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Effects of Methylthiodeoxyadenosine and Its Analogs on *in vitro* Invasion of Rat Ascites Hepatoma Cells and Methylation of Their Phospholipids

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The relationship between tumor invasiveness *in vitro* and methylation of plasma membrane phospholipids was investigated. For this purpose, two hepatoma cell lines, C1-30 and LC-AH, were used which show specific penetration to below cultured monolayers of mesothelial cells from rat mesentery and endothelial cells from calf pulmonary artery, respectively. Methylthiodeoxyadenosine (MTA) and five of its analogs, difluoro-MTA, deoxyadenosine, sinefungin, phenylthiodeoxyadenosine and fluorophenylthiodeoxyadenosine, inhibited the invasion of the tumor cells without affecting their proliferation. This inhibition was associated with reduction in the incorporation of radioactivity of [methyl-^{3}H]methionine into cellular phosphatidylethanolamine derivatives without changes in the labelings of RNA and DNA and carboxylmethylation of protein. These compounds also decreased the membrane fluidity of the tumor cells, measured by a steady-state fluorescence polarization method. Three other MTA analogs (fluorodideoxyadenosine, fluoroazidodideoxyadenosine and fluoroaminodideoxyuridine) did not affect the invasiveness of the tumor cells or alter their phospholipid methylation or membrane fluidity at concentrations that did not inhibit proliferation. These results suggest that the decrease in invasiveness of tumor cells by MTA and its analogs is due to alterations in the phospholipid composition and fluidity of the tumor cell membranes.

Key words: Methylthiodeoxyadenosine — Invasion — Phospholipid — Methylation — Fluidity

5′-Methylthio-5′-deoxyadenosine (MTA) is formed from decarboxylated *S*-adenosylmethionine in the biosynthesis of spermidine and spermine and is cleaved by MTA phosphorylase into adenine and 5-methylthio-5-deoxyribose-1-phosphate, which are used for the salvage of ATP and methionine, respectively, in mammalian cells.[1)]

We previously reported that 5′-difluoromethylthio-5′-deoxyadenosine (DFMTA), a synthetic analog of MTA, is a competitive inhibitor of MTA phosphorylase and exerts a cytostatic effect on various leukemia cells by inhibiting the MTA phosphorylase activity in the cells. On the other hand, growth inhibition by MTA was observed in MTA phosphorylase-deficient cells.[2)] A series of experiments showed that DFMTA and MTA inhibit the growth of rat ascites hepatoma cells (AH-130 cells) and its sublines (Cl-30 and LC-AH cells), which have high and specific abilities to invade to below cultured monolayers of cells derived from rat mesentery and calf pulmonary artery. We have also reported that DFMTA, MTA, 2′-deoxyadenosine (DA) and sinefungin (SINE) suppressed the *in vitro* invasiveness of Cl-30 cells at concentrations that did not inhibit cell growth.[3)] These findings suggest that the inhibitions of tumor cell invasion by these four nucleosides result not from impairment of general cellular metabolism, but rather from a site-specific disturbance of invasive behavior.

There are reports that MTA,[4)] DA and SINE[5)] inhibit *S*-adenosylmethionine-dependent transmethylation and that membrane phospholipids of highly metastatic B16 melanoma cells have a high phosphatidylcholine/phosphatidylethanolamine ratio.[6)] Therefore, in the present study, we investigated the effects of a variety of MTA analogs on *in vitro* invasiveness of two tumor cell lines, Cl-30 and LC-AH cells, and on methylation of their cellular components and membrane fluidity.

MATERIALS AND METHODS

Materials The sources of materials were as follows: MTA, 2-deoxyadenosine, sinefungin, phospholipids and nucleic acids from Sigma Chemical Co. (St. Louis, MO), DFMTA from Meito Sangyo Co. (Nagoya), other analogs of MTA from Asahi Glass Co. (Yokohama), 1,6-diphenyl-1,3,5-hexatriene from Wako Pure Chemical Industries (Osaka), [methyl-^{3}H]methionine (1.85 TBq/mmol) from ICN Biochemicals (Irvine, CA), fetal calf serum from Flow Laboratories (McLean, VA), silica gel plates (60A LK6D) for TLC from Whatman International (Maidstone, England) and calf pulmonary arterial endothelial cells from the Japanese Cancer Research Resources Bank.

The structural formulas and abbreviations of the compounds tested are shown in Table I.

