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Effects of Synthetic Sphingosine-1-Phosphate Analogs on Cytosolic Phospholipase $A_2\alpha$–Independent Release of Arachidonic Acid and Cell Toxicity in L929 Fibrosarcoma Cells: the Structure–Activity Relationship

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Abstract. Sphingolipid metabolites including ceramide, sphingosine, and their phosphorylated products [sphingosine-1-phosphate (S1P) and ceramide-1-phosphate] regulate cell functions including arachidonic acid (AA) metabolism and cell death. The development of analogs of S1P may be useful for regulating these mediator-induced cellular responses. We synthesized new analogs of S1P and examined their effects on the release of AA and cell death in L929 mouse fibrosarcoma cells. Among the analogs tested, several compounds including DMB-mC11S [dimethyl (2*S*,3*R*)-2-*tert*-butoxycarbonylamino-3-hydroxy-3-(3'-undecyl)phenylpropyl phosphate] and DMB-mC9S [dimethyl (2*S*,3*R*)-2-*tert*-butoxycarbonylamino-3-hydroxy-3-(3'-nonyl)phenylpropyl phosphate] released AA within 1 h and caused cell death 6 h after treatment. The release of AA was observed in C12 cells [a L929 variant lacking a type α cytosolic phospholipase A_2 ($cPLA_2\alpha$)] and L929-cPLAα–siRNA cells (L929 cells treated with small interference RNA for $cPLA_2\alpha$). Treatment with pharmacological inhibitors of secretory and Ca^{2+}-independent PLA_2s decreased the DMB-mC11S–induced release of AA. The effect of the S1P analogs tested on the release of AA was comparable to that on cell death in L929 cells, and a high correlation coefficient was observed. Two analogs lacking a butoxycarbonyl moiety [DMAc-mC11S (dimethyl (2*S*,3*R*)-2-acetamino-3-hydroxy-3-(3'-undecyl)phenylpropyl phosphate] and DMAm-mC11S [dimethyl (2*S*,3*R*)-2-amino-3-hydroxy-3-(3'-undecyl)phenylpropyl phosphate)] had inhibitory effects on the release of AA and cell toxicity induced by DMB-mC11S. Synthetic phosphorylated lipid analogs may be useful for studying PLA_2 activity and its toxicity in cells.
[Supplementary Fig. 1: available only at http://dx.doi.org/10.1254/jphs.08284FP]

Keywords: sphingosine-1-phosphate, ceramide-1-phosphate, synthetic analog, phospholipase A_2, cell death

Introduction

There is increasing evidence that sphingolipids are involved in regulating various cellular functions such as the actions of enzymes and receptors, membrane transport, and signaling transduction (1 – 4). Metabolites of sphingolipids such as ceramide, sphingosine, ceramide-1-phosphate (C1P), and sphingosine-1-phosphate (S1P) are produced in response to stress and the activation of receptors in cells. Once S1P is released into extracellular spaces, it acts as an endogenous agonist for the S1P receptors, a family of G protein–coupled cell surface receptors (5, 6). In addition, S1P acts as an intracellular

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messenger, which is important for the regulation of cellular functions and various kinases (2, 3). Also, ceramide, sphingosine, and C1P are reported to act as intracellular signaling molecules (1, 2, 7, 8). The development of synthetic analogs of S1P and/or C1P, which can mimic or inhibit the effects of these molecules, may be useful for studying the cellular functions of sphingolipid metabolites. Previously, we reported that some synthetic S1P analogs released arachidonic acid (AA) in a phospholipase A_2 (PLA_2)-dependent manner in L929 mouse fibrosarcoma cells (9). In the present study, we examined the effects of new synthetic S1P analogs on the release of AA in L929 cells and variant cells lacking type α cytosolic PLA_2 ($cPLA_2\alpha$, group IVA).

PLA_2 enzymes are esterases that hydrolyze at the *sn*-2 position of glycerophospholipids, releasing a free fatty acid and lysophospholipid. The released fatty acid is mainly AA, which can be metabolized to eicosanoids including prostaglandins and leukotrienes. The PLA_2 family consists of over 20 isoforms that differ in their Ca^{2+} requirements and cellular localization including secretion from cells; secretory PLA_2, Ca^{2+}-independent PLA_2, and Ca^{2+}-dependent and cytosolic PLA_2 ($cPLA_2$, group IV). $cPLA_2\alpha$ (group IVA) plays a pivotal role in providing AA because of its selectivity for phospholipds containing AA at the *sn*-2 position (4, 10). In group IV, $cPLA_2\beta$ (IVB), and $cPLA_2\gamma$ (IVC) require Ca^{2+} for their activity, and newly identified isoforms of $cPLA_2$ including δ, ε, and ζ subtypes did not appear to have a preference for AA in phospholipids (10, 11). So far, the Ca^{2+}-independent PLA_2 (group VIA and VIB) family and some of the Group IV family show Ca^{2+}-independent activity (12, 13). Secretory PLA_2s require Ca^{2+} at mM concentrations for their activity and contain an even number of cysteine residues at characteristic positions, each of which pairs with another specific cysteine to form a disulfide bridge, thus producing an adequate ternary structure (14). The PLA_2 enzymes and/or release of AA are involved in various cellular functions including cell survival and growth (11, 12, 15, 16). C1P triggered the translocation of $cPLA_2\alpha$ from the cytosol to the perinuclear region in cells via interaction with its C2 domain and increased the enzyme activity (17, 18). Several reports including those from our laboratory showed that sphingolipid-related compounds such as sphingomyelin, ceramide, C1P, and S1P analogs regulated the activities of PLA_2s, including secretory PLA_2 (19, 20) and $cPLA_2\alpha$ (9, 21 – 23). Thus, synthetic analogs of S1P/C1P that can regulate the release of AA are potential therapeutic agents for AA-related diseases, including immunological disorders and neuronal death (13, 16).

In the present study, we synthesized various new analogs of S1P and examined their effects on the release of AA in L929 cells. The effects of the synthetic compounds on cell death were also examined. The potency of the effect on the release of AA was comparable to that on cell death in L929 cells. The relation between the release of AA and cell death is discussed.

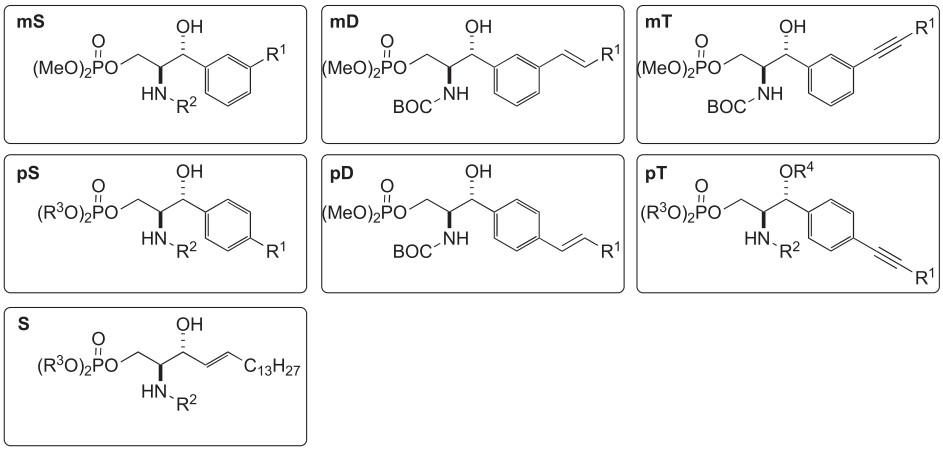
Materials and Methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (7.92 TBq/mmol) was from Amersham (Buckinghamshire, UK). Pyrrophenone was kindly provided by Dr. Hanasaki (Shionogi Pharm., Ltd., Osaka). Bromoenol lactone (BEL), thioetheramide-phosphorylcholine (TEAPC), secretory PLA_2III (No. 60500, from bee venom), and dithiothreitol (DTT) were from Cayman (Ann Arbor, MI, USA). 4β-Phorbol 12-myristate 13-acetate and A23187 were from Sigma (St. Louis, MO, USA). The S1P analogs tested were synthesized by standard methods in our laboratories and details of the synthesis will be reported elsewhere. The formula, structural abbreviation, and compound number (GS series shown as bold numbers, order of synthesis in our Lab., **No. 1 – 3** and **No. 11** were excluded) of each analog are shown in Fig. 1 (and Supplementary Fig. 1: available in the online version only). The effects of the compounds were examined at pharmacological concentrations (10 and 30 μM). The concentrations of other reagents including enzyme inhibitors were the same as those in previous reports (18, 24, 25). The S1P analogs having a dimethylated phosphate group were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide was under 0.5%. The S1P analogs having a dihydrogen phosphate group were dissolved in acetic acid and used after neutralization. The vehicles containing dimethyl sulfoxide and acetic acid did not cause release of AA for 1 h, cell toxicity for 6 h, or morphological changes in L929 cells.

