DNA adducts we chose 8-oxodG, CdG_1, and CdG_2. The results of quantification of oxidative DNA adducts are shown in Figure 5 (a) and (b). The levels of CdG_1 and CdG_2 were essentially unchanged following aqu-C_{60} treatment as compared to the control; a slight but nonsignificant increase in the level of 8-oxodG lesions was observed. In the same condition, the cell viability of HepG2 was not changed (Figure 4 (a)). Bulky DNA adducts were also measured by using the ^{32}P-postlabeling method, but we could not detect any bulky DNA adducts caused by aqu-C_{60} (Figure S1).

4. DISCUSSION

The size distribution shift by LB broth and DMEM observed in Figure 1(c) is thought to be due to salt in the media as reported previously.[25] The size of colloidal C_{60} particle in those test media (241−554 nm) was considerably big. Although mammalian cells may take it by endocytosis, it is hard to imagine that the bacterial cells can intake such a big particle as it is. So that, we feel that only a small portion of the aqu-C_{60} was taken up by the bacterial test strains. The availability of the particle aqu-C_{60} by bacterial and mammalian cells needs to be elucidated in further study.

In this study, we prepared aqu-C_{60} by the THF−water exchange method. It was reported that γ-butyrolactone (GBL), a toxic byproduct of THF, contaminated in the aqu-C_{60} was prepared by this method.[26] We checked the contamination by using LC/MS/MS, and the concentration of GBL in our aqu-C_{60} solution was 0.064% (v/v). We also checked if this concentration of GBL affected the test results (Figures S2 and S3). The results indicated that the influence of the contaminated GBL could be negligible.

A summary of the results of genotoxicity tests available in literature, together with our data, is presented in Table 1. Aqu-C_{60} elicited positive genotoxic responses in experimental systems which detect early events in mutagenesis, including DNA damage and DNA repair responses, such as the comet assay,[1,4,16] the micronucleus test,[4] the *Bacillus subtilis* Rec-assay, and the *umu* test. These lines of evidence support the hypothesis that aqu-C_{60} has DNA-damaging potential. On the other hand, the results from some other experimental systems have been negative or conflicting: bacterial reverse mutation assay (negative[6,7]), chromosomal aberration test (negative[6,7]), and transgenic mutagenesis systems (positive,[4,5] negative[4]). As describe above, the mutagenic effect of aqu-C_{60} is not conclusive. That gives us an impression that the mutagenic effect of aqu-C_{60} might be modest. The result of ^{32}P-postlabeling/polyacrylamide gel electrophoresis analysis indicates that aqu-C_{60} does not make covalent DNA adducts. So that C_{60} seems to make DNA damage by some indirect mechanisms.

ROS generation is considered an important property of C_{60}. Two mechanisms of ROS induction by C_{60} have been reported. First, C_{60} is excited from the singlet state to the triplet state by light. The excited triplet state of C_{60} produces singlet oxygen by energy transfer.[17] Second, the excited triplet state of C_{60} is converted to the reduced triplet state in the presence of reducing agents such as NADH. The reduced triplet state of C_{60} produces superoxide anion radical by electron transfer.[15] It was reported that exposure to aqu-C_{60} increased LPO in human dermal fibroblasts, HepG2 cells, human astrocytes, the brain of juvenile largemouth bass, rat lung, and adult male fathead minnows, as measured by the thiobarbituric acid assay for malondialdehyde.[20−23] These ROS and LPO products can oxidatively damage DNA to form DNA adducts such as 8-oxodG. Folkmann et al. reported that the levels of 8-oxodG were increased in the liver and lung but not the colon of rats after intragastric administration of C_{60} suspended in both saline and corn oil.[27] However, we did not observe significant changes in the levels of 8-oxodG in HepG2 cells after treatment with 0.46 mg/L aqu-C_{60} for 24 h despite the known ROS-generating ability of C_{60} (Figure 5). On the other hand, there are few studies on LPO-related DNA adducts in connection with particle toxicology. The levels of LPO-related DNA adducts CdG_1 and CdG_2 were also not significantly changed in this study (Figure 5(b)). One of the possible reasons for this discrepancy is that refined systems for removing 8-oxodG[28] and CdGs[29] in mammalian cells could have overcome the increased levels of these DNA adducts induced by aqu-C_{60}.

