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Deguanylation of Guanine Based-Nucleosides and Calf Thymus DNA Induced by Halogenated Alkanes at the Physiological Condition

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Massive deguanylation of guanine based-nucleosides induced by halogenated alkanes at the physiological condition have been observed. For the study of deguanylation effects by the different substituents and/or functionality in halogenated alkanes, diverse kinds of halogenated alkanes were incubated with guanine based-nucleosides (ddG, dG and guanosine) for 48 h at the physiological condition (pH 7.4, 37 °C), which were analyzed by HPLC and further confirmed by LC-MS. Among the sixteen different halogenated alkanes, we observed massive deguanylation of nucleosides by 2-bromo-2-methylpropane, 2,3-dibromopropene, 2-bromopropane, bromoethane and 2-iodopropane. The order of deguanylation rate was highest in 2-bromo-2-methylpropane followed by 2,3-dibromopropene, 2-bromopropane, bromoethane and 2-iodopropane. In addition, time and dose response relationship of deguanylation in guanine based-nucleosides induced by 2-bromo-2-methylpropane, 2,3-dibromopropene, 2-bromopropane, bromoethane and 2-iodopropane at the physiological condition were investigated. Deguanylation of calf thymus DNA induced by halogenated alkanes was also investigated. These results suggest that the toxic effect of certain halogenated alkanes might be from the depurination of nucleosides.

Key Words: Deguanylation, Depurination, Halogenated alkanes, Guanine base-nucleosides, Calf thymus DNA

Introduction

The depurination or deguanylation of nucleic acids, which involve the release of purine or guanine bases, respectively, from nucleic acids by hydrolysis of the *N*-glycosidic bond (Fig. 1), gives rise to alterations of the cell genome.[1,2] Although depurination occurs spontaneously under physiological conditions, the rate of depurination is accelerated at low pH, high temperature, or by alkylation.[3] Since the apurinic sites resulting from depurination have shown lethality[4,5] and base substitution errors,[5] depurination (deguanylation) could be one of the promising novel mechanisms of toxicity induced by the short chain halogenated alkanes.

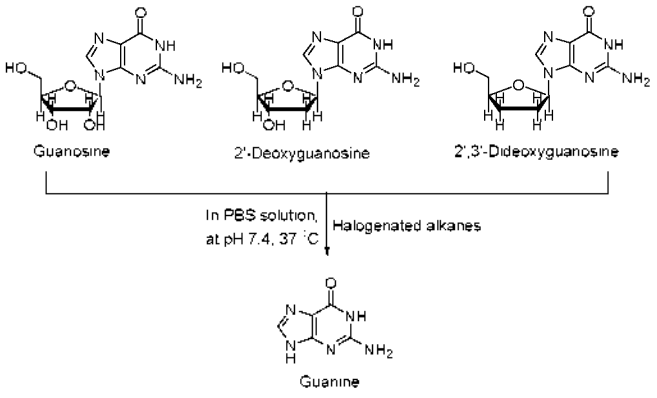

Figure 1. Scheme of deguanylation, the release of guanine bases from nucleic acids by hydrolysis of the *N*-glycosidic bond. Guanine based-nucleosides were incubated with 16 halogenated alkanes at the physiological condition (pH 7.4, 37 °C) for a certain period and analyzed by HPLC, LC-MS and UV.

Previously, we observed the massive depurination of nucleosides such as 2',3'-dideoxyadenosine (ddA), 2'-deoxyadenosine (dA), 2',3'-dideoxyguanosine (ddG), 2'-deoxyguanosine (dG), and calf thymus DNA with an excess amount of 2-bromopropane (2-BP) at the physiological condition.[6] Also we observed the massive deadenylation of adenine based-nucleosides such as ddA, dA, and calf thymus DNA with excess amount of halogenated alkanes at the physiological condition.[7] It would be very interesting to investigate deguanylation of guanine based-nucleosides induced by halogenated alkanes according to the difference of substituents and/or functionality in halogenated alkanes, which may provide valuable information for the mechanism of toxicity of halogenated alkanes. In addition, it would also be interesting to compare the rate of depurination between guanine based-nucleosides and adenine based-nucleosides, as guanine has been reported to release more rapidly than adenine.[3]

In connection with previous studies, diverse kinds of halogenated alkanes were incubated with guanine based-nucleosides (ddG, dG and guanosine) for 48 h at the physiological condition (pH 7.4, 37 °C), which were analyzed by HPLC and further confirmed by LC-MS for the study of deguanylation effects by different substituents of and/or functionality in halogenated alkanes. Short-chain halogenated alkanes, which we utilized for the study of deguanylation of guanine based-nucleosides, have been widely used industrially as chemical intermediates, extraction solvents, degreasing compounds, copolymer cross-linking agents, or have been reported to be mutagenic and carcinogenic.[8-17] Among the sixteen different halogenated alkanes, we observed massive deguanylation of nucleosides by 2-bromo-2-methylpropane (2-B-2-MP), 2,3-dibromopropene (2,3-dBPe), 2-bromopropane (2-BP), bromoethane (BE) and 2-iodopropane (2-IP).

Materials and Methods

Chemicals. Iodomethane (99.5%), bromoethane (≥ 99%), iodoethane (99%), 1,2-dibromoethane (99+%), 1,2-dichloroethane (99.8%), 1-bromopropane (99%), 2-bromopropane (99 %), 1-chloropropane (98%), 2-chloropropane (99+%), 1-iodopropane (99%), 2-iodopropane (2-IP, 99%), 1,2-dibromopropane (97%), 1,3-dibromopropane, 1,2,3-tribromopropane (97 %), 2,3-dibromopropene (> 99%), 2'-deoxyguanosine hydrate (99%), guanosine hydrate (98%), calf-thymus DNA (deoxyribonucleic acid sodium salt, from calf thymus), 5-fluorouracil (99%), phosphate buffered saline (pH 7.4) and ammonium acetate (99.995+%) were purchased from Sigma Aldrich Co. (ST. Louis, MO). 2',3'-Dideoxyguanosine and 2-bromo-2-methylpropane (≥ 97%) were obtained from Berry & Associates Inc and Fluka, respectively. 9-Methyl adenine (9-MA) was obtained by synthesis in the present lab. HPLC grade acetonitrile and methanol was purchased from World Science, Korea. All other chemicals, if not mentioned, were also obtained from Sigma Aldrich Co. (ST. Louis, MO).

Preliminary reactions. One mg of nucleoside (ddG, dG and guanosine) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Ten μL (5 mg in 1 mL PBS) of 5-flurouracil (5-FU) was added as an internal standard. It was then incubated with an excess amount (512 equivalents) of each of the sixteen halogenated alkanes listed in Table 1 at 37 °C for 48 h, respectively. It was analyzed by HPLC and further confirmed by LC-MS. All the reactions were repeated for three times.