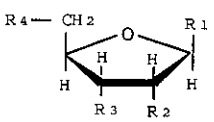
Culture of tumor cells The tumor cell lines used in this study were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air as reported previously.[7,8] Briefly, Cl-30 and LC-AH cells, which are subclones of rat ascites hepatoma cells, were cultured in suspension in medium A (Dulbecco's modified Eagle's medium containing 10% fetal calf serum). Cl-30 cells show high invasiveness of monolayers of mesothelial cells from rat mesentery,[7] whereas LC-AH cells are highly invasive of both mesothelial and calf pulmonary arterial endothelial cells.[8] For experiments on cell proliferation, tumor cells (1×10^5 cells/ml) were grown for 24 h and cell numbers were determined in a Coulter counter.

Invasion assay Mesothelial and endothelial cells grown to confluence in Corning Wells (diameter 22 mm) were cocultured with tumor cells (2×10^4 cells) in 1 ml of medium A. After 24 h, the number of single tumor cells and colonies that had penetrated to below the monolayer was counted with a phase contrast microscope as reported previously.[7]

Isolation of plasma membranes Plasma membrane ghosts were isolated from Cl-30 cells labeled with [methyl-^{3}H]-methionine as reported previously.[9] Briefly, the cells (4×10^7 cells) were cultured for 1 h in 5 ml of labeling medium (methionine-free modified Eagle's medium supplemented with 10 μM methionine, 370 KBq/ml [methyl-^{3}H]methionine and 10% fetal calf serum) and the labeling was stopped by addition of methionine (2 mM final concentration). The cells were collected by centrifugation, washed with ice-cold 1 mM $CaCl_2$ and homogenized in a stainless steel Dounce homogenizer. Plasma membrane ghosts were separated by centrifugation of the cell homogenate in 2.5% polyethylene glycol and were finally suspended in phosphate-buffered saline (PBS), (pH 7.4) for protein determination and lipid extraction.

Labeling and fractionation of cellular components The tumor cells (2×10^6 cells) were cultured for 1 h in 2 ml of the labeling medium and washed twice with cold PBS containing 2 mM methionine. Then they were suspended in the same solution and homogenized with an equal volume of cold 10% trichloroacetic acid-2.5% $MgCl_2$.[10] The mixture was stood in an ice bath for 20 min, and then the acid-insoluble fraction containing phospholipids, nucleic acids and proteins was precipitated by centrifugation and washed three times with cold 5% trichloroacetic acid-1.25% $MgCl_2$. Lipids were extracted from this fraction with acetone, 95% ethanol and 95% ethanol-ether (2:1, v/v)[11] and finally dissolved in chloroform-methanol (2:1, v/v). Phospholipids in the lipid fraction were separated by silica gel TLC using chloroform-propionic acid-*n*-propyl alcohol-H_2O (2:2:3:1, v/v) as a developing solvent[12] with egg yolk lecithin and dipalmitoylesters of phosphatidyl-N-monomethyl-and -N,N-dimethylethanolamines as standards. Phospholipids on silica gel were detected with Rhodamine,[12] and scaped off for measurement of their radioactivities. RNA, DNA and protein were fractionated from the lipid-free acid-insoluble fraction by the method of Schneider[13] and determined with orcinol (RNA),[13] diphenylamine (DNA)[14] and phenol reagent (protein)[15] using yeast RNA, calf thymus DNA and bovine serum albumin as standards. For assay of protein carboxylmethylation, methylester in the lipid-free, acid-insoluble fraction was hydrolyzed in sodium borate (pH 11) and volatile [^{3}H]methanol was extracted and counted by the method of Bartel

Table I. Structural Formulas of Methylthiodeoxyadenosine and Its Analogs



Compound (Abbreviation)	R_1	R_2	R_3	R_4
5′-Methylthio-5′-deoxyadenosine (MTA)	adenine	OH	OH	SCH_3
5′-Difluoromethylthio-5′-deoxyadenosine (DFMTA)	adenine	OH	OH	$SCHF_2$
2′-Deoxyadenosine (DA)	adenine	H	OH	OH
Sinefungin (SINE)	adenine	OH	OH	$CH(NH_2)CH_2CH_2$ $HOOC(H_2N)CH$
5′-Phenylthio-5′-deoxyadenosine (PTA)	adenine	OH	OH	SC_6H_5
3′-Fluoro-5′-phenylthio-3′,5′-dideoxyadenosine (FPTA)	adenine	OH	F	SC_6H_5
3′-Fluoro-2′,3′-dideoxyadenosine (FA)	adenine	H	F	OH
3′-Fluoro-5′-azido-3′,5′-dideoxyadenosine (AZFA)	adenine	OH	F	N_3
3′-Fluoro-5′-amino-3′,5′-dideoxyuridine (AFU)	uracil	OH	F	NH_2

and Borchardt.[16] The radioactivity of each fraction was counted in xylene-based scintillation cocktail.