Cell culture and measurement of release of [^{3}H]AA

L929 cells (a murine fibrosarcoma cell line) and their variant C12 cells lacking $cPLA_2\alpha$ were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum. A clone of L929 cells treated with small interference RNA directed against mouse $cPLA_2\alpha$ (L929-$cPLA_2\alpha$–siRNA cells) was established as described previously (26). When the cells achieved 70% – 80% confluence (sub-confluent stage), they were used for assays [release of AA and lactate dehydrogenase (LDH) leakage]. The morphological changes were examined when the cells achieved 40% – 50% confluence. The release of AA was determined as



Name			Class	MW	CLogP	R_1	R_2	R_3	R_4
S1P			S	379.47	4.89	-	H	H	-
GS-16	(DM B	-S1P)	S	507.64	6.79	-	BOC	Me	-
GS-20	(DM B	-m C9 S)	mS	501.59	5.66	$-C_9H_{19}$	BOC	-	-
GS-13	(DM B	-m C11 S)	mS	529.65	6.72	$-C_{11}H_{23}$	BOC	-	-
GS-22	(DM Ac	-m C11 S)	mS	471.57	5.04	$-C_{11}H_{23}$	Acetyl	-	-
GS-23	(DM Am	-m C11 S)	mS	429.53	4.84	$-C_{11}H_{23}$	H	-	-
GS-4	(DM B	-H)	pS	375.35	0.93	H	BOC	Me	-
GS-5	(DH Am	-H)	pS	247.18	-0.97	H	H	H	-
GS-19	(DM B	-p C9 S)	pS	501.59	5.66	$-C_9H_{19}$	BOC	Me	-
GS-6	(DM B	-p C11 S)	pS	529.65	6.72	$-C_{11}H_{23}$	BOC	Me	-
GS-7	(DH Am	-p C11 S)	pS	401.48	4.82	$-C_{11}H_{23}$	H	H	-
GS-27	(DM B	-m C9 D)	mD	499.58	4.94	$-C_7H_{15}$	-	-	-
GS-18	(DM B	-m C11 D)	mD	527.63	6.41	$-C_9H_{19}$	-	-	-
GS-21	(DM B	-m C11 DF5)	mD	617.58	5.91	$-C_7H_{14}(C_2F_5)$	-	-	-
GS-24	(DM B	-p C9 D)	pD	499.58	5.35	$-C_7H_{15}$	-	-	-
GS-17	(DM B	-p C11 D)	pD	527.63	6.41	$-C_9H_{19}$	-	-	-
GS-15	(DM B	-m C6 T)	mT	455.48	3.31	$-C_4H_9$	-	-	-
GS-14	(DM B	-m C9 T)	mT	497.56	4.90	$-C_7H_{15}$	-	-	-
GS-26	(DM B	-m C11 T)	mT	525.61	5.96	$-C_9H_{19}$	-	-	-
GS-10	(DM B	-p C6 T)	pT	455.48	3.31	$-C_4H_9$	BOC	Me	H
GS-12	(DM B	-OBn-p C9 T)	pT	587.68	7.13	$-C_7H_{15}$	BOC	Me	Benzyl
GS-8	(DM B	-p C9 T)	pT	497.56	4.90	$-C_7H_{15}$	BOC	Me	H
GS-9	(DH Am	-p C9 T)	pT	369.39	3.00	$-C_7H_{15}$	H	H	H
GS-25	(DM B	-p C11 T)	pT	525.61	5.96	$-C_9H_{19}$	BOC	Me	H

Fig. 1. Formula, structural abbreviation, and compound number of each S1P analog. [DM] shows the dimethylated phosphate group. [B], [Ac], and [Am] show the butoxycarbonyl (Boc) moiety, acetylated form, and free form on the amino group at C2, respectively. C11, C9, and C6 show the length of the carbon chain on the phenyl group. The analogs are classified by the following criteria (Class). [m] and [p] show the position (*meta* and *para*) of the carbon chain on the phenyl group. [S] shows the saturated form, and [D] and [T] show the existence of double and triple bonds, respectively, in a carbon chain. [OBn] and [F] show benzylether on the OH group and F on a carbon chain, respectively. MW: molecular weight of the analog and CLogP: the log *P* values of the analogs calculated by the CLOG P program (parameter of lipophilicity).

described previously (18, 26). Briefly, cells on 12-well plates were loaded overnight with medium containing 0.2% serum, 0.1% fatty acid-free bovine serum albumin, and [^{3}H]AA. The labeled and washed cells were stimulated with the tested compounds at 30 μM for 1 h in the medium. In some cases, cells were cultured with the respective inhibitors, such as pyrrophenone, for 30 min before the assay. Then, the medium was collected and centrifuged at 8,000 × *g* for 2 min. The ^{3}H content of the supernatant was estimated, and data are calculated as percentages of all the radioactivity incorporated (20,000 – 30,000 dpm per well). The release of AA without stimuli was dependent on each experiment and was 2% – 4% of the total incorporated in cells. For quantitative analyses of the data, in some cases, the values of fold-stimulation were normalized as a percent-

age of the respective value without stimuli. As a positive control, release of AA induced by 100 nM 4β-phorbol 12-myristate 13-acetate plus 10 μM A23187 was measured (25). It is reported that there is release of ^{3}H-labeled membrane vesicles and fragments as components of apoptotic bodies in apoptotic cells (27). In some experiments, the supernatant was further centrifuged at 10,000 $\times$ g for 30 min in order to precipitate the pellet containing the vesicles and fragments as described previously (27). In the GS**13**-treated cells, however, a large part of the radioactivity was detected in the supernatant that was collected by the high-speed centrifugation (data not shown). Thus, the ^{3}H content of the supernatant appeared to be non-esterified AA or the soluble metabolites.

Cell viability and morphological changes

The quantification of cell viability was based on the LDH leakage method as previously described (9). Since almost all the tested S1P analogs at 30 μM slightly or markedly caused LDH leakage 6 h after treatment, we examined the effects at 10 μM. None of the analogs at 10 and 30 μM caused LDH leakage from L929 cells 2 h after treatment. For observations of morphological change, L929 cells were cultured with the analogs at 30 μM for 2 h and then washed and cultured with compound-free medium for 4 h. The morphological changes were observed 6 h after the stimulation. Treatment with the S1P analogs tested at 30 μM for 6 h caused morphological changes without exceptions, and the changes induced by the analogs at 10 μM for 6 h were quite variable and depended on the experiment. Thus, we changed the method of application of the S1P analogs for observations of morphological change.

PLA_2 assay in vitro

PLA_2 activity in bee venom secretory PLA_2 (type III, Cayman, No 60500; Cayman Chemical Co., Ann Arbor, MI, USA) was measured using phospholipid vesicles containing 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (1776 MBq/mmol; Perkin Elmer, Boston, MA, USA) as described previously (25).

Data presentation

Values are means $\pm$ S.E.M. for more than three independent experiments, and the number of experiments is shown in parentheses. Some data are means $\pm$ S.D. for a typical experiment, representative of two or three independent experiments. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance followed by the Bonferroni test. For pairwise comparisons, Student's two-tailed t-test was used. P-values <0.05 were considered significant. The Pearson correlation coefficient and Spearman ρ were used for measuring the linear and rank correlation, respectively, between two variables (28).

