Abstract

It was reported that sphingosine kinase (SPHK) gene expression was increased in tumor tissues and that overexpression of SPHK1 in solid tumor cell lines was involved in anti-cancer drug resistance. Here, we reported the increase in sphingosine kinase 1 (*SPHK1*) and decrease in neutral sphingomyelinase 2 (*NSMase2*) gene expression in myelodysplastic syndromes and acute leukemia. This alteration is supposed to change the cellular sphingolipid rheostat and effects anti-cancer drug sensitivity. However, positive correlations were observed between daunorubicin (DA)-IC_{50} and the *SPHK1* message but not between DA-IC_{50} and *NSMase2* messages, when 16 different leukemia cell lines were used to analyze the relationship between gene expressions and the chemosensitivity against DA. Using two cell lines with either the highest or the lowest *SPHK1* expression, cellular ceramides and sphingosine 1-phosphate (S1P) were quantified by liquid chromatography/mass spectrometry. Increased ceramide was observed in DA-sensitive, but not in DA-resistant cell lines treated with low doses of DA. Upon DA treatment, S1P decreased more in the sensitive cell line than in the resistant cell lines. A SPHK inhibitor recovered the DA-sensitivity of DA-resistant cells. Furthermore, the modulation of SPHK1 gene expression by either overexpression or using siRNA affected the DA sensitivity of representative cell lines. Our results clearly show that the sphingolipid rheostat plays a significant role in DA-induced cytotoxicity of leukemia cells, and suggest that SPHK1 is both a good marker to predict DA-sensitivity of leukemia cells and a potential therapeutic target for leukemia with high SPHK1 expression.

Key words: Sphingolipid metabolic enzyme, Sphingosine kinase 1, Neutral sphingomyelinase 2, quantitative RT-PCR, Daunorubicin, Chemosensitivity, Ceramide, Sphingosine 1-phosphate

Introduction

Sphingholipid and its metabolites recently appeared a potent class of regulators of cell proliferation, survival and apoptosis[1]. The ceramide/S1P rheostat has been proposed as the model to determine the cell's fate[2]. This rheostat is being increasingly recognized as critical element of tumor cell proliferation and cancer chemotherapy. Ceramide, which is located at the central position of the sphingolipid metabolic pathway, is the degradation product of sphingomyelin. Ceramide is also metabolized to glucosyl ceramide, the precursor of several glucosylsphingolipids such as lactosyl ceramides/gangliosides at the cell surface. Sphingomyelinase is responsible for the first step of the sphingomyelin-catabolic pathway and produce ceramide, which plays mostly as the proapoptic factor in response to various reagents including anti cancer drug or radiation. On the contrary, sphingosine kinase (SPHK) is the enzyme that produces sphingosine 1-phosphate (S1P) from sphingosine. S1P binds to five G-protein coupled receptors called as S1P receptors. S1P promotes cell survival or motion as the first or second cellular messenger in response to various agonists.

Interestingly, overexpression of SPHK1 is thought to be oncogenic, and renders transfected cells chemoresistant[2]. SPHK1 mRNA in various cancer tissues was significantly higher than that in their normal counterparts[3]. In prostate cancer cell lines, we recently reported the inverse relationship between SPHK1 level and anti-cancer drug sensitivity[4]. The quantity of each cellular sphingolipid metabolite was thought to be determined by the complex balance between each metabolic enzyme activity and substrates. Enzymes in this pathway might be regarded as the potential target of new anticancer drugs. In contrast to SPHK1, neutral sphingomyelinase 2 (*NSMase2)* was originally cloned as the gene whose expression is increased in cell cycle arrest[5]. Overexpression of *NSMase2* is thought to be pro-apoptotic[6]. However, the analysis of gene expression of sphingolipid metabolizing including SPHK1 of acute leukemia or related diseases has not been reported.

Bonhoure *et al.*[7] reported that targeting *SPHK1* overcomes the multi-drug- resistant gene (*MDR*)-associated chemoresistance of HL60 cells. Most previous studies by other investigators have analyzed sphingolipid rheostat models using only one or two representative cell lines and/or their genetically manipulated subclones.

In the current study, we examined, for the first time, gene expression of sphingolipid

metabolic enzymes in clinical samples including myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). We obtained interesting finding that increase in SPHK1 message and decrease in NSMase2 were observed in MDS and AML bone marrows. However, measurement of sphingolipid metabolites and evaluation of chemosensitivity were not possible in clinical samples. The data obtained from total bone marrow RNA might not be free from the population effect because we did not examine pure leukemic clone.

Therefore we examined 16 leukemia clones with obvious chemosensity to analyze issues described above. The combination of data from clinical samples and those from cell lines clearly suggests the important of SPHK1 expression and the involvement of sphingolipid rheostat (i.e. ceramide/S1P) in anti-cancer drug sensitivity. The relationship between SPHK1 message, protein, SPHK1 enzyme activity, and sphingolipid rheostat were discussed. Our results also strongly suggested that sphingolipid rheostat may be a novel target of leukemia treatment.

Materials and Methods

Clinical samples

After obtaining the informed consent, bone marrow cells were collected from 19 patients with acute leukemia and 60 patients with myelodysplastic syndromes (MDS) (28 RA, 21 RAEB and 11 RAEB-t according to FAB classification) mostly at their initial diagnosis or before any treatment. For the normal control, bone marrow samples for the disease staging were used from 11 patients with non Hodgkin's lymphoma without bone marrow invasion. Mononuclear cells were collected and RNA was extracted. The first strand cDNA was prepared by using the Super Script First-Strand System (Invitrogen).

Cell Lines and Reagents

Human leukemia cell lines (HL60, NB4, K562, HEL, KU812F, CMK86, CMK115, U937, Jurkat, NALL-1, BALL-1, NALM-1, NALM-17, THP-6, PRMI8402, KOPT-K1) [11] were cultured in RPMI1640 containing 10% FCS. Daunorubicin (DA) was purchased from Sigma (St. Louis, MO, USA). SiRNA of human *SPHK1* was from Sigma Genosys (Hokkaido, Japan) as described previously[12]. Scrambled siRNA was purchased from Dharmacon (GE Healthcare Sciences, Tokyo, Japan). The 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, non-ATP competitive SPHK inhibitor, was purchased from Calbiochem (Merck, Darmstadt, Germany). D-*Erythro*-sphingosine 1-phosphate, D-*erythro*-dihydrosphingosine 1-phosphate, *N*-hexadecanoyl-D-*erythro*-sphingosine (C16 ceramide), N-octadecanoyl-D-erythro-sphingosine (C18 ceramide) and *N*-tetracosanoyl-D-*erythro*-sphingosine (C24 ceramide) were from Matreya, Inc. (Pleasant Gap, PA, USA). C17-*erythro*-sphingosine 1-phosphate was from Avanti Polar Lipid, Inc. (Alabaster, AL, USA).

Quantitative RT-PCR of SPHK1, SPHK2, NSMase2, BCL-2 and MDR

Quantitative PCR was performed with Power SYBR Green master mix (Applied Biosystems, Foster City, CA) in duplicate using primer sets described in Table 1, and ABI PRISM 7000 sequence detection systems (Applied Biosystems.) were used for the measurement. ABL gene expression was measured as the internal control with Taqman probe

as shown in Table 1 according to the recommendation by Beillard *et al.*[13] The specificity of PCR product was confirmed in the preliminary experiments using cell lines. Standard curve was created using cDNA fragment of each enzyme which was produced by the PCR method and then inserted into the cloning vector. The relative gene expression level in clinical samples was calculated as the ratio of each gene expression /ABL gene expression, and those in cell lines were calculated as the ratio of the respective message divided by the GAPDH message.

Cell Viability and Measurement of IC_{50} of DA

Viable cells were counted by trypan blue dye exclusion in triplicate. IC_{50} of DA was examined by counting viable cells after 24 h with or without various concentrations of DA. The viable cell number without DA treatment was determined as 100% of the viable cell number.

