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Development of a sphingosine kinase 1 specific small-molecule inhibitor

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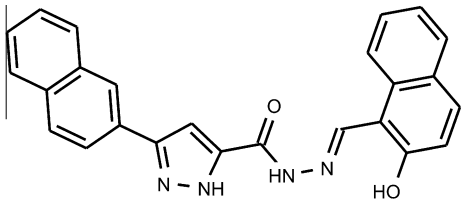
ABSTRACT

The sphingolipid metabolic pathway represents a potential source of new therapeutic targets for numerous hyperproliferative/inflammatory diseases. Targets such as the sphingosine kinases (SphKs) have been extensively studied and numerous strategies have been employed to develop inhibitors against these enzymes. Herein, we report on the optimization of our novel small-molecule inhibitor SKI-I (*N*′-[(2-hydroxy-1-naphthyl)methylene]-3-(2-naphthyl)-1*H*-pyrazole-5-carbohydrazide) and the identification of a SphK1-specific analog, SKI-178, that is active in vitro and in vivo. This SphK1 specific small-molecule, non-lipid like, inhibitor will be of use to elucidate the roles of SphK1 and SphK2 in the development/progression of hyperproliferative and/or inflammatory diseases.

Sphingosine kinase (SphK) is an oncogenic lipid kinase and key regulator of the sphingolipid metabolic pathway (reviewed in 1–4). The SphK isoforms (SphK1 and SphK2) catalyze the conversion of the pro-apoptotic substrate D-*erythro*-sphingosine (Sph) to the pro-growth/survival product sphingosine-1-phosphate (S1P). Accumulation of S1P, a potent mitogenic/migratory signaling lipid, has been linked to the development/progression of numerous hyperproliferative and/or inflammatory diseases including, but not limited to, cancer, asthma, atherosclerosis, inflammatory bowel disease, sepsis, rheumatoid arthritis and diabetic nephropathy.[5–7] Blocking SphK activity, therefore, has been postulated to inhibit the uncontrolled growth associated with hyperproliferative/inflammatory diseases while simultaneously inducing apoptosis of the targeted cells. Hence, the SphKs have become burgeoning drug targets for a variety of hyperproliferative/inflammatory diseases.

While a number of sphingosine kinase inhibitors have been described (extensively reviewed by Pitman and Pitson 2010[8]), the roles of the SphKs and S1P in the progression and pathophysiology of hyperproliferative diseases were first elucidated by the discovery of the sphingosine kinase inhibitors *N*,*N*-dimethylsphingosine and D,L-*threo*-sphingosine.[9,10] However, their lipid properties made these lipid-substrate analogs less than ideal 'drug-like' molecules and limited their therapeutic potential. To overcome these limitations, we conducted a small-molecule library screen, identified four classes of specific small-molecule inhibitors of SphK and demonstrated their in vivo effectiveness in a BALB/c immunocompetent mouse JC mammary adenocarcinoma model.[11,12]

Herein, we describe our structure–activity relationship (SAR) studies of our previously identified SphK inhibitor (SKI) chemotype I (SKI-I; *N*′-[(2-hydroxy-1-naphthyl)methylene]-3-(2-naphthyl)-1*H*-pyrazole-5-carbohydrazide) lead compound.[11] In this study, we report the identification of a novel SphK1-specific, non-lipid substrate, small-molecule inhibitor. We believe, this SphK1 specific compound will enable further quantitative structure–activity relationship (QSAR) studies aimed at improvement of efficacy and facilitate the evaluation of therapeutic strategies specifically targeting SphK1 for hyperproliferative diseases such as cancer.



1

Our initial chemical library screen for small-molecule inhibitors of SphK1 yielded four distinct classes of 'lead' compounds.[11] Of these four lead compounds, SKI-I (*N*′-[(2-hydroxy-1-naphthyl)methylene]-3-(2-naphthyl)-1*H*-pyrazole-5-carbohydrazide: **1**) was found to be the most cytotoxic toward a panel of tumor cell lines ($IC_{50} \sim 1–3$ μM) including multi-drug resistant cell lines and active in a BALB/c immunocompetent mouse JC mammary adenocarcinoma model.[11,12] Our preliminary studies also indicate that SKI-I is

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capable of inhibiting SphK2 albeit at slightly lower levels than SphK1 (data not shown). However, poor correlation to Lipinski's Rule-of-Five (i.e., *c* Log *P* = 6.2; ACS/PhysChem[13]) and poor oral bioavailability dampened the prospects for use of **1** as an anti-cancer therapeutic agent.