Overall, our data suggest that aqu-C_{60} has DNA-damaging potential and that the DNA damage is not due to covalent DNA adduct formation by C_{60} itself. The mechanism by which aqu-C_{60} induces DNA damage and the resulting mutation needs to be elucidated in further study.

■ ASSOCIATED CONTENT

Supporting Information. Material and methods and Figures S1-S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Application of the DNA adductome approach to assess the DNA-damaging capability of *in vitro* micronucleus test-positive compounds

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ABSTRACT

The *in vitro* micronucleus (MN) test is widely used for screening genotoxic compounds, but it often produces false-positive results. To consider the significance of positive results, it is important to know whether DNA adducts are formed in the cells treated with the test compound. Recently, Matsuda et al. developed the DNA adductome approach to detect DNA adducts comprehensively ([4] Kanaly, et al., Antioxid. Redox Signal., 2006, 8, 993–1001). We applied this method to assess the DNA-damaging capability of *in vitro* MN test-positive compounds. CHL/IU cells were treated with compounds from three categories: (1) carcinogens causing DNA alkylation, ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; (2) carcinogens producing DNA bulky adducts, 2-amino-6-phenyl-1-methylimidazo[4,5-*b*]pyrene, benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, and 4-nitroquinoline-1-oxide, and (3) non-carcinogens, caffeine, maltol, and sodium chloride, with or without metabolic activation. With the conditions in which all test compounds gave positive results in the MN tests, DNA was extracted from the cells and hydrolyzed to deoxyribonucleosides, which were subsequently subjected to LC/ESI-MS/MS analysis. All carcinogens (categories 1 and 2) produced various DNA adduct peaks, and some of the *m/z* peak values corresponded to known adducts. No non-carcinogens produced DNA adducts, indicating that these compounds produced MN through different mechanisms from the adduct formation. These results indicate that the adductome approach is useful to demonstrate DNA damage formation of MN test-positive compounds and to understand their mechanisms of action.

1. Introduction

In regulatory science, *in vitro* genotoxicity tests are used for examinations of gene mutations and chromosomal alterations due to DNA damage caused by chemicals. The tests can predict carcinogenic potential of new chemicals applicable as pharmaceuticals, industrial materials, food additives, and cosmetic ingredients. If a compound shows a positive result from these tests, further *in vitro* studies to clarify the mechanism of its action (MOA) or *in vivo* genotoxicity tests are required to assess the risk for human health. Kirkland et al. demonstrated recently that the results from *in vitro* genotoxicity tests, especially the chromosome aberration assay and the micronucleus test in Chinese hamster cells and the mouse lymphoma tk locus assay, are highly discrepant from the results from rodent *in vivo* carcinogenicity tests [1].

Direct or indirect DNA reaction with a compound is an example of MOA, and should be first considered after a positive result is obtained in *in vitro* genotoxicity tests [2]. Direct DNA-reactive compounds are considered to have a non-effective threshold in the dose–response relationship in carcinogenesis; however, non-DNA-reactive (indirect) compounds have a threshold. It is considered that there is no cancer risk below the threshold level exposure; therefore, evidence of direct or indirect reaction of the genotoxicity test-positive compound is important for its cancer risk evaluation. A rapid, sensitive, and accurate method to measure cellular DNA damage, that is, direct DNA reactivity in cells, at the same experimental condition as the genotoxicity test will be required to clarify the MOA of the compound.

DNA damage formation can be measured using various analytical methods [3]. The amount of DNA adducts can be determined by measuring radioactive decay or accelerator mass spectrometry of radiolabeled adduct residues in DNA of the cells treated with radiolabeled chemicals. When the labeled compounds are not available, adducts can be measured by ^{32}P post-labeling analysis, physicochemical methods including mass spectrometry, fluorescence

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spectrometry, and electrochemical detection, or by immunochemical methods. Each of these approaches has different merits and limitations, and the measurement of DNA adduct formation needs a specific experimental protocol that is dependent on the reactivity and characteristics of each compound. None of these methods is very sensitive and accurate to quantitate the amount of DNA damage at the low concentration used in *in vitro* genotoxicity tests.

Recently, Kanaly et al. developed the "DNA adductome" approach to detect DNA adducts comprehensively using high-performance liquid chromatography equipped with tandem mass spectrometry (LC–MS/MS) [4]. The technique allows comprehensive monitoring of multiple types of DNA adducts that have different molecular weights even though their molecular structures are unknown. The technique can detect adducts in cellular DNA with extremely high sensitivity by comparing the "adductome maps" of treated and untreated cells, and is applicable to the analysis of DNA damage produced in various experimental protocols *in vivo* and *in vitro*.