Western Blotting

Western blotting of SPHK1 was performed using anti-human SPHK1 antibody[14]. Anti-human phospho-SPHK1 specific antibody (Ser-225, ECM Biosciences, Versailles, KY, USA) and anti-β-actin antibody (Cytoskeleton Inc., Denver, Co, USA) were also used.

SPHK Enzyme Activity

Because SPHK has two isoforms, SPHK1 and SPHK2, we tried to measure these SPHK enzyme activities separately. SPHK activity determined in the presence of 0.25% Triton X-100 or in 1 M KCl is only an operational differentiation method developed with overexpressed SPHK1 and SPHK2, respectively[15]. SPHK1 activity was examined as described previously[16] in the presence of 50 μM sphingosine and [γ-^{32}P] ATP (10 μCi, 1 mM) containing 10 mM $MgCl_2$ in 0.25% Triton X-100, which inhibits SPHK2. SPHK2 activity was determined with sphingosine added as a complex with 4 mg/ml BSA and [γ-^{32}P] ATP containing 10 mM $MgCl_2$ in the presence of 1 M KCl, conditions in which SPHK2 activity is optimal and SPHK1 strongly inhibited. Labeled S1P was extracted and separated by TLC on silica gel G60 with 1-butanol/acetic acid/H_2O (3:1:1) as solvent. The spot of S1P on TLC plate was visualized by autoradiography, and was quantified using a densitometer (Atto,

Tokyo, Japan). The activity was expressed as pmol/ min /mg proteins. Assay was performed in triplicate.

Lipid Extraction and LC-MS/MS Analysis of S1P and Ceramides

Leukemic cells were lysed by sonication in the lysis buffer (20 mM HEPES pH 7.4, 10 μg/ml protease inhibitor, 1 mM dithiothreitol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM sodium fluoride, and 0.5 mM 4-deoxypyridoxine). Cellular lipids were extracted by a modified Bligh & Dyer procedure[17] under acidic conditions using 0.1M HCl. C17-S1P (50 ng) employed as an internal standard, was added during the lipid extraction step. The lipid extracts were dissolved in 20% acetonitrile (100 μl) containing 0.1% trifluoroacetic acid (TFA), and subjected to combined liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS analyses were performed using a Waters 2695 HPLC system (Milford, MA, USA) coupled to a Micromass Quattromicro^{TM} API triple-quadrapole mass spectrometer (Micromass, Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization (ESI) source in positive ion mode (ESI+) with multiple reaction monitoring (MRM). Chromatographic separations were obtained under gradient conditions using a Cadenza HS-C18 column (3-μm porous silica, 2.0 mm i.d. x 50 mm, Imtakt Co., Kyoto, Japan). The mobile phase consisted of solvent A (water/TFA, 100:0.1 v/v), solvent B (acetonitrile/TFA, 100:0.1 v/v) and solvent C (acetone/TFA, 100:0.1 v/v).

The gradient was as follows: From time 0 to 0.5 min, a linear gradient from A/B 80:20 to B/C 50:50, time 0.5 to 10 min, B/C 50:50. The program included two washing cycles by solvent B to eliminate the previous sample carryover. The flow rate was set at 0.3 mL/min. The column and autosampler-tray temperatures were stabilized at 30^{o}C and 4^{o}C, respectively. A volume of 10 μL was injected, and the LC effluent was directed to the ESI source without splitting. The analytical run time was 10 min. The capillary voltage was maintained at 3.39 kV. The respective optimal cone voltages for C16 ceramide, C18 ceramide, C24 ceramide, dihydro-S1P, S1P and C17-S1P were 25, 25, 20, 30, 30 and 25 V. The extractor voltage was set to 3 V. Nebulizing gas and electrospray source temperatures were stabilized at 350 and 100 ^{o}C, respectively.

Detection of the ions was performed by monitoring the transitions of *m/z* 380.21 to 264.35

for S1P (collision energy 17 eV), *m/z* 382.21 to 284.34 for dihydro-S1P (17 eV), *m/z* 538.41 to 520.35 for C16 ceramide (11 eV), *m/z* 566.47 to 548.38 for C18 ceramide (11 eV), *m/z* 650.58 to 632.52 for C24 ceramide (11 eV), and *m/z* 366.22 to 250.35 for C17 S1P (17 eV). Peak areas for all components were automatically integrated using Masslynx^{TM} NT 4.0 software (Micromass, Manchester, UK). Losses were accounted for by dividing the each sphingolipid peak area by the percent recovery of the C17-S1P internal standard in each sample. Each loss-adjusted sphingolipid peak area was converted into a concentration using a loss-adjusted standard curve of each sphingolipid, which was linear in the relevant range. We did not measure C22 ceramide because its level was reported to be undetectable in K562 cells[18].

Overexpression and Suppression of SPHK1

Transient overexpression of SPHK1 of NALM-17 cells (DA-sensitive) was performed by SPHK1 expression vector[9] with FUGENE 6 (Roche, Manheim, Germany). SiRNA of SPHK1 was transfected to K562 cells (DA-resistant) with oligofectamine (Invitrogen). The change of SPHK1 expression was confirmed by Western blotting.

Statistical analysis

The correlation index was calculated by Microsoft Excel and its affiliated software. The statistical significance was analyzed by one-way factorial analysis of variance and multiple comparison test (Fisher's method) using Stat view ver5 (SAS Institute Inc., Cary, NC, USA).

Results

Gene expression of sphingolipid metabolic enzymes in normal, MDS and Acute leukemia (AL) bone marrow

Figure 1 shows the message levels of sphingolipid metabolic enzymes as well as *BCL2* and *MDR*. We mainly focused on the statistical difference between AL and normal control. In addition, we also analyzed both high and low risk MDS samples.

Among enzymes examined, AL also showed noticeable increases of *SPHK1* message as compared to normal control. Another isoforn, *SPHK2* did not show significant differences between groups analyzed. It is also of note that *SPHK1* expression of RAEB-t is also significantly higher than normal. There was no correlation between *SPHK1* level and abnormal karyotypes with poor prognosis (data not shown). Sphingomyelin synthase as well as glucosylceramide synthase were reported to be involved with chemoresistance[19,20], and the GM3 level was shown to be higher in chemoresistant cells than chemosensitive cells[21]. In our analysis, however, the message level of these genes did not show significant difference between AL and normal control. Similarly, ASMase, acid ceramidase, and sphingosine 1 phosphate lyase (SPL) gene expression also were not significantly different between AL and normal control.

Interestingly, *NSMase2* was decreased in AL, RAEB and RA samples as compared to normal control, suggesting the alterations of the sphingolipid rheostat. Unexpectedly, statistical significance was also observed in sphingosine 1-phosphate phosphatase 1 (SPP1) only between AL and normal control but not between MDS and normal.

MDR gene expression showed no significant difference between AL and normal control. On the contrary, statistical significance was observed in *BCL2* between AL and normal, but not between MDS and normal control. Because *BCL2* gene expression was reported to locate upstream of *SPHK1*[22], we analyzed the relationship between *SPHK1* and *MDR* or *BCL2* gene expression among total cases. There was no relationship between *SPHK1* gene expression level and other two well recognized genes of chemoresistance, *MDR* and *BCL2*, of the same sample (precise data not shown). It was also revealed that there are no relationship between *SPHK1* and *NSMase2* or between *SPHK1* and *SPP1* gene expression as well as the relationship between *SPHK1* and *SPHK2* gene expression (data not shown). We used RNA

from total mononuclear cells of bone marrow aspirates instead of purified hematopoietic stem cells or blast cells. Therefore, the heterogeneity of bone marrow component might have affected our results.

Relationship between Gene Expression Levels and IC_{50} of DA

Because we could not analyze the pure leukemia cell population from clinical sample, we extended our analysis to 16 established leukemia cell lines to determine whether the DA sensitivity and expression levels of *SPHK1* and/or *NSMase2* in pure leukemia cell population are correlated. Figure 2 shows the relationship between IC_{50} of DA and the expression levels of *SPHK1*, *NSMase2* and *MDR*. We also examined the *SPHK2* message level because *SPHK2* might also affect chemosensitivity of leukemia cells. A significant relationship with IC_{50} of DA was only observed in *SPHK1* but not in others. High *SPHK1* message levels were observed in K562 and KU812F, while low expression of *SPHK1* message was observed in NALM-17 and NB4 cells. We did not examine ceramide synthase and glucosly ceramide synthase, because clinical samples did not show significant involvement of these enzymes in MDS progression and leukemogensis[2].

