Deadenylation of Adenine Based-Nucleosides and Calf thymus DNA Induced by Halogenated Alkanes at the Physiological Condition

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Massive deadenylation of adenine based-nucleosides induced by halogenated alkanes at the physiological condition have been observed. For the study of deadenylation effects by the different substituents and/or functionality in halogenated alkanes, diverse kinds of halogenated alkanes were incubated with adenine based-nucleosides (ddA, dA and adenosine) 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 deadenylation of nucleosides by 2-bromo-2-methylpropane, 2,3-dibromopropene, 2-bromopropane, bromoethane and 2-iodopropane. The order of deadenylation 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 deadenylation in adenine based-nucleosides induced by 2-bromo-2-methylpropane, 2,3-dibromopropene, 2-bromopropane, bromoethane and 2-iodopropane at the physiological condition were investigated. In addition, deadenylation 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: Deadenylation, Depurination, Halogenated alkanes, Adenine base-nucleosides, Calf thymus DNA

Introduction

The depurination or deadenylation of nucleic acids, the release of purine or adenine bases, respectively, from nucleic acids by hydrolysis of the *N*-glycosidic bond (Figure 1), gives rise to alterations of the cell genome.[1,2] Deadenylation as well as depurination occurs spontaneously with a relatively high frequency under physiological conditions. However, it has been reported that depurination is accelerated at low pH and high temperatures, and guanine is released slightly more rapidly than adenine.[3] The rate of depurination is accelerated by 10^6-old by alkylation, and is four-fold greater in single-stranded DNA than double-stranded DNA.[3] The apurinic sites resulting from depurination have been shown lethality[4,5] and base substitution errors.[5] Depurination (deadenylation) could be one of the promising novel mechanisms of toxicity induced by the short chain halogenated alkanes. It has been reported that formation of adduct on DNA leads to depurination.[5] For instance, *N*-nitrosation at the *N*-7 atom of guanine or adenine residues and/or the *N*-3 atom of adenine imparts a destabilizing positive charge on the purine ring system which is neutralized by cleavage of the *N*-glycosidic bond giving rise to depurination.

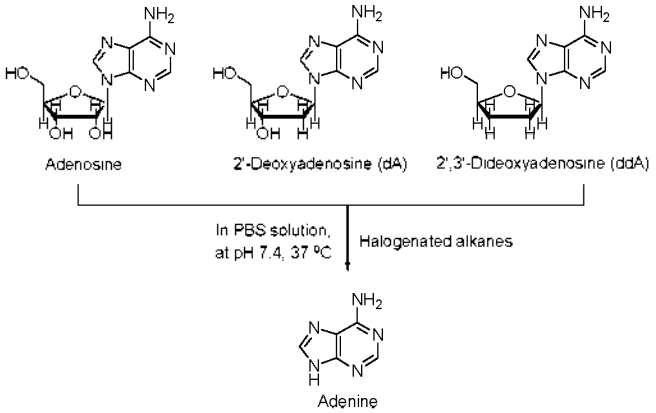


Figure 1. Scheme of deadenylation, the release of adenine bases from nucleic acids by hydrolysis of the *N*-glycosidic bond. Adenine based-nucleosides were incubated with 16 halogenated alkanes at the physiological condition (pH 7.4, 37 °C) for a certain period and analyzed by HPLC and confirmed by LC-MS 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 when the nucleosides and calf thymus DNA were incubated with an excess amount of 2-bromopropane (2-BP) at the physiological condition.[6] Regarding the rate of depurination, dideoxy showed the highest reactivity followed by deoxy and then ribose nucleosides. We also found that 2-BP showed faster rate of depurination than 1-BP, which indicated that secondary alkyl halide displayed greater reactivity than primary alkyl halide regarding the rate of depurination.[7] It would be very interesting to investigate depurination of nucleosides induced by halogenated alkanes according to the difference of substituents of and/or functionality in halogenated alkanes, which may provide valuable information for the mechanism of toxicity of halogenated alkanes.

In connection with previous studies, diverse kinds of halogenated alkanes were incubated with adenine based-nucleosides (ddA, dA and adenosine) 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 deadenylation effects by the different substituents of and/or functionality in halogenated alkanes. Short-chain halogenated alkanes which we utilized for the study of deadenylation of adenine based-nucleosides, have been widely used industrially for chemical intermediates, extraction solvents, degreasing compounds, copolymer cross-linking agents, or have been reported to be

mutagenic and carcinogenic.[7-17] Among the sixteen different halogenated alkanes, we observed massive deadenylation 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'-deoxyadenosine (99 - 100%), adenosine (99%), calf-thymus DNA (deoxyribonucleic acid sodium salt, from Calf thymus), 5-flurouridine, phosphate buffered saline (pH 7.4) and ammonium acetate (99.995+%) were purchased from Sigma Aldrich Co. (ST. Louis, MO). 2',3'-Dideoxyadenosine and 2-bromo-2-methylpropane (≥ 97%) were obtained from Berry & Associates Inc and Fluka, respectively. 9-Methyl adenine (9-MA) was synthesized in our lab. HPLC grade acetonitrile and methanol was purchased from World Science, Korea. All the other chemicals, if not mentioned, were also obtained from Sigma Aldrich Co. (ST. Louis, MO).

Preliminary reactions. One mg of nucleoside (ddA, dA or adenosine) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Ten μL (5 mg in 1 mL PBS) of 5-flurouridine (5-FUri) 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 (ddA and dA) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial separately. Ten μL (5 mg in 1 mL PBS) of 5-flurouridine (5-FUri) was added as an internal standard. It was then incubated with an excess amount (512 equivalents) of 2,3-dBPe, 2-BP, BE and 2-IP for ddA and dA, 32 equivalents of 2-B-2-MP for ddA, 64 equivalents of 2-B-2-MP for dA, respectively, at 37 °C. About 10 μL of samples were withdrawn at certain time interval and analyzed by HPLC until 100% deadenylation occurred. All the reactions were repeated for three times.

Dose response reaction. One mg of nucleoside (ddA and dA) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial separately. Ten μL (5 mg in 1 mL PBS) of 5-flurouridine (5-FUri) 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% deadenylation occurred. For incubation with 2-B-2-MP, different amounts (0, 0.5, 1, 2, 4, 8, 16 and 32 equivalents for ddA), (0, 0.5, 1, 2, 4, 8, 16, 32 and 64 equivalents for dA) 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 the condition mentioned below.