Results

Effects of S1P analogs on release of AA from L929 cells

First, we examined the effects of the indicated S1P analogs (Fig. 1 and Supplementary Fig. 1), having a dimethylated phosphate group at position C1, a butoxycarbonyl (Boc) moiety on the amino group at C2, and a phenyl group containing a saturated or unsaturated alkyl chain at C3 of 2-amino-1,3-propanediol as a mimic of the *trans* double bond in S1P, at 30 μM on the release of [^{3}H]AA for 1 h from labeled L929 cells (Table 1). The log P values of the analogs calculated by the CLOG P program (Bio-Loom for Windows, ver. 1.0; BioByte) as parameters of lipophilicity are shown in Fig. 1. DMB-mC11S (GS**13**) and DMB-pC11S (GS**6**), which have a C_{11} saturated alkyl chain, and DMB-mC11T (GS**26**) and DMB-pC11T (GS**25**), which have a C_{11} side chain with a triple bond, significantly ($P<0.01$, about 2-fold) released AA. The responses to DMB-mC11D (GS**18**) and DMB-pC11D (GS**17**), which have a double bond in the C_{11} chain, were much less extensive compared with those to (GS**13**), (GS**6**) etc. Among the C_9 chain compounds, DMB-mC9S (GS**20**), DMB-pC9S (GS**19**), DMB-mC9T (GS**14**), and DMB-pC9T (GS**8**) significantly released AA, and the responses to DMB-mC9D (GS**27**) and DMB-pC9D (GS**24**) were limited. DMB-mC6T (GS**15**) and DMB-pC6T (GS**10**), which have a triple bond in the C_6 chain, had no effect. DMB-H (GS**4**), which has a phenyl group without a carbon chain, had no effect. The release of AA induced by 30 μM DMB-mC11S (GS**13**) was not marked for 15 and 30 min after stimulation, and treatment with DMB-mC11S (GS**13**) at 10 μM for 1 h showed a moderate response (150% – 200% of the control response), although the response at 5 μM was marginal (data not shown). The responses by the effective S1P analogs including DMB-mC11S (GS**13**) were less than that of the positive control, which is co-stimulation with 4β-phorbol 12-myristate 13-acetate and A23187, a strong releaser of AA via cPLA$_2\alpha$ activation in L929 cells (25). To examine a possible interaction between the compounds, DMB-mC11S (GS**13**) was used in the following experiments since that was a strongest compound in the tested analogs.

In the presence of 30 μM DMB-mC11S (GS**13**), treatment with DMB-mC11T (GS**26**), but not DMB-pC11T (GS**25**), released AA over the additive value. Treatment with DMB-pC11S (GS**6**) did not enhance the

Table 1. Effects of S1P analogs on the release of AA in L929 cells

Compounds (**GS-No**)	Alone		With 30 μM DMB-mC11S (GS**13**)	
	Release of AA (% of control)			
Vehicle	100%	(15)	270 ± 16[a]	(10)
DMB-H (**4**)	103 ± 7	(5)	209 ± 20	(3)
C_{11} compounds				
DMB-mC11S (**13**)	270 ± 16[a]	(10)	—	
DMB-pC11S (**6**)	163 ± 10[a]	(10)	246 ± 23	(6)
DMB-mC11D (**18**)	129 ± 5	(6)	329 ± 64	(3)
DMB-pC11D (**17**)	120 ± 5	(4)	227 ± 32	(3)
DMB-mC11T (**26**)	241 ± 27[a]	(3)	540 ± 68	(3)
DMB-pC11T (**25**)	193 ± 13[a]	(4)	375 ± 39	(3)
C_9 compounds				
DMB-mC9S (**20**)	234 ± 10[a]	(10)	692 ± 73[b]	(7)
DMB-pC9S (**19**)	213 ± 13[a]	(6)	611 ± 78[b]	(3)
DMB-mC9D (**27**)	136 ± 14	(3)	358 ± 41	(3)
DMB-pC9D (**24**)	124 ± 3.2	(4)	394 ± 66	(3)
DMB-mC9T (**14**)	169 ± 11[a]	(6)	650 ± 82[b]	(6)
DMB-pC9T (**8**)	171 ± 7[a]	(10)	578 ± 34[b]	(4)
C_6 compounds				
DMB-mC6T (**15**)	115 ± 7	(5)	201 ± 17	(5)
DMB-pC6T (**10**)	119 ± 7	(5)	196 ± 34	(4)
DMB-mC11S analogs				
DMAc-mC11S (**22**)	100 ± 6	(7)	154 ± 3[c]	(6)
DMAm-mC11S (**23**)	91.1 ± 5.3	(6)	169 ± 13[c]	(6)
DMB-mC11D analog				
DMB-mC11DF5 (**21**)	179 ± 13[a]	(5)	308 ± 11	(3)
DMB-pC9T analog				
DMB-OBn-pC9T (**12**)	173 ± 17[a]	(5)	254 ± 30	(4)
Native S1P analog				
DMB-S1P (**16**)	136 ± 8	(4)	194 ± 21	(5)
Dihydrogen phosphate analogs				
DHAm-H (**5**)	107 ± 11	(3)	216 ± 13	(3)
DHAm-pC11S (**7**)	120 ± 9	(5)	314 ± 65	(4)
DHAm-pC9T (**9**)	106 ± 12	(5)	317 ± 55	(4)
4β-PMA + A23187	463 ± 36 (Ref. 25)		Not determined	

Labeled L929 cells were treated with the indicated S1P analogs at 30 μM in the presence or absence of 30 μM DMB-mC11S (GS**13**) for 1 h. Data are means ± S.E.M. for the indicated number (n) of experiments, each performed in duplicate or triplicate. [a]$P<0.01$, significantly different from the control (vehicle-treated) cells; [b]$P<0.05$, significantly different from the additive values; [c]$P<0.05$, significantly different from DMB-mC11S (GS**13**)-induced values. As a positive control for release of AA, labeled L929 cells were stimulated with 100 nM 4β-phorbol 12-myristate 13-acetate (4β-PMA) plus 10 μM A23187 for 30 min (Ref. 25).

DMB-mC11S (GS**13**)-induced response. In the presence of DMB-mC11S (GS**13**), treatment with DMB-mC9S (GS**20**), DMB-pC9S (GS**19**), DMB-mC9T (GS**14**), and DMB-pC9T (GS**8**), which have a C_9 carbon chain (saturated or triple bond), synergistically released AA ($P<0.05$). DMB-mC11D (GS**18**), DMB-pC11D (GS**17**), DMB-mC9D (GS**27**), and DMB-pC9D (GS**24**), which have a double bond in the C_{11} or C_9 carbon chain, did not change the DMB-mC11S (GS**13**)-induced release of AA. Treatment with DMB-mC6T (GS**15**) or DMB-pC6T (GS**10**) inhibited the DMB-mC11S (GS**13**)-induced release of AA, although the effect was not

Table 2. Effects of S1P analogs on release of AA in L929-cPLA$_2\alpha$–siRNA cells and C12 cells

Compounds (**GS-No**)	L929-cPLA$_2\alpha$–siRNA cells				C12 cells	
	Alone		with DMB-mC11S (**13**)		Alone	
	Release of AA (% of control)					
Vehicle	100%	(12)	197 ± 5[a]	(12)	100%	
DMB-mC11S (**13**)	197 ± 5[a]	(12)	—		254, 148	(2)
DMB-pC11S (**6**)	148 ± 14	(6)	208 ± 13	(3)	190 ± 21[a]	(6)
DMB-mC11T (**26**)	178, 151	(2)	328, 264	(2)	ND	
DMB-pC11T (**25**)	129 ± 8[a]	(6)	230 ± 30	(5)	ND	
DMB-mC9S (**20**)	182 ± 23[a]	(10)	414 ± 34[b]	(6)	ND	
DMB-pC9S (**19**)	190 ± 15[a]	(4)	441 ± 39[b]	(4)	ND	
DMB-mC9D (**27**)	139, 124	(2)	253, 260	(2)	ND	
DMB-mC9T (**14**)	127 ± 4[a]	(6)	335 ± 30	(6)	141	(1)
DMB-pC9T (**8**)	132 ± 3[a]	(5)	391 ± 47[b]	(3)	180 ± 11[a]	(5)
DMB-mC6T (**15**)	109 ± 2	(4)	199 ± 9	(5)	103	(1)
DMB-pC6T (**10**)	116 ± 11	(5)	240 ± 10	(3)	117 ± 10	(3)
DMAc-mC11S (**22**)	93.9 ± 5.1	(5)	116 ± 8[c]	(5)	90.6	(1)
DMAm-mC11S (**23**)	80.4 ± 2.4[a]	(5)	130 ± 8[c]	(5)	ND	
DMB-mC11DF5 (**21**)	155 ± 17	(4)	232 ± 20	(3)	165	(1)
DMB-OBn-pC9T (**12**)	125 ± 9	(3)	213 ± 17	(3)	143	(1)
DMB-S1P (**16**)	111, 128	(2)	183, 165	(2)	ND	

