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# Structural Requirements and Docking Analysis of Amidine-Based Sphingosine Kinase 1 Inhibitors Containing Oxadiazoles

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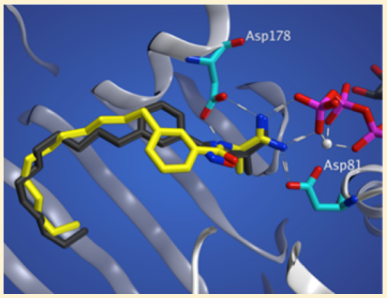
Supporting Information

**ABSTRACT:** Sphingosine 1-phosphate (S1P) is a potent growth-signaling lipid that has been implicated in cancer progression, inflammation, sickle cell disease, and fibrosis. Two sphingosine kinases (SphK1 and 2) are the source of S1P; thus, inhibitors of the SphKs have potential as targeted cancer therapies and will help to clarify the roles of S1P and the SphKs in other hyperproliferative diseases. Recently, we reported a series of amidine-based inhibitors with high selectivity for SphK1 and potency in the nanomolar range. However, these inhibitors display a short half-life. With the goal of increasing metabolic stability and maintaining efficacy, we designed an analogous series of molecules containing oxadiazole moieties. Generation of a library of molecules resulted in the identification of the most selective inhibitor of SphK1 reported to date (705-fold selectivity over SphK2), and we found that potency and selectivity vary significantly depending on the particular oxadiazole isomer employed. The best inhibitors were subjected to *in silico* molecular dynamics docking analysis, which revealed key insights into the binding of amidine-based inhibitors by SphK1. Herein, the design, synthesis, biological evaluation, and docking analysis of these molecules are described.




**KEYWORDS:** *Sphingosine 1-phosphate, sphingosine kinase, cancer, inhibitor, oxadiazoles*

Sphingosine 1-phosphate (S1P) is a potent signaling lipid that acts on five membrane-bound receptors (S1P1−5)[1] and various intracellular targets.[2] S1P has been shown to regulate a host of processes that affect the growth and fate of cells,[3] and aberrant S1P signaling has been linked to numerous diseases including cancer,[2,4] asthma,[5] fibrosis,[6] and sickle cell disease.[7] Cellular synthesis of S1P is exclusively dependent on the action of two sphingosine kinases (SphK1 and 2) that directly phosphorylate sphingosine (Sph).[3] As the sole sources of cellular S1P, the SphKs influence cell survival[8,9] and have been identified as targets of interest in pharmacology and the pharmaceutical industry.[4,10] Therefore, SphK inhibitors, by affecting S1P levels, have therapeutic potential and are necessary to clarify the many roles of S1P and each SphK in physiology and disease.[10−12]

S1P signaling is complex, and the roles of SphK1 and 2 in various pathways differ significantly. These differences are due, in large part, to subcellular compartmentalization; SphK2 possesses an N-terminal nuclear localization sequence not present in SphK1.[13] Indeed, SphK2 is located primarily in the nucleus and other organelles and may influence gene expression through S1P-dependent inhibition of histone deacetylase 1 and 2 (HDAC1 and 2).[14] Additionally, S1P produced by SphK2 in the endoplasmic reticulum and mitochondria has the effect of stimulating apoptosis.[15] In contrast, SphK1 is located primarily in the cytosol, and SphK1-derived S1P generally elicits prosurvival and proliferative effects through interactions with S1P1−5. Upon phosphorylation by the extracellular-regulated kinases, SphK1 is activated and translocated to the inner leaflet of the plasma membrane.[16] Then, in an inside-out signaling mechanism, S1P is shuttled outside the cell by the membrane-spanning spinster 2 protein (or by members of the ABC transport family), where it may bind with any of the G protein-coupled S1P1−5. Agonism at these receptors initiates a cascade of signaling events affecting growth, motility, immune cell trafficking, metastasis, and angiogenesis.[12]