Time response reaction. One mg of nucleoside (ddG, dG and guanosine) was dissolved separately in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Ten μL (5 mg in 1 mL PBS) of 5-flurouracil (5-FU) was added as an internal standard. It was then incubated with 512 equivalents of 2,3-dBPe, 2-BP, BE and 2-IP for ddG and dG, 4 equivalents of 2-B-2-MP for ddG, 32 equivalents of 2-B-2-MP for dG, 512 equivalents of 2-B-2-MP for guanosine, respectively, at 37 °C. About 10 μL of samples were withdrawn at certain time intervals and analyzed by HPLC until 100% deguanylation occurred. All the reactions were repeated for three times.

Dose response reaction. One mg of nucleoside (ddG, dG and guanosine) was dissolved separately in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Ten μL (5 mg in 1 mL PBS) of 5-fluorouracil (5-FU) was added as an internal standard. It was then incubated with different amounts (0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents) of 2,3-dBPe, 2-BP, BE and 2-IP at 37 °C for a time period in which 100% deguanylation occurred. For incubation with 2-B-2-MP, different amounts (0, 1, 2, 3 and 4 equivalents for ddG, 0, 2, 4, 8, 16 and 32 equivalents for dG, 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents for guanosine) were employed. Again the samples were analyzed by HPLC and repeated for three times.

Reactions with Calf-thymus DNA (ct-DNA). Two mg of ct-DNA was dissolved in 20 mL of PBS solution and 40 μL of 9-methyl adenine (0.5 mg in 1 mL PBS) was added as an internal standard and stirred to mix properly. One mL of the above prepared solution of ct-DNA was taken in 5 mL vial and incubated with 128 μL of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP at the physiological condition for 48 h as a preliminary reaction. At the end of the reaction 300 μL of 1 M HCl was added and centrifuged for 10 min at 13,000 rpm. Then it was analyzed by LC-MS under the condition mentioned below.

Time response reaction was performed with 128 μL of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP at a time interval of 8h for 0, 8, 16, 24, 32, 40 and 48 h at the physiological condition, respectively. At the end of the reaction 300 μL of 1 M HCl was added and centrifuged for 10 min at 13,000 rpm. Then it was analyzed by LC-MS under the condition mentioned below.

Dose response reaction was performed with 2, 4, 8, 16, 32, 64 and 128 μL of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP for 48 h at the physiological condition, respectively. At the end of the reaction 300 μL of 1 M HCl was added and centrifuged for 10 min at 13,000 rpm. Then it was analyzed by LC-MS under

Table 1. List of halogenated alkanes with their chemical structure and molecular weight

No.	Halogenated alkanes	Structure	Mol. wt.
1	Iodomethane (IM)		141.94
2	Bromoethane (BE)		108.97
3	Iodoethane (IE)		155.97
4	1,2-dibromoethane (1,2-dBE)		187.86
5	1,2-dichloroethane (1,2-dCE)		98.96
6	1-bromopropane (1-BP)		122.99
7	2-bromopropane (2-BP)		122.99
8	1-chloropropane (1-CP)		78.54
9	2-chloropropane (2-CP)		78.54
10	1-iodopropane (1-IP)		169.99
11	2-iodopropane (2-IP)		169.99
12	1,2-dibromopropane (1,2-dBP)		201.89
13	1,3-dibromopropane (1,3-dBP)		201.89
14	2-bromo-2methylpropane (2-B-2-MP)		137.03
15	1,2,3-tribromopropane (1,2,3-tBP)		280.78
16	2,3-dibromopropene (2,3-dBPe)		199.8

the condition mentioned below.

Calculation for deguanylation ratio in nucleosides. Deguanylation ratio (DR, %) was calculated on the basis of the decreased amount of nucleosides in percentage by comparing the integration value of the nucleosides in HPLC using the formula below:

$$\text{Deguanylation ratio (\%)} = \frac{\frac{A_o}{IS_o} - \frac{A_t}{IS_t}}{\frac{A_o}{IS_o}} \times 100\%$$

where 'A_o' is the initial amount of nucleoside; 'A_t' is the amount of nucleoside after time, t ; 'IS_o' is the initial amount of internal standard and 'IS_t' is the amount of nucleoside after time, t.

Calculation for deguanylation ratio in ct-DNA. Deguanylation ratio was calculated on the basis of the increased amount of guanine compared to the internal standard (IS) by comparing the integration value in EIC from LC-MS using the formula below:

$$\text{Deguanylation ratio} = \text{guanine} / \text{IS}$$

Apparatus. HPLC analyses were performed using two Shimadzu LC-10AT pumps gradient-controlled HPLC system equipped with Shimadzu photo diode array detector (Model SPD-M10A) and dual channel UV detection at 280 nm. Analytes were eluted with a 4.6 × 250 mm, 5 μm Waters XTerra® C_{18} reverse phase analytical column using the following HPLC condition: Isocratically with 4% acetonitrile in water with 50 mM ammonium formate at pH 6.9, 1 mL/min flow rate and 10 μL injection volume for guanine-based nucleosides.

ESI LC/MS analyses were performed with a Finnigan LCQ Advantage® LC-MS/MS spectrometry utilizing Xcalibur® program. The samples were analyzed using 2.1 × 150 mm, 3.5 μm Waters XTerra® C_{18} reverse phase analytical column using the following LC condition: Isocratically with 3% acetonitrile in water with 50 mM ammonium formate at pH 6.9, 0.18 mL/min flow rate and 2 μL injection volume. The mass spectrometer was operated in the positive polarity mode with ESI source type. Capillary voltage was controlled at 10 V and 270 °C and nitrogen gas was used as sheath gas.

Centrifugation was done using Hanil Micro-12 (made in Korea) with maximum capacity 1.5 mL × 12, maximum speed 13,000 rpm, maximum RCF 10,770 × g, and power AC 110 V, 60 Hz.

Statistical analysis. All the reactions were performed at least three times ($n \geq 3$). The mean value ± standard error (SE) was determined for each test. Student's t-test was used to compare statistical significance of data. The significant values at either $P < 0.05$(*) or $P < 0.01$(**) are represented by asterisks.

Results

The deguanylation effect of the sixteen halogenated alkanes on guanine based-nucleosides was analyzed by HPLC and further confirmed by LC-MS. Table 1 shows the list of halogenated alkanes with chemical structure and molecular weight, which were treated with guanine based-nucleosides. From the preliminary reaction, it was found that 100% deguanylation occurred in ddG and dG with excess amount (512 equivalents) of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP at the physiological condition for 48 h (Table 2), which is described in Figure 2 and 3. It is interesting to notice that 100% deguanylation occurred in guanosine with treatment of excess amount (512 equivalents) of 2-B-2-MP at the physiological condition for 48 h, whereas almost no change was observed for adenosine by treatment of all of the sixteen halogenated alkanes at the same condition.[7] In addition, ddG, dG and guanosine were not affected by the other halogenated alkanes.