In this study, we combined this adductome approach with the *in vitro* micronucleus (MN) test to examine whether adductome analysis is useful in regulatory science. Chinese hamster lung (CHL) cells were treated with representative MN-inducing compounds with different MOA, and the increase in the MN incidence was confirmed. Following chemical treatment with the identical condition to the MN test, DNA was extracted from the cells, and DNA adducts were measured by adductome analysis. DNA adducts should not be detected in cells treated with non-DNA-reacting compounds such as caffeine, maltol, and sodium chloride, whereas DNA adducts should be detected in cells treated with directly DNA-reacting compounds such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 4-nitroquinoline-1-oxide (4-NQO). If the adductome analysis in the MN test condition is valid in this pilot study, newly found MN-positive compounds would be rapidly evaluated in terms of whether they are directly or indirectly reactive to DNA by adductome analysis, which may become a new standard method for the MOA evaluation of *in vitro* genotoxic compounds.

2. Materials and methods

2.1. Test chemicals and reagents

Nine compounds were selected for the MN test and adductome analysis, which were classified into three categories: group A, carcinogens known to produce alkyl residues including ethylmethanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG); group B, carcinogens known to make bulky DNA adducts including 2-amino-6-phenyl-1-methylimidazo[4,5-b]pyrene (PhIP), benzo[*a*]pyrene (B[*a*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA), and 4-nitroquinoline-1-oxide (4-NQO), and group C, non-carcinogens including caffeine, maltol, and sodium chloride (NaCl). EMS, B[*a*]P, 4-NQO, and caffeine were purchased from Sigma Co. (St. Louis, MO, USA), and the other chemicals were purchased from Wako Chemical (Osaka, Japan). They were dissolved in distilled water (DW), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), physiological saline (saline), or minimum essential medium with 10% calf serum (MEM), immediately before treatment (Table 1). The solvent for each test chemical was used as a negative control. If a chemical required metabolic activation to exert its genotoxicity, rat liver S9 mix, which was designed for the *in vitro* chromosomal aberration test (Kikkoman Corporation, Noda, Japan), was added simultaneously during the treatment period (Table 1).

O^6-methyl deoxyguanosine was purchased from Chemsyn Science Laboratories (Kansas, USA). N^7-methyl deoxyguanosine was synthesized according to the method reported by Yang et al. [5]. [$^{15}N_5$, $^{13}C_{10}$]-2-(2'-deoxyguanosine-8yl)-3-aminobenzanthrone ([$^{15}N_5$, $^{13}C_{10}$]-dG-8-ABA) was kindly supplied by Dr. Takamura of Kanagawa Institute of Technology. These compounds were used for chromatogram standards for the LC/ESI-MS/MS analysis.

2.2. Cells

CHL/IU cells were obtained from DS Pharma Biomedical Co. Ltd. (Osaka, Japan) and used in all experiments. The cells were maintained in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated (56 °C for 30 min) calf serum (CS; Hana-Nesco-Bio Co., Tokyo, Japan) in a 5%-CO_2 incubator at 37 °C.

2.3. MN test

The cells were seeded in ϕ60 mm plastic dishes at 1.6×10^4 cells/dish for the micronucleus tests. The cells were treated with the test chemicals for 6 h in the absence or presence of S9 mix followed by a 20-h recovery period (Fig. 1). Then, the cells were trypsinized and counted. Cytotoxicity was evaluated using the relative cell survival rate, which was defined as the number of chemical-treated cells divided by the number of solvent-treated cells. The cells were spun down and then resuspended in KCl hypotonic solution (75 mM) for 5 min at room temperature. The hypotonized cells were fixed twice in methanol:glacial acetic acid (3:1). Finally, the cells were suspended in methanol containing 1% acetic acid and dropped onto glass slides. After drying, the cells were stained with 0.04% acridine orange solution and subjected to microscopic examination. One thousand intact interphase cells were observed using a microscope, and the incidence of the MN cells was calculated. Fisher's exact test was performed for a statistical analysis.