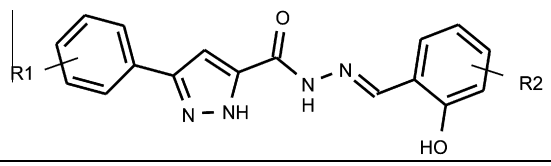
Currently, the X-ray crystal structures/NMR solution structures of the SphKs are not known. Therefore, rational drug design and/or molecular modeling strategies for the optimization of SKIs are not possible. To gain insight into the structural features of **1** critical for SphK inhibition, we initiated this study by obtaining a series of SKI-I analogs.[14] We first sought to eliminate the naphthyl rings of **1** (R^1 and R^2), due to their ease of metabolism and propensity of their electrophilic epoxy and quinone metabolites to form DNA adducts.[15–18]

To determine whether these naphthyl rings were necessary for inhibition of SphK1, we tested a series of analogs in which these rings were replaced with substituted phenyl rings. SphK1 in vitro activity assays were performed using affinity purified His_{6X}-tagged SphK1 protein recombinantly over-expressed in HEK293 cells.[19] All data are reported as percent inhibition of SphK1 at 10.7 μM which is the calculated IC_{50} of **1** under these assay conditions. As shown in Table 1, the R^2 2,3-phenyl ring was dispensable for inhibition of SphK1 (**2–5**). However, the position of the R^2-OH group affects SphK1 inhibition with compound **2** demonstrating that the *ortho* position was preferred over *meta* and *para* (**2**: 59.3% inhibition; R^2 = 2-OH). Similarly, the R^1 3,4-phenyl ring was not required for inhibition of SphK1 as demonstrated by compound **6** (**6**: 52.5% inhibition; R^1 = 4-OMe).

Having determined that the naphthyl rings of compound **1** are not necessary for SphK1 inhibition, we identified 4-OMe and 2-OH as preferable substitutions in the R^1 and R^2 positions, respectively. We next sought to determine whether the chain length of the R^1 position affected the inhibitory activity toward SphK1. As shown in Table 2, we directly compared R^1 = 4-H, 4-Me and 4-OMe substitutions (**7–9**). Interestingly, although R^1 = 4-OMe and R^2 = 2-OH were identified above (**2** and **6**) as substitutions that retained the inhibitory capacity of compound **1**, in combination, they decreased the SphK1 inhibitory activity of compound **9**.

We next decided to further optimize R^2 while holding R^1 constant. As demonstrated in Table 3 and consistent with the results obtained above (**3** and **4**), single substitutions of R^2 resulted in lower percent inhibition than **1** (R^2 = 3-OH **10:** 15.5% inhibition; R^2 = 4-OH or 4-F **11–12:** 14.3% and 27.4% inhibition, respectively). Larger R^2 substitutions were, however, more favorable. In particular, compound **17** (R^2 = 3,4-di-OMe: 44.5% inhibition) was similar to the inhibition observed for compound **1**. Thus, while the naphthyl rings of compound **1** were not required for SphK1 inhibition, it appears that larger groups are preferred at the R^2 position.

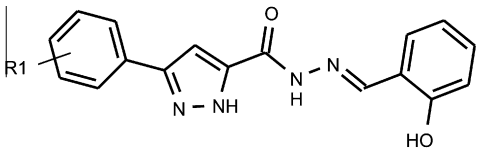
Table 1
SphK1 inhibition for naphthyl ring substitutions of compound **1**



Compound	R^1	R^2	SphK1 % inhib[a]
2	3,4-Phenyl	2-OH	59.3
3	3,4-Phenyl	3-OH	25.1
4	3,4-Phenyl	4-OH	28
5	3,4-Phenyl	2,4-Di-OH	39.5
6	4-OMe	2,3-Phenyl	52.5

[a] Values are percent inhibition of SphK1 at 10.7 μM, averages of two separate experiments, standard deviations were ±5%.