Membrane fluidity measurement Fluid changes were monitored by measuring fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene as a probe.[17] Briefly, tumor cells that had been cultured with MTA analogs for 24 h in medium A were suspended in a equal volume of PBS containing 1.5 μM fluorescence probe and incubated at 37°C for 2 h. Fluorescence polarization was calculated from the fluorescence intensity (ex=366 nm, em=430 nm) measured at 37°C in a Hitachi fluorescence spectrophotometer equipped with a polarization accessory.

Statistics Statistical comparisons were made by using Student's t test. A P value of 0.05 or less was defined as significant.

RESULTS

Effects of DFMTA on invasion and proliferation of tumor cells Previously we reported that the invasiveness of Cl-30 cells decreased in the presence of DFMTA and that pretreatment of Cl-30 cells with 300 μM DFMTA for 1 h caused 52% inhibition of their invasiveness. In the present study, we examined this inhibitory effect of DFMTA on two tumor cell lines, Cl-30 and LC-AH cells. As shown in Experiment A of Table II, pretreatment of the tumor cells with DFMTA for 3 h resulted in dose-dependent inhibition of their invasiveness in the absence of DFMTA, without any effect on their growth. Longer pretreatment for 24 h resulted in greater inhibition of invasiveness, but significant growth inhibitions of both cell lines were observed at a concentration of 300 μM DFMTA (Experiment B of Table II). This growth inhibition was reversible because the growth of DFMTA-treated cells recovered completely during subsequent culture for 24 h in the absence of DFMTA (data not shown). Treatment of the mesothelial or endothelial cell layer with 300 μM DFMTA for 3 h had not effect on the invasiveness of untreated tumor cells (data not shown), indicating that DFMTA acts on the tumor cells.

Effects of MTA and its analogs on tumor invasion We previously reported that additions of MTA, DA and SINE to the assay medium caused dose-dependent depression of the invasion of Cl-30 cells.[3] As on pretreatment with DFMTA (Experiment B of Table II), pretreatments with these three nucleosides inhibited the invasiveness of Cl-30 cells (Experiment A of Table III). The effects of five other synthetic nucleosides on LC-AH

Table II. Effects of DFMTA on *in vitro* Invasiveness and Proliferation of Tumor Cells

Tumor cell	Treatment with DFMTA		Invasion[c]		Proliferation[c]	
	Concentration (μM)	Time (h)	No. cells and colonies/cm^2	%	$\times 10^{-5}$ cells/ml	%
Experiment A[a]						
Cl-30	0 (control)	3	619±37	100	2.01±0.13	100
	100	3	379±35[e]	61	2.03±0.21	101
	300	3	241±22[d]	39	1.97±0.11	98
LC-AH	0 (control)	3	1312±17	100	1.62±0.08	100
	100	3	971±40[d]	74	1.54±0.09	95
	300	3	771±66[d]	59	1.50±0.11	93
Experiment B[b]						
Cl-30	0 (control)	24	619±37	100	2.01±0.13	100
	50	24	520±97	84	1.93±0.05	96
	100	24	303±59[e]	49	1.91±0.03	95
	150	24	117±8[d]	19	1.76±0.14	88
	300	24	56±8[d]	9	1.40±0.12[f]	70
LC-AH	0 (control)	24	1312±17	100	1.62±0.08	100
	100	24	879±33[d]	67	1.60±0.12	99
	300	24	197±14[d]	15	1.21±0.05	75

a) The tumor cells (1.0×10^5 cells/ml) were cultured with DFMTA for 3 h in medium A, and their proliferation and invasion in the absence of DFMTA were measured 21 h and 24 h later, respectively.
b) The tumor cells (1.0×10^5 cells/ml) were cultured with DFMTA for 24 h and then proliferation was measured as increase in cell numbers. The invasiveness of the DFMTA-treated cells was then assayed in the absence of DFMTA.
c) Values are means±SD for 3 or 4 experiments.
d) $P<0.001$, *e*) $P<0.01$, *f*) $P<0.05$, compared with the corresponding control.

