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NOTES

Inhibition of Recombinant Sphingosine Kinases by Novel Inhibitors of Microbial Origin, F-12509A and B-5354c

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Sphingosine-1-phosphate (SPP) was initially described as an intermediate in the metabolic pathway of long-chain sphingoid bases[1]. However, it is now widely accepted as a unique bioactive lipid messenger. It acts as a second messenger to regulate proliferation and survival intracellularly, and as a ligand for G protein-coupled receptors of the EDG-1 subfamily extracellularly[2~4]. SPP is involved in a variety of cellular functions, including vascular maturation[5], angiogenesis[6], TNF-α signaling[7], FcεRI signaling in mast cells[8], nerve growth factor-mediated neuronal survival and differentiation[9], regulation of cell motility[10,11], platelet activation[12], activation of muscarinic K^+ currents[13], neurite retraction[14] airway remodeling in asthma[15], and cell proliferation, especially in signal transduction pathways of platelet-derived growth factor (PDGF)[16].

Sphingosine (SPH) kinase, which catalyzes phosphorylation of SPH on its primary hydroxyl group, is a key enzyme that regulates the cellular level of SPP. Recently, we cloned two isoforms (SPHK1 and SPHK2) of mammalian SPH kinase and characterized their biochemical properties[17~19]. However, precise individual physiological functions and regulatory mechanisms of the two isoforms remain unclear. Therefore, selective inhibitors of each SPH kinase isoform are desirable for illuminating this.

We previously screened for inhibitors of SPH kinase and isolated novel SPH kinase inhibitors F-12509A[20] and B-5354c[21,22] from microbial metabolites. F-12509A was isolated from a culture broth of a discomycete, *Trichopezizella barbata* SANK 25395. The structure of F-12509A is a new sesquiterpene quinone consisting of a drimane moiety and a dihydroxybenzoquinone (Fig. 1). B-5354c was isolated from a culture broth of a novel marine bacterium, SANK 71896. The structure of B-5354c is a new ester of 4-amino-3-hydroxybenzoic acid with a long-chain unsaturated alcohol (Fig. 1). Previously we reported the kinetic analysis of the inhibitory effects of these two inhibitors on crude SPH kinase from rat liver cytosol fraction. PCR analysis indicated that rat liver SPH kinase contains SPHK1 and SPHK2 (data not shown). F-12059A inhibits rat liver SPH kinase competitively with respect to SPH with a K_i value of 18 μM[20]. On the other hand, B-5354c inhibits the enzyme noncompetitively with a K_i value of 12 μM[22]. However, the inhibitory properties of these inhibitors on recombinant SPHK1 and SPHK2 had not been clarified. Therefore, we investigated the inhibitory effects of F-12509A and B-5354c on recombinant human SPHK1 and SPHK2.

SPH kinase assay using recombinant enzymes, prepared from HEK293 cells (ATCC CRL-1573) transfected with vectors containing hSPHK1 or hSPHK2 constructs, was performed by the method previously reported[18,19] with

Fig. 1. Structures of SPH kinase inhibitors.