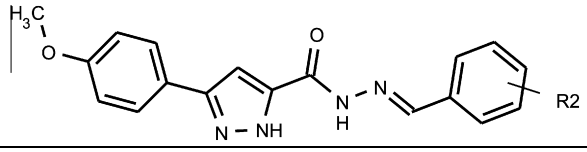
Table 2
SphK1 inhibition for various R^1 substituted phenyl groups of compound **2**



Compound	R^1	SphK1 % inhib[a]
7	4-H	11.9
8	4-Me	9.8
9	4-OMe	33.5

[a] Values are percent inhibition of SphK1 at 10.7 μM, averages of two separate experiments, standard deviations were ±5%.

Table 3
SphK1 inhibition for various R^2 substituted phenol groups of compound **9**



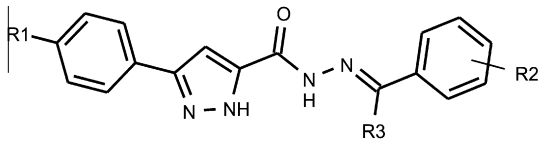
Compound	R^2	SphK1 % inhib[a]
10	3-OH	15.5
11	4-OH	14.3
12	4-F	27.4
13	3,5-Di-OH	12.5
14	4-*iso*-Propy	15.3
15	2,4-Di-OH	30.8
16	4-*N*,*N*-diethyl	38
17	3,4-Di-OMe	44.5

[a] Values are percent inhibition of SphK1 at 10.7 μM, averages of two separate experiments, standard deviations were ±5%.

Finally, we examined the interconnecting region between the R^1 and R^2 substituted phenyl rings. To determine whether substitutions within this region would affect SphK1 inhibition, we tested a series of compounds with a methyl substitution (Table 4; R^3). Addition of a methyl group at R^3 resulted in improved SphK1 inhibitory activity (46.8% inhibition, **19**; versus 27.4% inhibition, **12**; and 59.6% inhibition, **20**; vs 44.5% inhibition, **17**). Also, consistent with the results obtained in Table 2, R^1 = 4-Me substitutions negatively impact SphK1 inhibitory activity (21.8% inhibition, **18**; versus 59.6% inhibition, **20**), suggesting that 4-OMe is the preferred substitution of the R^1 phenyl ring. Thus, through SAR studies, we have identified a promising new 'lead' compound (**20**) with improved pharmacological properties. Specifically, we have succeeded in lowering the calculated Log *P* (*c* Log P) value from 6.2 (**1**) to 4.2 (**20**) while simultaneously retaining approximately equal % inhibition of SphK1 activity. Importantly, the *c* Log D of **20** has also been lowered to 3.2 (pH 7.4) indicating that **20** has an increased likelihood of oral bioavailability.[13]

Having identified **20** as an effective inhibitor of SphK1 under in vitro assay conditions at 10.7 μM, we next wanted to determine the K_i and mechanism of inhibition for this compound. We therefore developed a chemical synthesis strategy for the production of **20**. The synthesis of **20** was carried out following our recently reported method[20] as outlined in Scheme 1. The reaction of 4-methoxyacetophenone **21** and dimethyl oxalate in the presence of sodium methoxide led to the formation of ester **22** which on treatment with anhydrous hydrazine led to the formation of pyrazole hydrazide **23**. The condensation of **23** with 3′,4′-dimethoxyacetophenone in the presence of catalytic amount of acetic acid furnished the desired product **20** in good yield. Compound **20** and all other intermediates were characterized on the basis of NMR and mass spectral analysis.[21]

Table 4
SphK1 inhibition for various R^3 methyl substituted compounds



Compound	R^1	R^2	R^3	SphK1 % inhib[a]
18	4-Me	3,4-Di-OMe	Me	21.8
19	4-OMe	4-Cl	Me	46.8
20	4-OMe	3,4-Di-OMe	Me	59.6

[a] Values are percent inhibition of SphK1 at 10.7 μM, averages of two separate experiments, standard deviations were ± 5%.