Labeled L929-cPLA$_2\alpha$–siRNA cells and C12 cells were treated with the indicated S1P analogs at 30 μM in the presence and absence of 30 μM DMB-mC11S (GS**13**) for 1 h. Data are means ± S.E.M. for the indicated number (n) of experiments, each performed in duplicate or triplicate. In some cases, values from one or two independent experiments are shown (n = 1 – 2). [a]P<0.01, significantly different from the control (vehicle-treated) value in the respective cells; [b]P<0.05, significantly different from the additive value; [c]P<0.05, significantly different from the DMB-mC11S (GS**13**)-induced value. ND, not determined.

significant because of large variation.

Next, we investigated several modified S1P analogs (Table 1). DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**), which are analogs of DMB-mC11S (GS**13**) having different structures on the amino group at position C2, did not release AA. Interestingly, both the compounds significantly (P<0.05) inhibited the DMB-mC11S (GS**13**)-induced release of AA. Treatment with 10 μM A23187 (Ca^{2+} ionophore) released AA via activation of cPLA$_2\alpha$ in L929 cells (18, 24). Treatment with 30 μM DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**) did not inhibit the A23187-induced release of AA in L929 cells; the release was 199 ± 24 (% of control, n = 6) on treatment with A23187 alone, 276 ± 22% (n = 3) with A23187/DMAc-mC11S, and 181 ± 8% (n = 3) with A23187/DMAm-mC11S. Treatment with DMB-mC11DF5 (GS**21**, an analog of DMB-mC11D) and DMB-OBn-pC9T (GS**12**, DMB-pC9T analog) alone released AA from L929 cells, and the response by DMB-S1P (GS**16**, native S1P analog) was limited. Treatment with DMAm-H (GS**5**), a simplified analog of DMB-H (GS**4**), had no effect on the release of AA with and without DMB-mC11S (GS**13**). DHAm-pC11S (GS**7**) and DHAm-pC9T (GS**9**), which are lacking a *N*-Boc moiety, and the non-methylated forms of DMB-pC11S (GS**6**) and DMB-pC9T (GS**8**), respectively, had no effect.

S1P analog-induced release of AA via PLA$_2$, but not cPLA$_2\alpha$

Previously, we reported that some synthetic S1P analogs at pharmacological concentrations released AA from cells in a PLA$_2$-dependent, but not cPLA$_2\alpha$-dependent, manner (9). Next, we examined the S1P analog-induced release of AA in two types of L929 variants expressing no and/or extremely low levels of cPLA$_2\alpha$. We have established a clone of L929 cells lacking cPLA$_2\alpha$ by the siRNA method (L929-cPLA$_2\alpha$–siRNA cells, Ref. 26). Table 2 shows the analog-induced release of AA from L929-cPLA$_2\alpha$–siRNA cells. The analogs that released AA from native L929 cells including DMB-mC11S (GS**13**) and DMB-mC9S (GS**20**) significantly released AA from L929-cPLA$_2\alpha$–siRNA cells, and the less effective compounds in L929 cells such as DMB-mC6T (GS**15**), DMB-pC6T (GS**10**), DMAc-mC11S (GS**22**), and DMAm-mC11S (GS**23**) did not release AA from the siRNA cells. The release of AA induced by the S1P analogs was very similar between

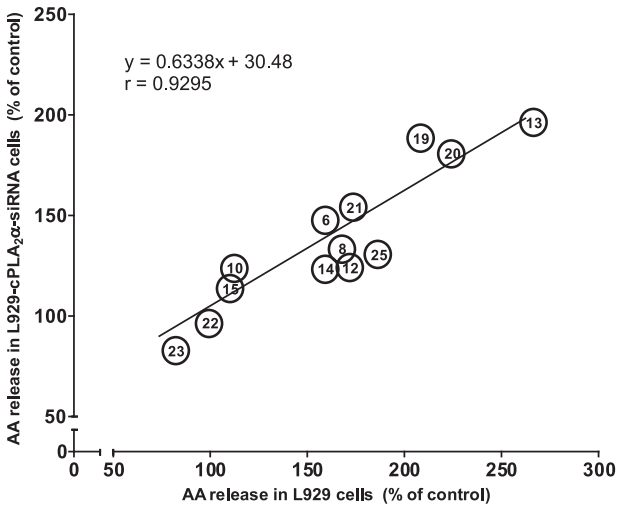


Fig. 2. Positive correlation between S1P analog–stimulated release of AA in L929 cells and that in L929-cPLA$_2\alpha$–siRNA cells. The release of AA stimulated by the analogs at 30 μM for 1 h in L929 cells and the release in L929-cPLA$_2\alpha$–siRNA cells are shown on the x axis and y axis, respectively. Data are presented as a percentage of the control (vehicle-treated) value, and data from over three independent experiments were used for the analysis.

L929-cPLA$_2\alpha$–siRNA cells and native L929 cells, with a high correlation coefficient [Pearson r = 0.929 (Fig. 2) and Spearman $\rho = 0.895$]. Treatment with DMB-mC9S (GS**20**), DMB-pC9S (GS**19**), and DMB-pC9T (GS**8**) synergistically enhanced the DMB-mC11S (GS**13**)-induced release of AA, and both DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**) significantly inhibited the DMB-mC11S (GS**13**)-induced response in the siRNA cells. The L929 variant C12 cell line expresses no and/or undetectable levels of cPLA$_2\alpha$ (24, 26). DMB-mC11S (GS**13**), DMB-pC11S (GS**6**), and DMB-pC9T (GS**8**) markedly or significantly released AA from C12 cells, like from L929 cells. Treatment of L929 cells with 10 μM pyrrophenone, which is reported to be a selective inhibitor of cPLA$_2\alpha$ (26, 29) and to inhibit cPLA$_2\alpha$-mediated release of AA in L929 cells (25), did not inhibit the DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-induced release of AA (Table 3). The data shown above exclude the involvement of cPLA$_2\alpha$ in the S1P analog–induced release of AA from cells.

Ca^{2+}-independent PLA$_2$ enzymes are inhibited by BEL, which does not inhibit secretory PLA$_2$ or cPLA$_2\alpha$ at similar concentrations (12). Treatment with 10 μM BEL partially, but significantly, inhibited the DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-induced release of AA from L929 cells (Table 3). Treatment with 10 μM TEAPC, an inhibitor of secretory PLA$_2$ (30), inhibited the analog-induced release of AA. Also, treatment with the inhibitors decreased the S1P analog-induced responses in L929-cPLA$_2\alpha$–siRNA cells. Treatment with 2 mM DTT, which inhibited secretory PLA$_2$

Table 3. Effects of PLA$_2$ inhibitors on S1P analog–induced release of AA in L929 cells and L929-cPLA$_2\alpha$–siRNA cells

Inhibitors	Release of AA (% of net release by the analogs)	
	DMB-mC11S (GS**13**)	DMB-mC9S (GS**20**)
L929 cells		
Vehicle	100% (4)	100% (4)
BEL	42.8 ± 3.4[a] (3)	57.0 ± 7.6[a] (3)
TEAPC	64.6 ± 2.4[a] (3)	69.8 ± 7.0[a] (3)
Pyrrophenone	92.8 ± 9.4 (3)	119 ± 3 (3)
L929-cPLA$_2\alpha$–siRNA cells		
Vehicle	100% (4)	100% (3)
BEL	14.3, 14.2 (2)	24.2, 44.9 (2)
TEAPC	50.4 ± 6.2[a] (3)	21.2, 52.1 (2)
Pyrrophenone	126 ± 19 (3)	Not determined

Labeled and washed L929 cells or L929-cPLA$_2\alpha$–siRNA cells were incubated for 30 min with vehicle or the respective inhibitor (10 μM BEL, 10 μM TEAPC, or 10 μM pyrrophenone) and then stimulated with 30 μM DMB-mC11S (GS**13**) or 30 μM DMB-mC9S (GS**20**) for 1 h in the presence of the same concentrations of the inhibitors. The release of AA was 206 ± 24 (% of control, n = 4) and 216 ± 15 (n = 4) in the DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-treated L929 cells, respectively, and the values without the analogs in the inhibitor alone–treated cells were 95 – 105 (% of control, n = 6). The release of AA was 187 ± 13 (% of control, n = 4) and 248 ± 47 (n = 3) in the DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-treated L929-cPLA$_2\alpha$–siRNA cells, respectively. In order to clarify the effects of the inhibitors, the net increase of S1P analog–induced release of AA release is normalized as a percentage of the control value in each experiment. Data are means ± S.E.M. for the indicated number (n) of experiments, each performed in duplicate or triplicate. [a]$P<0.05$, significantly different from the control value without the inhibitors.