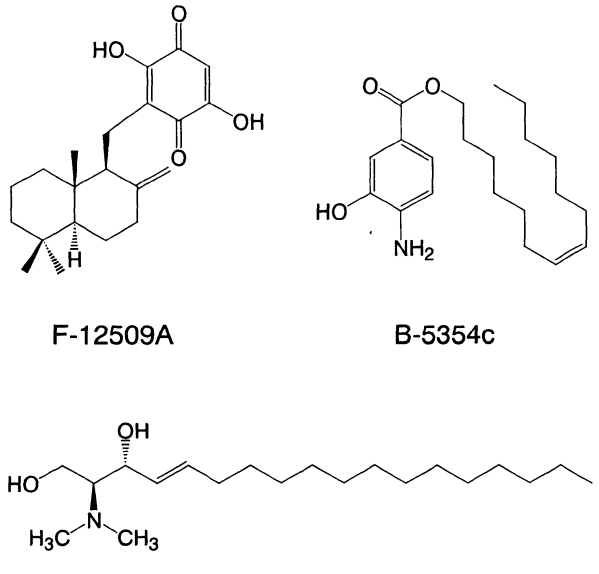

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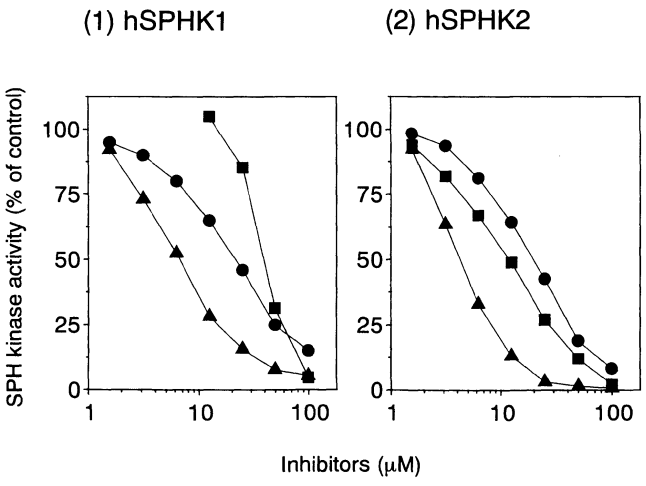
Fig. 2. Dose-dependent inhibition of recombinant hSPHK1 and hSPHK2 by F-12509A, B-5354c and DMS.



SPH kinase activities in cytosol from HEK293 cells transfected with hSPHK1 (1) and hSPHK2 (2) were measured with 20 μM SPH in the presence of increasing concentrations of F-12509A (■), B-5354c (▲) and DMS (●). The IC_{50} values calculated from the dose-dependent curves were: (1) 41, 7.8, 21 μM, and (2) 11, 5.0, 19 μM, for F-12509A, B-5354c and DMS, respectively.