We performed in vitro SphK1 activity assays with varying concentrations of D-*erythro*-sphingosine (Sph) as substrate in the presence and absence of several concentrations of **20**. The calculated K_i for **20** ranges from 1.26 to 1.55 μM and as shown in Figure 1A, **20** appears to inhibit SphK1 in a competitive manner with respect to the Sph binding site. The K_i of **20** as obtained above is in agreement with the K_i value of **20** (1.33 μM) derived from our IC_{50} studies by the Cheng–Prussoff equation and compares favorably to the calculated K_i value of **1** (1.06 μM; K_m of Sph = 2.75 μM[22,23]). The close agreement between the K_i values of **1** and **20** is consistent with our % inhibition data indicating that **20** is equally effective to **1** at inhibiting SphK1. Importantly, **20** inhibits SphK1 in a non-competitive manner with respect to ATP binding (Fig. 1B). This is also consistent with the mechanism of inhibition of **1**[11] indicating that modification of the SKI-I chemotype has not altered the manner in which the inhibitor binds to SphK1.

As stated above, **1** inhibits both SphK1 and SphK2. To determine whether the optimization of **1** affected the inhibitory activity toward SphK2, we next determined the SphK2 inhibitory activity of **20**. As shown in Figure 2, **20** does not inhibit SphK2 at concentrations up to 25 μM which is near the solubility limit of **20** in the aqueous SphK assay environment. In contrast, the parent compound SKI-I (**1**) significantly inhibits SphK2 activity in a dose dependent manner. This indicates that **20** is the first small-molecule non-lipid specific inhibitor of SphK1.

The data above indicate that **20** inhibits SphK1 catalytic activity in vitro. To demonstrate that **20** is also active in vivo, we next determined the in vivo cytotoxicity of **20** by performing sulforhodamine B (SRB) cytotoxicity assays in the lung adenocarcinoma cell line A549. As shown in Figure 3A, treatment of A549 cells with compound **20** significantly increases the percent cytotoxicity (1.37-fold; p <0.02) relative to **1.** Importantly, to demonstrate the specificity of this cytotoxic effect we also examined a compound

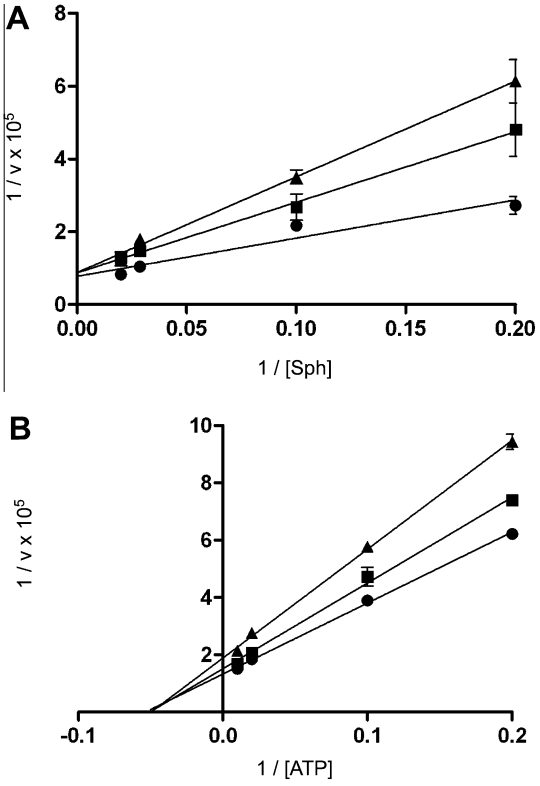
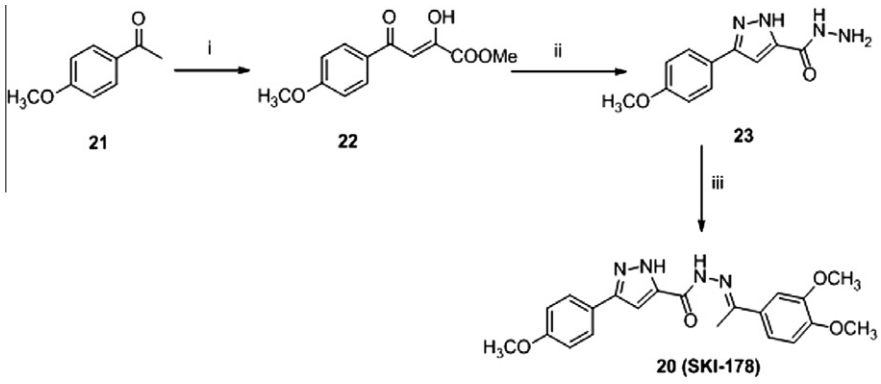


