

Effect of alkyl chain length on sphingosine kinase 2 selectivity

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ABSTRACT

The conversion of sphingosine to sphingosine-1-phosphate is catalyzed by sphingosine kinase (SphK), which has been implicated in disease states such as cancer and fibrosis. Because SphK exists as two different isoforms, SphK1 and SphK2, understanding the physiological function of each isoenzyme is important. Of the two isoenzymes, SphK2 is significantly less understood, which is evident by the lack of selective small molecule inhibitors. Building on our initial work that focused on the structure–activity relationship study on an FTY720-derived cyclohexylamine scaffold, we report that varying the alkyl chain length on the hydrophobic tail can impart selectivity toward SphK2 over SphK1.

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Sphingosine-1-phosphate (S1P) is an endogenous lipid that signals through specific G-protein coupled receptors (S1P_{1–5}) to mediate cellular processes ranging from cell motility to angiogenesis.¹ Levels of S1P, sphingosine, and ceramide are tightly regulated and sphingosine kinase (SphK), the enzyme that phosphorylates sphingosine to form S1P, sits at a critical junction in the sphingolipid metabolism pathway. Two isoforms of SphK have been identified, SphK1 and SphK2.² The importance of the S1P metabolism pathway in the treatment of disease states is highlighted by the approval of a sphingosine analogue FTY720 (Fingolimod, GilenyaTM) by the Food and Drug Administration as an oral medication for multiple sclerosis.^{3,4} FTY720 is specifically phosphorylated by SphK2 to (S)-FTY720 phosphate, which binds to S1P receptors inducing the internalization and degradation of S1P₁ receptor. FTY720 acts as an immunosuppressant by the blockade of S1P/S1P₁ signals, decreasing the egress of T-lymphocytes from lymph nodes.

Both SphK1 and SphK2 produce S1P in vivo; however, it is unclear whether they have similar functions. While cytosolic SphK1 migrates to the plasma membrane when phosphorylated, SphK2 is located mostly in the nucleus and endoplasmic reticulum. Between the two isoenzymes, SphK1 is more studied. SphK1 is overexpressed in many tumor types and numerous reports support its anti-apoptotic role.⁵ Hence, it has been a target for drug discovery. In contrast, SphK2 is less understood.

Histone deacetylases (HDAC1 and HDAC2) in repressor complexes are inhibited by SphK2-derived S1P in the nucleus, which results in increased histone acetylation and enhanced gene transcription of *p21* (a cyclin-dependent kinase inhibitor) and *c-fos* (a transcriptional regulator).⁶ Also, overexpression of SphK2 suppresses growth and promotes apoptosis; the latter is likely because of its BH3 domain that interacts with BCLX_L.⁷ In tumor cells, downregulation of SphK2 by RNA interference inhibits glioblastoma cell proliferation,⁸ reduces doxorubicin-induced expression of *p21* and G2/M arrest,⁹ increases doxorubicin-induced apoptosis,⁹ and eliminates epidermal growth factor-induced migration of HEK 293 cells.¹⁰ Furthermore, knockdown of SphK2 suppressed cell proliferation and migration/invasion more when compared to SphK1 knockdown in A498, Caki-1 and MDA-MB-453 cells.¹¹ Surprisingly, in this particular study, ablation of SphK2 increased mRNA, protein and activity levels of SphK1 as well as S1P.

In addition to the possible roles of SphK2 in cancer, SphK2 may also be involved in other diseases. For example, SphK2 is hyperactive in the brains of patients with sporadic Alzheimer's disease and the S1P generated in neuronal cells is suggested to bind to β -site APP cleaving enzyme-1 and modulate its proteolytic activity.¹² In a separate study, SphK2 inhibition is shown to improve mitochondrial function and survival in liver injury caused by ischemia-reperfusion.¹³

It is likely that other functions for SphK2 are yet to be unraveled; selective inhibitors will be useful tools to dissect its role in biology, and drug-like molecules have the potential to provide therapy for diseases wherein SphK2 activity is misregulated.

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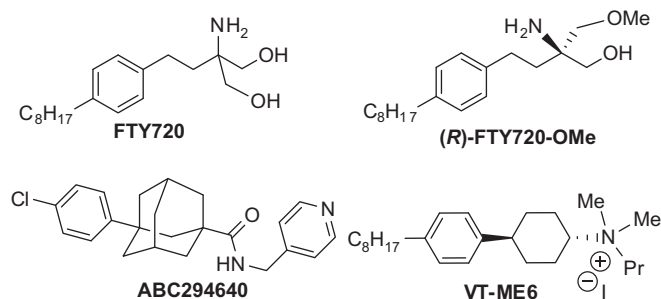


Figure 1. Structures of FTY720 and SphK2-selective inhibitors.