Table III. Effects of MTA and Its Analogs on Tumor Invasion

Tumor cell	Addition		Invasion[c] No. cells and colonies/cm^2	Invasion[c] %
Experiment A[a]				
Cl-30	None	(Control)	554±27	100
	MTA	(300 μM)	33±11[d]	6
	DA	(100 μM)	116±17[d]	21
	SINE	(400 μM)	371±29[e]	67
Experiment B[b]				
LC-AH	None	(Control)	1319±30	100
	DFMTA	(100 μM)	870±66[d]	66
	PTA	(50 μM)	831±85[e]	63
	FPTA	(30 μM)	818±53[d]	62
	FA	(100 μM)	1301±22	99
	AZFA	(100 μM)	1236±46	94
	AFU	(100 μM)	1248±39	95

a) Cl-30 cells were treated with MTA or its analogs for 24 h and then the invasion was determined in the absence of the analogs.
b) The analogs were added to the medium for assay of invasion.
c) Values are means±SD for 4 experiments.
d) $P<0.002$, *e*) $P<0.005$, compared with the croresponding control.

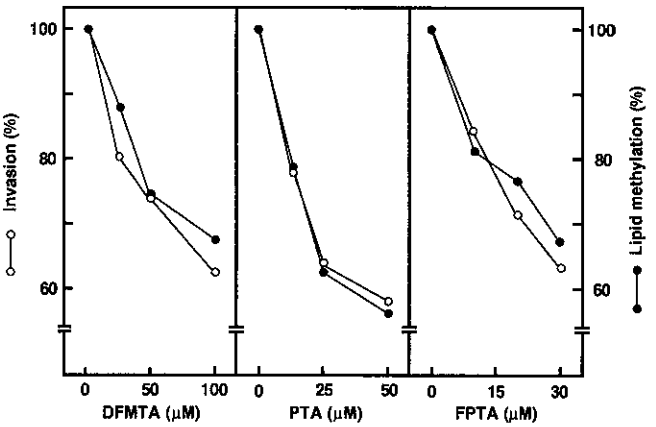


Fig. 1. Parallel inhibitions by MTA analogs of invasion by LC-AH cells and their lipid methylation. Invasiveness was determined in the presence of MTA analogs. Radioactive lipids were extracted from LC-AH cells cultured in the presence of the MTA analogs for 23 h in medium A and then for 1 h in labeling medium. Values (% of control) are means for 2 or 3 experiments. The control values were 1350 invading cells and colonies/cm^2 (invasion) and 141 dpm/μg protein (methylation).

cells are shown in Experiment B of Table III, with that of DFMTA for comparison. In this experiment, the nucleosides were added to the medium for assay of invasion at concentrations that did not inhibit growth. The phenylthio derivatives PTA and FPTA were as effective as DFMTA, but three other analogs, FA, AZFA and AFU, were ineffective.

Effects of MTA analogs on methylation of cellular components of tumor cells MTA, DA and SINE are reported to be inhibitors of *S*-adenosylmethionine-mediated transmethylation, which is suggested to be closely related to the *in vivo* metastatic properties of tumor cells[17-19] Consistent with these reports, we have observed that DFMTA-induced depression of invasion was greater in methionine-depleted assay medium.[3] To investigate whether methylation reactions are involved in the inhibition of tumor cell invasion by MTA analogs, we examined the effects of these compounds on the incorporation of radioactivity from [methyl-^{3}H]methionine into cellular components. The results are presented in Tables IV and V and Fig. 1. In these experiments, tumor cells were treated with MTA analogs under the same conditions as in Table III, except that the tumor cells were cultured with [methyl-^{3}H]methionine for the last hour of the treatments. As shown in Table IV, the incorporation of radioactivity into the lipid fraction (lipid methylation) of the tumor cells was inhibited by all six nucleosides that inhibited tumor cell invasion. Similar inhibition was observed in the experiments with a shorter labeling period of 30 min instead of 1 h (data not shown). On the other hand, three other nucleosides, FA, AZFA and AFU, that had no effect on invasiveness (Experiment B of Table III) also had no effect on the lipid methylation (Experiment B of Table IV). On treatment of Cl-30 cells with various doses of DFMTA, PTA or FPTA, decrease of invasiveness was roughly proportional to decrease of lipid methylation (Fig. 1). Under the conditions employed, none of the MTA analogs significantly inhibited the incorporations of radioactivity into RNA and DNA, and MTA and DFMTA did not alter the carboxylmethylation of protein in Cl-30 cells (Table IV).