Figure 1. Kinetic evaluation of compound **20**. (A) Sphk1 catalytic activity was measured in the absence (filled circles) or presence of **20** (10 μM filled squares or 17 μM filled triangles) a varying concentration of Sph. Lineweaver–Burk representation indicates the **20** is a competitive inhibitor of Sph binding. (B) Sphk1 catalytic activity was measured in the absence (filled circles) or presence of **20** (10 μM filled squares or 17 μM filled triangles) a varying concentration of ATP. Lineweaver-Burk representation indicates the **20** is a non-competitive inhibitor of ATP binding.

(**5**) with reduced SphK1 inhibitory activity. This compound was less cytotoxic than **1** indicating that the increased cytotoxicity of **20** is due to the inhibition of SphK1 activity and not due to non-specific cytotoxic effects of the pyrazole-5-carbohydrazide backbone. To further demonstrate that **20** directly inhibits SphK1 catalytic activity in vivo, we examined the cellular levels of S1P production in A549 cells treated with **1** or **20** (1 μM) for 48 h, by thin layer chromatography. As shown in Figure 3B, **1** and **20** both reduce cellular S1P levels relative to DMSO controls further indicating that **20** directly inhibits SphK1 in vivo. The lower band observed in the control and compound treated lanes likely represents a lipid kinase present in the cell lysate as confirmed



Scheme 1. Reagents and conditions: (i) sodium, MeOH, dimethyl oxalate, C_6H_6, 0 °C, rt, overnight; (ii) anhydrous hydrazine, EtOH, reflux, 6 h; (iii) 3′,4′-dimethoxyacetophenone, cat. AcOH, DMSO.

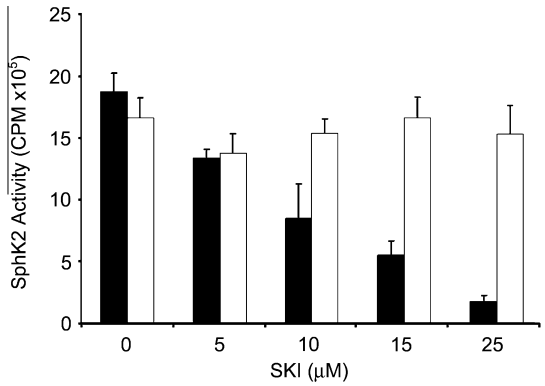


Figure 2. Inhibition of SphK2 by **20.** Sphk2 catalytic activity was measured in the presence of increasing concentrations of **1** (black bars) and **20** (white bars). Lack of inhibition indicates that **20** does not inhibit SphK2 catalytic activity.