Because of the complexity of S1P signaling, a large and diverse set of pharmacological tools is necessary to probe the underlying physiological pathways and conclusively validate both SphK1 and 2 in different disease models. Small-molecule SphK inhibitors are an important example of such a tool, and ideally should have the following characteristics: single-digit nanomolar potency, at least 100-fold selectivity for either of the SphK isoforms, and high metabolic stability.[10] Currently, no single molecule fulfills each criterion simultaneously, but a new generation of inhibitors is nearing this mark. In particular, recent efforts to improve our highly potent (but unstable) amidine-based inhibitors have yielded a class of very effective guanidine-based, oxadiazole-containing molecules.[17−19] These compounds are the most potent and selective inhibitors that elicit prolonged shifts in blood S1P concentrations; the ability

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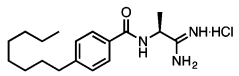
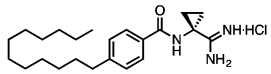
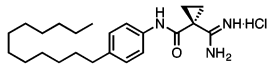
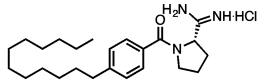
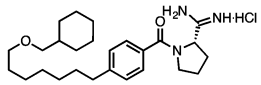
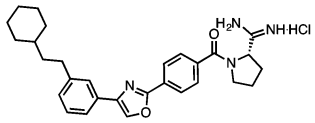
to lower or raise blood S1P levels is an important biomarker for inhibition of SphK1 or 2, respectively.[20]

To further improve SphK inhibitors, particularly regarding potency, it is necessary to continue to probe the Sph-binding pockets of SphK1 and 2 with structurally diverse sets of molecules. To this end, we have developed and characterized a small library of molecules combining an amidine headgroup with various oxadiazole linkers, elaborating on our earlier studies.[21,22] Herein, we report an inhibitor with double-digit nanomolar potency and 705-fold selectivity for SphK1 over SphK2. Additionally, we reveal two key insights into the structure−activity relationships governing SphK1 inhibition, namely, that potency and selectivity are greatly affected by oxadiazole subtypes and alkyl tail positioning.

Our previous work yielded a series of amidine-based molecules displaying high potency and selectivity for SphK1. The lead molecule, **1**, was modified to optimize tail length, amide orientation, and headgroup identity (**2**−**4**, Table 1).[21]

**Table 1. Previously Described Amidine-Based SphK Inhibitors[20,21]**

| Compound | Structure | $K_I$ (μM)[a] SphK1 | $K_I$ (μM)[a] SphK2 | SphK1 Selectivity[b] |
|---|---|---|---|---|
| 1 (VPC94075) |  | 55 | 20 | 0.7 |
| 2 |  | 0.2 | 0.5 | 5 |
| 3 |  | 0.3 | 6 | 40 |
| 4 |  | 0.125 | 1.5 | 24 |
| 5 |  | 0.075 | 3.0 | 80 |
| 6 |  | 0.047 | 4.2 | 180 |

[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. [b] Selectivity = $(K_I/K_M)^{SphK2}/(K_I/K_M)^{SphK1}$.

Further improvements were the result of *in silico* screening of a library of molecules with varied, rigidified tails. This analysis was carried out with a SphK1 homology model based on diacylglycerol kinase beta, and yielded **6**, a SphK1 inhibitor with a $K_I$ of 47 nM and 180-fold selectivity over SphK2 (Table 1).[22]

In the work described presently, we followed a similar approach by optimizing a lead molecule with a library of synthetic variants and then following with *in silico* modeling. In this new series, the amide moiety was replaced with three distinct oxadiazoles; the reasoning for this is 3-fold: (1) our previous inhibitors have displayed poor half-lives in vivo and oxadiazoles are well-documented amide surrogates,[23−27] (2) oxadiazoles have been effective structural subunits in our previous work with SphK2 substrates,[28] and (3) oxadiazole linkers improved the efficacy of guanidine-based SphK1 inhibitors.[17]

As a starting point, **11c**, an analogue of **2** and **3**, was synthesized as follows (Scheme 1). First, 1-dodecene was reduced to the alkyl borane with 9-BBN and coupled to 4-bromobenzonitrile using Suzuki conditions to p-alkylbenzonitrile **7c**. Next, conversion to aryl amidoxime **8c** was achieved using hydroxylamine hydrochloride and triethylamine in ethanol. PyBop-mediated coupling of the amidoxime to 1-cyano-1-cyclopropanecarboxylic acid yielded **9c**. Cyclization of the coupled amidoxime with TBAF[29] gave oxadiazole **10c** and was subjected to base-catalyzed Pinner conditions[30] to yield amidine **11c**.