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

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

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

$$\text{Deadenylation ratio} = \text{adenine} / \text{IS}$$

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% and 5% 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 and adenine- based nucleosides, respectively.

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.

Result

The deadenylation effect of the sixteen halogenated alkanes on adenine based-nucleosides was analyzed by HPLC and further confirmed by LC-MS. Table 1 shows the list of halo-

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

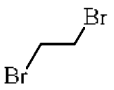
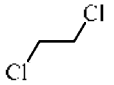
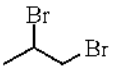
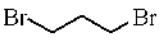
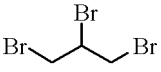
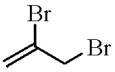
No.	Halogenated alkanes	Structure	Mol. wt.	Density
1	Iodomethane (IM)		141.94	2.28
2	Bromoethane (BE)		108.97	1.46
3	Iodoethane (IE)		155.97	1.93
4	1,2-dibromoethane (1,2-dBE)		187.86	2.18
5	1,2-dichloroethane (1,2-dCE)		98.96	1.256
6	1-bromopropane (1-BP)		122.99	1.354
7	2-bromopropane (2-BP)		122.99	1.311
8	1-chloropropane (1-CP)		78.54	0.892
9	2-chloropropane (2-CP)		78.54	0.86
10	1-iodopropane (1-IP)		169.99	1.743
11	2-iodopropane (2-IP)		169.99	1.703
12	1,2-dibromopropane (1,2-dBP)		201.89	1.937
13	1,3-dibromopropane (1,3-dBP)		201.89	1.989
14	2-bromo-2methylpropane (2-B-2-MP)		137.03	1.22
15	1,2,3-tribromopropane (1,2,3-tBP)		280.78	2.398
16	2,3-dibromopropene (2,3-dBPe)		199.8	1.934

Table 2. Preliminary reaction of ddA, dA and adenosine with an excess amount of halogenated alkanes at the physiological condition for 48 h.

S. No.	halogenated alkanes	DR (%) in ddA	DR (%) in dA	DR (%) in adenosine
1	Iodomethane	*	*	*
2	Bromoethane	**100.00**	**100.00**	−0.13
3	Iodoethane	−1.90	9.43	5.54
4	1,2-dibromoethane	−2.26	1.34	2.27
5	1,2-dichloroethane	−1.34	2.34	0.97
6	1-bromopropane	−1.62	1.37	1.74
7	2-bromopropane	**100.00**	**100.00**	4.60
8	1-chloropropane	0.94	1.24	2.34
9	2-chloropropane	−1.82	2.72	1.07
10	1-iodopropane	1.44	2.30	0.35
11	2-iodopropane	**100.00**	**100.00**	1.48
12	1,2-dibromopropane	−0.66	0.96	1.96
13	1,3-dibromopropane	−2.37	−0.23	−0.44
14	2-bromo-2methylpropane	**100.00**	**100.00**	*
15	1,2,3-tribromopropane	0.31	−0.22	−0.31
16	2,3-dibromopropene	**100.00**	**100.00**	8.10

One mg of nucleoside (ddA, dA and adenosine) was dissolved in 1 mL phosphate buffered saline solution (PBS) in 5 mL vial. Ten μL (5 mg in 1 mL PBS) of 5-flurouridine was added as an internal standard. It was incubated with excess amount (512 equivalents) of halogenated alkanes at the physiological condition for 48 h, which was analyzed by HPLC and further confirmed by LC-MS and UV. DR: deadenylation rate. *Only small amount of deadenylated product (adenine) was observed with many adducts formation.

genated alkanes with chemical structure, molecular weight and density, which were treated with adenine based-nucleosides. From the preliminary reaction, it was found that 100% deadenylation was occurred in ddA and dA 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 was described in Figure 2 and 3. However, ddA and dA were not affected by the other halogenated alkanes. In addition, almost no change was observed for adenosine by treatment of all of the sixteen halogenated alkanes.

Analysis of deadenylation of ddA 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 deadenylation of ddA 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 adenine at retention time of 6.20 min, and chromatogram 2 indicates the mixture of ddA and 5-fuorouridine (5-FUri) utilized as an internal standard at retention times of 16.51 min and 4.69 min, respectively. Adenine, ddA and 5-fuorouridine 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 ddA 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 indicated the peak of retention time at 16.51 min which

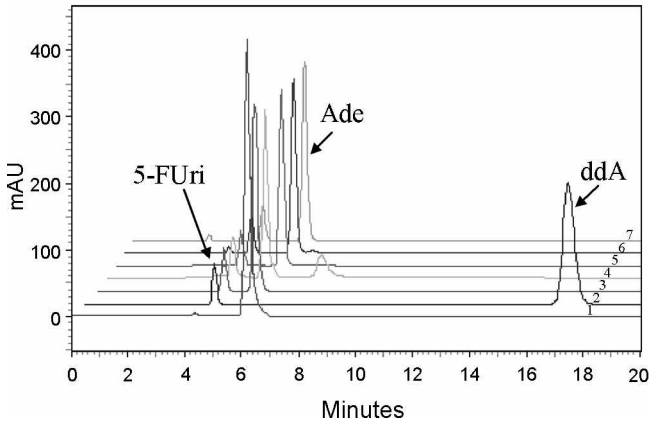


Figure 2. HPLC chromatogram of (1) adenine (Ade), (2) ddA + 5-FUri, (3) ddA + 5-FUri + 2-B-2-MP (48 h), (4) ddA + 5-FUri + 2,3-dBPe (48 h), (5) ddA + 5-FUri + 2-BP (48 h), (6) ddA + 5-FUri + BE (48 h), (7) ddA + 5-FUri + 2-IP (48 h). Retention time for 5-FUri, Ade and ddA were 4.69, 6.20 and 16.51 min, respectively, under the HPLC condition mentioned in materials and methods.