Correlation between SPHK1 Message Level and SPHK Enzyme Activity

Figure 3A and B reveals that the SPHK1 enzyme activity and *SPHK1* message level were well correlated and that there is also a positive relationship between IC_{50} of DA and SPHK1 enzyme activity. In order to examine the correlation between SPHK1 message, SPHK1 protein, SPHK1 enzyme activity and IC50 of DA further, we selected 4 cell lines as the representative with high and low SPHK1 expression. K562 and KU812F showed the highest SPHK1 enzyme activity and highest IC_{50} of DA, whereas in parallel with the message, NALM-17 and NB4 cells showed the lowest SPHK1 enzyme activity. There was no significant difference in SPHK2 enzyme activity among these four cell lines examined (Figure 3D).

SPHK1 Phosphorylation in Cell Lines with High or Low SPHK1 Enzyme Activity

SPHK1 is reportedly activated by serine phosphorylation[8,9]. Figure 3C illustrated that the cell lines with high SPHK enzyme activity and *SPHK1* message (K562 and KU812F) showed

a higher amount of SPHK1 protein associated with a higher amount of phosphorylation than those (NALM-17, NB4) with low *SPHK1* message and low SPHK enzyme activity, suggesting that *SPHK1* message, total SPHK1 protein, phosphorylated SPHK1 protein, and SPHK enzyme activity are well correlated in these leukemia cell lines analyzed. Densitometric analysis of Western blotting revealed that the ratio of phosphorylated SPHK1/total SPHK1 did not differ significantly between high SPHK1 and low SPHK1 expressing cells (data not shown). We also examined the change of SPHK1 protein after DA treatment. SPHK1 degraded gradually after DA treatment of K562 cells (data not shown), which is consistent with the previous report[23].

Changes of Cellular Levels of Ceramide and Sphingosine 1-Phosphate with DA Treatment

Figure 4A illustrates the respective position of each metabolite (of control K562 cells) by our mass spectrometry system. A clear separation of each metabolite was possible under our experimental conditions. Based on this system, cellular ceramides and sphingosine 1-phosphate (S1P) contents of untreated cells were quantified (Table 2). Besides this quantification, we examined the ceramide contents in the presence of DA at the IC_{50} levels of DA-sensitive cell lines: NB4, 0.03 μM; NALM-17, 0.01 μM; K562 and KU812F, 0.05 μM, respectively. These concentrations are less than 1/20 of DA-IC_{50} of the DA-resistant cells (K562 and KU812F), and DA-resistant cell lines did not show apoptosis at this DA concentration.

Under these conditions, we measured the amount of C24, C18 and C16 ceramide. The most obvious changes were observed in C24 ceramide, which was the major ceramide component of these cell lines. Upon DA treatment, the increase of C24 ceramide was higher in DA-sensitive than in DA-resistant cell lines. C18 ceramide level also showed moderate alterations in both DA-sensitive and DA-resistant cell lines. The changes of C16 ceramide in DA-sensitive cell lines were less remarkable. In contrast, S1P levels showed a remarkable decrease when DA-sensitive cell lines were treated with DA. It is of note that cells with high SPHK1 did not always show high cellular S1P at their control condition, suggesting that the total cellular S1P level was regulated by multi-sphingolipid metabolic enzymes. However, after low-dose DA-treatment, the decrease of S1P was more remarkable in DA-sensitive cell lines, which is consistent with the decrease of SPHK1 protein described above. For the

purpose of comprehensiveness, the ceramide/S1P ratio was calculated with the respective control (non-treated) value as 100% rather than the absolute normalized values (pmol/0.5 mg protein) shown in Table 2. As shown in Fig. 4B, changes in the ceramide/S1P ratio with DA were well correlated with the DA-sensitivity of these cell lines: NALM-17 and NB4, the most sensitive cell lines, showed more than a 5-fold increase (in case of C24/S1P) while K562 and KU812F barely doubled.

When an SPHK inhibitor, 2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl) thiazole, was added together with DA to K562 cells, DA-induced cytotoxicity, the ceramide/S1P ratio and the amount of ceramide increased significantly as compared to DA-treated cells (Fig. 5A and B, data not shown). This SPHK inhibitor showed at most a 40% decrease of viable cells even when its concentration was increased up to 40 μg/ml (twice the recommended concentration[3]) and induced the mild increase of ceramide/S1P ratio. Further increase of the SPHKI concentration did not increase cell death. The isobologram analysis was performed to examine the cooperative effects of DA and SPHK inhibitor according to Kanzawa *et al*.[24] Fig. 5D suggests that these two reagents (DA and SPHKI) showed the additive effect.

Because the specificity of this SPHK inhibitor against SPHK isoform is not completely defined yet, we examined the specificity against SPHK1 and SPHK2 enzyme activity using K562 cells. Our analysis showed that SPHK1 inhibitor inhibited SPHK1 but not SPHK2 enzyme activity of K562 cells and that DA + SPHK inhibitor decreased SPHK1 enzyme activity more compared with DA- or inhibitor-treated cells (Fig. 5C and data not shown). However, according to our preliminary experiments, this SPHK inhibitor did inhibit SPHK2 activity of other cancer cell line (data not shown). Therefore, the inhibitory effects of this SPHK inhibitor against the two SPHK isozymes might depend on cell lines and endogeneous enzyme level.

Effects of Modulation of SPHK1 on DA-Sensitivity of K562 Cells (DA-Resistant) and NALM-17 Cells (DA-Sensitive)

Previous studies have suggested that the modulation of sphingolipid metabolic enzyme affects chemosensitivity[25]. Based on these reports, we analyzed how the modulation of SPHK1 affects the daunorubicin (DA) sensitivity of a human leukemia cell line, K562, which is DA-resistant and NALM-17 cells which is DA-sensitive. We knocked down the *SPHK1*

mRNA of K562 cells by siRNA. siRNA of SPHK1 but not scramble siRNA increased the DA sensitivity of K562 cells (Fig. 6A). In contrast, overexpression of *SPHK1* rendered NALM-17 cells more resistant to DA (Fig. 6B). Overexpression of *SPHK1* rendered K562 cells more resistant to DA than control K562 cells (data not shown). In common with a previous report on sphingosine 1-phosphate lyase[26], the present results also suggest that modulation of the gene expression level of a sphingolipid metabolic enzyme may affect the anti-cancer drug sensitivity of leukemia cells.

Discussion

Involvement of the sphingolipid metabolism in the process of leukemogenesis and disease progression has recently been reported[25,26]. Furthermore, the cellular ceramide level is thought to determine a cell's fate, *i.e.*, differentiation, senescence, and apoptosis[28,29]. Procedures that increase the ceramide level of leukemia cells might lead to the induction of cell death. In this study, we reported a change in the gene expression levels of *SPHK1* and *NSMase2* in bone marrow from cases of MDS and AL. Some AL showed three log order higher SPHK1 gene expression as compared to the normal control. SPHK2 did not show significant differences between groups analyzed. There was no correlation between the SPHK1 gene expression level and abnormal karyotypes with poor prognosis such as 7 monosomy or complex abnormality (data not shown), suggesting that SPHK1 gene expression is independent from karyotype abnormality. Although we did not have the sequential analysis of the same MDS patient. SPHK1 might be a candidate of the surrogate marker of AL and MDS, because its expression gradually increased during the progression of MDS and high in AL. Others reported the relationship between the changes in glucosyl ceramide synthase, sphingomyelin synthase and S1P lyase to chemosensitivity[27-29]. Since we found no significant changes in the expression level of these enzymes between AML and normal control, or between MDS and normal controls, we did not further examine the gene expression level of these genes and the relationship between gene expression and sphingolipid metabolites.