Table 2. Preliminary reaction of ddG, dG and guanosine with halogenated alkanes at the physiological condition for 48 h

No.	halogenated alkanes	DR (%) in ddG	DR (%) in dG	DR (%) in guanosine
1	Iodomethane	*	*	*
2	Bromoethane	**100.00**	**100.00**	5.28
3	Iodoethane	6.30	0.84	3.49
4	1,2-dibromoethane	6.50	1.17	7.40
5	1,2-dichloroethane	4.80	-0.42	5.34
6	1-bromopropane	6.90	-0.18	6.70
7	2-bromopropane	**100.00**	**100.00**	13.66
8	1-chloropropane	5.40	-0.22	7.27
9	2-chloropropane	10.10	0.43	6.35
10	1-iodopropane	4.80	-0.08	5.96
11	2-iodopropane	**100.00**	**100.00**	7.30
12	1,2-dibromopropane	5.90	0.01	5.69
13	1,3-dibromopropane	2.00	2.00	-0.44
14	2-bromo-2methylpropane	**100.00**	**100.00**	**100.00**
15	1,2,3-tribromopropane	4.20	-3.49	4.69
16	2,3-dibromopropene	**100.00**	**100.00**	*

One mg of nucleoside (ddG, dG and guanosine) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Twenty μL of 5-flurouracil (10 mg in 1 mL PBS) was added as an internal standard. It was then incubated with excess amount (512 equivalents) of halogenated alkanes at the physiological condition for 48 h. It was analyzed by HPLC and LC-MS. *Only small amount of deguanyled product (guanine) was observed with many adducts formation.

Analysis of deguanylation of ddG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP by HPLC. Figure 2 shows the HPLC chromatograms under the chromatographic condition described in material and methods for the analysis of deguanylation of ddG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP at 37 °C for 48 h.

In Figure 2, chromatogram 1 indicates authentic guanine (Gua) at retention time of 4.87 min, and chromatogram 2 indicates the mixture of ddG and 5-fuorouracil (5-FU) utilized as an internal standard at retention times of 12.83 min and 4.12 min, respectively. Guanine, ddG and 5-fuorouracil were well separated from the biological background under the described chromatographic condition. Chromatogram 3, 4, 5, 6 and 7 indicates the mixture after incubation of ddG and excess amount (512 equivalent) of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP

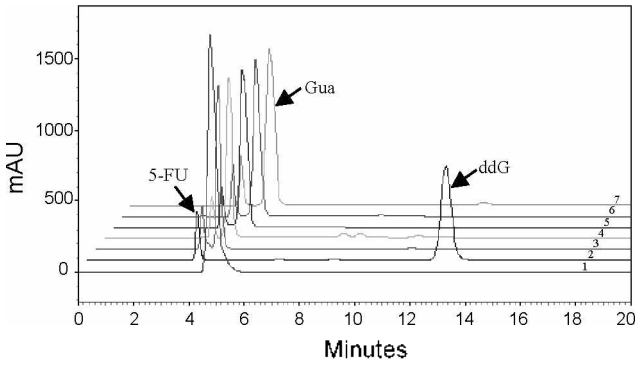

Figure 2. HPLC chromatogram of (1) guanine (Gua), (2) ddG + 5-FU, (3) ddG + 5-FU + 2-B-2-MP (48 h), (4) ddG + 5-FU + 2,3-dBPe (48 h) (5) ddG + 5-FU + 2-BP (48 h) (6) ddG + 5-FU + BE (48 h) (7) ddG + 5-FU + 2-IP (48 h). Retention time for 5-FU, Gua and ddG were 4.12, 4.87 and 12.83 min, respectively, under the HPLC condition mentioned in materials and methods.

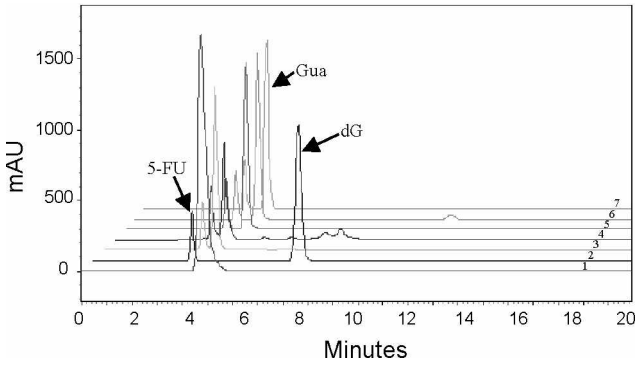

Figure 3. HPLC chromatogram of (1) Gua, (2) dG + 5-FU, (3) dG + 5-FU + 2-B-2-MP (48 h), (4) dG + 5-FU + 2,3-dBPe (48 h) (5) dG + 5-FU + 2-BP (48 h) (6) dG + 5-FU + BE (48 h) (7) dG + 5-FU + 2-IP (48 h). Retention time for 5-FU, Gua and dG were 4.08, 4.82 and 8.38 min, respectively, under the HPLC condition mentioned in materials and methods.

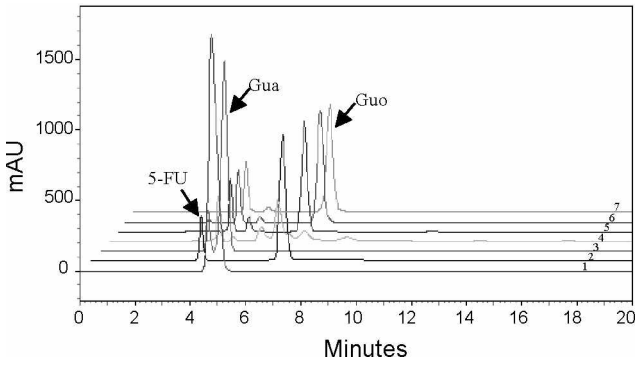

Figure 4. HPLC chromatogram of (1) Gua, (2) guanosine (Guo) + 5-FU, (3) Guo + 5-FU + 2-B-2-MP (48 h), (4) Guo + 5-FU + 2,3-dBPe (48 h) (5) Guo + 5-FU + 2-BP (48 h) (6) Guo + 5-FU + BE (48 h) (7) Guo + 5-FU + 2-IP (48 h). Retention time for 5-FU, Gua and Guo were 4.06, 4.76 and 6.70 min, respectively, under the HPLC condition mentioned in materials and methods.

for 48 h at the physiological condition, respectively, which indicates the peak of retention time at 12.83 min, which corresponds to the complete disappearance of ddG and a peak of retention time at 4.87 min, corresponding to the new appearance of guanine. These results indicated that complete deguanylation occurred when ddG was incubated with excess amount of 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP at 37 °C for 48 h.

Analysis of deguanylation of dG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP by HPLC. Figure 3 shows the HPLC chromatograms under the chromatographic condition described in material and methods. for the analysis of deguanylation of dG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP at 37 °C for 48 h.