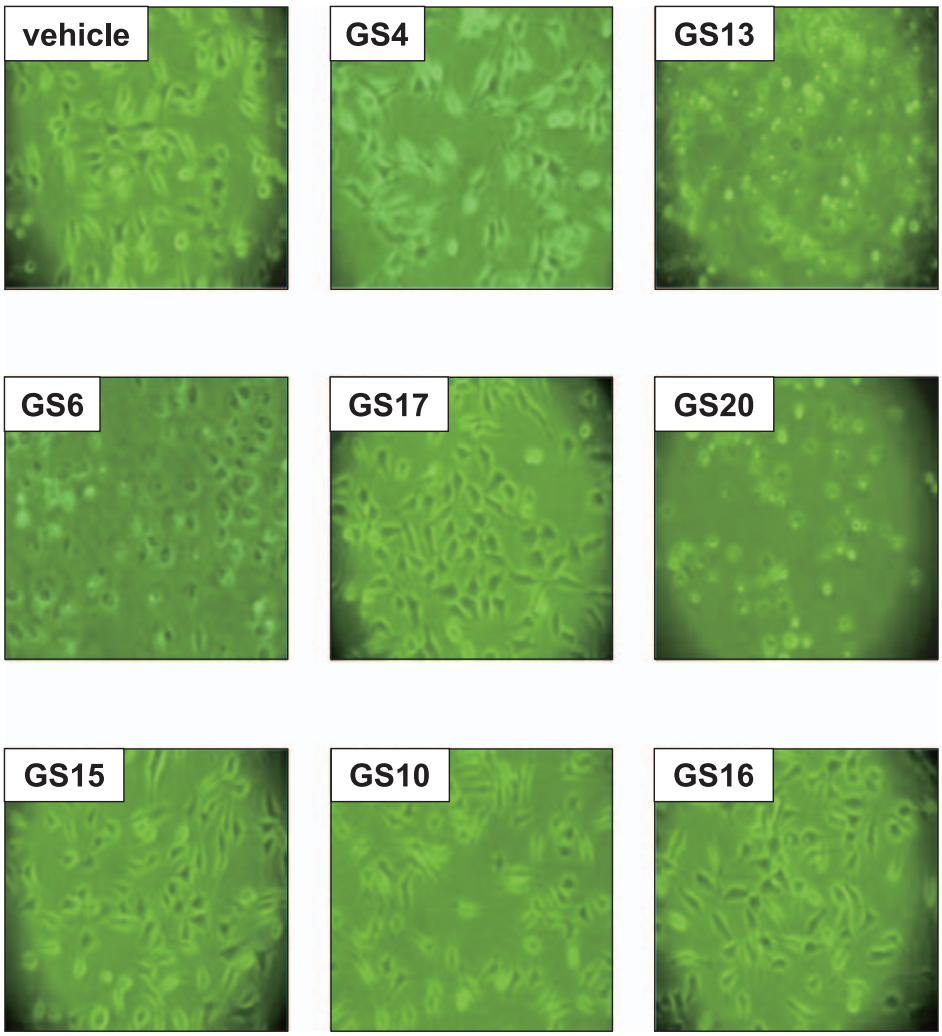


Fig. 3. S1P analog-induced morphological changes in L929 cells. L929 cells were treated with vehicle or the indicated analogs at 30 μM for 2 h. Then, the cells were washed and cultured with fresh medium without the analogs for 4 h. The morphological changes were observed by phase contrast microscopy. Data are from a representative experiment repeated three times with similar results. The results concerning morphological changes induced by other analogs are summarized in Table 4.

activity in vitro (14) and the release of AA via activation of secretory PLA_2 in intact cells (31), markedly inhibited the analog-induced release of AA; the values were 224 ± 31 (% of control, mean ± S.D. of three determinations) by 30 μM DMB-pC11S (GS**6**), 124 ± 4% by DMB-pC11S/DTT, 200 ± 24% by DMB-pC9T (GS**8**), 108 ± 4% by DMB-pC9T/DTT, and 96.1 ± 8.3% by DTT alone in a typical experiment. These findings suggest that analogs such as DMB-mC11S (GS**13**) are likely to release AA in secretory PLA_2– and/or Ca^{2+}-independent PLA_2–mediated pathways, although the exact mechanism(s) of activation induced by the analogs remained to be solved. Whether the analogs directly interact with the PLA_2 enzymes or not remained to be elucidated. In preliminary experiments, addition of DMB-mC11S (GS**13**) at 5 – 30 μM did not change the activity of secretory PLA_2 (type III, 0.01 μg/mL) from bee venom under our experimental conditions using phospholipids vesicles. In a typical experiment, the values were 6800 ± 155 dpm/tube with vehicle and 7500 ± 250 with 10 μM DMB-mC11S (GS**13**).

Morphological changes and LDH leakage induced by synthetic S1P analogs

Figure 3 shows the morphological changes induced by the S1P analogs in L929 cells. In this experiment, L929 cells were treated with 30 μM of analog or vehicle for 2 h, and then the medium was removed and further cultured with fresh medium without analogs for 4 h. The morphological changes were observed by phase contrast microscopy. The cells treated with DMB-mC11S (GS**13**), DMB-pC11S (GS**6**), and DMB-mC9S (GS**20**)

Table 4. Effects of S1P analogs on LDH leakage and morphological changes in L929 cells

Compounds (**GS-No**)	LDH leakage (% of total) 6 h		LDH leakage (% of total) 24 h		Morphological changes
Vehicle	3.58 ± 0.34	(9)	3.65 ± 0.58	(9)	–
DMB-H (**4**)	4.01 ± 1.18	(3)	6.76 ± 1.29	(3)	–
C_{11} compounds					
DMB-mC11S (**13**)	53.0 ± 5.7[b]	(4)	over 90	(4)	+++
DMB-pC11S (**6**)	13.6 ± 5.3	(4)	77.6 ± 7.4[b]	(4)	++ ~ +++
DMB-mC11D (**18**)	30.1	(1)	~ 90	(1)	–
DMB-pC11D (**17**)	55.5	(1)	~ 80	(1)	–
DMB-mC11T (**26**)	68.8, 76.9	(2)	over 90	(2)	+++
DMB-pC11T (**25**)	58.9 ± 11.3[b]	(4)	over 90	(4)	+++
C_9 compounds					
DMB-mC9S (**20**)	87.7 ± 1.7[b]	(3)	over 90	(3)	+++
DMB-pC9S (**19**)	70.5 ± 8.4[b]	(3)	over 90	(3)	++ ~ +++
DMB-mC9D (**27**)	9.91, 4.34	(2)	57.0 ± 2.2[b]	(3)	+ ~ ++
DMB-pC9D (**24**)	36.9 ± 2.2[a]	(3)	~ 80	(3)	–
DMB-mC9T (**14**)	29.7 ± 12.2	(7)	67.7 ± 16.5[b]	(6)	–
DMB-pC9T (**8**)	29.8 ± 12.0	(4)	84.3 ± 7.2[b]	(4)	+/–
C_6 compounds					
DMB-mC6T (**15**)	5.66 ± 1.55	(4)	7.63 ± 4.13	(4)	–
DMB-pC6T (**10**)	4.40 ± 1.31	(4)	5.37 ± 1.27	(4)	–
DMAc-mC11S (**22**)	8.63 ± 2.52	(4)	29.7 ± 9.8[a]	(4)	–
DMAm-mC11S (**23**)	4.72 ± 1.28	(3)	3.90 ± 0.39	(3)	–
DMB-mC11DF5 (**21**)	56.7 ± 3.0[b]	(3)	over 90	(3)	++
DMB-OBn-pC9T (**12**)	20.9 ± 4.8[a]	(4)	85.2 ± 4.5[b]	(4)	– ~ +/–
DMB-S1P (**16**)	9.65 ± 3.0	(4)	75.4 ± 3.8[b]	(4)	–
DHAm-H (**5**)	1.39 ± 0.66	(3)	5.64 ± 1.58	(3)	–
DHAm-pC11S (**7**)	ND		ND		+/– ~ +
DHAm-pC9T (**9**)	ND		ND		–