$K_I$ values were determined by a [$\gamma$-$^{32}$P]ATP *in vitro* assay of SphK enzymatic activity (Table 2).[31] Compound **11c** maintains potency and SphK1 selectivity, validating the oxadiazole as a suitable replacement for the amide group.

The incorporation of an oxadiazole into the molecular scaffold significantly alters the angle between the tail and head groups. With this in mind, a variant of **11c** was synthesized with *meta*-substitution about the phenyl ring. Interestingly, upon attaching the tail at the *meta* position, potency and selectivity increased dramatically, with **11g** displaying a $K_I$ of 40 nm and 705-fold selectivity for SphK1 over SphK2. As a control, a *meta*-substituted analogue of our amide compounds, **13**, was synthesized (Scheme SI-1). This compound lost potency proportionately at both kinases (Table 3). *meta*-Substituted analogues present the amidine to the $\gamma$-phosphate of ATP such that the amidine is no longer antiperiplanar with the aromatic ring. Deletion of the cyclopropane ring alpha to the amidine resulted in a loss of activity in oxadiazole **15** (Scheme SI-2, Table SI-1). The unique torsional angle of the cyclopropane

**Scheme 1. Synthesis of Oxadiazole Amidine 11c**

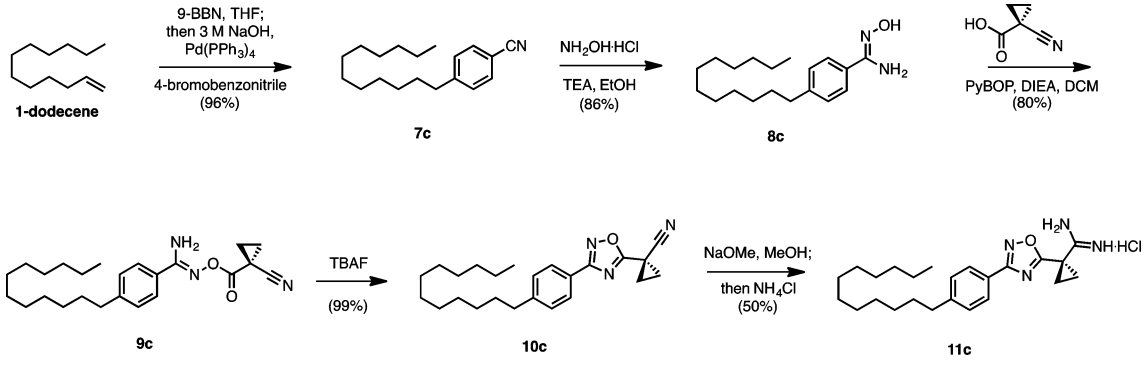





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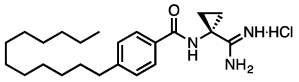
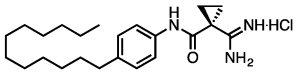
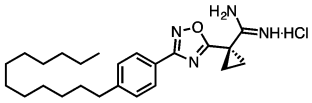
**Table 2. $K_I$ Values of Oxadiazole 11c Compared to Amides 2 and 3**

| Compound | Structure | $K_I$ (μM)[a] SphK1 | SphK2 | SphK1 Selectivity[b] |
|---|---|---|---|---|
| 2 |  | 0.2 | 0.5 | 5 |
| 3 |  | 0.3 | 6 | 40 |
| 11c |  | 0.32 | 8 | 50 |

[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. [b] Selectivity = $(K_I/K_M)^{SphK2}/(K_I/K_M)^{SphK1}$.

ring provides improved presentation of the amidine in the active site.