Using sixteen human leukemia cell lines, it was demonstrated that *SPHK1* but not *NSMase2* was correlated with chemosensitivity against DA (Fig. 2). *SPHK2* and *MDR* were also not found to be related with the chemosensitivity of these cell lines. Therefore, *SPHK1* gene was the focus in further study. Figure 3A clearly showed that *SPHK1* message and SPHK1 enzyme activity were well correlated. Intriguingly, increased phosphorylated SPHK1 protein was observed in cell lines with a high *SPHK1* message level (Fig. 3C). Although the localization of SPHK1 protein was not examined, it can be presumed that SPHK1 activated through serine phospholyration was located in the plasma membrane of those DA-resistant cell lines as reported previously[19,20].

After DA treatment, cellular ceramide levels (C16, C18 and C24 ceramide) were changed, although the changes in each ceramide species were heterogeneous among the cell lines tested

(Table 2). It should be noted that, among the ceramide species analyzed, C24 ceramide was most closely related to chemosensitivity against DA. We previously reported an increase of C24 ceramide but not C16 or C18 ceramide in ATRA-treated NB4 cells[28]. The C24 change suggests the involvement of sphingomyelinase whereas the change of C16 or C18 is related to ceramide synthase. Recent reports showed that C18 ceramide increases with imatinib treatment in K562 cells[18], and that C16 ceramide is important in inducing apoptosis[30]. We could not completely rule out the involvement of ceramide synthase in the present study, and we are unable to determine which ceramide species is of primary importance for apoptosis induction. The changes in ceramide species after various treatments may depend on the cell type and drug. Further analysis is necessary to examine the preferred involvement of each ceramide species in the apoptotic process, such as the relationship between each ceramide species and caspase activation.

Based on Table 2, cellular S1P levels in control culture conditions did not depend solely on SPHK1 enzyme activity. Although previous reports showed that the extremely high expression of SPHK1 by stable transfection of expression vector increased cellular S1P level[1,31], we assumed that the cellular S1P level of these cell lines in the current study was determined through the balance of total sphingolipid metabolic enzymes. Another possible explanation is that S1P is released into the culture medium, and that the amounts released are not the same among cell lines. In the current study, we did not examine S1P levels in culture medium. However, because the cellular S1P level decreased in parallel with decreased SPHK1 protein level by DA treatment, it is less likely that the release of S1P into the culture medium is the origin of our current observation. The decrease of cellular S1P was much higher in DA-sensitive cell lines than those of DA-resistant cell lines, suggesting that the basal SPHK1 level is important in determining the S1P level after DA treatment. There are discrepancies regarding the SPHK1 protein level by anti-cancer drug treatment. Taha *et al.*[23] reported that SPHK1 protein was degraded by actinomycin D, doxorubicin, and etoposide, whereas Akao *et al.*[4] reported a transient increase in SPHK1 activity after camptothecin treatment. The changes in SPHK1 expression by chemotherapy could be cell line- and/or anti-cancer drug-specific. Based on our RT-PCR and SPHK2 enzyme activity measurement, the involvement of SPHK2 in DA sensitivity was less likely.

The sphingolipid rheostat is defined as the ratio of total cellular ceramide/S1P. Recently,

Zabielski *et al*.[32] reported a change in the S1P/ceramide ratio after a partial hepatectomy. In the present study, we calculated the ceramide/S1P ratio as shown in Figure 4B. The rheostat ratio (ceramide/S1P) was increased by treatment with DA in DA-sensitive cell lines more than in DA-resistant cell lines. Interestingly, the cellular C24 ceramide/cellular S1P ratio showed the most remarkable change between DA-sensitive and DA-resistant cell lines. Using imatinib-resistant K562 cells, Baran *et al*.[18] reported that the acquisition of imatinib resistance was associated with increased SPHK1 expression and also S1P production, thus resulting in changes in the ceramide/S1P ratio. The SPHK inhibitor showed the additive effect when used in combination with DA (Fig. 5A and D). Furthermore, our results showed that siRNA of *SPHK1* augmented the cytotoxic effect of DA in DA-resistant K562 cells and overexpression of SPHK1 rendered DA-sensitive NALM-17 cells more DA-resistant.

Taken together, our results demonstrate the adequacy of the sphingolipid rheostat model in leukemia cells after the anti-cancer drug, DA. Further study may lead to the possibility that the strategy of sphingolipid rheostat modulation can be applied not only to chemoresistant patients after various chemotherapy regimens but also to predictions of the outcome of a patient’s initial treatment. A combination of the SPHK inhibitor (including siRNA) and an anti-cancer drug such as DA might also be a possible choice in the future chemotherapy.

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Figure legends

Figure 1. **Relative message expression levels of sphingolipid metabolic enzymes in AL, MDS and normal control.**

Quantitative RT-PCR was performed with total bone marrow RNA. The relative expression level was calculated as the enzyme gene expression /ABL gene expression level and was shown in the log scale. The classification of MDS was according to the FAB classification. Horizontal short bar denotes the mean value of the group. Statistical significances were analyzed by using one-way factorial analysis of variance and multiple comparison test (Bonferroni/Dunn's method). * means $p< 0.01$.

Figure 2. Correlation between IC_{50} of DA and gene expression levels.

Gene expressions of *SPHK1, NSMase2, SPHK2* and *MDR* were examined in 16 leukemia cell lines and expressed as the relative ratio of each enzyme message/*GAPDH* message in log scale. IC_{50} of DA was determined as described in Materials and methods. The correlation of IC_{50} of DA and each message level was calculated, with correlation efficiency (R^2) shown in each figure. The relative gene expression level of 1.E-05 of *SPHK1*, 1.E-06 of *NSMase2*, 1.E-08 of *SPHK2* and 1.E-08 of *MDR* gene were the detection limit of our assay conditions.

Figure 3. Relationship between IC_{50} of DA, SPHK activity and SPHK1 message.

SPHK1 enzyme activity was measured as described in Materials and Methods, and was shown as pmol/min/mg protein. Relationships between SPHK enzyme activity and *SPHK1* message level (A), and the IC_{50} of DA and SPHK enzyme activity (B) were illustrated. Correlation efficiency (R^2) was shown in each figure. (C) SPHK1 and serine phosphorylated SPHK1 protein levels of K562, KU812F, NALM-17 and NB4 cells were examined using anti-SPHK1[14] and anti-p-SPHK1 antibody (ser-225, ECM Biosciences), respectively. (D)

SPHK2 enzyme activity of 4 representative cell ines were shown.

Figure 4. Measurement of cellular ceramide and S1P contents and calculation of ceramide/S1P ratio in DA-sensitive and -resistant cell lines after DA treatment.

Cellular ceramides (C16, C18 and C24) and S1P of representative cell lines (K562 and KU812F as DA-resistant and NALM-17 and NB4 as DA-sensitive) were analyzed using LC-MS/MS as described in Materials and methods. (A) Respective chromatograms of sphingolipid in lipid extract from control K562 cells. Lipid extracts from cell lysate corresponding to 0.5 mg protein were dissolved in 20% acetonitril containing 0.1% TFA (100 μl) as described in the Materials and Methods, and 10 μl was injected for analyses of S1P, dihydro-S1P, C16-ceramide, C18-ceramide and C24-ceramide. C17-S1P (50 ng/ml) was used as the internal standard. Peaks are displayed with their respective retention times. The measurement was repeated at least twice. (B) Ceramide/S1P ratios of four representative cell lines are calculated from Table 2. The ratio in non-treated cells was regarded as 100%. Statistical significance was calculated using a one-way factorial analysis of variance and a multiple comparison test (Fisher's method): **, $p<0.01$; *, $p<0.05$

Figure 5. Effect of SPHK inhibitor on DA-induced cytotoxicity and ceramide/S1P ratio.