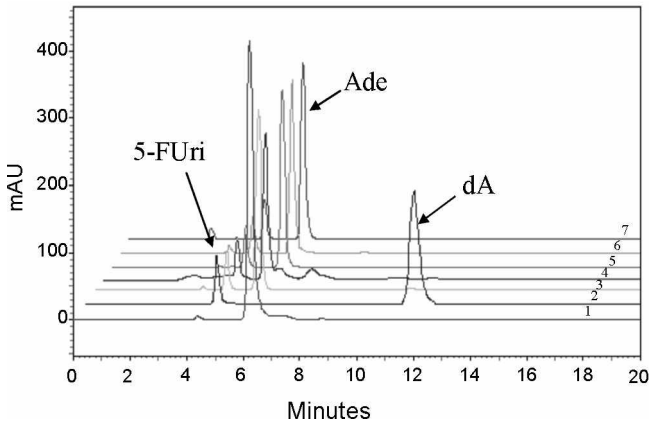


Figure 3. HPLC chromatogram of (1) Ade, (2) dA + 5-FUri, (3) dA + 5-FUri + 2-B-2-MP (48 h), (4) dA + 5-FUri + 2,3-dBPe (48 h), (5) dA + 5-FUri + 2-BP (48 h), (6) dA + 5-FUri + BE (48 h), (7) dA + 5-FUri + 2-IP (48 h). Retention time for 5-FUri, Ade and dA were 4.69, 6.20 and 11.40 min, respectively, under the HPLC condition mentioned in materials and methods.

corresponds to ddA completely disappeared and a peak of retention time at 6.20 min corresponding to adenine newly appeared. These results indicated that complete deadenylation occurred when ddA 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 deadenylation of dA 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 deadenylation of dA 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 adenine (Ade) at retention time of 6.20 min, and chromatogram 2 indicates the mixture of dA and 5-fuorouridine (5-FUri) utilized as an internal standard at retention times of 11.40 min and 4.69 min, respectively. Adenine, dA and 5-fuorouridine 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 dA 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 indicated the peak of retention time at 11.40 min which corresponds to dA completely disappeared and a peak of retention time at 6.20 min corresponding to adenine newly appeared. These results indicated that complete deadenylation occurred when dA 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 deadenylation of adenosine 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 deadenylation of adenosine 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 adenine (Ade) at retention time of 6.20 min, and chromatogram 2 indicates the mixture of adenosine and 5-fuorouridine (5-FUri) utilized as an internal standard at retention times of 11.34 min and 4.69 min, respectively. Adenine, adenosine and 5-fuorouridine 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 adenine 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,

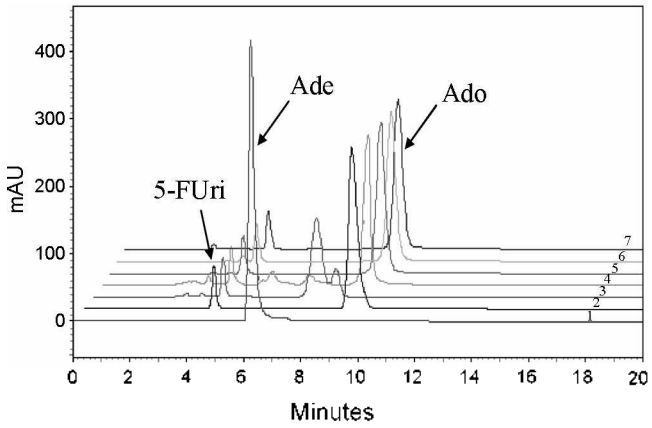


Figure 4. HPLC chromatogram of (1) Ade, (2) adenosine (Ado) + 5-FUri, (3) Ado + 5-FUri + 2-B-2-MP (48 h), (4) Ado + 5-FUri + 2,3-dBPe (48 h), (5) Ado + 5-FUri + 2-BP (48 h), (6) Ado + 5-FUri + BE (48 h), (7) Ado + 5-FUri + 2-IP (48 h). Retention time for 5-FUri, Ade and Ado were 4.69, 6.20 and 11.34 min, respectively, under the HPLC condition mentioned in materials and methods.

respectively, which informs almost no change in amount of adenosine and no production of adenine at that condition by treatment of 2,3-dBPe, 2-BP, BE and 2-IP. In chromatogram 3, which corresponds to treatment of 2-B-2-MP, the peak of adenosine completely disappeared, and new peaks at retention