Because the incorporation of oxadiazoles increases the overall molecular length, we altered the length of the alkyl tail to accommodate this change. Compounds with tails ranging from 10 to 13 carbons in length, both *para*- and *meta*-substituted, were synthesized through the same methods illustrated in Scheme 1 ($K_I$ values summarized in Table 3). The tail derivatives follow the same trend as our previous amidine-based inhibitors.[21,22] Potency increases with tail length before dropping off at a length of 13 carbons. Tail substitution at the *para* position reaches a maximum potency and selectivity for SphK1 at a length of 11 carbons, with a $K_I$ of 0.26 μM (**11b**). Because an oxadiazole is one atom longer than an amide, it is expected that the 11-carbon tail fills out the sphingosine-binding pocket similarly to a 12-carbon tail on an amide analogue.

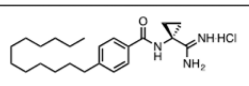
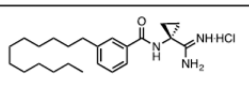
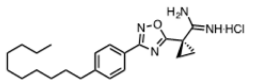
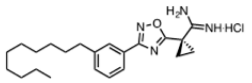
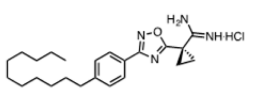
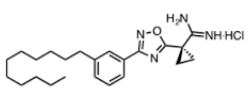
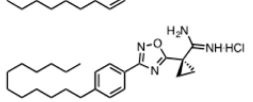
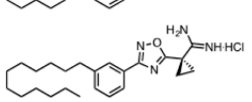
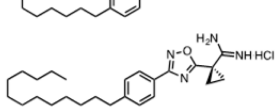
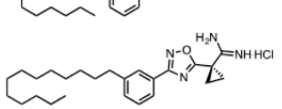
By integrating our previous work in the development of a SphK1 homology model[22] with the recently determined SphK1 crystal structure,[32] we have identified several key binding interactions between **11g** and SphK1. On docking compounds **11g** and **13** into a SphK1 model (generated with the crystal structure, PDB 4L02), one can see that *meta* amide (**13**) and *meta* oxadiazole (**11g**) adopt very different configurations (Figure 1). The oxadizaole inhibitor fills out the tail-binding region of the pocket to a greater extent than the amide. Further analysis reveals three cationic attractions of the amidine headgroup: to the γ-phosphate of ATP (as previously reported), and to Asp 81 and Asp178. Additionally, π-stacking between the oxadiazole and Phe192, which forms the roof of the pocket above the linker region, contributes to binding, as well as a favorable arene−H interaction between the phenyl ring and Ile-174.

Following the evaluation of **11c and 11g**, four additional compounds were synthesized to determine the relative efficacy of the three oxadiazole subtypes, as it has been reported in the literature that each individual regioisomer exhibits unique physical and pharmacological properties.[33] For each oxadiazole subtype, both *meta*- and *para*-substituted variants were synthesized (Schemes SI-3 and SI-4).

Biological evaluation revealed substantial differences in the inhibitory qualities of molecules containing isomeric oxadiazoles (Table 4). Surprisingly, the largest distinction occurred between compounds containing the 3-aryl (**11c** and **11g**) and 5-aryl (**21a** and **21b**) 1,2,4-oxadiazole isomers. Inhibitors **11c** and **11g** display significantly higher potency and selectivity for SphK1 than do **21a** and **21b**. Compounds containing 1,3,4-oxadiazoles (**26a** and **26b**) display intermediate potency. Substitution about the phenyl ring again proved to be a very important factor, and all compounds with *meta*-substituted alkyl tails are much more potent and selective than *para*-substituted analogues.