(A) K562 cells were treated with various concentrations of DA either with (square) or without (rectangle) 20 μg/ml of SPHK inhibitor (2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole). After 24 h, viable cell numbers were counted. Statistical significance was examined by Student t' test. *, $p<0.01$. (B) Cellular ceramides and the S1P content of K562 cells with or without various treatments were measured. Ceramide and S1P contents were measured using LC-MS/MS. Ceramide/S1P ratios were calculated in the SPHK inhibitor (20 μg/ml)-treated, DA-treated (0.05 μM), or DA+SPHK inhibitor-treated cells 24 h after each treatment. The ratio of ceramide/S1P of control K562 cells was defined as 100%. Each measurement was

performed in triplicate and repeated at least twice. Statistical significance was examined using a one-way factorial analysis of variance and a multiple comparison test (Fisher's method); *, $p<0.01$. (C) The inhibitory effect of SPHK inhibitor on SPHK1 enzyme activity. Samples of control, SPHK inhibitor (20 μg/ml), DA (0.05 μM), DA+SPHK inhibitor-treated K562 cells were collected and their SPHK1 enzyme activity was measured according to the Materials and Methods in triplicate. Experiments were repeated twice with the similar results. * $p<0.01$ (D) Isobologram evaluation of DA and SPHK inhibitor was performed according to Kanzawa *et al*.[24] Because the highest inhibition of the viable cell number was at most 40% by 40 μg/ml of SPHK inhibitor. Figure showed the analysis of 40% inhibition of viable cell number. Broken line denotes that 40% inhibition was obtained by either 0.8 μM of DA or 40 μg/ml of SPHK inhibitor. Solid line shows the effect of the combination of various concentrations of these two drugs. The left-shift of the curve denotes the additive effect of DA and SPHK inhibitor.

Figure 6. Modulation of SPHK1 gene expression and DA-sensitivity.

(A) DA (shown as μM) sensitivity of K562 cells was examined with or without siRNA of *SPHK1*, or scramble siRNA. For the transfection of siRNA, oligofectamine (Invitrogen) was used according to the manufacturer's instructions. Viable cell numbers were counted by trypan blue dye exclusion 24 h after DA addition (0.5 and 5 μM, respectively). The percentage of viable cell numbers was expressed as 100% compared to non-treated cells. (B) NALM-17 cells were transiently transfected with *SPHK1*- or mock-expression vector. Cell numbers were counted 48 h after DA addition (0.5 and 5 μM). In panels A and B, statistical significance was analyzed by Fisher's method using Stat View Ver 5; **, $p<0.01$. (C) (D) Western blotting showed the effect of siRNA of SPHK1 of K562 and SPHK1 overexpression of NALM-17 cells.

Table 1. Design and location of the oligonucleotides used for real-time PCR

Hugo gene nomenclature	chromosome	Alternate name	GenBank Accession No.	Amplicon location relative to transcription	%GC	Forward primer sequence	Reverse primer sequence	Annealing Temp.
SPHK1	17	sphingosine kinase 1	NM_021972	+821/+1060	58.6	TCCTGGCACTGCT GCACTC	TAACCATCAATTC CCCATCCAC	61.0
SPHK2	19	sphingosine kinase 2	NM_020126	+26/+186	70.9	AGCAGCAGGACC AGAGGCCA	GGTGAGGGCAAA GCGTGGG	67.0
SPL	10	sphingosine-1-phosphate lyase	NM_003901	+794/+934	51.5	TGGAGGTGGATGT GCGGGCAA	CCCAGACAAGCGT CGACATGAAG	62.0
SPP1	4	sphingosine-1-phosphate	NM_030791	+637/+772	44.9	ACCGCCATCCCCA TTTCT	AGGAATCCAGCAA TAATATCCAG	59.0
ASMase	11	acid sphingomyelinas	NM_000543	+1073/+1220	56.0	AAGCCCTGCGCAC CCTCAGAA	CCTGAAGCTCCCC CACCAGCC	64.0
NSMase2	2	neutral sphingomyelinas	NM_018667	+1535/+1645	60.0	ACTTTGATAACTG CTCCTCTGAC	TTCGTGTCCAGCA GAGTACC	63.0
ACDase	8	acid ceramidase	NM_177924	+875/+968	47.8	GATATTGGCCCCA GCCTACTTT	ACCCTGCTTAGCA TCGAGTTCA	60.0
GlcCer synthase	9	glucosylceramide synthase	NM_003358	+144/+341	37.5	CAAGCTCCCAGGT GTCTCTCTTC	GATTAATGCCAAC TTTTTTACCACCTA	64.0
SM synthase1	10	sphingomyelin synthase 1	NM_147156	+757/+894	48.4	GAAGCCCAACTGC GAAGAATAA	AGAGTCGCCGAG GGGAATAC	60.0
MDR	7	multidrug resistance 1	NM_000927	+1141/+1349	48.7	AGTGGGCACAAAC CAGATAA	CTGTCCATCAACA CTGACCA	63.0
BCL2	18	BCL2 (B-cell lymphoma type 2)	NM_000633	+337/+583	61.7	GCCGAGATGTCCA GCCAG	AGTTCCACAAAGG CATCCCA	62.0
GAPDH	12	glyceraldehyde-3-phosphate	NM_002046	+307/+401	60.7	CAGGAGCGAGATC CCTCCAA	CCCCCTGCAAATG AGCCC	57.2
ABL	22	PCR primer	sense	CCCAACCTTTTCGTTGCACTGT				
			antisense	CGGCTCTCGGAGGAGACGTAGA				
		Taqman probes	sense	ACTAAAGGTGAAAAGCTCCGGGTC-FITC				
			antisense	LCRed640-TAGGCTATAATCACAATGGGGAATGG				

Table 2. Changes in Cellular Ceramides and S1P Content after Daunorubicin Treatment

Cell	ceramide						S1P	
	C16		C18		C24		S1P	
Daunorubicin	-	+	-	+	-	+	-	+
	(pmol/0.5 mg protein)							
K562	375.1	464.1	19.6	34.8	726.2	715.2	6.1	5.4
+/-SD	75.7	159	3.8	11.7	12.8	43	1.1	1.2
KU812F	120.0	185.8	7.2	12.5	757.2	847.7	10.2	12.3
+/-SD	2.4	9.3	1.7	0.6	103	241	0.9	1.9
NALM-17	1118.0	1678.0	498.5	1047.0	1761.0	4692.0	24.3	11.5
+/-SD	508	214	16.6	66.2	238	739	4.3	1.7
NB4	579.1	702.6	141.4	207.8	670.9	1436.0	8.2	3.5
+/-SD	80.7	41.5	35.5	8.2	109	254	3.3	1.3

Samples were prepared from K562, KU812F, NALM-17 and NB4 cells with or without DA treatment, respectively. DA concentration used was described in the Results section. Ceramide species and S1P were measured in triplicate by LC-MS/MS according to the Materials and Methods. Results were shown as the mean +/- SD.