In Figure 3, chromatogram 1 indicates authentic guanine (Gua) at retention time of 4.82 min, and chromatogram 2 indicates the mixture of dG and 5-fluorouracil (5-FU) utilized as an internal standard at retention times of 8.38 min and 4.08 min, respectively. Guanine, dG, and 5-fuorouracil were well separated from the biological background under the described chromatographic condition. Chromatogram 3, 4, 5, 6, and 7 indicates the mixture after incubation of dG and excess amount (512 equivalent) of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP for 48 h at the physiological condition, respectively, which indicates the peak of retention time at 8.38 min, which corresponds to the complete disappearance of dG and a peak of retention time at 4.82 min, corresponding to the new appearance of guanine. These results indicated that complete deguanylation occurred when dG was incubated with excess amount of 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP at 37 °C for 48 h.

Analysis of deguanylation of guanosine induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP by HPLC. Figure 4 shows the HPLC chromatograms under the chromatographic condition described in material and methods. for the analysis of deguanylation of guanosine (Guo) induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP at 37 °C for 48 h.

In Figure 4, chromatogram 1 indicates authentic guanine (Gua) at retention time of 4.76 min, and chromatogram 2 indicates the mixture of guanosine and 5-fuorouracil (5-FU) utilized as an internal standard at retention times of 6.70 min and 4.06 min, respectively. Guanine, guanosine and 5-fluorouracil were well separated from the biological background under the described chromatographic condition. Chromatogram 3, 4, 5, 6, and 7 indicates chromatogram of the mixture after incubation of guanosine and excess amount (512 equivalent) of 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP for 48 h at the physiological condition, respectively, which informs almost no change in amount of guanosine and no production of guanine at that condition by treatment of 2,3-dBPe, 2-BP, BE, and 2-IP (chromatogram 4, 5, 6 and 7). However, in chromatogram 3, which corresponds to treatment of 2-B-2-MP, the peak of guanosine completely disappeared and a peak of retention time at 4.76 min corresponding to guanine newly appeared. The results indicated that deguanylation only occurred when guanosine was incubated with excess amount of 2-B-2-MP for 48 h at the physiological condition.

In Figures 2, 3, and 4, any change in amount of 5-fluorouracil during incubation of ddG, dG and guanosine with 2-B-2-MP,

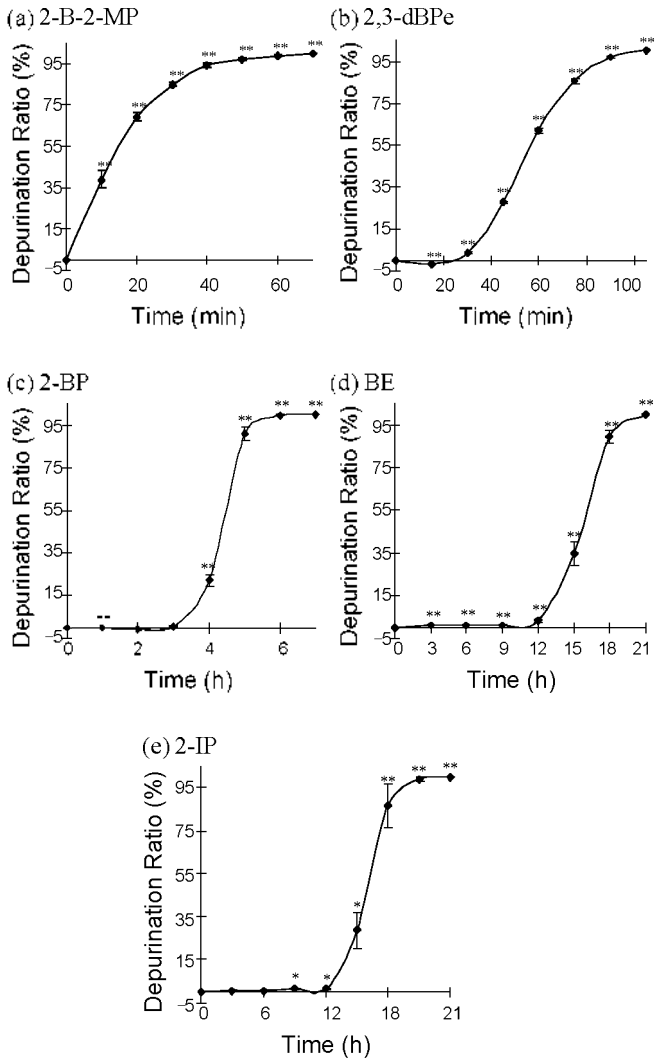

Figure 5. Time response curves of ddG with (a) 2-B-2-MP (b) 2,3-dBPe (c) 2-BP (d) BE and (e) 2-IP. Time response reactions were performed with 4 equivalents of 2-B-2-MP and 512 equivalents of 2,3-dBPe, 2-BP, BE and 2-IP respectively until the time at which 100% deguanylation occurred. Then it was analyzed by HPLC following the condition mentioned in the materials and methods.

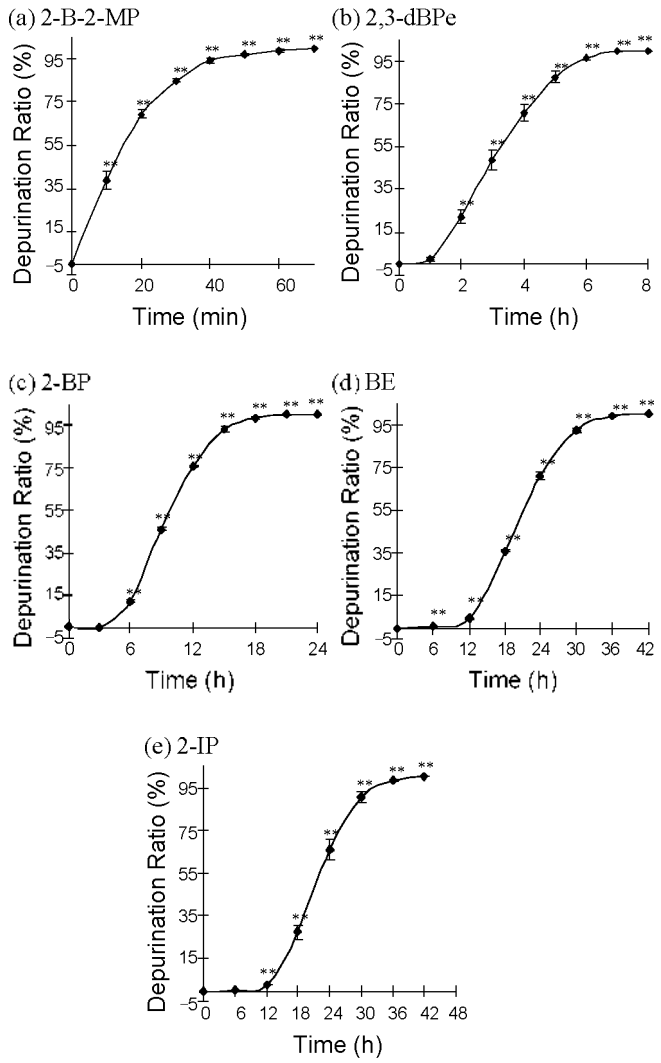

Figure 6. Time response curves of dG with (a) 2-B-2-MP (b) 2,3-dBPe (c) 2-BP (d) BE and (e) 2-IP. Time response reactions were performed with 32 equivalents of 2-B-2-MP and 512 equivalents of 2,3-dBPe, 2-BP, BE and 2-IP respectively until the time at which 100% deguanylation occurred. Then it was analyzed by HPLC following the condition mentioned in the materials and methods.