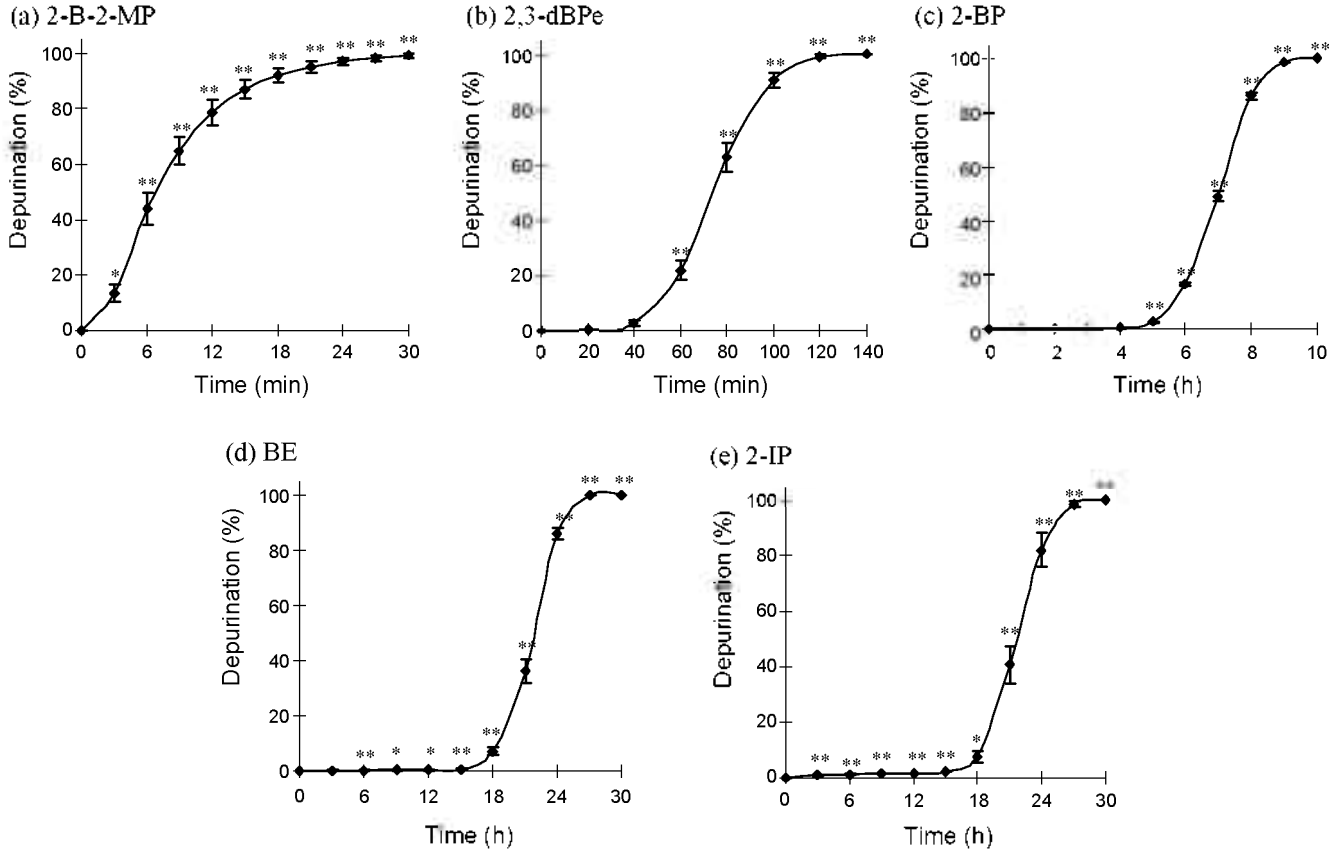


Figure 5. Time response curves of ddA with (a) 2-B-2-MP, (b) 2,3-dBPe, (c) 2-BP, (d) BE and (e) 2-IP. Time response reactions were performed with 32 equivalents of 2-B-2-MP and 512 equivalents of 2,3-dBPe, 2-BP, BE and 2-IP, respectively until the time at which 100% deadenylation occurred. Then it was analyzed by HPLC following the condition mentioned in the materials and methods.

time of 8.61 min and 9.43 min appeared which may indicate the formation of adducts with adenosine and 2-B-2-MP. However, no formation of adenine was observed in chromatogram 3. The results indicated that no deadenylation occurred when adenosine was incubated with excess amount of 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP for 48 h at the physiological condition.

In Figures 2, 3 and 4, any change of amount in 5-fuorouridine during incubation of ddA, dA and adenosine with 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP was not observed, which indicates the concentration of 5-fuorouridine was consistently well maintained and 5-fuorouridine was not affected by 2-B-2-MP, 2,3-dBPe, 2-BP, BE, 2-IP or nucleosides.

Analysis of time response deadenylation of ddA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 5 shows time response curves of deadenylation rate of ddA 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 deadenylation after incubation of ddA and 512 dose equivalent of 2-B-2-MP at the physiological condition at a time interval of 6 min. Deadenylation begin to occur at 1 min, and increase until 30 min in time dependent manner. Complete deadenylation occurred at 30 min. In Figure 5(b), corresponding to treatment with 2,3-dBPe, deadenylation begin to occur at 40 min, and drastically increase until 100 min in time dependent manner. Complete deadenylation occurred at 120 min. In Figure 5(c), corresponding to treatment with 2-BP, deadenylation begin to occur at 5 h, and drastically increase until 9 h in time dependent manner. Complete deadenylation occurred at 10 h. In Figure 5(d), corresponding to treatment with BE, deadenylation begin to occur at 15 h, and drastically increase until 24 h in time dependent manner. Complete deadenylation occurred at 27 h. In Figure 5(e), corresponding to treatment with 2-IP, deadenylation begin to occur at 15 h, and drastically increase until 27 h in time dependent manner. Complete deadenylation occurred at 30 h. Compared to deadenylation rates in ddA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP > 2,3-dBPe > 2-BP > BE ≈ 2-IP. Especially, the deadenylation 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 deadenylation of dA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 6 shows time response curves of deadenylation rate of dA 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 deadenylation after incubation of dA and 512 dose equivalent of 2-B-2-MP at the physiological condition at a time interval of 20 min. Deadenylation begin to occur at 10 min, and drastically increase until 80 min in time dependent manner. Complete deadenylation occurred at 120 min. In Figure 6(b), corresponding to treatment with 2,3-dBPe, deadenylation begin to occur at 2 h, and drastically increase until 6 h in time dependent manner. Complete deadenylation occurred at 7 h.