Next we treated tumor cells with MTA analogs under the same conditions as in Experiments A and B of Table IV, and then extracted their lipid fraction and separated it by TLC. About 30% of the total radioactivity in the lipid fraction was recovered in the spots of three methylated phosphatidylethanolamines. The other labeled compounds have not yet been identified, but most of the radioactivity remained at the origin on chromatography. As shown in Table V, the incorporation of radioactivity into phosphatidylcholine (PC) was depressed by all six inhibitors of tumor cell invasion, MTA, DFMTA, DA, SINE, PTA and FPTA. Some of these compounds significantly reduced the incorporations of

Table IV. Effects of MTA and Its Analogs on Incorporation of Radioactivity from [methyl-^{3}H]Methionine into Cellular Components in Tumor Cells

Tumor cell	Addition		Radioactivity incorporated (% of control)[b]			
			Lipid	RNA	DNA	Protein (carboxyl-methylation)
Experiment A[a]						
Cl-30	None	(Control)	100[f]	100[g]	100[h]	100[i]
	MTA	(300 μM)	66±13[d]	85±17	92±16	98±5
	DFMTA	(300 μM)	63±9[d]	92±13	95±6	110±13
	DA	(100 μM)	71±5[d]	89±6	91±8	ND[e]
	SINE	(400 μM)	71±9[d]	95±4	97±10	ND
Experiment B[a]						
LC-AH	None	(Control)	100[j]	100[k]	100[l]	ND
	DFMTA	(100 μM)	66±8[d]	87±5	85±4	
	PTA	(50 μM)	56±2[c]	93±2	93±6	
	FPTA	(30 μM)	64±5[c]	79±10	76±5	
	FA	(100 μM)	102±9	95±5	94±3	
	AZFA	(100 μM)	103±7	101±10	103±5	
	AFU	(100 μM)	103±4	96±6	106±8	

a) Tumor cells (2×10^6 cells) were cultured with the indicated compounds in 2 ml of medium A for 2 and 23 h in Experiments A and B, respectively. Then, labeling with [methyl-^{3}H]methionine was carried out for 1 h in the presence of MTA or its analogs in 2 ml of labeling medium. The cellular components were isolated as described in "Materials and Methods."

b) Values are means±SD for 4 experiments.

c) $P<0.005$, *d*) $P<0.02$, compared with the corresponding control.

e) ND, not determined.

The control values were: *f*) 152±9 dpm/μg protein, *g*) (12.5±1.7)$\times10^3$ dpm/μg RNA, *h*) (56.1±7.1)$\times10^2$ dpm/μg DNA, *i*) (54.5±7.0)$\times10^2$ dpm/mg protein, *j*) 148±9 dpm/μg protein, *k*) (10.5±1.0)$\times10^3$ dpm/μg RNA, and *l*) (46.4±7.8)$\times10^2$ dpm/μg DNA.

radioactivity into phosphatidyl-N,N-dimethyl-ethanolamine (PDE) and phosphatidyl-N-monomethylethanolamine (PME) as well. In contrast, FA, AZFA and AFU, which had no effect on tumor cell invasiveness (Table III) or lipid methylation (Table IV), had no effect on the methylation of phospholipids.

Effects of MTA and its analogs on methylation of plasmalemmal phospholipids and membrane fluidity of tumor cells There is suggestive evidence that methylation of plasma membrane phospholipids influences membrane fluidity[20] and that change in membrane fluidity of tumor cells is closely related with metastatic activity.[17-19, 21] Therefore, we examined the alterations of these two molecular characteristics of the plasma membrane in tumor cells treated with MTA and its analogs. For this, plasma membrane ghosts were prepared from Cl-30 cells that had been cultured under the same conditions as for Experiment A of Table IV, and plasma membrane phospholipids were extracted and separated by TLC. As shown in Table VI, the labelings of both PC and PDE by [methyl-^{3}H]methionine were markedly inhibited in tumor cells treated with MTA or DFMTA. The extents (40–50%) of inhibition were comparable with those observed in phospholipids extracted from the whole cells (Experiment A of Table V).

The changes in cell membrane fluidity of tumor cells treated with MTA analogs were assayed under the same conditions as for Experiment B of Table V. The fluidity is shown as fluorescence polarization in Table VII. All the anti-invasive analogs tested, DFMTA, PTA and FPTA, significantly increased the membrane fluorescence polarization of LC-AH cells, indicating decrease of cell membrane fluidity, whereas FA and AZFA had no effect on the fluorescence polarization.