Unfortunately, SphK2-selective inhibitors are limited and the design of inhibitors is hampered by the lack of a crystal structure of the enzyme. The first SphK2-selective compound reported, **ABC294640**, was discovered from the screening of a chemical library which featured an aryladamantane scaffold (Fig. 1).¹⁴ **ABC294640** has a K_i of 9.8 μM and it has been deployed in a variety of disease models. Recently, Pyne and co-workers synthesized an FTY720 derivative wherein the pro-(R)-hydroxyl group was methylated. The resulting compound, **(R)-FTY720-OMe**, is indeed a selective SphK2 inhibitor with a K_i of 16.5 μM .¹⁵

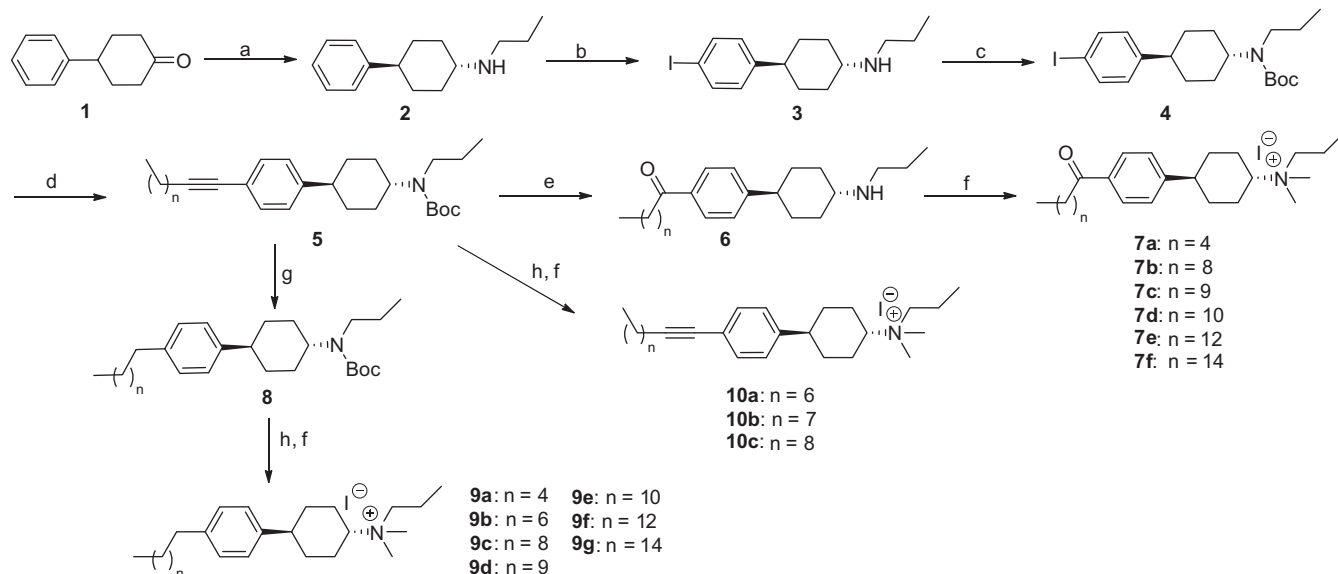
In continuation of our effort in discovering SphK2-selective inhibitors, we sought to define further the structure–activity profile of SphK2. We recently disclosed the identification of a novel SphK2-selective scaffold that featured a cyclohexylammonium head group.¹⁶ Structure–activity relationship studies focused on the head group (cyclohexylamine moiety) of this series revealed an inhibitor, **VT-ME6**, with a K_i of 8 μM and modest SphK2-selectivity. A logical next step is to examine if the alkyl tail influences SphK isoenzyme selectivity. Hence, we synthesized **VT-ME6** derivatives with varying linkages and lipid tail length. Herein, we report that the length of the alkyl chain in **VT-ME6** can impart SphK2 selectivity over SphK1.

The compounds were prepared using the general method illustrated in Scheme 1. Reductive amination of 4-phenylcyclohexanone **1** with propylamine and LiBH_4 afforded secondary amine **2** with the *trans* isomer as the major product. Selective iodination