2.4. DNA extraction

The cells were seeded in ϕ150 mm plastic dishes at 10×10^4 cells/dish for DNA extraction. The cells were treated with test chemicals for 6 h in the absence or presence of S9 mix (Fig. 1). The treatment was carried out with the same experimental protocol as the MN test. The cells were detached by trypsinization, and cellular DNA was extracted according to the method described previously [1]. Purified DNA was suspended in distilled water, and the DNA concentration was determined by measuring absorbance at 260 nm using a UV-vis spectrophotometer. An aliquot of DNA (100 μg) was transferred to a 1.5 mL Eppendorf tube and subjected to evaporation.

2.5. Digestion of DNA samples

DNA was enzymatically hydrolyzed to nucleosides by the micrococcal nuclease/spleen phosphodiesterase (MCN/SPD) method or the nuclease P1 method as described below. In the MCN/SPD method, DNA (100 μg) was enzymatically hydrolyzed to 2'-deoxyribonucleoside-3'-monophosphates for 3 h at 37 °C by the addition of 45 μL of buffer (17 mM sodium succinate and 8 mM $CaCl_2$ at pH 6.0) and 9 μL of MCN/SPD mix consisting of 7.5 units/μL MCN (Worthington Biochemical, Lakewood, NJ) and 0.025 units/μL SPD (Sigma, St. Louis, MO). Then, 3 units of alkaline phosphatase, 30 μL of 0.5 M Tris–HCl (pH 8.5), 15 μL of 20 mM $ZnSO_4$, and 200 μL of water were added and further incubated for 3 h at 37 °C.

In the nuclease P1 method, DNA (100 μg) was enzymatically hydrolyzed to 2'-deoxyribonucleoside-5'-monophosphates by the addition of 300 μL of buffer (30 mM sodium acetate at pH 5.3 and 10 mM 2-mercaptoethanol), 15 μL of 20 mM $ZnSO_4$, 15 μL of water, 3 units of alkaline phosphatase (Wako, Osaka, Japan), and 6 units of nuclease P1 (Wako, Osaka, Japan) for 3 h at 37 °C. Then, 60 μL of 0.5 M Tris–HCl (pH 8.5) was added and incubated for another 3 h at 37 °C.

The digested samples were extracted twice with methanol. The resultant methanol fraction was completely evaporated, and the remaining 2'-deoxyribonucleosides were dissolved in 160 μL of 30% DMSO containing an internal standard (11.5 nM [$^{15}N_5$, $^{13}C_{10}$]-dG-8-ABA).

2.6. Adductome analysis by LC/ESI-MS/MS

The analysis was performed using the Shimadzu HPLC System (Shimadzu), which consists of LC-10ADvp bipumps, a SIL-10ADvp autosampler, a Shim-pack XR-ODS (3.0 mm × 75 mm, 2.2 μm, Shimadzu), and a SPD-10 ADvp UV–Vis detector. The HPLC mobile phases A and B were water and methanol, respectively. The HPLC flow rate was set at 0.2 mL/min. The HPLC gradient started at 5% B, was increased linearly to 80% B over 20 min, and returned to the initial condition over 1 min, which was maintained for a further 10 min. The HPLC system was interfaced with a Quattro Ultima Pt (Waters-Micromass) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 130 °C, and the desolvation temperature was maintained at 380 °C. Nitrogen was used as the desolvation gas (700 L/h), and the cone gas was set to 30 L/h. The capillary voltage was set at 3.5 kV. The collision cell pressure and collision energy were set to 3.8×10^{-3} mBar and 15 eV, respectively. The adducts were analyzed by MS/MS using multiple reaction monitoring (MRM). Ion transition was set at $[M+H]^+ \rightarrow [M+H-116]^+$, the [M+H] of which ranged from *m/z* 250 to *m/z* 702. The LC/ESI-MS/MS was set to monitor 32 ion transitions simultaneously in each injection and 10 μL of each sample was injected 15 times. The ion transitions for an internal standard (*m/z* 526→ *m/z* 405) were monitored in each injection. The absorbance at 254 nm was also monitored with a UV–Vis detector to monitor DNA digestion, and the peak area of 2'-deoxyguanosine (dG) was used for data analysis peak normalization as described below.

2.7. Data analysis

DNA adduct peaks were extracted by comparing chromatograms between the controls (solvent-treated samples) and chemical-treated samples using the following criteria: the signal to noise (S/N) ratio of the detected peak should be more than 3, and the peak area should be 3 times larger than the control peak. When a possible adduct peak was detected, a repeated MN test and adductome analysis were