DISCUSSION

We previously reported that DFMTA, a non-metabolizable analog of MTA, inhibited the growth of a variety of human leukemia cells by inhibiting the MTA phosphorylase activity in the cells. Fifty percent growth inhibitions of Raji and HL-60 cells were observed at concentrations of 10 and 44 μM MTA, respectively.[2] In contrast to leukemia cells, highly invasive subclones of rat ascites hepatoma, Cl-30 and LC-AH cells, were resis-

Table V. Effects of MTA and Its Analogs on Methylation of Phospholipids in Tumor Cells

Tumor cell	Addition		Radioactivity incorporated (% of control)[b]		
			PC	PDE	PME
Experiment A[a]					
Cl-30	None	(Control)	100[f]	100[g]	100[h]
	MTA	(300 μM)	60±6[d]	68±5[e]	80±9
	DFMTA	(300 μM)	47±6[c]	61±13[e]	62±13[e]
	DA	(100 μM)	50±4[c]	84±9	79±11
	SINE	(400 μM)	73±11[e]	78±12	87±14
Experiment B[a]					
LC-AH	None	(Control)	100[f]	100[j]	100[k]
	DFMTA	(100 μM)	64±7[d]	75±7[e]	67±11[e]
	PTA	(50 μM)	55±1[c]	68±7[e]	42±6[e]
	FPTA	(30 μM)	67±8[d]	70±13[e]	52±10[d]
	FA	(100 μM)	101±5	97±11	93±13
	AZFA	(100 μM)	97±6	105±8	92±2
	AFU	(100 μM)	103±3	100±6	107±5

a) The experimental conditions were the same as for Table IV.
b) Phospholipids [PC; phosphatidylcholine, PDE; phosphatidyl-N,N-dimethylethanolamine, PME; phosphatidyl-N-monomethylethanolamine] were separated by TLC from the lipids in Table IV. Values are means±SD for 4 experiments.
c) $P<0.005$, *d*) $P<0.02$, *e*) $P<0.05$, compared with the corresponding control.
Control values ($\times 10^{-3}$ dpm/mg protein) are *f*) 32.1±3.0, *g*) 11.8±1.4, *h*) 6.20±0.88, *i*) 30.9±1.8, *j*) 10.1±1.3, and *k*) 4.74±0.53.

Table VI. Effects of MTA and DFMTA on Incorporation of Radioactivity from [methyl-^{3}H]Methionine into Plasma Membrane Phospholipids of Cl-30 Cells

Addition[a]		Radioactivity in phospholipids[b]					
		PC		PDE		PME	
		dpm/μg protein	%	dpm/μg protein	%	dpm/μg protein	%
None	(control)	184.7	100	43.5	100	48.0	100
MTA	(300 μM)	98.1	53	24.9	57	45.8	95
DFMTA	(300 μM)	91.1	49	22.3	51	42.7	89

a) Treatment of Cl-30 cells (4×10^7 cells) with MTA or DFMTA for 3 h and labeling with [methyl-^{3}H]-methionine for 1 h were carried out in 5 ml of the medium as for Experiment A of Table IV except that the labeling medium contained 1.11 MBq/ml [methyl-^{3}H]methionine. Phospholipids were separated by TLC from the lipids in Table IV.
b) Values are means for two separate experiments.

tant to this analog, showing no detectable growth inhibition, in spite of a marked decrease in invasiveness when cultured with 100 μM DFMTA for 24 h (Table II). Under these conditions, DFMTA-dependent accumulation of MTA in the culture medium, determined by the method described in the previous paper[2] was observed (data not shown), indicating that the MTA phosphorylase activities of these subclones, like that of Raji cells,[2] were inhibited by DFMTA and that the intracellular MTA was secreted into the medium. As MTA inhibits tumor cell invasion as effectively as DFMTA (Tables II and III), intracellular MTA might be responsible for the effect of DFMTA. This possibility, however, seems unlikely because the accumulation of MTA in the medium was less than 2 μM and no significant accumulation of MTA was detected when the invasion of the subclone cells was inhibited by treatment with 300 μM DFMTA for 3 h (data not shown). Further investigations, e.g., on the intracellular level of MTA in DFMTA-treated cells, would be desirable.

Table VII. Effects of MTA Analogs on Membrane Fluorescence Polarization in LC-AH Cells

Treatment (24 h)[a]		Fluorescence polarization[b]
None		0.189±0.002
DFMTA	(100 μM)	0.202±0.005[c]
PTA	(25 μM)	0.197±0.003[c]
FPTA	(30 μM)	0.199±0.004[c]
FA	(100 μM)	0.190±0.003
AZFA	(100 μM)	0.190±0.003

a) LC-AH cells (2×10^6 cells) were cultured with MTA analogs for 24 h at 37°C in 2 ml of medium A and then fluorescence polarization was determined as described in "Materials and Methods."
b) Values are means±SD for 6 or 8 experiments.
c) $P<0.002$, compared with the corresponding control.