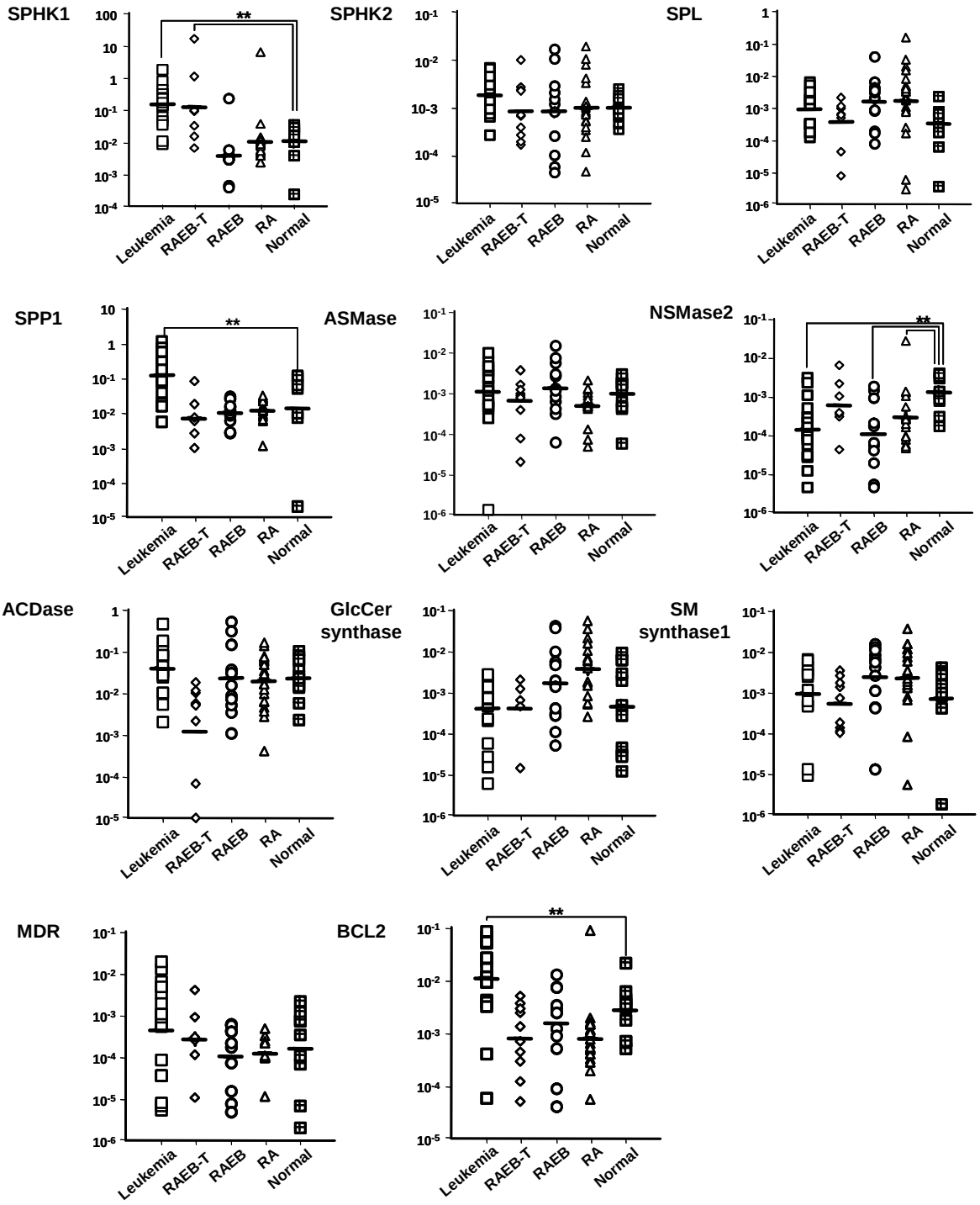
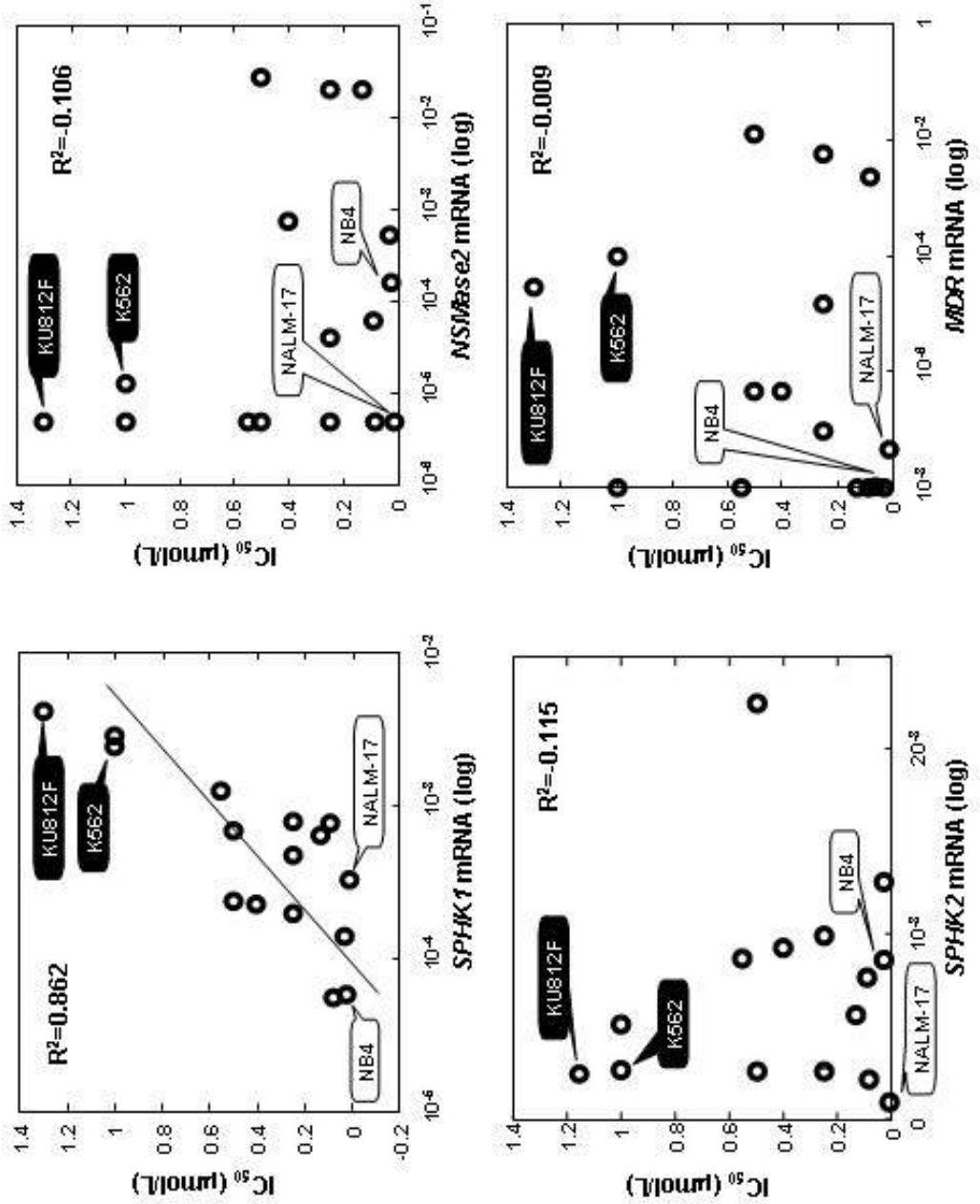

SPHK1
SPHK2
SPL
SPP1
ASMase
NSMase2
ACDase
GlcCer synthase
SM synthase1
MDR
BCL2
**
Leukemia
RAEB-T
RAEB
RA
Normal

Figure 1.

Figure 2.


R²=0.862
R²=-0.106
R²=-0.009
R²=-0.115
KU812F
K562
NALM-17
NB4
IC50 (μmol/L)
SPHK1 mRNA (log)
NSMase2 mRNA (log)
MDR mRNA (log)
SPHK2 mRNA (log)