2,3-dBPe, 2-BP, BE, and 2-IP was not observed, which indicates the concentration of 5-fluorouracil was consistently well maintained and 5-fluorouracil was not affected by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, 2-IP, or nucleosides.

Analysis of time response deguanylation of ddG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP. Figure 5 shows time response curves of deguanylation rate of ddG induced by 512 dose equivalent of 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP according to time. Figure 5(a) indicates time response curve of deguanylation after incubation of ddG and 512 dose equivalent of 2-B-2-MP at the physiological condition at a time interval of 15 min. Deguanylation begin to occur at 15 min, and increase until 45 min in a time dependent manner. Complete deguanylation occurred at 60 min. In Figure 5(b), corresponding to treatment with 2,3-dBPe, deguanylation begin to occur at 30 min, and drastically increase until 80 min in a time dependent manner. Complete deguanylation occurred at 100 min. In Figure 5(c), corresponding to treatment with 2-BP, deguanylation begin to occur at 3 h, and drastically increase until 5 h in a time dependent manner. Complete deguanylation occurred at 6 h. In Figure 5(d), corresponding to treatment with BE, deguanylation begin to occur at 12 h, and drastically increase until 18 h in a time dependent manner. Complete deguanylation occurred at 21 h. In Figure 5(e), corresponding to treatment with 2-IP, deguanylation begin to occur at 12 h, and drastically increase until 18 h in a time dependent manner. Complete deguanylation occurred at 21 h. Compared to deguanylation rates in ddG among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2,3-dBPe > 2-BP > BE ≈ 2-IP. Especially, the deguanylation rates of 2-B-2-MP and 2,3-dBPe were much faster than that of 2-BP, BE and 2-IP.

Analysis of time response deguanylation of dG induced by

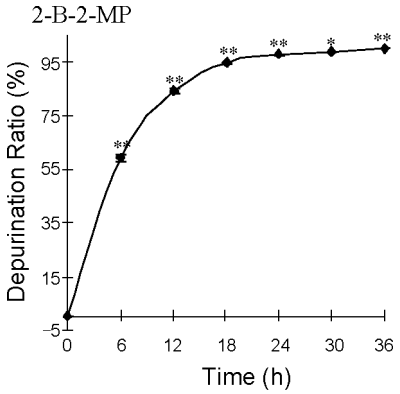

Figure 7. Time response curves of guanosine with 2-B-2-MP. Time response reactions were performed with the 512 equivalents of 2-B-2-MP at a time interval of 6 h until the time at which 100% deguanylation occurred. Then it was analyzed by HPLC following the condition mentioned in the materials and methods.

2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP. Figure 6 shows time response curves of deguanylation rate of dG induced by 512 dose equivalent of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP according to time. Figure 6(a) indicates time response curve of deguanylation after incubation of dG and 512 dose equivalent of 2-B-2-MP at the physiological condition at a time interval of 10 min. Deguanylation begin to occur at 10 min, and drastically increase until 40 min in a time dependent manner. Complete deguanylation occurred at 60 min. In Figure 6(b), corresponding to treatment with 2,3-dBPe, deguanylation begin to occur at 1 h, and drastically increase until 6 h in a time dependent manner. Complete deguanylation occurred at 7 h. In Figure 6(c), corresponding to treatment with 2-BP, deguanylation begin to occur at 6 h, and drastically increase until 15 h in a time dependent manner. Complete deguanylation occurred at 21 h. In Figure 6(d), corresponding to treatment with BE, deguanylation begin to occur at 12 h, and drastically increase until 30 h in a time dependent manner. Complete deguanylation occurred at 36 h. In Figure 6(e), corresponding to treatment with 2-IP, deguanylation begin to occur at 12 h, and drastically increase until 30 h in a time dependent manner. Complete deguanylation occurred at 42 h. Compared to deguanylation rates in dG among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2,3-dBPe > 2-BP > BE ≈ 2-IP. Especially, deguanylation rate of 2-B-2-MP was much faster than that of 2-BP, BE, and 2-IP. Comparing deguanylation rates of ddG and dG, the rate of ddG was faster than dG. In addition, the rate of deguanylation of guanine based-nucleosides was generally faster than deadenylation of adenine based-nucleosides.[7]

Analysis of time response deguanylation of guanosine induced by 2-B-2-MP. Figure 7 shows time response curve of deguanylation rate of guanosine induced by 512 dose equivalent of 2-B-2-MP at the physiological condition at a time interval of 6 h. Deguanylation begin to occur within 6 h, and drastically increase until 12 h in a time dependent manner. Complete deguanylation occurred at 24 h. The rate of deguanylation of guanosine was much lower than that of ddG or dG.

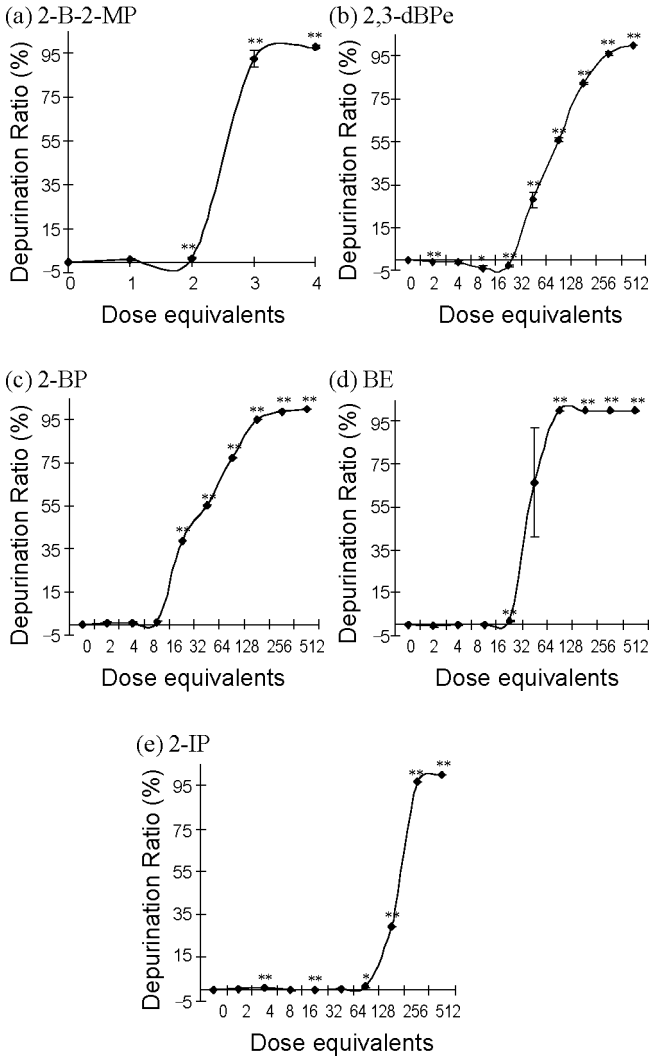

Figure 8. Dose response curves of ddG with (a) 2-B-2-MP (b) 2,3-dBPe (c) 2-BP (d) BE and (e) 2-IP. Dose response reactions were performed with the 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents of halogenated alkanesfor a fixed time at which 100% deguanylation occurred. Then it was analyzed by HPLC under the condition mentioned in the materials and methods.