A variety of adenosine analogs[5] including MTA[22] and 2′-deoxyadenosine[5] are inhibitors or inactivators of *S*-adenosylhomocysteine hydrolase. These analog-induced inhibitions of this hydrolase activity have been suggested to lead to intracellular accumulation of *S*-adenosylhomocysteine, which is both a product and a potent inhibitor of various *S*-adenosylmethionine-mediated transmethylases.[5, 23]

All the inhibitors of tumor cell invasion tested in this study decreased the methylation of phospholipids but not the methylation of nucleic acids or growth of the two sublines in the concentration ranges used. Carboxymethylation of protein was also not affected by MTA or DFMTA (Table IV). These results suggest that MTA analogs do not affect either the uptake or the activation of methionine by the cells and that phospholipid methylation is important for the high invasiveness of the two tumor cell lines. This view was supported by the following results. 1) None of the MTA analogs that had no effect on tumor cell invasion inhibited phospholipid methylation (Tables III and IV). 2) The invasiveness and phospholipid methylation were depressed in parallel by MTA analogs (Fig. 1). Hirata and Axelrod reported similar effects of 3-deazaadenosine, an inhibitor of *S*-adenosylhomocysteine hydrolase. They found that when the receptor-mediated biological responses of HeLa cells were reduced by 3-deazaadenosine, methylation of phospholipid was inhibited much more than the methylation of proteins or nucleotides.[20] These results also indicate that the transmethylases of phospholipids are more susceptible than those of RNA, DNA and protein to MTA and its analogs used in this study and 3-deaza-adenosine.

There are reports that tumor cell membranes play crucial roles in the metastatic process and that modifications of a variety of cell membrane components may alter the invasiveness of tumor cells. The lipid composition of the membrane influences both cell motility and membrane fluidity. For example, Liotta *et al.*[24] demonstrated that methylation of membrane phospholipids affects tumor cell motility using a stimulant, autocrine motility factor, and an inhibitor, 3-deazaadenosine. Grimstad[25] reported that highly motile tumor cells have a high invasive capacity. Schroeder and Gardiner[26] reported that, compared with the cell membranes of low-metastatic melanoma cells, those of a high-metastatic variant show differences in their lipid compositions including lower cholesterol/phospholipid ratios and higher phosphatidylcholine/phosphatidylethanolamine ratios. These two ratios are considered to be closely related to membrane fluidity because both methylation of phosphatidylethanolamine in erythrocyte membranes[27] and treatment of tumor cells with phosphatidylcholine increase membrane fluidity, but treatment of tumor cells with cholesteryl hemisuccinate decreases membrane fluidity.[17] Increase in membrane fluidity has also been suggested to enhance motility and invasion of tumor cells.[17–19, 21]

The present results are consistent with these reports. MTA and DFMTA inhibited the methylations of phospholipids in cell membranes of Cl-30 cells (Table VI) to almost the same extents to those of total cellular phospholipids (Table V), suggesting that the latter value represents methylations of phospholipids in the cell membrane. In addition, only MTA analogs that decreased the invasiveness of LC-AH cells decreased the membrane fluidity (Table VII). From these results it appears that MTA and its analogs tested in this study lower the invasiveness of tumor cells by decreasing the membrane fluidity due to hypomethylation of membrane phospholipids.

Additional experiments are necessary to understand the molecular bases for inhibition of phospholipid methylation by MTA and its analogs and the role of membrane fluidity in invasive processes.