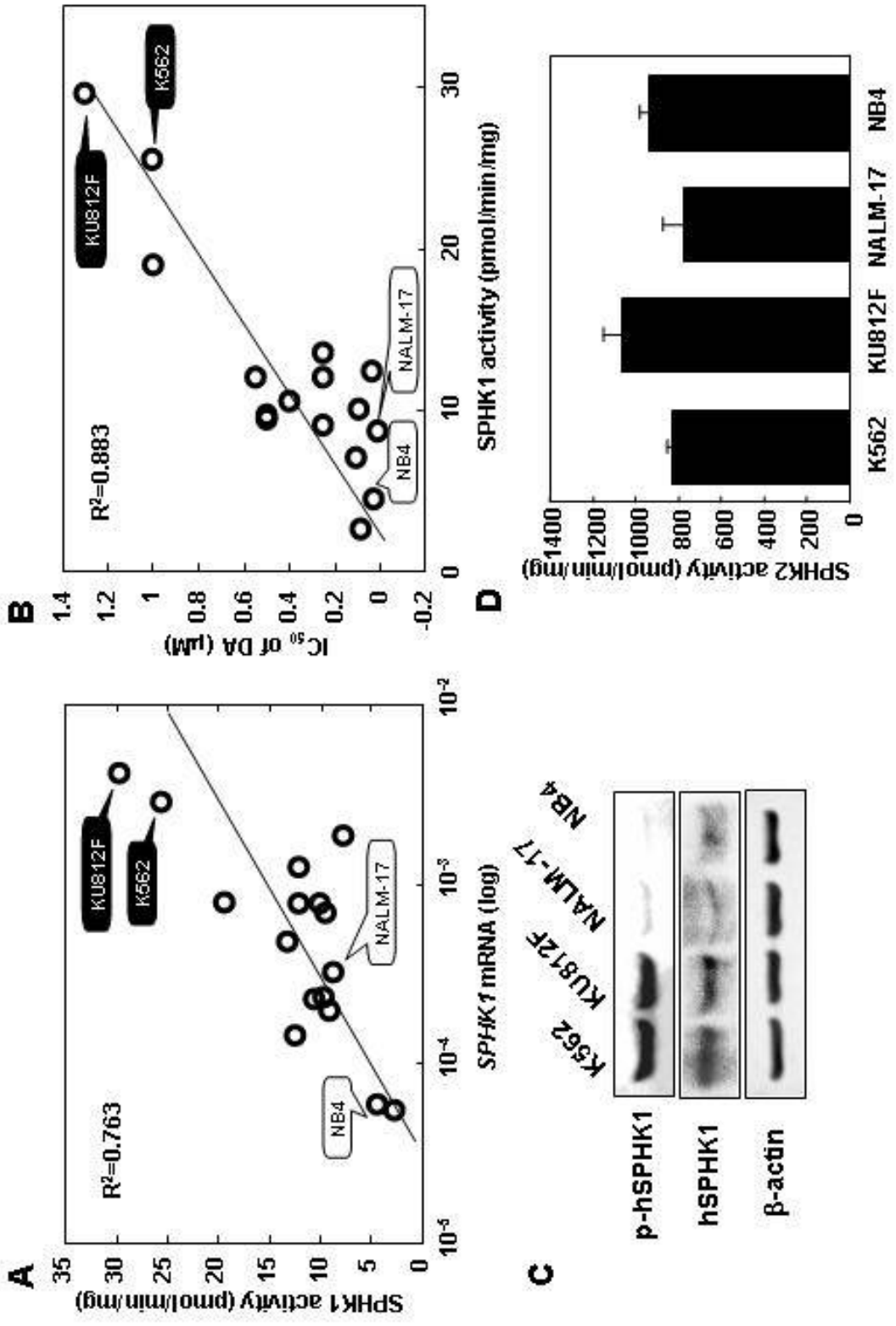

A
SPHK1 activity (pmol/min/mg)
R²=0.763
KU812F
K562
NALM-17
NB4
SPHK1 mRNA (log)
B
IC50 of DA (μM)
R²=0.883
KU812F
K562
NALM-17
NB4
SPHK1 activity (pmol/min/mg)
C
K562
KU812F
NALM-17
NB4
p-hSPHK1
hSPHK1
β-actin
D
SPHK2 activity (pmol/min/mg)
K562
KU812F
NALM-17
NB4

Figure 3.

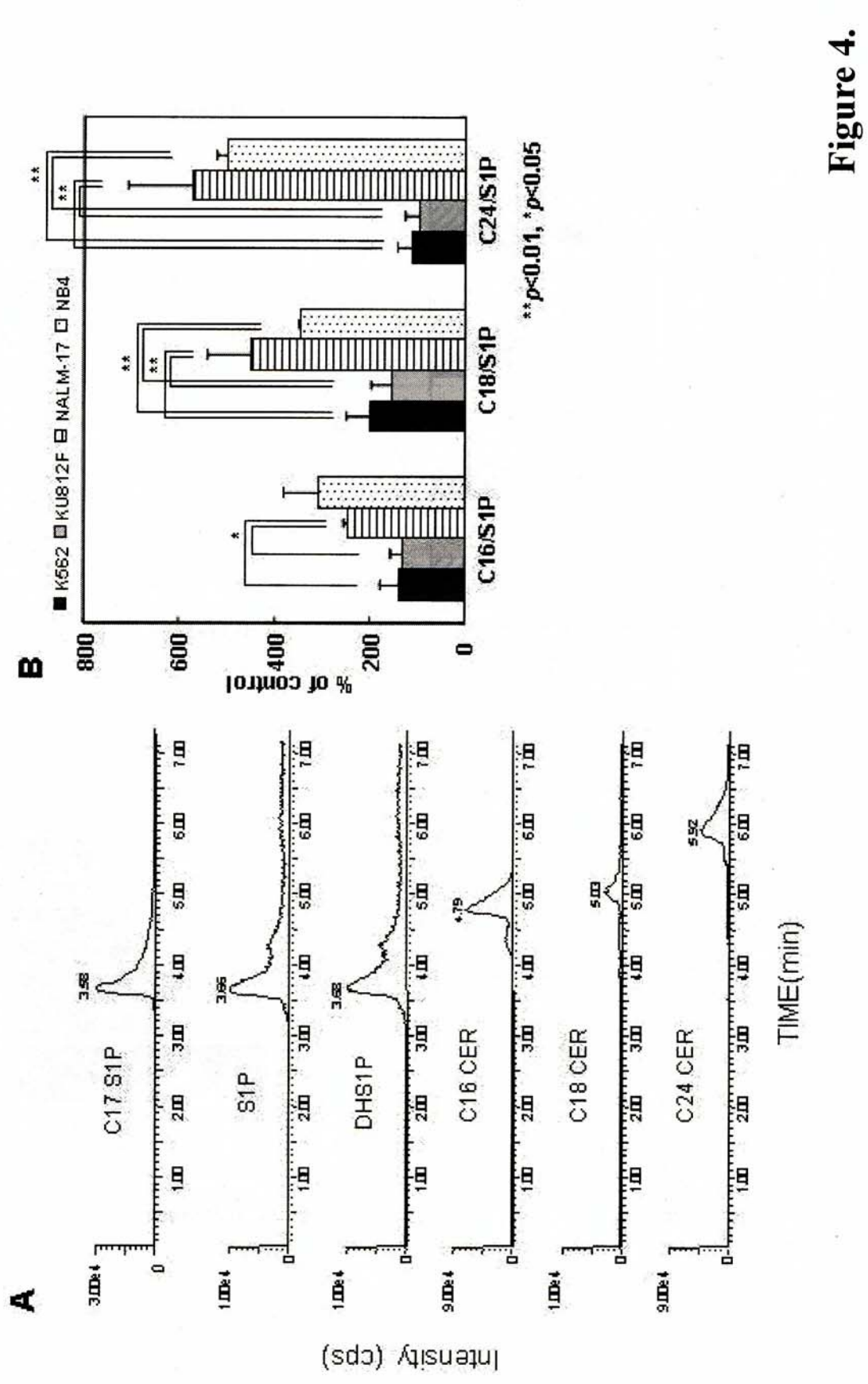

A
C17 S1P
S1P
DHS1P
C16 CER
C18 CER
C24 CER
Intensity (cps)
TIME(min)
B
K562 KU812F NALM-17 NB4
% of control
C16/S1P
C18/S1P
C24/S1P
**p<0.01, *p<0.05

Figure 4.