For the LDH leakage assay, L929 cells were treated with the indicated S1P analogs at 10 μM for 6 and 24 h. Data are means ± S.E.M. for the indicated number (n) of experiments, each performed in duplicate or triplicate. In some cases, values from one or two independent experiments are shown (n = 1 – 2). [a]P<0.05, [b]P<0.01, significantly different from control (vehicle-treated) cells. ND, not determined. For the analysis of morphological changes, L929 cells were treated with the analogs as described in Experimental procedures. The changes were expressed as (–) absent, (+/–) inconclusive, (+) weak, (++) moderate, and (+++) strong.

appeared round with vacuoles. Treatment with DMB-H (GS**4**), DMB-pC11D (GS**17**), DMB-mC6T (GS**15**), DMB-pC6T (GS**10**), and DMB-S1P (GS**16**) had no effect on morphology at 6 h after treatment. Table 4 shows the degree of morphological change induced by the analogs. Most of the analogs that caused morphological changes released AA within 1 h and LDH leakage after 6 h by treatment in L929 cells, although some compounds such as DMB-OBn-pC9T (GS**12**) and DMB-S1P (GS**16**) caused the release of AA but not morphological changes. Similar morphological changes were observed in C12 cells; DMB-mC11S (GS**13**) showed a strong effect, but DHAm-pC11S (GS**7**) did not (data not shown).

Next, we investigated LDH leakage from L929 cells at 6 h and 24 h after treatment with the S1P analogs at 10 μM (Table 4). Treatment with DMB-mC11S (GS**13**), DMB-mC11T (GS**26**), DMB-pC11T (GS**25**), DMB-mC9S (GS**20**), and DMB-pC9S (GS**19**) markedly and/or significantly caused LDH leakage within 6 h. Although the effects of DMB-pC11S (GS**6**), DMB-mC9T (GS**14**), and DMB-pC9T (GS**8**) were not clear within 6 h, these compounds markedly caused leakage 24 h after treatment. DMB-mC11D (GS**18**), DMB-pC11D (GS**17**), DMB-mC9D (GS**27**), and DMB-pC9D (GS**24**), which have a double bond in the carbon chain on the phenyl group and were less effective in the release of AA, appeared to cause LDH leakage markedly and/or

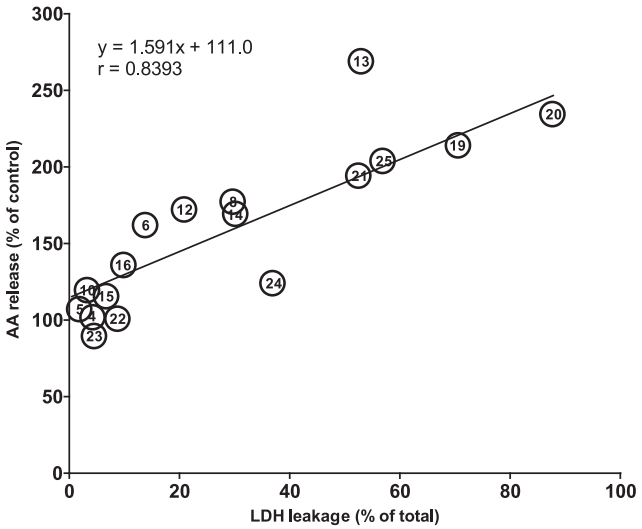


Fig. 4. Positive correlation between S1P analog–stimulated release of AA and LDH leakage in L929 cells. The release of AA for 1 h and the LDH leakage for 6 h induced by the S1P analogs are shown on the y axis and x axis, respectively. Data for the release of AA and leakage of LDH were presented as a percentage of the control value and as a percentage of the total LDH activity, respectively. Data from over three independent experiments were used for the analysis.

significantly 24 h after treatment. By contrast, DMB-H (GS**4**), DMB-mC6T (GS**15**), and DMB-pC6T (GS**10**) did not show toxicity even 24 h after treatment. The effects of DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**) on LDH leakage were limited at 6 and 24 h after treatment, and those of DMB-mC11DF5 (GS**21**), DMB-OBn-pC9T (GS**12**), and DMB-S1P (GS**16**) were marked at 24 h after treatment. In the case of treatment for 6 h, the degree of LDH leakage induced by the effective S1P analogs seemed to correspond to the degree of morphological change. Figure 4 shows that there is a strong positive correlation between the analog-induced release of AA and the LDH leakage in L929 cells; the coefficient values were 0.839 (Pearson r) and 0.877 (Spearman ρ). The cytotoxicity 24 h after treatment appeared to be complex, since DMB-S1P (GS**16**), which had no effect on release of AA and morphological changes in the short periods (1 – 6 h), caused LDH leakage.

The LDH leakage induced by 10 μM DMB-mC11S (GS**13**) for 6 h was significantly inhibited by 10 μM TEAPC, but not by 10 μM pyrrophenone (Table 5). The leakage induced by 10 μM DMB-mC9S (GS**20**) was also inhibited by the TEAPC treatment. The effect of 10 μM BEL on the DMB-mC11S (GS**13**)-induced LDH leakage was not determined since the treatment with BEL alone caused marked leakage 6 h after the treatment (data not shown). Treatment with 10 μM DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**) at least half inhibited the

Table 5. Effects of PLA_2 inhibitors on DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-induced LDH leakage from L929 cells

Inhibitors	LDH leakage (% of total)	
	DMB-mC11S (GS**13**)	DMB-mC9S (GS**20**)
Vehicle	60.3 ± 9.8 (3)	79.8 ± 4.7 (4)
TEAPC	27.0 ± 12.0[a] (3)	38.9 ± 9.0[a] (4)
Pyrrophenone	50.4 ± 11.4 (3)	53.5 ± 10.7 (4)

L929 cells were incubated for 30 min with vehicle, 10 μM TEAPC, or 10 μM pyrrophenone and then treated with 10 μM DMB-mC11S (GS**13**) or 10 μM DMB-mC9S (GS**20**) for 6 h in the presence of the same concentrations of the inhibitors. Treatment with TEAPC or pyrrophenone alone had no effect on the LDH leakage; the values were 4% – 6%. Data are means ± S.E.M. for the indicated number (n) of experiments, each performed in duplicate or triplicate. [a]$P<0.01$, significantly different from the control (vehicle-treated) cells.

LDH leakage induced by 10 μM DMB-mC11S (GS**13**). In a typical experiment from two representative experiments, LDH leakage (% of total) for 4 h was 20.1 ± 3.0 (mean ± S.D. of three determinations) in 10 μM DMB-mC11S–treated cells, 6.7 ± 0.9 in DMB-mC11S/DMAc-mC11S–treated cells, and 12.6 ± 1.2 in DMB-mC11S /DMAm-mC11S–treated cells. The addition of 100 μM AA caused LDH leakage from L929 cells 6 h after treatment; the values in two independent experiments were 58% and 45%, respectively. The response to 10 μM AA was limited. The release of AA induced by the effective S1P analogs such as DMB-mC11S (GS**13**) and DMB-mC9S (GS**20**) was detected in the washed L929 cells that were treated with the analogs for 2 h and then cultured for 4 h without the analogs (data not shown). Thus, the S1P analog–induced release of AA appears to be irreversible, although the release for 4 h (total 6 h after the treatment) may induce spontaneous release from the damaged cells.