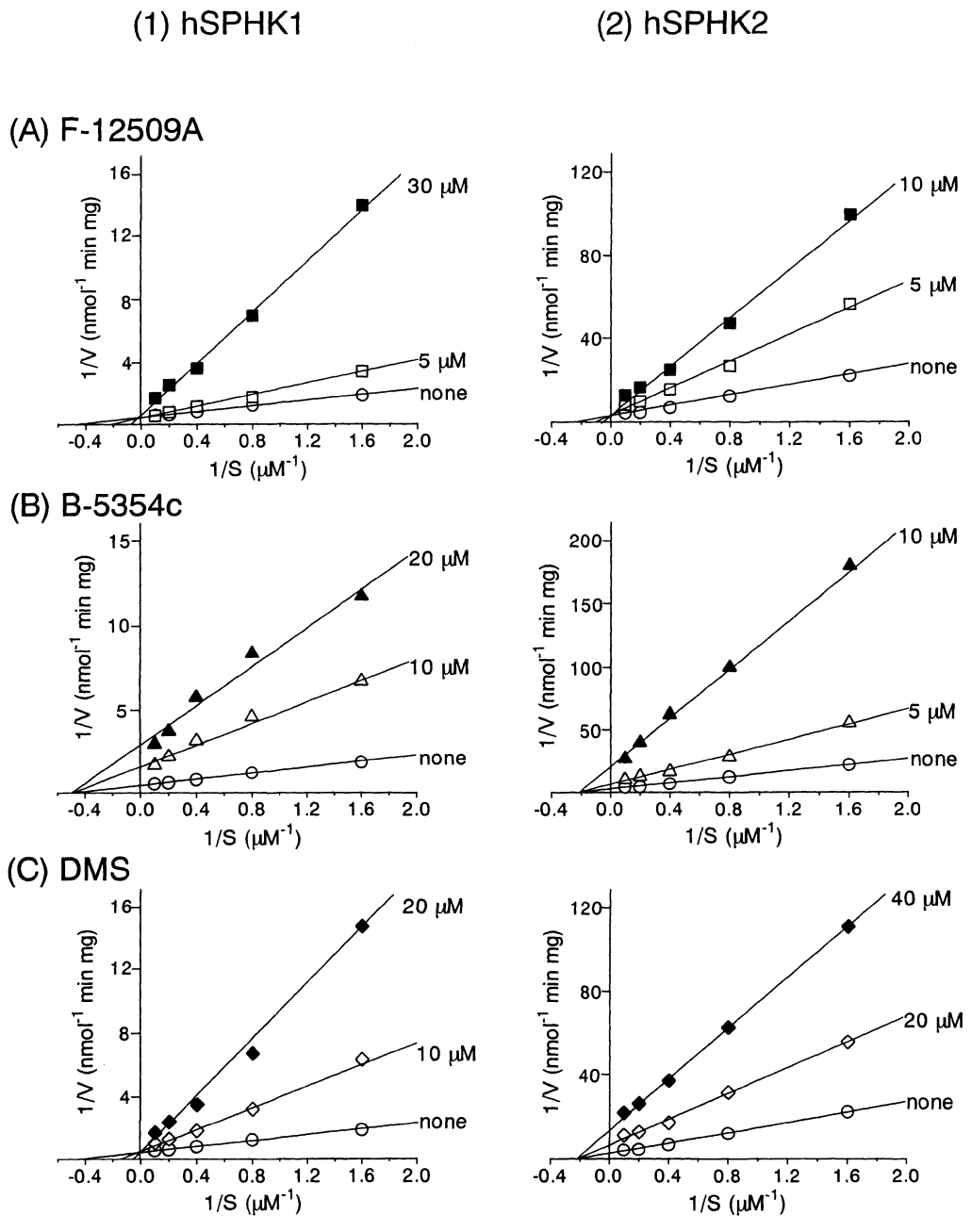
some modifications in order to adapt human SPH kinase homologs. Briefly, SPH kinase activities in cytosol from HEK293 cells transfected with hSPHK1 and hSPHK2 were measured by incubation, using 20 μM SPH (Matreya) prepared as a complex with bovine serum albumin (essential fatty acid free; Sigma), [γ-^{32}P]ATP (100 μCi/assay; Amersham Pharmacia Biotech) and inhibitors dissolved in DMSO, for 20 minutes at 37°C in reaction buffer (100 mM potassium phosphate [pH 7.4], 10 mM $MgCl_2$, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). After stopping the reactions, radiolabeled lipids were extracted and separated by thin layer chromatography, then analyzed by an imaging analyzer (BAS2000; Fuji Film). The specific activities of the recombinant hSPHK1 and hSPHK2 preparations were 1225 and 225 pmol SPP/min/mg protein, respectively. These activities were satisfactory and approximately 150 and 30 times higher than the control preparation (8 pmol SPP/min/mg protein) from vector alone-transfected HEK293 cells, respectively.

The inhibitory effects of F-12509A and B-5354c on recombinant human SPH kinase isoforms were compared with *N*,*N*-dimethylsphingosine (DMS, Fig. 1), a well-characterized SPH kinase inhibitor[17,19,23]. As shown in Fig. 2, all compounds inhibited both SPH kinase isoforms in a dose-dependent manner. At 100 μM, they inhibited both isoforms completely in our assay system. F-12509A inhibited hSPHK2 about four times more potently than hSPHK1. The IC_{50} values of F-12509A for hSPHK1 and hSPHK2 were 41 μM and 11 μM, respectively. A steep slope in the dose-response curve was observed in F-12509A inhibition of hSPHK1, but a relatively moderate slope was observed in that of hSPHK2. On the other hand, B-5354c inhibited both isoforms equally and more potently than DMS. The IC_{50} values of B-5354c for hSPHK1 and hSPHK2 were 7.8 and 5.0 μM, respectively. Regarding hSPHK2 inhibition, B-5354c was about four times more potent than DMS.