Analysis of dose response deguanylation of ddG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 8 shows dose response curves of deguanylation rates of ddG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP according to dose. Figure 8(a) indicates dose response curve of deguanylation after incubation of ddG and different dose equivalents of 2-B-2-MP at the physiological condition for 24 h. Deguanylation begin to occur at 1 dose equivalent of 2-B-2-MP, and drastically increase until 3 dose equivalent of 2-B-2-MP in a dose dependent manner. Complete deguanylation occurred at 4 dose equivalent of 2-B-2-MP. In Figure 8(b), corresponding to treatment with 2,3-dBPe, deguanylation begin to occur at 32 dose equivalent, and drastically increase until 128 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 256 dose equivalent. In Figure 7(c), corresponding to treatment with 2-BP, deguanylation begin to occur at 16 dose equivalent, and

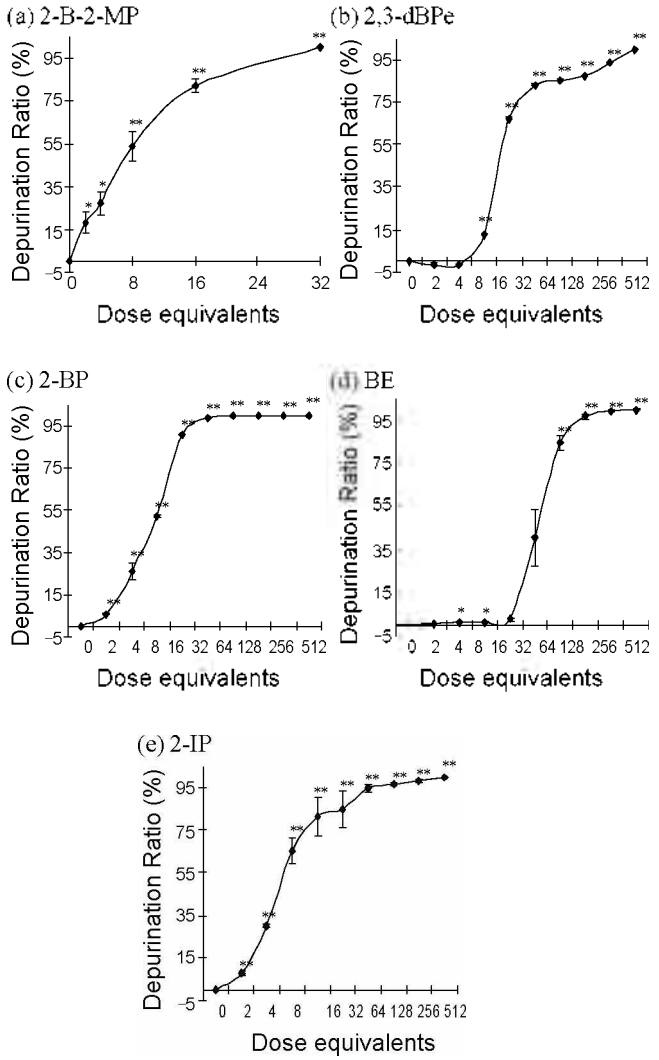

Figure 9. Dose response curves of dG with (a) 2-B-2-MP (b) 2,3-dBPe (c) 2-BP (d) BE and (e) 2-IP. Dose response reactions were performed with the 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents of halogenated alkanes for a fixed time at which 100% deguanylation occurred. Then it was analyzed by HPLC under the condition mentioned in the materials and methods.

drastically increase until 128 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 256 dose equivalent. In Figure 7(d), corresponding to treatment with BE, deguanylation begin to occur at 16 dose equivalent, and drastically increase until 64 dose equivalent in dose dependent manner. Complete deguanylation occurred at 64 dose equivalent. In Figure 7(e), corresponding to treatment with 2-IP, deguanylation begin to occur at 64 dose equivalent, and drastically increase until 256 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 256 dose equivalent. Compared to deguanylation rates in ddG among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2-IP $\approx$ 2-BP $\approx$ 2,3-dBPe $\geq$ BE.

Analysis of dose response deguanylation of dG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP. Figure 9 shows dose response curves of deguanylation rates of dG induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP according to dose. Figure 9(a) indicates the dose response curve of deguanylation after incubation of dG and different dose equivalents of 2-B-2-MP at the physiological condition for 24 h. Deguanylation begin to occur at 2 dose equivalent of 2-B-2-MP, and drastically increase until 16 dose equivalent of 2-B-2-MP in a dose dependent manner. Complete deguanylation occurred at 32 dose equivalent of 2-B-2-MP. In Figure 9(b), corresponding to treatment with 2,3-dBPe, deguanylation begin to occur at 8 dose equivalent, and drastically increase until 32 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 64 dose equivalent. In Figure 8(c), corresponding to treatment with 2-BP, deguanylation begin to occur at 2 dose equivalent, and drastically increase until 16 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 32 dose equivalent. In Figure 8(d), corresponding to treatment with BE, deguanylation begin to occur at 16 dose equivalent, and drastically increase until 64 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 128 dose equivalent. In Figure 7(e), corresponding to treatment with 2-IP, deguanylation begin to occur at 2 dose equivalent, and drastically increase until 16 dose equivalent in a dose dependent manner. Complete deguanylation occurred at 64 dose equivalent. Compared to deguanylation rates in ddA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2-IP $\approx$ 2-BP $\approx$ 2,3-dBPe $\geq$ BE.

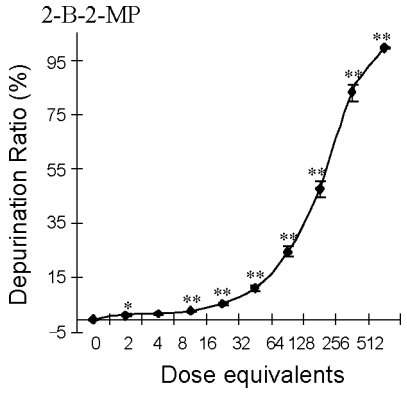

Figure 10. Dose response curves of guanosine with 2-B-2-MP. Dose response reactions were performed with the 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents of 2-B-2-MP for 36 h. Then it was analyzed by HPLC underthe condition mentioned in the materials and methods.