Discussion

Structure–activity relationship of synthetic S1P analogs for release of AA

Previously, we reported that synthetic S1P analogs including D-*erythro*-*N,O,O*-trimethyl-S1P that had no affinity for S1P receptors released AA from C12 cells and from L929 cells in a $cPLA_2\alpha$-independent manner (9). In the present study, we synthesized several S1P analogs in order to study the regulation of this release by sphingolipid-related molecules. The analogs used in this study have several modifications; dimethylation of the C1 phosphate group, *N*-Boc on the C2 amino-moiety, and substitution of the C3 phenyl group with or without a carbon chain that mimics a double bond of S1P. For example, DMB-mC11S (GS**13**) having a dimethylated

phosphate group, a *N*-Boc group, and a phenyl group with a meta C_{11} saturated carbon chain, could markedly release AA from L929 cells. D-*erythro*-*O,O*-Dimethyl-S1P is inactive upon binding to cells expressing S1P receptors such as $S1P_{1\text{-}3}$ receptors (32), and the protonated amino group of S1P is critical for the recognition of S1P by the $S1P_1$ receptor (33). Methylation of the C1 phosphate group and the C2 amino-group of S1P eliminate S1P's ability to interact with S1P receptors. Thus, DMB-mC11S (GS**13**) is not likely to interact with S1P receptors on L929 cells if the receptors exist in the cells. Unlike S1P, the compounds tested including DMB-mC11S (GS**13**) did not cause a rapid increase in the intracellular Ca^{2+} concentration in HeLa cells expressing $S1P_1$ receptors (data not shown). We could not rule out the possibility of the involvement of unknown cell-surface receptors.

The release of AA induced by DMB-mC11S (GS**13**), DMB-pC11S (GS**6**), DMB-mC11T (GS**26**), and DMB-pC11T (GS**25**) was much greater than that by DMB-mC11D (GS**18**) and DMB-pC11D (GS**17**) having a double bond in the C_{11} carbon chain. Among the analogs having a C_9 chain, similarly, the response to DMB-mC9S (GS**20**) and DMB-mC9T (GS**14**) was greater than that to DMB-mC9D (GS**27**) or DMB-pC9D (GS**24**) having a double bond in the alkyl chain. The double bond may disturb the conformation and/or flexibility of the compounds for interaction with the target molecule(s) resulting in the release of AA. Furthermore, the specified carbon chain, which is longer than C_6 and does not have a double bond, appeared to have a critical role in the analog-induced release of AA. The reasons were 1) DMB-H (GS**4**), DMB-mC6T (GS**15**), and DMB-pC6T (GS**10**) had no effect, and 2) analogs such as DMB-mC11T (GS**26**), DMB-mC9S (GS**20**), and DMB-mC9T (GS**14**) synergistically enhanced the DMB-mC11S (GS**13**)-induced response. The position of the carbon group (*meta* or *para*) on the phenyl group of S1P analogs did not appear to have a critical role in the release of AA. Treatment with DMB-OBn-pC9T (GS**12**) having a benzylether group on the C3 hydroxy group released AA and did not affect the DMB-mC11S (GS**13**)-induced response. Interestingly, modification of the C2 amino group of DMB-mC11S [DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**)] had an antagonistic and/or inhibitory effect on the DMB-mC11S (GS**13**)-induced response. These results suggest that the synthetic S1P analogs regulate the release of AA in cells with a structure-activity relationship.

S1P analog induced release of AA via secretory and/or Ca^{2+}-independent PLA_2, not via $cPLA_2\alpha$

S1P analogs such as DMB-mC11S (GS**13**), DMB-pC11S (GS**6**), DMB-mC11T (GS**26**), and DMB-pC9T (GS**8**) released AA not only from L929 cells but also from L929 cells lacking $cPLA_2\alpha$ (L929-$cPLA_2\alpha$–siRNA cells and C12 cells). The response in L929-$cPLA_2\alpha$–siRNA cells was very similar to that in native L929 cells, and a higher correlation coefficient was observed. The release of AA induced by the effective analogs was at least partially decreased by the inhibitors of PLA_2 including DTT and TEAPC, but not by an inhibitor of $cPLA_2\alpha$ (pyrrophenone). Treatment with DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**), which alone had no effect, inhibited the DMB-mC11S (GS**13**)-induced release of AA from cells. The analogs that released AA within 1 h did not cause LDH leakage 2 h after treatment. Thus, the release of AA induced by the effective S1P analogs for 1 h after treatment is mediated by PLA_2-, but not $cPLA_2\alpha$-, dependent pathways, and the response is not due to cell toxicity.

Treatment with DTT or TEAPC, general inhibitors of secretory PLA_2s, decreased the DMB-mC11S (GS**13**)- and DMB-mC9S (GS**20**)-induced release of AA (Table 3). It is reported that ceramide activated secretory PLA_2s including types IIa, V, and X, and the enzymes with ceramide prefer AA-containing phospholipids (19, 20). Under our conditions with type III secretory PLA_2 from bee venom, however, we could not detect direct activation of the enzyme by DMB-mC11S (GS**13**). Since treatment with 10 μM BEL, which does not inhibit secretory PLA_2 or $cPLA_2\alpha$ (12), inhibited the S1P analog–induced responses, the analogs may stimulate Ca^{2+}-independent PLA_2, resulting in the release of AA. It is reported that free cholesterol loading, which might be attributable to induction of endoplasmic reticulum stress, released AA and caused apoptosis of mouse macrophages in a β-isoform Ca^{2+}-independent PLA_2-mediated pathway (34). The effective S1P analogs for the release of AA such as DMB-mC11S (GS**13**), which are lipophilic compounds with larger log *P* values, may interact with intracellular organelles, resulting in the activation of secretory and Ca^{2+}-independent PLA_2s. Further identification of the molecular target(s) of the effective S1P analogs including the subtypes of secretory and Ca^{2+}-independent PLA_2s remains to be conducted. In addition, possible roles of other subtypes of cytosolic PLA_2s such as β and γ types on S1P analogs–induced release of AA should be determined.

Possible role of release of AA in cell death

In the present study, a higher correlation coefficient was observed between the S1P analog–induced release of AA for 1 h and cell death at 6 h after treatment in L929 cells (Fig. 4). Lipophilicity of the analogs did not appear to account for variations of AA release, morpho-

logical changes, and LDH leakage 6 h after treatment. S1P analogs released AA, which was followed by LDH leakage in L929 cells (and in C12 cells), and treatment with some analogs [DMAc-mC11S (GS**22**) and DMAm-mC11S (GS**23**)] inhibited both the AA release and leakage of LDH induced by DMB-mC11S (GS**13**). Treatment with TEAPC at least partially but significantly inhibited both the release of AA and leakage of LDH induced by DMB-mC11S (GS**13**). The addition of 100 μM AA caused marked toxicity in L929 cells. Thus, the S1P analog–induced release of AA appears to be linked with cell death in L929 cells. AA has been known to regulate the activity and function of many proteins and cell growth, differentiation, and survival in direct and indirect manners (16, 34). In L929 cells, AA is reported to increase ceramide levels via the activation of sphingomyelinase and resulting cell death (35). Ca^{2+}-independent PLA_2 (β-isofrom) is reported to participate in endoplasmic reticulum stress-induced apoptosis in insulinoma cells (36) and in macrophages (34). Secretory PLA_2s play important roles in cell damage under pathological conditions including inflammation and atherogeneis (14, 16, 20). These findings including our data suggest the cytotoxicity induced by AA. Under our conditions, we measured extracellular ^3H content and have not identified the molecules derived from AA. The eicosanoids produced from AA metabolism in cells may play a role in cell death. In addition, many enzymes responsible for metabolizing AA produce hydroperoxides and other oxidative species, which are highly reactive and show cytotoxicity at low intracellular concentrations (4, 37). The identification of the molecule(s) that is derived from AA and shows cytotoxicity is in progress in our laboratory. The metabolism of AA and/or sphingolipids has important roles in physiological and pathophysiological aspects including immune responses (1, 8). Synthetic analogs of S1P may be useful for regulating the release of AA and cell survival.