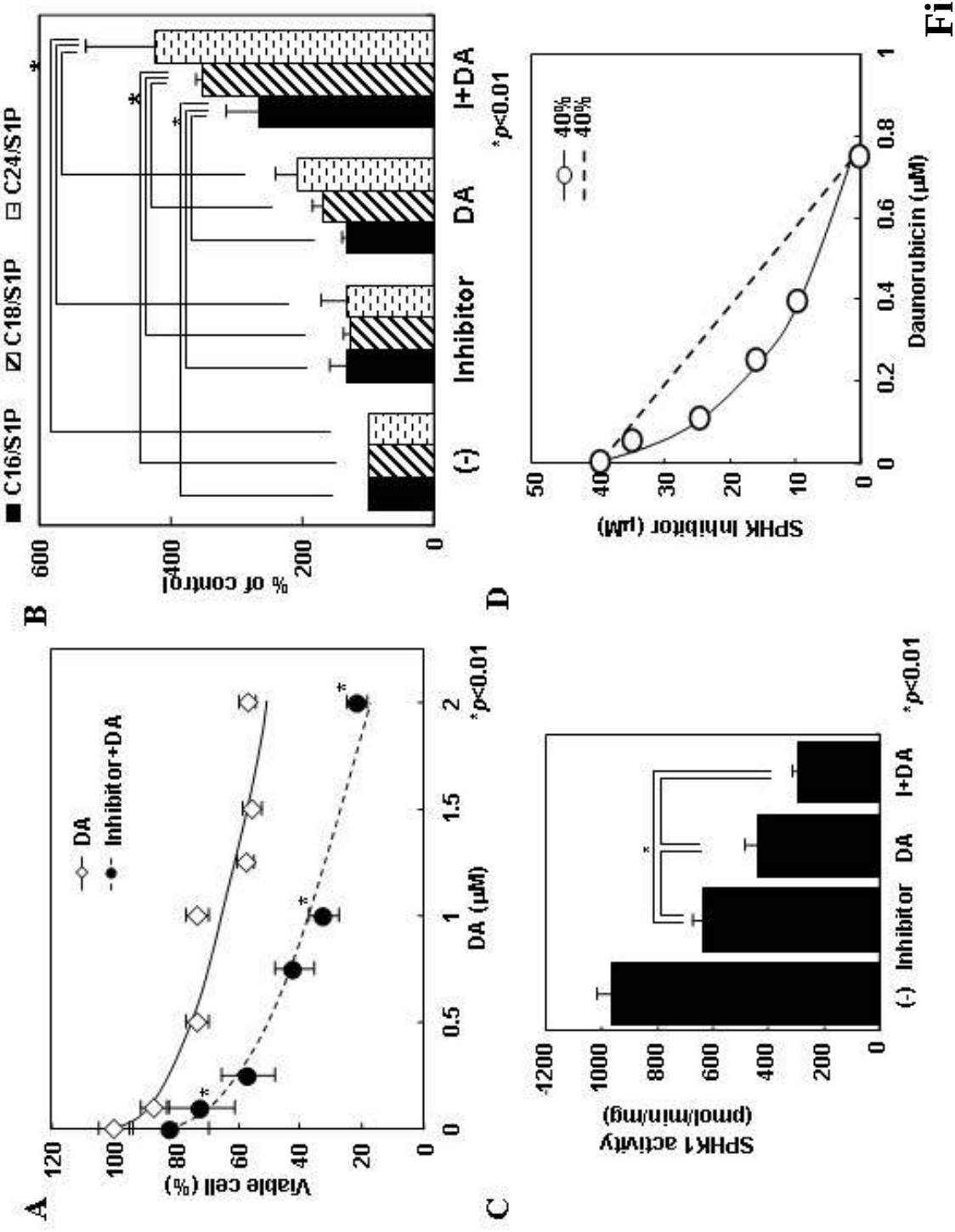

A
Viable cell (%)
DA
Inhibitor+DA
DA (μM)
*p<0.01
B
% of control
C16/S1P
C18/S1P
C24/S1P
(-)
Inhibitor
DA
I+DA
*p<0.01
C
SPHK1 activity (pmol/min/mg)
(-)
Inhibitor
DA
I+DA
*p<0.01
D
SPHK Inhibitor (μM)
40%
40%
Daunorubicin (μM)

Figure 5

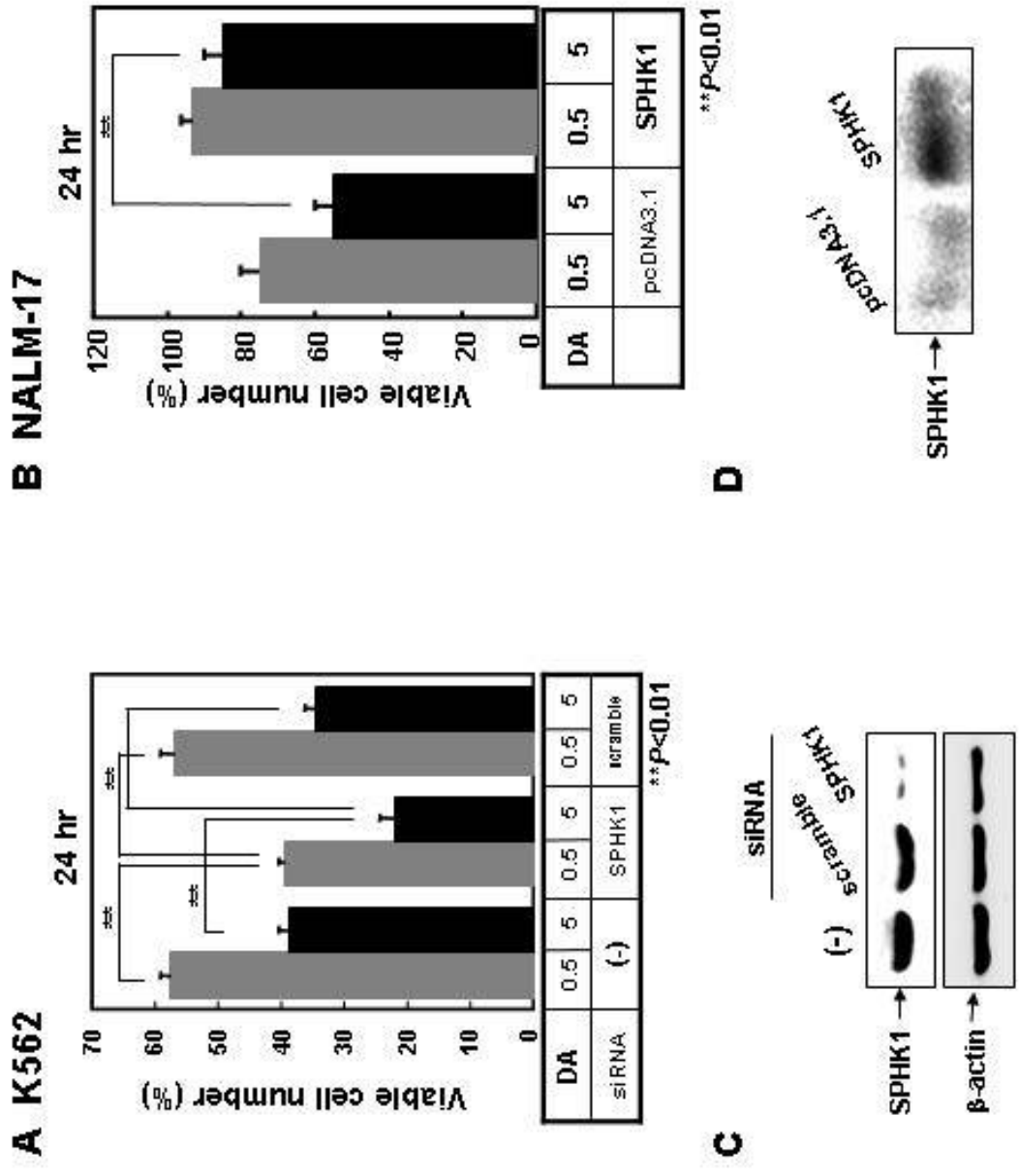

A K562
24 hr
Viable cell number (%)
70
60
50
40
30
20
10
0
DA 0.5 5 0.5 5 0.5 5
siRNA (-) SPHK1 scramble
**P<0.01
B NALM-17
24 hr
Viable cell number (%)
120
100
80
60
40
20
0
DA 0.5 5 0.5 5
pcDNA3.1 SPHK1
**P<0.01
C
siRNA
(-) scramble SPHK1
SPHK1
β-actin
D
pcDNA3.1 SPHK1
SPHK1

Figure 6