Analysis of dose response deguanylation of guanosine induced by 2-B-2-MP. Figure 10 shows dose response curves of the deguanylation rate of guanosine induced by 2-B-2-MP according to dose at the physiological condition for 36 h. Deguanylation begin to occur at 8 dose equivalent of 2-B-2-MP, and drastically increase until 256 dose equivalent of 2-B-2-MP in a dose dependent manner. Complete deguanylation occurred at 512 dose equivalent of 2-B-2-MP. The rate of deguanylation of guanosine was much lower than that of ddG or dG.

LC-MS depurination analysis of calf thymus DNA induced by 2-B-2-MP. Figure 11 shows LC-MS depurination analysis of calf thymus DNA induced by 128 μL of 2-B-2-MP for 48 h at the physiological condition under the LC-MS condition men-

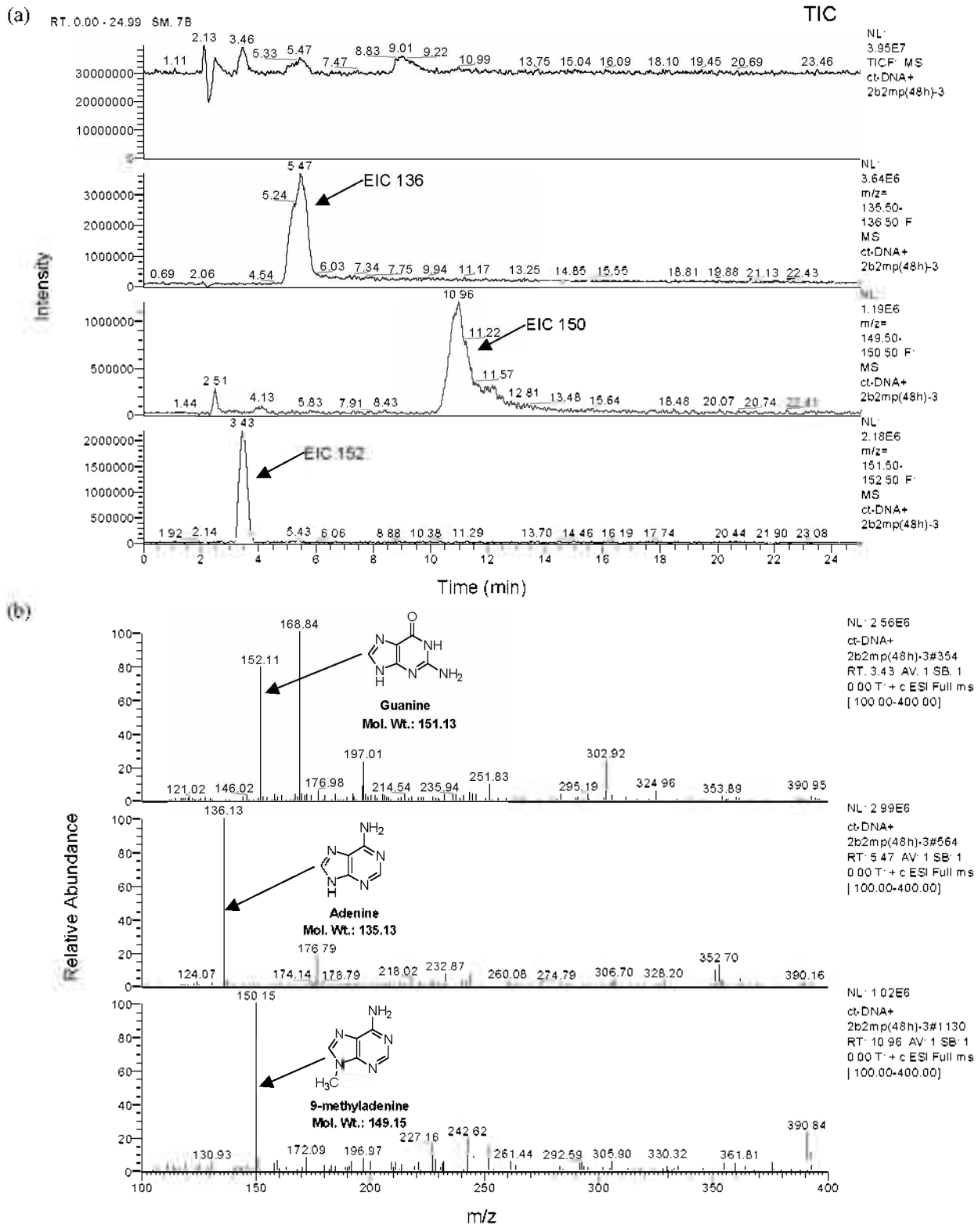

Figure 11. LC-MS analysis of Ct-DNA with 128 μL 2-B-2-MP for 48 h at the physiological condition under the LC-MS condition mentioned in materials and methods. (a) LC-MS chromatogram. RT for Ade, Gua and 9-MA (internal standard) are 5.47, 3.43 and 10.96 min, respectively. (b) ESI MS spectrogram. $[M+H]^+$ peaks for Ade, Gua and 9-MA are 136.13, 152.11 and 150.15, respectively.

tioned in materials and methods. Figure 11(a) shows LC-MS chromatogram of calf thymus DNA treated by 2-B-2-MP under the above condition. In the total ion chromatogram (TIC), it is difficult to identify peaks of corresponding adenine, guanine, and 9-methyl adenine as an internal standard because of the formation of relatively small amounts of products along with many

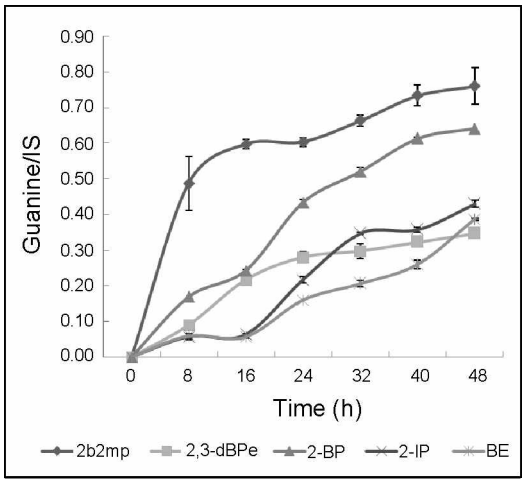

Figure 12. Time response curves of Ct-DNA for deguanylationwith 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Time response reactions were performed with the 128 μL of halogenated alkanes at a time interval of 8 h until 48 h.