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References

1 Baumruker T, Bornancin F, Billich A. The role of sphingosine and ceramide kinases in inflammatory responses. Immunol Lett. 2005;96:175–185.
2 Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. Sphingosine kinases, sphigosine 1-phosphate, apoptosis and diseases. Biochim Biophys Acta. 2006;1758:2016–2026.
3 Taha TA, Mullen TD, Obeid LM. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. Biochim Biophys Acta. 2006;1758:2027–2036.
4 Farooqui AA, Horrocks LA, Farooqui T. Interactions between neural membrane glycerophospholipid and sphingolipid mediators: a recipe for neural cell survival or suicide. J Neurosci Res. 2007;85:1834–1850.
5 Brinkmann V. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. Pharmacol Ther. 2007;115:84–105.
6 Oskeritzian CA, Milstien S, Spiegel S. Sphingosine-1-phosphate in allergic responses, asthma and anaphylaxis. Pharmacol Ther. 2007;115:390–399.
7 Gómez-Muñoz A. Ceramide-1-phosphate: a novel regulator of cell activation. FEBS Lett. 2004;562:5–10.
8 Schenck M, Carpinteiro A, Grassmé H, Lang F, Gulbins E. Ceramide: physiological and pathophysiological aspects. Arch Biochem Biophys. 2007;462:171–175.
9 Nakamura H, Takashiro Y, Hirabayashi T, Horie S, Koide Y, Nishida A, et al. Effects of synthetic sphingosine-1-phosphate analogs on arachidonic acid metabolism and cell death. Biochem Pharmacol. 2004;68:2187–2196.
10 Hirabayashi T, Murayama T, Shimizu T. Regulatory mechanism and physiological role of cytosolic phospholipase A_2. Biol Pharm Bull. 2004;27:1168–1173.
11 Kita Y, Ohta T, Uozumi N, Shimizu T. Biochemical properties and pathophysiological roles of cytosolic phospholipase A_2s. Biochim Biophys Acta. 2006;1761:1317–1322.
12 Blsinde J, Pérez R, Balboa MA. Calcium-independent phospholipase A_2 and apoptosis. Biochim Biophys Acta. 2006;1761:1344–1350.
13 Schaloske RH, Dennis EA. The phospholipase A_2 superfamily and its group numbering system. Biochim Biophys Acta. 2006;1761:1246–1259.
14 Kudo I, Murakami M. Phospholipase A_2 enzymes. Prostaglandins Other Lipid Mediat. 2002;68/69:3–58.
15 Taketo MM, Sonoshita M. Phospholipase A_2 and apoptosis. Biochim Biophys Acta. 2002;1585:72–76.
16 Farooqui AA, Ong WY, Horrocks LA. Inhibitors of brain phospholipase A2 activity; their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. Pharmacol Rev. 2006;58:591–620.
17 Pettus BJ, Bielawska A, Subramanian P, Wijesinghe DS, Machael M, Leslie CC, et al. Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A_2. J Biol Chem. 2004;279:11320–11326.
18 Nakamura H, Hirabayashi T, Shimizu M, Murayama T. Ceramide-1-phosphate activates cytosolic phospholipase $A_2\alpha$ directly and by PKC pathway. Biochem Pharmacol. 2006;71:850–857.
19 Singh DK, Subbaiah PV. Modulation of the activity and arachidonic acid selectivity of group X secretory phospholipase A_2 by sphingolipids. J Lipid Res. 2007;48:683–692.
20 Singh DK, Gesquiere LR, Subbaiah PV. Role of sphingomyelin and ceramide in the regulation of the activity and fatty acid specificity of group V secretory phospholipase A_2. Arch Biochem Biophys. 2007;459:280–287.
21 Nakamura H, Hirabayashi T, Someya A, Shimizu M, Murayama T. Inhibition of arachidonic acid release and cytosolic phospholipase $A_2\alpha$ activity by D-*eryhtro*-sphingosine. Eur J Pharmacol.

2004;484:9–17.

22 Stahelin RV, Subramanian P, Vora M, Cho W, Chalfant CE. Ceramide-1-phosphate binds group IVA cytosolic phospholipase A_2 via a novel site in the C2 domain. J Biol Chem. 2007; 282:20467–20474.

23 Payne SG, Oskeritzian CA, Griffiths R, Subramanian P, Barbour SE, Chalfant CE, et al. The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. Blood. 2007;109:1077–1085.

24 Shimizu M, Azuma C, Taniguchi T, Murayama T. Expression of cytosolic phospholipase $A_2\alpha$ in murine C12 cells, a variant of L929 cells, induces arachidonic acid release in response to phorbol myristate acetate and Ca^{2+} ionophores, but not to tumor necrosis factor-α. J Pharmacol Sci. 2004;96:324–332.

25 Taniguchi T, Shimizu M, Nakamura H, Hirabayashi T, Fujino M, Murayama T. Hydrogen peroxide-induced arachidonic acid release in L929 cells; roles of Src, protein kinase C and cytosolic phospholipase $A_2\alpha$. Eur J Pharmacol. 2006;546:1–10.

26 Shimizu M, Matsumoto Y, Kurosawa T, Azuma C, Enomoto M, Nakamura H, et al. Release of arachidonic acid induced by tumor necrosis factor-α in the presence of caspase inhibition: evidence for a cytosolic phospholipase $A_2\alpha$-independent pathway. Biochem Pharmacol. 2008;75:1358–1369.

27 Zhang J, Driscoll TA, Hannun YA, Obeid LM. Regulation of membrane release in apoptosis. Biochem J. 1998;334:479–485.

28 Zou KH, Tuncali K, Silverman SG. Correlation and simple linear regression. Radiology. 2003;227:617–628.

29 Ono T, Yamada K, Chikazawa Y, Ueno M, Nakamoto S, Okuno T, et al. Characterization of a novel inhibitor of cytosolic phospholipase $A_2\alpha$, pyrrophenone. Biochem J. 2002;363:727–735.

30 Morioka N, Takeda K, Kumagai K, Hanada T, Okoma K, Hide I, et al. Interleukin-1β-induced substance P release from rat cultured primary afferent neurons driven by two phospholipase A_2 enzymes; secretory type IIA and cytosolic type IV. J Neurochem. 2002;80:989–997.

31 Nabemoto M, Ohsawa K, Nakamura H, Hirabayashi T, Saito T, Okuma Y, et al. Reversible activation of secretory phospholipase A_2 by sulfhydryl reagents. Arch Biochem Biophys. 2005;436: 145–153.

32 Lim HS, Oh YS, Sun PG, Chung SK. Syntheses of sphingosine-1-phosphate stereoisomers and analogues and their interaction with EDG receptors. Bioorg Med Chem Lett. 2003;13:237–240.

33 Parrill AL, Wang D, Bautista DL, Van Brocklyn JR, Lorincz Z, Fischer DJ, et al. Identification of Edg 1 receptor residues that recognize sphingosine 1-phosphate. J Biol Chem. 2000;275: 39379–39384.

34 Bao S, Li Y, Lei X, Wohltmann M, Jin W, Bohrer A, et al. Attenuated free cholesterol loading-induced apoptosis but preserved phospholipid composition of peritoneal macrophages from mice that do not express group VIA phospholipase A_2. J Biol Chem. 2007;282:27100–27114.

35 Hayter HL, Pettus BJ, Ito F, Obeid LM, Hannun YA. TNFα-induced glutathione depletion lies downstream of $cPLA_2$ in L929 cells. FEBS Lett. 2005;507:151–156.

36 Lei X, Zhang S, Bohrer A, Bao S, Song H, Ramanadham S. The group VIA-calcium-independent phospholipase A_2 participates in ER stress-induced INS-1 insulinoma cell apoptosis by promoting ceramide generation via hydrolysis of sphingomyelins by neutral sphingomyelinase. Biochemistry. 2007;46:10170–10185.

37 Caro AA, Cederbaum AI. Role of cytochrome P450 in phospholipase A_2- and arachidonic acid-mediated cytotoxicity. Free Radic Biol Med. 2006;40:364–375.