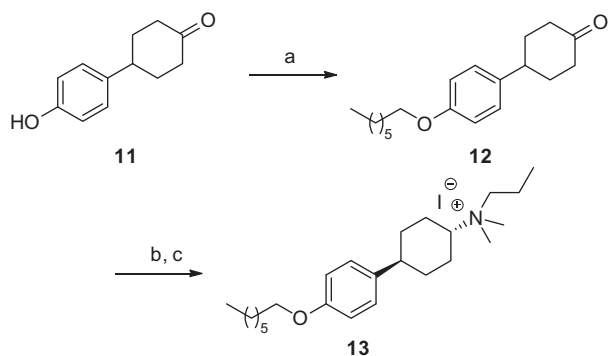
of **2** in the *para*-position using H_2SO_4 , NaIO_3 and I_2 provided the desired product **3** in good yield;¹⁷ however, the *ortho*-iodinated side product was difficult to separate by chromatography. Hence, we opted for an alternative route that used $\text{PhI}(\text{O}_2\text{CCF}_3)_2$ and I_2 , which yielded **3** in 65% as the only isomer observed.¹⁸ Protection of the corresponding amine with the Boc group followed by Sonogashira cross-coupling with alkynes of varying chain length led to the formation of **5**. Key intermediate **5** was used as a point of diversification to synthesize compounds that explore the effect of the nature and length of various linkers (ketone, alkynyl and alkyl). For example, treatment of **5** with wet trifluoroacetic acid resulted in the concomitant removal of the Boc group and hydration of the alkyne moiety to generate compound **6**. Arylketones **7a–f** were obtained by exhaustive methylation of **6** under refluxing conditions. Alternatively, catalytic hydrogenation of **5** with 10% Pd/C under a balloon of H_2 provided **8**. Deprotection by bubbling HCl gas afforded the deprotected material, which was hypermethylated to form the desired ammonium salts **9a–g**. Finally, alkynes **10a–c** were synthesized from **5** by a sequential deprotection-methylation protocol as shown in Scheme 1.

A derivative (**13**) containing an ether linkage was also synthesized as shown in Scheme 2. Heptyl bromide was first converted to the iodide using the Finkelstein reaction and then reacted with 4-(4-hydroxyphenyl)cyclohexanone **11** to afford **12**, which was then transformed to the ammonium salt **13** by reductive amination and methylation.

With the target compounds in hand, their inhibitory activities against SphK1 and SphK2 were evaluated using a medium throughput assay developed in our laboratory.¹⁹ Briefly, assay ingredients (lysate, Sph, ATP, and ^{32}P -ATP with or without inhibitors) were assembled in flash plates pre-coated with poly-lysine using 10 and 5 μM Sph for SphK1 and SphK2, respectively. Because the ^{32}P -labeled S1P generated bound to the plate (Sph does not), kinase inhibition could be detected by measuring the amount of radioactivity by scintillation as a function of inhibitor concentration. We initially screened the compounds at 10 μM to determine which lipid tail linkage—i.e., ketone, alkynyl or alkyl—is worth pursuing further (Table 1). Each of these linkers aimed to probe solubility properties as well as geometry of the group adjacent to the aryl ring.



Scheme 1. Reagents and conditions: (a) n -PrNH $_2$, MeOH, rt, 30 min; then LiBH_4 , THF, -78°C to rt, 3 h, *trans* 80%, *cis* 10%; (b) $\text{PhI}(\text{O}_2\text{CCF}_3)_2$, I_2 , CH_3CN , 2 h, 65%; (c) Boc_2O , NEt_3 , THF, rt, 4 h, 85%; (d) $\text{RC}\equiv\text{CH}$, $\text{PdCl}_2(\text{PPh}_3)_2$, CuI, NEt_3 , DMF, 90°C , 58–66%; (e) TFA, 1% H_2O , MeOH, rt, 1 h, >95%; (f) MeI, K_2CO_3 , MeOH, reflux, 3 h, 25–85%; (g) 10% Pd/C, H_2 , MeOH, rt, 2 h, >95%; (h) HCl (g), THF, 10 min, >95%.



Scheme 2. Reagents and conditions: (a) *n*-heptylBr, K₂CO₃, KI, acetone, reflux, 68%; (b) *n*-PrNH₂, MeOH, rt, 30 min; then LiBH₄, THF, –78 °C to rt, 76%; (c) MeI, K₂CO₃, MeOH, reflux, 3 h, 68%.

In general, the FTY720-derived scaffold provided compounds that were selective toward SphK2 using this single concentration assay. We defined <40% SphK2 activity as a threshold level that is sufficiently stringent to generate inhibitors in the low μM range. Because the alkyl series **9a–g** had more compounds below the 40% threshold value compared to the ketone and alkynyl series (entries 1–6 and 14–17 vs entries 7–13), the *K_i* values for this series of compounds were determined as listed in Table 2. The data suggests that these compounds inhibit both SphK1 and SphK2 with **9f** as the best inhibitor in the series (36 and 7 μM, respectively). Of note is that the *K_m* values of SphK1 (10 μM) and SphK2 (5 μM) are different. Hence, to compare selectivity, the inhibitory activities at each enzyme were normalized by the *K_m*. As shown in Figure 2, only the compounds bearing the octyl (**VTME6**)¹⁶ and tetradecyl (**9f**) groups showed selectivity toward SphK2 while the rest were non-selective. These compounds are approximately threefold selective for SphK2 over SphK1. Indeed, there is a difference of six methylene units between **VTME6** and **9f** while their selectivity and affinity are identical (Table 2). We hypothesize that SphK2 has a binding site that can be accessed by a 'short' tail but one that can also reveal a larger cavity that can accommodate a tetradecyl