These marked differences in *in vitro* activity between oxadiazole subtypes can be rationalized, in part, with additional *in silico* analyses. Compounds **11g**, **21b**, and **26b** were docked into the substrate-binding domain of SphK1, where the respective heterocycles appear to adopt different binding orientations (Figure 2). Most notably, electrostatic repulsion between the nonbonding electrons of the oxadiazole isomers and the backbone carbonyl of Leu268 destabilizes binding of compounds **21b** and **26b**. This interaction alters the positioning of the amidine headgroup, limiting binding interactions with the γ-phosphate of kinase-bound ATP. Furthermore,

**Table 3. $K_I$ Values of *para*- and *meta*-Substituted Oxadiazoles 11a−h Compared to Amide Analogues 2 and 13**

| Compound | Structure | $K_I$ (μM)[a] SphK1 | SphK2 | SphK1 Selectivity[b] | Compound | Structure | $K_I$ (μM)[a] SphK1 | SphK2 | SphK1 Selectivity[b] |
|---|---|---|---|---|---|---|---|---|---|
| 2 |  | 0.2 | 0.5 | 5 | 13 |  | 4.6 | 10.9 | 4.7 |
| 11a |  | 0.44 | 6 | 27 | 11e |  | 0.10 | 10.3 | 206 |
| 11b |  | 0.26 | 43 | 330 | 11f |  | 0.070 | 9.9 | 283 |
| 11c |  | 0.32 | 8 | 50 | 11g |  | 0.04 | 14.1 | 705 |
| 11d |  | 2.5 | 11 | 9 | 11h |  | 0.98 | 11.7 | 24 |

[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. [b] Selectivity = $(K_I/K_M)^{SphK2}/(K_I/K_M)^{SphK1}$.



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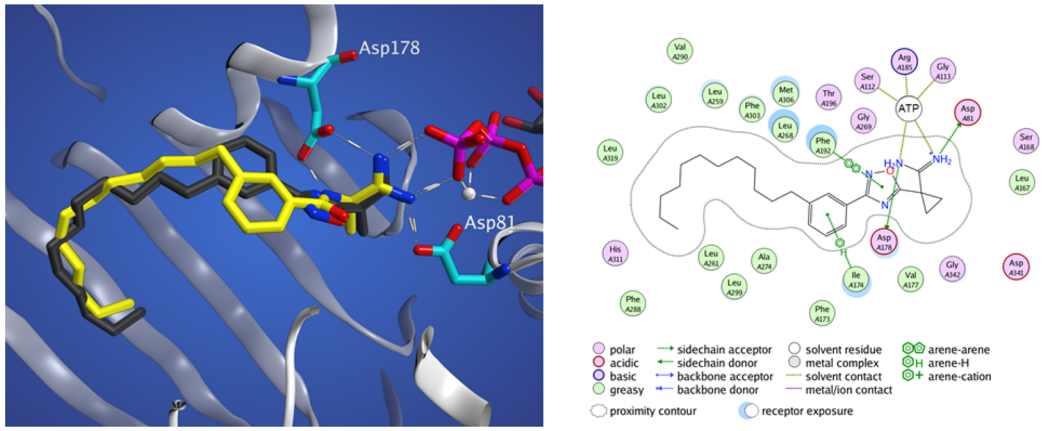
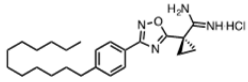
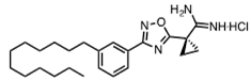
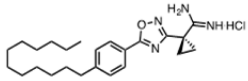
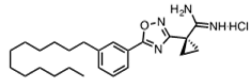
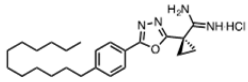
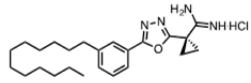



**Figure 1.** Docking studies with SphK1. (left) *meta*-Substituted oxadiazole (**11g**, black) and amide (**13**, yellow) derivatives docked into the crystal structure of SphK1. (right) Two-dimensional representation of **11g** and its side chain interactions.