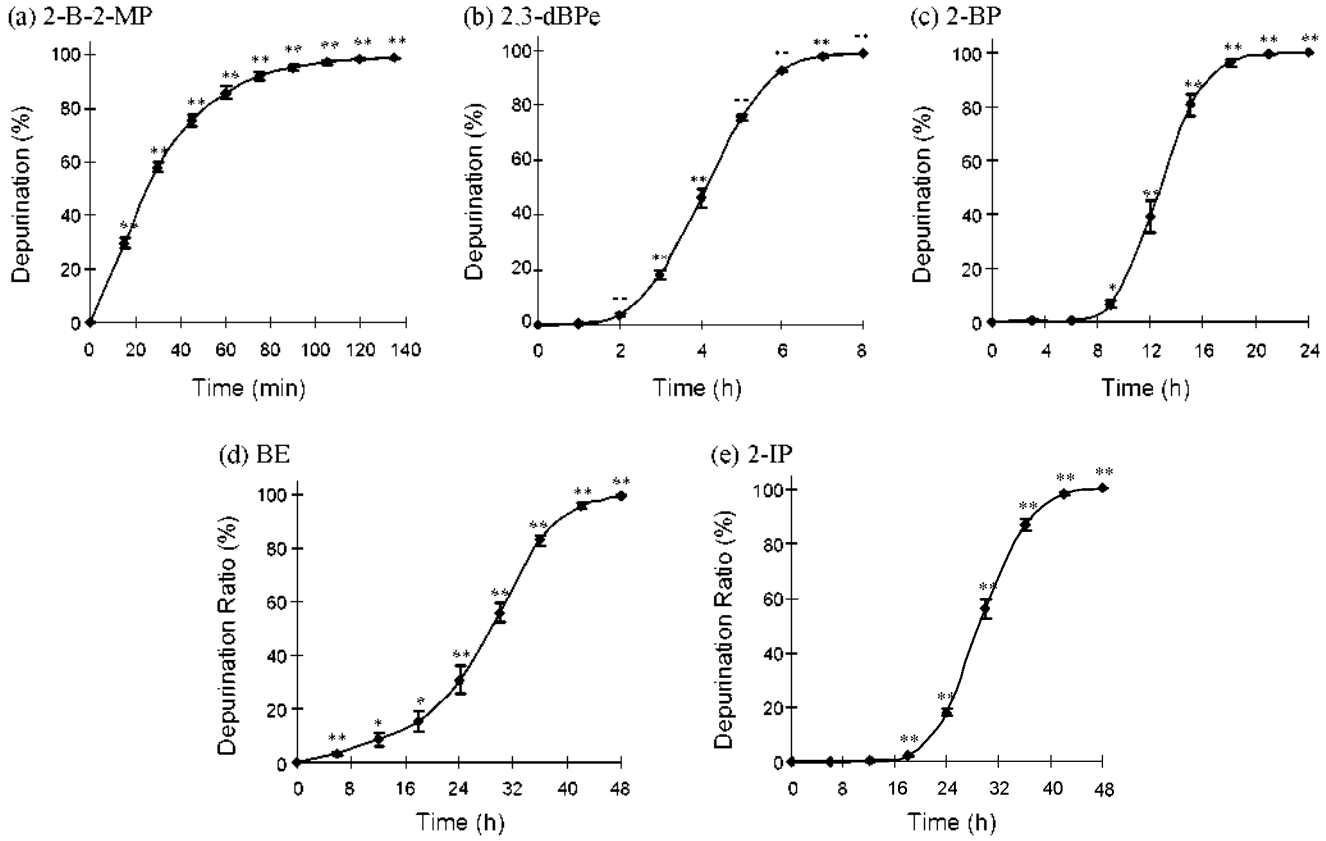


Figure 6. Time response curves of dA with (a) 2-B-2-MP, (b) 2,3-dBPe, (c) 2-BP, (d) BE and (e) 2-IP. Time response reactions were performed with 64 equivalents of 2-B-2-MP and 512 equivalents of 2,3-dBPe, 2-BP, BE and 2-IP, respectively until the time at which 100% deadenylation occurred. Then it was analyzed by HPLC following the condition mentioned in the materials and methods.

In Figure 6(c), corresponding to treatment with 2-BP, deadenylation begin to occur at 8 h, and drastically increase until 18 h in time dependent manner. Complete deadenylation occurred at 20 h. In Figure 6(d), corresponding to treatment with BE, deadenylation begin to occur at 8 h, and drastically increase until 40 h in time dependent manner. Complete deadenylation occurred at 48 h. In Figure 6(e), corresponding to treatment with 2-IP, deadenylation begin to occur at 16 h, and drastically increase until 40 h in time dependent manner. Complete deadenylation occurred at 48 h. Compared to deadenylation rates in dA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP > 2,3-dBPe > 2-BP > BE > 2-IP. Especially, deadenylation rate of 2-B-2-MP was much faster than that of 2-BP, BE and 2-IP. Comparing deadenylation rates of ddA and dA, the rate of ddA was faster than dA.

Analysis of dose response deadenylation of ddA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 7 shows dose response curves of deadenylation rates of ddA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP according to dose. Figure 7(a) indicates dose response curve of deadenylation after incubation of ddA and different dose equivalents of 2-B-2-MP at the physiological condition for 24 h. Deadenylation begin to occur at 0.5 dose equivalent of 2-B-2-MP, and drastically increase until 16 dose equivalent of 2-B-2-MP in dose dependent manner. Complete deadenylation occurred at 32 dose equivalent of 2-B-2-MP. In Figure 7(b), corresponding to treatment with 2,3-dBPe, deadenylation begin to occur at 32 dose equivalent, and drastically increase until 128 dose equivalent in dose dependent manner. Complete deadenylation occurred at 256 dose equivalent. In Figure 7(c), corresponding to treatment with 2-BP, deadenylation begin to occur at 4 dose equivalent, and drastically increase until 16 dose equivalent in dose dependent manner. Complete deadenylation occurred at 32 dose equivalent. In Figure 7(d), corresponding to treatment with BE, deadenylation begin to occur at 16 dose equivalent, and drastically increase until 64 dose equivalent in dose dependent manner. Complete deadenylation occurred at 128 dose equivalent. In Figure 7(e), corresponding to treatment with 2-IP, deadenylation begin to occur at 2 dose equivalent, and drastically increase until 8 dose equivalent in dose dependent manner. Complete deadenylation occurred at 64 dose equivalent. Compared to deadenylation rates in ddA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP > 2-IP ≈ 2-BP ≈ 2,3-dBPe ≈ BE.

Analysis of dose response deadenylation of dA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 8 shows dose response curves of deadenylation rates of dA 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 deadenylation after incubation of dA and different dose equivalents of 2-B-