Table 1
Inhibitory effects of various analogs on SphK1 and SphK2

Entry	Compound	Tail length	Linkage X	SphK activity level ^a (%)	
				SphK1	SphK2
1	7a	6	Ketone	89 ± 2	71 ± 3
2	7b	10	Ketone	93 ± 7	75 ± 4
3	7c	11	Ketone	79 ± 11	40 ± 3
4	7d	12	Ketone	88 ± 2	41 ± 3
5	7e	14	Ketone	73 ± 7	50 ± 5
6	7f	16	Ketone	77 ± 1	58 ± 3
7	9a	6	Alkyl	98 ± 3	98 ± 7
8	9b	9	Alkyl	100 ± 12	64 ± 2
9	9c	10	Alkyl	67 ± 7	26 ± 6
10	9d	11	Alkyl	44 ± 3	50 ± 4
11	9e	12	Alkyl	63 ± 3	26 ± 8
12	9f	14	Alkyl	76 ± 1	38 ± 2
13	9g	16	Alkyl	94 ± 2	70 ± 3
14	10a	8	Alkynyl	87 ± 6	100 ± 11
15	10b	9	Alkynyl	61 ± 4	78 ± 7
16	10c	10	Alkynyl	62 ± 3	28 ± 7
17	13	8	Ether	100 ± 12	70 ± 9

^a Values are percent activity of SphK1 or SphK2 with 10 and 5 μM Sph, respectively, in the presence of 10 μM inhibitor.

Table 2
K_i and *K_m* normalized values for the alkyl series

Entry	Compound	Tail length	<i>K_i</i> ^a (μM)		<i>K_i/K_m</i>	
			SphK1	SphK2	SphK1	SphK2
1	9a	6	35 ± 5	43 ± 6	3.5	8.6
2	VTME6	8	47 ± 4	8 ± 1	4.7	1.6
3	9b	9	22 ± 3	21 ± 3	2.2	4.2
4	9c	10	35 ± 3	43 ± 1	3.5	8.6
5	9d	11	15 ± 1	11 ± 1	1.5	2.2
6	9e	12	13 ± 2	8 ± 1	1.3	1.6
7	9f	14	36 ± 3	7 ± 1	3.6	1.4
8	9g	16	27 ± 2	29 ± 2	2.7	5.8

^a *K_i* = [I]/([K'_m/K_m – 1]); *K_m* of sphingosine at SphK1 = 10 μM; *K_m* of sphingosine at SphK2 = 5 μM.

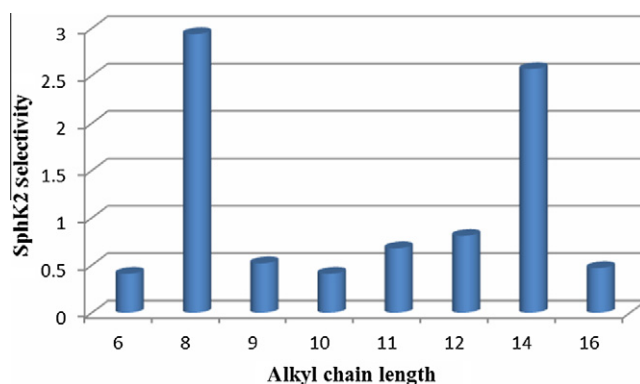


Figure 2. Effect of lipid tail length (**9a–g**) on SphK2 selectivity.

group. Although the selectivity observed here appears to be modest, it is not surprising because SphK2 is thought to be more substrate sensitive—i.e., different *K_m* for Sph—and could be a reason why there is a paucity of SphK2-selective inhibitors.

In conclusion, we describe our effort in further defining the structure–activity profile of SphK2 by varying the alkyl chain on a lead compound. Indeed, we demonstrated that the 'tail' can impart SphK2-selectivity. In combination with further modification and optimization on other regions of the scaffold discussed here, we suspect that more potent and selective compounds can be generated. We are currently pursuing these strategies as well as generating a homology model to aid in our understanding of kinase selectivity.

Acknowledgments

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Supplementary data

Supplementary data (¹H, ¹³C NMR and mass spectral data for **7a–f**, **9a–g**, **10a–c** and **13**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.050.

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