**Table 4. $K_I$ Values of Heterocyclic Isomers with *meta*- and *para*-Substitution about the Phenyl Ring**

| Compound | Structure | $K_I$ (μM)[a] SphK1 | SphK2 | SphK1 Selectivity[b] | Compound | Structure | $K_I$ (μM)[a] SphK1 | SphK2 | SphK1 Selectivity[b] |
|---|---|---|---|---|---|---|---|---|---|
| **11c** |  | 0.32 | 6 | 50 | **11g** |  | 0.04 | 14.1 | 705 |
| **21a** |  | 3 | 3.4 | 2.3 | **21b** |  | 0.40 | 10.2 | 51 |
| **26a** |  | 1.6 | 1.2 | - | **26b** |  | 0.2 | 8.1 | 81 |

[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. [b] Selectivity = $(K_I/K_M)^{SphK2}/(K_I/K_M)^{SphK1}$.

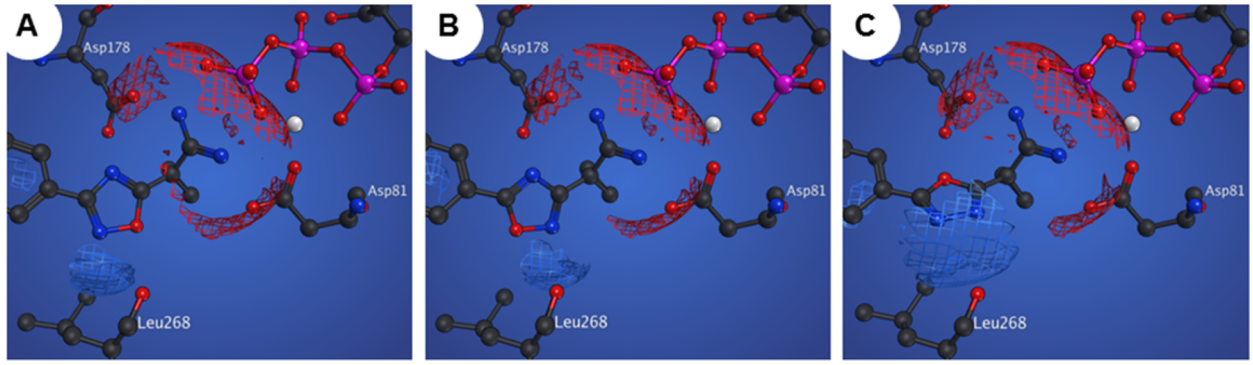



**Figure 2.** *In silico* docking of **11g** (A), **21b** (B), and **26b** (C). White sphere = $Mg^{2+}$.

nitrogen atoms on the oxadiazoles of **21b** and **26b** likely undergo a repulsive interaction with the Asp81 residue.

These simulations suggest that, relative to isoforms present in other compounds, the particular 1,2,4-oxadiazole of **11g** is subjected to minimal repulsive interactions in the SphK1 active site. This favorable binding orientation, coupled with *meta*-substitution about the phenyl ring, results in a significantly enhanced inhibitory profile. Compound **11g** is not only highly potent but, with 705-fold selectivity for SphK1, is the most selective SphK1 inhibitor reported to date.

In summary, we have generated a library of amidine-based SphK1 inhibitors with *in vitro* potencies in the nanomolar range. Surprisingly, different oxadiazole subtypes elicited dramatically different effects regarding potency and selectivity; also, *meta*-substitution about the phenyl ring increased efficacy for all isomers. Docking analysis revealed important interactions between **11g** and specific amino acid residues in the SphK1 active site, and provided justification for the favorable binding of this compound. This study contributes to the growing understanding of the structural requirements for SphK1 inhibition, and compounds such as **11g** provide the ideal scaffold for future inhibitors. Simultaneous optimization of potency, selectivity, and pharmacokinetic properties will be necessary to further interrogate both SphKs in pathophysiology and to identify suitable therapeutic candidates in the fight against cancer and hyperproliferative diseases.



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## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00002.

Experimental details for the synthesis of all compounds, compound characterization, and description of the SphK assay (PDF)

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### Author Contributions

All authors have given approval to the final version of the manuscript.


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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

HDAC, histone deacetylace; S1P, sphingosine 1-phosphate; Sph, sphingosine; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2; SphKs, sphingosine kinases

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