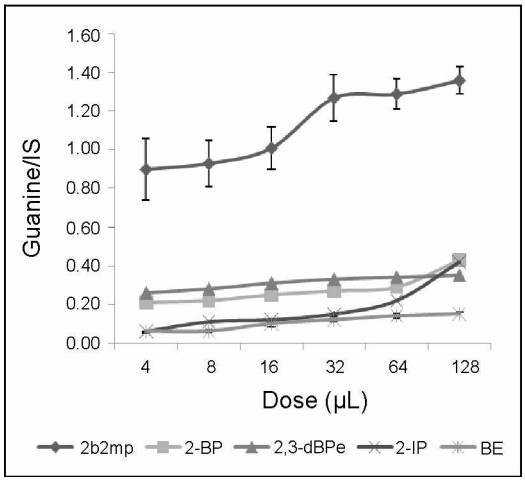

Figure 13. Dose response curves of Ct-DNA for deguanylationwith 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP Dose response reactions were performed with the 2, 4, 8, 16, 32, 64 and 128 μL of halogenated alkanes for 48 h.

impurities. However, in the extracted ion chromatogram (EIC), the peak of retention time at 5.47 corresponding to adenine (EIC 136), the peak of retention time at 10.96 corresponding to 9-methyl adenine (EIC 150), and the peak of retention time at 3.43 corresponding to guanine (EIC 152), were well separated and displayed in a single peak in each EIC chromatogram, which was utilized for the analysis of time and dose response relationship. Figure 11(b) shows ESI MS spectrograms at EIC 152, EIC 136, and EIC 150, which corresponds to guanine, adenine, and 9-methyl adenine, respectively. The $[M+H]^+$ peaks for adenine, guanine, and 9-methyl adenine are 136.13, 152.11, and 150.15, respectively. With the same methods, LC/MS analyses of other haloalkanes were performed, and time and dose response deguanylation relationships induced by haloalkanes were additionally performed.

Analysis of time response deguanylation of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP. Figure 12 shows time response curves of the deguanylation rate of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, according to time. The formation of guanine from calf thymus DNA by treatment with 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP was observed in a time response manner. Compared to deguanylation rates in calf thymus DNA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2-BP > 2,3-dBPe ≈ 2-IP > BE. Compared to the previously reported results,[7] the rate of deguanylation in calf thymus DNA was much lower than that of deadenylation.

Analysis of dose response deguanylation of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP. Figure 13 shows dose response curves of deguanylation rate of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, according to dose. The formation of guanine from calf thymus DNA by treatment with 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP was observed in a dose response manner for 48 h. Compared to deguanylation rates in calf thymus DNA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, the order of deguanylation rate was observed in 2-B-2-MP > 2-BP ≈ 2,3-dBPe > 2-IP ≈ BE. Also the rate of deguanylation in calf thymus DNA was much lower than that of deadenylation.

Discussion

Deguanylation ratios (%) in guanine based-nucleosides were calculated on the basis of the decreased amount of nucleosides in percentage by comparing the integration value of the nucleosides in HPLC using the formula mentioned in materials and methods. Since the solubility of guanine is relatively low at physiological condition, the guanine formed after deguanylation precipitates in a pH 7.4 buffer solution, which decreases the accuracy of deguanylation ratios, if we apply the increasing amount of deguanylated product (guanine) for the determination of deguanylation ratios. Therefore, we applied the decreasing amounts of nucleosides for the determination of deguanylation ratios. Meanwhile, deguanylation ratios in calf thymus DNA were calculated on the basis of the increased amount of guanine by comparing the integration value between increased guanine and the internal standard in EIC from LC-MS/MS using the formula mentioned in materials and methods. Since guanine is soluble in acidic condition, 1 M aqueous HCl was added to dissolve the precipitated guanine before analysis.

In Figures 2, 3, and 4, chromatogram 1 shows the peak of authentic guanine as a reference, chromatogram 2 shows that of guanine based-nucleosides, ddG, dG, or guanosine, along with the peak of internal standard, and chromatogram 3, 4, 5, 6, and 7 show the peaks of products formed after incubation of ddG, dG, or guanosine with 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP for 48 h, respectively. It is evident that the peaks of ddG and dG completely disappeared and the peaks of guanine have appeared after incubation with 2-B-2-MP, 2,3-dBPe, 2-BP, BE, or 2-IP for 48 h (chromatogram 3, 4, 5, 6, and 7 in Figures 2 and 3). However, almost no change of chromatogram was observed after incubation of guanosine with 2,3-dBPe, 2-BP,

BE, and 2-IP for 48 h (chromatogram 4, 5, 6, and 7 in Figure 4). Meanwhile, in chromatogram 3 in Figure 4, which corresponds to treatment of 2-B-2-MP and guanosine, the peak of guanosine completely disappeared, and the peak of guanine appeared, which indicates that deguanylation only occurred when guanosine was incubated with excess amount of 2-B-2-MP for 48 h at the physiological condition, whereas almost no deadenylation was observed for adenosine by treatment of 2-B-2-MP at the same condition.[7]

Time and dose response reaction with ddG and dG by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP, and also with guanosine by 2-B-2-MP indicate that deguanylation increased in a time and dose dependent manner (Figures 5, 6, 7, 8, 9, and 10). According to time (Figures 5 and 6), the order of deguanylation rate among halogenated alkanes was observed as 2-B-2-MP > 2,3-dBPe > 2-BP > BE ≈ 2-IP in both ddG and dG. Especially, deguanylation rate of 2-B-2-MP was much faster than that of 2-BP, BE, and 2-IP. Comparing deguanylation rates of ddG, dG, and guanosine, the rate of ddG was faster than dG, followed by guanosine.[18-20] According to dose (Figures 8 and 9), the order of deguanylation rate among halogenated alkanes was observed as 2-B-2-MP > 2-IP ≈ 2-BP ≈ 2,3-dBPe ≥ BE in both ddA and dA. Similary, deguanylation rate of 2-B-2-MP was much faster than that of 2-BP, BE and 2-IP, which indicated that the rate of deguanylation in tertiary halide was highest, followed by secondary and primary halide.

Time and dose response reaction with calf thymus DNA by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, and 2-IP indicated that deguanylation increased in a time and dose dependent manner (Figures 12 and 13). The order of deguanylation rate was observed in 2-B-2-MP > 2-BP > 2,3-dBPe ≈ 2-IP > BE in time response reactions, and 2-B-2-MP > 2-BP ≈ 2,3-dBPe > 2-IP ≈ BE in dose response reactions. Comparing rates of deadenylation and deguanylation in calf thymus DNA, the rate of deadenylation was much higher than that of deguanylation.[7] This result may be explained by the fact that guanine is bound to cytosine with three H-bonds whereas adenine is bound to thymine with two H-bonds in double stranded DNA, which makes adenine easier to detach from the chain.[3]

Conclusion

Among the sixteen halogenated alkanes, we observed that five halogenated alkanes (2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP) induced deguanylation of ddG and dG, and 2-B-2-MP also induced deguanylation of guanosine as a probable mechanism of toxicity. 2-B-2-MP showed the highest deguanylation rate compared to the other halogenated alkanes, which indicates that tertiary alkyl halides display greater reactivity than secondary alkyl halides, followed by primary alkyl halides. It was also observed that ddG showed the highest reactivity, followed by dG and guanosine. Although the exact mechanism of deguanylation is not known, our results show that the hydroxyl group in the sugar moiety of the nucleosides plays an important role regarding the rate of deguanylation. The study which aims to elucidate the mechanism of deguanylation induced by halogenated alkanes is in progress.

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