Kinetic analysis of the inhibitory effects of F-12509A and B-5354c on recombinant human SPHK1 and SPHK2 was evaluated using the classical Michaelis-Menten method. Lineweaver-Burk plot analysis indicated that F-12509A increased the apparent K_m values for SPH, while the V_{max} values remained unaltered (Fig. 3A). Thus, F-12509A inhibited both human SPH kinase isoforms competitively with respect to SPH. This result suggests that the sesquiterpene moiety of F-12509A may mimic the conformation of SPH binding the active sites of SPH kinase isoforms. On the other hand, B-5354c decreased the apparent V_{max} values, while the K_m values for SPH remained unaltered (Fig. 3B). Thus, B-5354c showed noncompetitive-type inhibition with respect to SPH. This result suggests that B-5354c may interact with domains distinct from the SPH binding sites and regulate SPH kinase activity. It was reported previously that when using inactive derivatives of B-5354c, the double bond in the aliphatic chain of B-5354c is important for its inhibitory activity[22]. Interestingly, phosphatidylserine stimulates the activities of both SPH kinase isoforms in a noncompetitive manner and the lipid also needs double bonds in its aliphatic chain to regulate SPH kinase activity[19,24]. These findings raise the possibility that B-5354c modulates SPH kinase activity by a similar mechanism to phosphatidylserine.

In addition, DMS inhibited hSPHK1 competitively, whereas it inhibited hSPHK2 noncompetitively (Fig. 3C), both results being similar to those of murine SPH kinase isoforms reported previously[17,19]. The K_i values of these inhibitors are shown in Table 1. Comparing the K_i values, inhibitory potencies of F-12059A and B-5354c for

Fig. 3. Lineweaver-burk plots of inhibition of recombinant hSPHK1 and hSPHK2 by F-12509A, B-5354c and DMS.



SPH kinase activities in cytosol from HEK293 cells transfected with hSPHK1 (1) and hSPHK2 (2) were measured with varying concentrations of SPH in the presence of F-12509A (A), B-5354c (B) and DMS (C). K_m values for SPH were: (1) 2.0 μM and (2) 5.6 μM. V_{max} values were: (1) 2.3 nmol min^{-1} mg^{-1} and (2) 0.45 nmol min^{-1} mg^{-1}.

hSPHK1 were almost the same as that of DMS. However, those for hSPHK2 were about two and four times more potent than DMS, respectively. DMS, an SPH analog, has been used as an SPH kinase inhibitor[7,9,10,17,19,23]. However, due to its structural similarity to SPH, DMS is also reported to have several other pharmacological functions that may not be involved in SPH kinase inhibition. For example, DMS, like SPH, acts as a potent inhibitor of PKC[25,26] and induces apoptosis in a variety of human cell lines[27]. Since F-12509A and B-5354c are not structural analogs of SPH,

Table 1. Kinetic constants for inhibition of recombinant hSPHK1 and hSPHK2 by F-12509A, B-5354c and DMS.

(1) hSPHK1

Inhibitors	Inhibition type	K_i (μM)
F-12509A	competitive	4.0
B-5354c	noncompetitive	3.7
DMS	competitive	3.2

(2) hSPHK2

Inhibitors	Inhibition type	K_i (μM)
F-12509A	competitive	5.5
B-5354c	noncompetitive	2.2
DMS	noncompetitive	8.6

The K_i values of recombinant hSPHK1 (1) and hSPHK2 (2) for F-12509A, B-5354c and DMS were calculated from the Lineweaver-Burk plots shown in Fig. 3.

they are not expected to exhibit such unfavorable activities as observed in SPH analogs. Indeed, F-12509A and B-5354c do not inhibit PKC at all[20,22]. Thus, F-12509A and B-5354c have more desirable characteristics than SPH analogs.

Here we have evaluated the inhibitory properties of F-12509A and B-5354c on recombinant human SPH kinases. These structurally unique inhibitors can now be used to determine the physiological functions and regulatory mechanisms of SPH kinase isoforms.

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