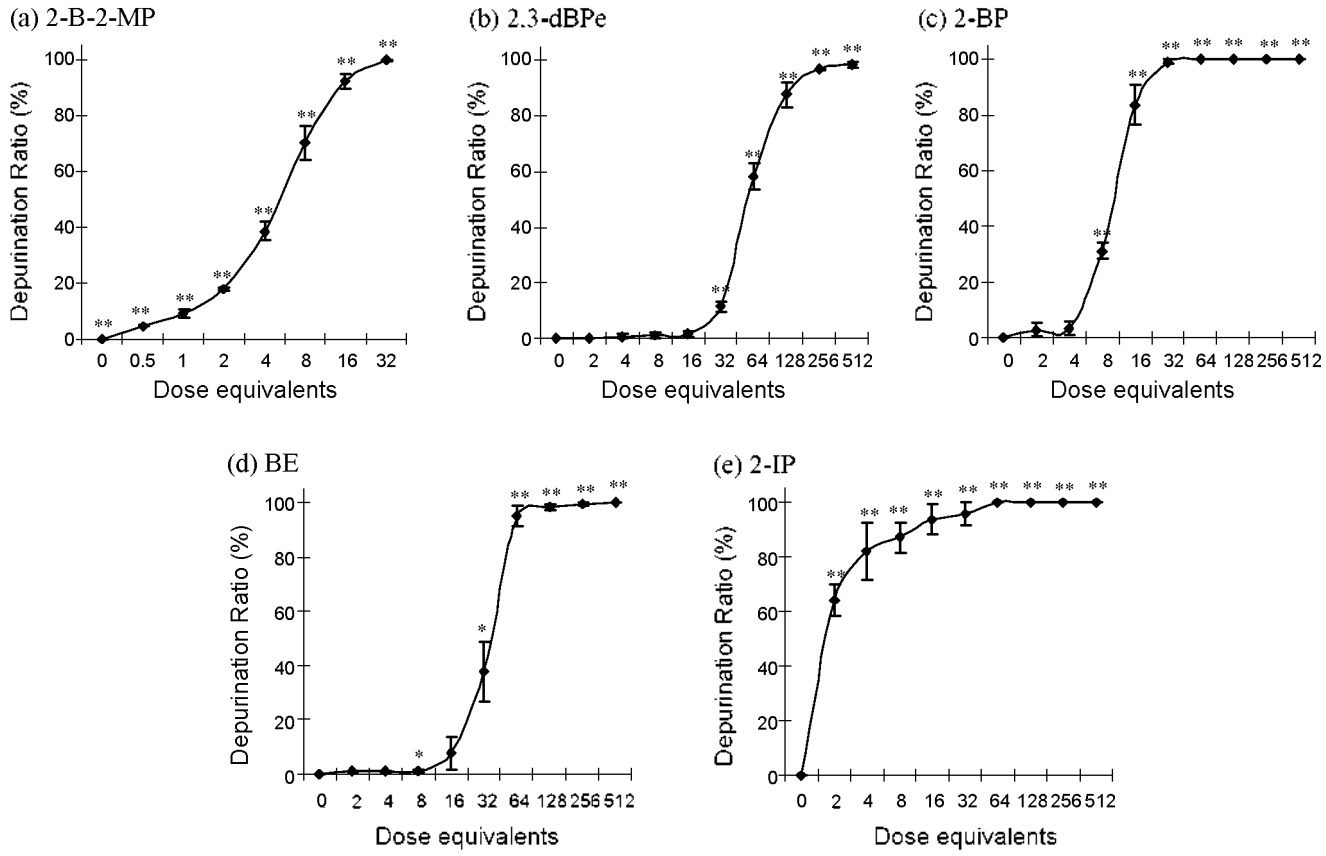


Figure 7. Dose response curves of ddA with (a) 2-B-2-MP, (b) 2,3-dBPe, (c) 2-BP, (d) BE and (e) 2-IP. Dose response reactions were performed with the 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents of halogenated alkanes for a fixed time at which 100% deadenylation occurred. Then it was analyzed by HPLC under the condition mentioned in the materials and methods.

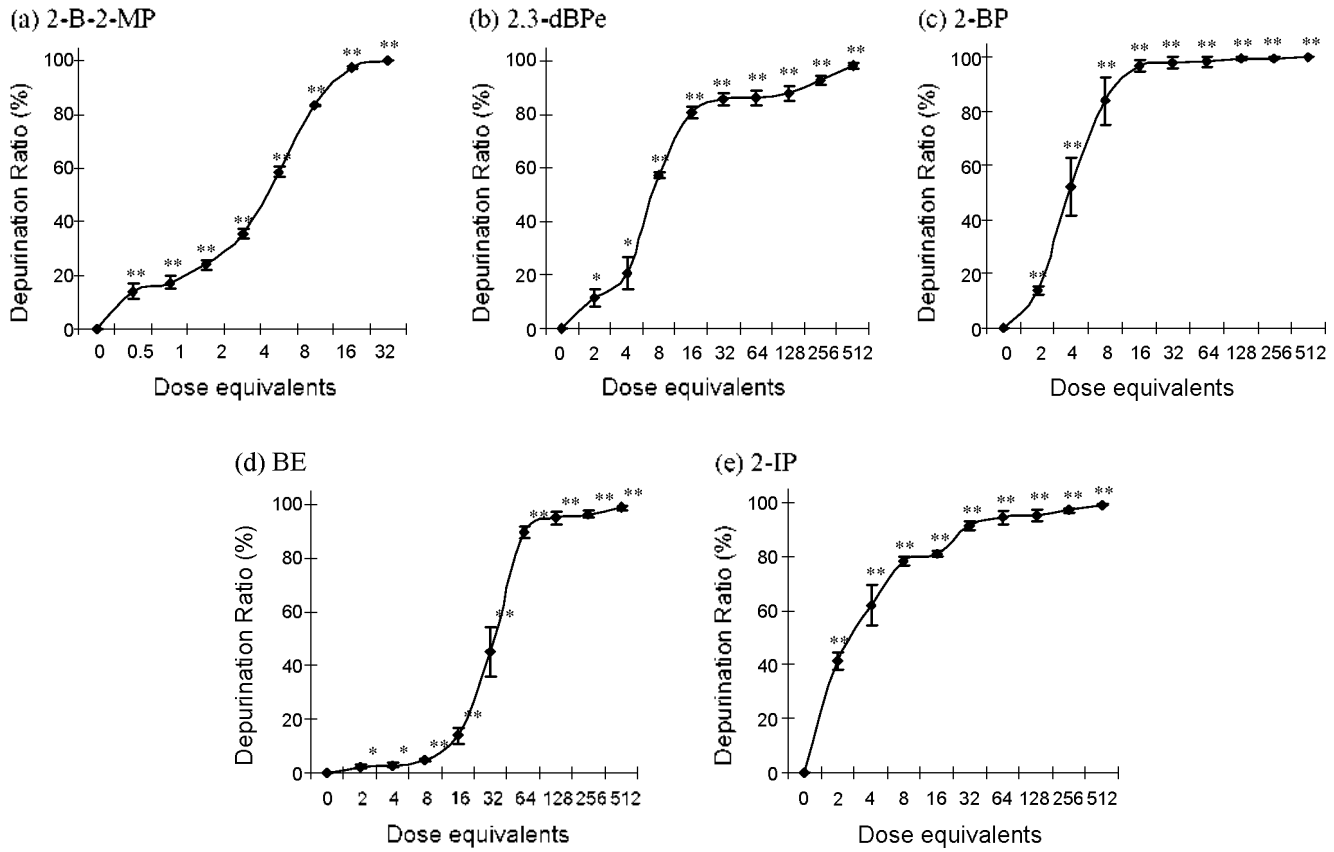


Figure 8. Dose response curves of dA with (a) 2-B-2-MP, (b) 2,3-dBPe, (c) 2-BP, (d) BE and (e) 2-IP. Dose response reactions were performed with the 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 equivalents of halogenated alkanes for a fixed time at which 100% deadenylation occurred. Then it was analyzed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