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REFERENCES

1) Savarese, T. M., Ghoda, L. Y., Dexter, D. L. and Parks, R. E., Jr. Conversion of 5′-deoxy-5′-methylthioadenosine and 5′-deoxy-5′-methylthioinosine to methionine in cultured human leukemic cells. *Cancer Res.*, **43**, 4699–4702 (1983).
2) Nishikawa, S., Ueno, A., Inoue, H. and Takeda, Y. Effect of 5′-difluoromethylthioadenosine, an inhibitor of methylthioadenosine phosphorylase, on proliferation of cultured cells. *J. Cell. Physiol.*, **133**, 372–376 (1987).
3) Shinkai, K., Mukai, M., Horai, T., Ohigashi, H., Nishikawa, S., Inoue, H., Takeda, Y. and Akedo, H. Inhibition of *in vitro* tumor cell invasion by transmethylation inhibitors. *Jpn. J. Cancer Res.*, **80**, 716–719 (1989).
4) Ferro, A. J., Vandenbark, A. A. and MacDonald, M. R. Inactivation of *S*-adenosylhomocysteine hydrolase by 5′-deoxy-5′-methylthioadenosine. *Biochem. Biophys. Res. Commun.*, **100**, 523–531 (1981).
5) Ueland, P. M. Pharmacological and biochemical aspects of *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase. *Pharmacol. Rev.*, **34**, 223–253 (1982).
6) Schroeder, F. and Gardiner, J. M. Membrane lipids and enzymes of cultured high- and low-metastatic B16 melanoma variants. *Cancer Res.*, **44**, 3262–3269 (1984).
7) Akedo, H., Shinkai, K., Mukai, M., Mori, Y., Tateishi, R., Tanaka, K., Yamamoto, R. and Morishita, T. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. *Cancer Res.*, **46**, 2416–2422 (1986).
8) Ohigashi, H., Shinkai, K., Mukai, M., Ishikawa, O., Imaoka, S., Iwanaga, T. and Akedo, H. *In vitro* invasion of endothelial cell monolayer by rat ascites hepatoma cells. *Jpn. J. Cancer Res.*, **80**, 818–821 (1989).
9) Akedo, H., Shinkai, K., Mukai, M., Mori, Y. and Okada, M. Plasma membrane-bound tubulin in rat hepatoma ascites tumor cells. *Biochem. Int.*, **2**, 355–365 (1981).
10) Artom, C. Labeled compounds in the study of phospholipid metabolism. *Methods Enzymol.*, **4**, 809–840 (1957).
11) Entenman, C. General procedures for separating lipid components of tissue. *Methods Enzymol.*, **3**, 299–317 (1957).
12) Hirata, F., Viveros, H., Diliberto, E. J., Jr. and Axelrod, J. Identification and properties of two methyltransferases in conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc. Natl. Acad. Sci. USA*, **75**, 1718–1721 (1978).
13) Schneider, W. C. Determination of nucleic acid in tissues by pentose analysis. *Methods Enzymol.*, **3**, 680–684 (1957).
14) Giles, K. W. and Myers, A. An inproved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature*, **206**, 93 (1965).
15) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
16) Bartel, R. L. and Borchardt, R. T. Effects of adenosine dialdehyde on *S*-adenosylhomocysteine hydrolase and *S*-adenosylmethionine-dependent transmethylations in mouse L929 cells. *Mol. Pharmacol.*, **25**, 418–424 (1984).
17) Taraboletti, G., Perin, L., Bottazzi, B., Mantovani, A., Giavazzi, R. and Salmona, M. Membrane fluidity affects tumor-cell motility, invasion and lung-colonizing potential. *Int. J. Cancer*, **44**, 707–713 (1989).
18) Breillout, F., Poupon, M. F., Blanchard, P., Lascaux, V., Echinard-Garin, P. and Robert-Gero, M. Association of SIBA treatment and a Met-depleted diet inhibits *in vitro* growth and *in vivo* metastatic spread of experimental tumor cell lines. *Clin. Expl. Metastasis*, **6**, 3–16 (1988).
19) Nakagawa, I. and Iwaizumi, M. A role of the cancer cell membrane fluidity in the cancer metastases: an ESR study. *Tohoku J. Exp. Med.*, **157**, 193–198 (1989).
20) Hirata, F. and Axelrod, J. Phospholipid methylation and biological signal transmission. *Science*, **209**, 1082–1090 (1980).
21) Kier, A. B. Plasma membrane properties of cultured local LM cell tumors and metastases from athymic (nude) mice. *Cancer Lett.*, **50**, 19–30 (1990).
22) Ferro, A. J., Vandenbark, A. A. and MacDonald, M. R. Inactivation of *S*-adenosylhomocysteine hydrolase by 5′-deoxy-5′-methylthioadenosine. *Biochem. Biophys. Res. Commun.*, **100**, 523–531 (1981).
23) Kredich, N. M. and Hershfield, M. S. Perturbations in *S*-adenosylhomocysteine and *S*-adenosylmethionine metabolism: effects on transmethylation. *Adv. Enzyme Regul.*, **18**, 181–191 (1981).
24) Liotta, L. A., Mandler, R., Murano, G., Katz, D. A. Gordon, R. K., Chiang, P. K. and Schiffmann, E. Tumor cell autocrine motility factor. *Proc. Natl. Acad. Sci. USA*, **83**, 3302–3306 (1986).
25) Grimstad, I. A. Direct evidence that cancer cell locomotion contributes importantly to invasion. *Exp. Cell Res.*, **173**, 515–523 (1987).
26) Schroeder, F. and Gardiner, J. M. Membrane lipids and enzymes of cultured high- and low-metastatic B16 melanoma variants. *Cancer Res.*, **44**, 3262–3269 (1984).
27) Hirata, F. and Axelrod, J. Enzymatic methylation of phosphatidylethanolamine increases erythrocyte membrane fluidity. *Nature*, **275**, 219–220 (1978).