2-MP at the physiological condition for 24 h. Deadenylation begin to occur at 0.5 dose equivalent of 2-B-2-MP, and drastically increase until 32 dose equivalent of 2-B-2-MP in dose dependent manner. Complete deadenylation occurred at 64 dose equivalent of 2-B-2-MP. In Figure 8(b), corresponding to treatment with 2,3-dBPe, deadenylation begin to occur at 2 dose equivalent, and drastically increase until 16 dose equivalent in dose dependent manner. Complete deadenylation occurred at 128 dose equivalent. In Figure 8(c), corresponding to treatment with 2-BP, deadenylation begin to occur at 2 dose equivalent, and drastically increase until 16 dose equivalent in dose dependent manner. Complete deadenylation occurred at 32 dose equivalent. In Figure 8(d), corresponding to treatment with BE, deadenylation begin to occur at 8 dose equivalent, and drastically increase until 64 dose equivalent in dose dependent manner. Complete deadenylation occurred at 128 dose equivalent. In Figure 8(e), corresponding to treatment with 2-IP, deadenylation begin to occur at 2 dose equivalent, and drastically increase until 8 dose equivalent in dose dependent manner. Complete deadenylation occurred at 64 dose equivalent. Compared to deadenylation rates in ddA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP > 2-BP ≈ 2-IP ≈ 2,3-dBPe > BE.

LC-MS deadenylation analysis of calf thymus DNA induced by 2-B-2-MP. Figure 9 shows LC-MS deadenylation 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 mentioned in materials and methods. Figure 9(a) shows LC-MS chromatogram of calf thymus DNA treated by 2-B-2-MP under the above condition. In total ion chromatogram (TIC), it is difficult to identify peaks of corresponding adenine, guanine and 9-methyl adenine as an internal standard because of formation of relatively small amounts of products along with many impurities. However, in 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 9(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 analysis of other haloalkanes were performed, and time and dose response deadenylation relationships induced by haloalkanes were also performed.

Analysis of time response deadenylation of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 10 shows time response curves of deadenylation rate of calf

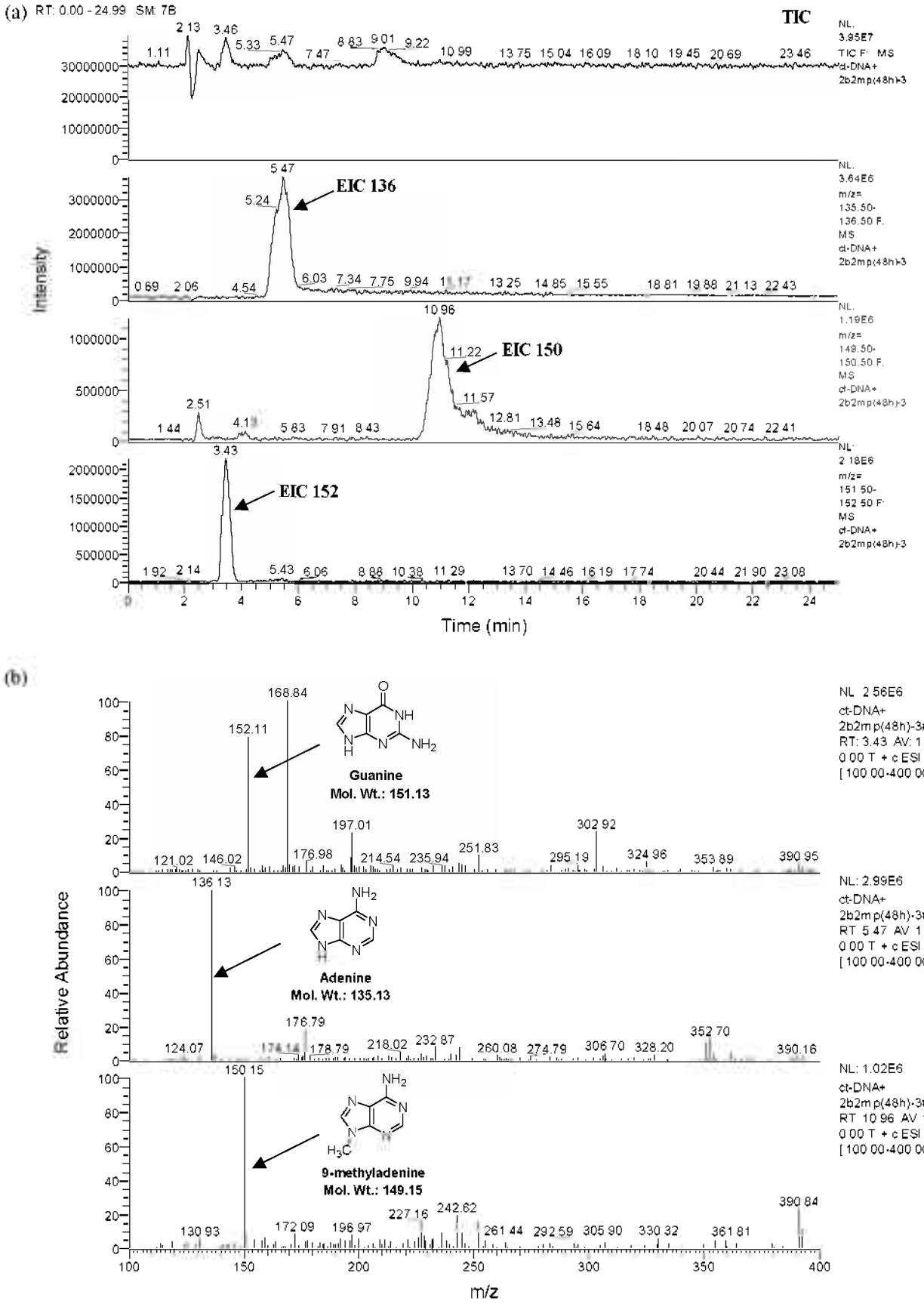


Figure 9. LC-MS analysis of calf thymus DNA with 128 μL of 2-B-2-MP for 48 h at the physiological condition under the LC-MS condition mentioned in materials and methods. (a) LC-MS chromatogram. Retention time for Ade, Gua and 9-MA are 5.47, 3.43 and 10.96 min, respectively. (b) ESI MS spectrogram. $[M+H]^+$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

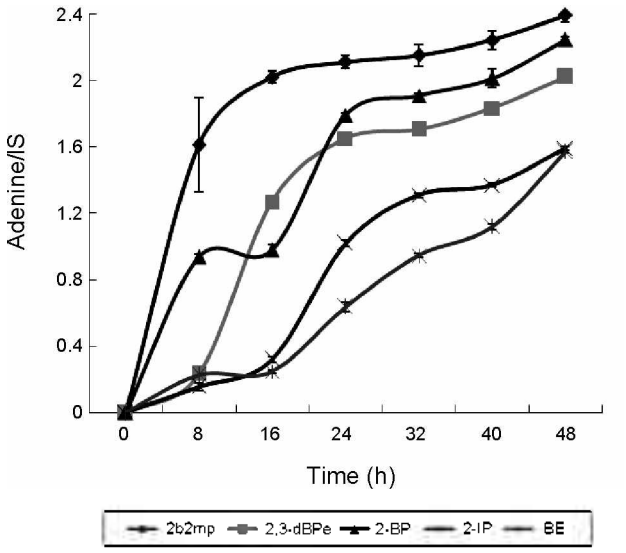


Figure 10. Time response deadenylation curves of calf thymus DNA with 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Time response reactions were performed with the 128 μL of halogenated alkanes at a time interval of 8 h until 48 h.

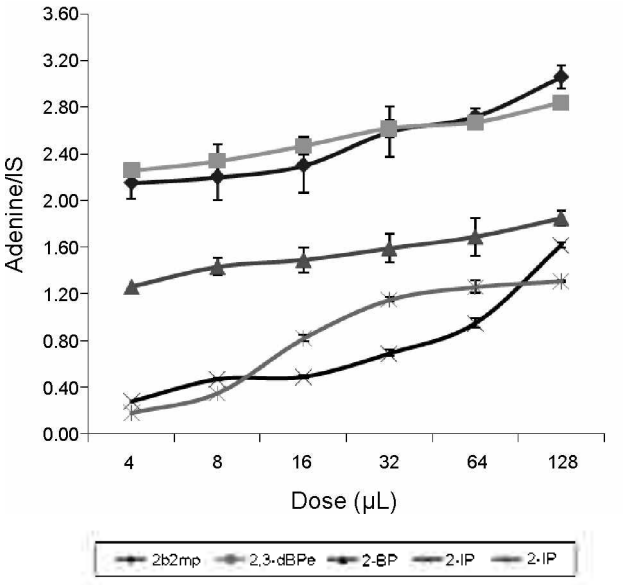


Figure 11. Dose response deadenylation curves of calf thymus DNA with 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Dose response reactions were performed with the 2, 4, 8, 16, 32, 64 and 128 μL of halogenated alkanes for 48 h.

thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP according to time. The formation of adenine from calf thymus DNA by treatment with 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP was observed in time response manner. Compared to deadenylation rates in calf thymus DNA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP > 2-BP ≈ 2,3-dBPe > 2-IP ≈ BE.

Analysis of dose response deadenylation of calf thymus DNA induced by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP. Figure 11 shows dose response curves of deadenylation 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 adenine from calf thymus DNA by treatment with 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP was observed in dose response manner for 48 h. Compared to deadenylation rates in calf thymus DNA among 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP, the order of deadenylation rate was observed in 2-B-2-MP ≈ 2-BP > 2,3-dBPe > 2-IP ≈ BE.

Discussion

Deadenylation ratios (%) in adenine 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.[7] Similarly, deadenylation ratios in calf thymus DNA were calculated on the basis of the increased amount of adenine by comparing the integration value between increased adenine and internal standard in EIC from LC-MS/MS using the formula mentioned in materials and methods.

In Figures 2, 3 and 4, chromatogram 1 shows the peak of authentic adenine as a reference, chromatogram 2 shows that of adenine based-nucleosides, ddA, dA or adenosine, along with the peak of the internal standard, chromatogram 3, 4, 5, 6 and 7 show the peaks of products formed after incubation of ddA, dA or adenosine 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 ddA and dA completely disappeared and the peaks of adenine 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 adenosine 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 adenosine, the peak of adenosine completely disappeared, and new peaks at retention time of 8.61 min and 9.43 min appeared which probably indicate the formation of adducts with adenosine and 2-B-2-MP. However, no formation of adenine was observed in chromatogram 3. These results indicated that 100% deadenylation occurred in ddA and dA, ddG by 2-B-2-MP, 2,3- dBPe, 2-BP, BE and 2-IP, but practically no deadenylation occurred in adenosine by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP (Table 2). Time and dose response reaction with ddA and dA by 2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP indicated that deadenylation increased in time and dose dependent manner (Figures 5, 6, 7 and 8). According to time (Figures 5 and 6), the order of deadenylation rate among halogenated alkanes was observed as 2-B-2-MP > 2,3-dBPe > 2-BP > BE ≥ 2-IP in both ddA and dA. Especially, deadenylation rate of 2-B-2-MP was much faster than that of 2-BP, BE and 2-IP. Of deadenylation rates between ddA and dA, the rate of ddA was faster than dA.[18,19,20] According to dose (Figures 7 and 8), the order of deadenylation rate among halogenated alkanes was observed as 2-B-2-MP > 2-BP ≈ 2-IP ≈ 2,3-dBPe ≥ BE in both ddA and dA. Similary, deadenylation rate of 2-B-2-MP was much faster than that of 2-BP, BE and 2-IP, which indicated that the rate of deadenylation 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 deadenylation increased in time and dose dependent manner (Figures 10 and 11). The order of deadenylation rate was observed in 2-B-2-MP ≥ 2-BP ≈ 2,3-dBPe ≥ 2-IP ≈ BE in both time and dose response reactions.

Conclusion

Among the sixteen halogenated alkanes, we observed five halogenated alkanes (2-B-2-MP, 2,3-dBPe, 2-BP, BE and 2-IP) induced deadenylation of ddA and dA as a probable mechanism of toxicity. 2-B-2-MP showed the highest deadenylation rate compared to the other halogenated alkanes. It was reported that 2-BP showed faster rate of deadenylation than 1-BP,[7] which indicated that the tertiary alkyl halide displayed greater reactivity than secondary followed by primary alkyl halides. It was also observed that ddA showed the highest reactivity followed by dA and adenosine. Although the exact mechanism of deadenylation is not known, our results show that hydroxyl group in the sugar moiety of the nucleosides plays an important role in the rate of deadenylation. The study which aims elucidate the mechanism of deadenylation induced by halogenated alkanes is in progress.

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