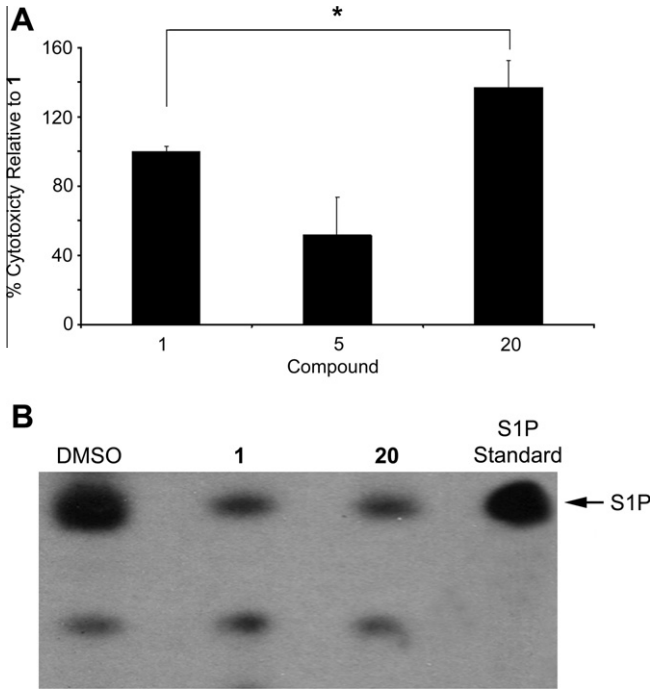


Figure 3. In vivo cytotoxicity and inhibition of S1P formation by **20**. (A) In vivo cytotoxicity of A549 cells was determined by SRB assay[24] treated with 1 μM compounds **1**, **20** and the inactive conformer **5**. Cytotoxicity is reported as the % increase or decrease in cell cytotoxicity, after 48 h treatment, relative to **1** as 100%. (B) SphK1 activity assays[19] were performed on whole cell lysates of A549 cells treated with 1 μM **1** or **20** for 48 h. Lipids were separated by thin layer chromatography as described previously.[36]

by its absence from the S1P standard lane which was prepared by in vitro SphK1 activity assay using recombinant SphK1 and D-*erythro*-sphingosine as a substrate.

Our previous studies have demonstrated that **1** induces apoptosis in T24, MCF7 and NCI-Adr cancer cell lines.[11] We next confirmed that **20** was cytotoxic toward a panel of cancer cell lines, including multi-drug resistant cell lines, by employing sulforhodamine B cytotoxicity assays.[24] Indeed as shown in Table 5, **20** was cytotoxic at IC_{50} concentrations ranging from 1.8 to 0.1 μM in both drug sensitive and multi-drug resistant cancer cell lines (i.e., MTR-3, NCI-ADR and HL60/VCR[25,26]). These data also demonstrate that specific inhibition of SphK1 is sufficient to induce cell death as has been demonstrated using siRNA approaches.[27]

We previously reported the identification of four novel classes of SphK inhibitor 'lead' compounds.[11] One of these classes, SKI-II

Table 5
Cytotoxicity of SKI-178 toward a panel of cancer cell lines

Cancer cell line	**20** (IC_{50} μM)[a]	Cancer cell line	**20** (IC_{50} μM)[a]
MCF-7	1.3 ± 0.4	Panc-1	0.1 ± 0.1
MTR-3	1.8 ± 0.4	Mia-PaCa2	0.1 ± 0.1
NCI/ADR	1.5 ± 0.3	BxPC3	0.2 ± 0.1
A549	0.3 ± 0.2	KG1a	0.5 ± 0.2
H460	0.3 ± 0.2	Kusami-3	0.2 ± 0.1
H226	0.3 ± 0.1	HL-60	0.2 ± 0.1
U251	0.5 ± 0.2	HL60/VCR	0.2 ± 0.1
U87-MG	0.6 ± 0.2	SH-N-SY	0.3 ± 0.2

[a] Values are averages of three experiments.

(4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol, CAS 312636-16-1), has been used extensively in studies of the in vitro and in vivo action of SphK.[28–30] While SKI-II has been independently validated as a SphK inhibitor, it was not the most potent of the four classes of lead compounds identified. SKI-I **(1)** was more potent in vitro as well as in vivo.[11] SKI-I was also highly cytotoxic toward several multi-drug resistant cell lines making it an attractive scaffold for further drug development. However, due to its high lipophilicity it was unsuitable as a candidate drug target. We therefore began a SAR study with the hope of optimizing the pharmacological properties of SKI-I.

The results of this SAR study yielded an improved compound, **20** (hereafter referred to as SKI-178), with enhanced pharmacological properties. While the improvement in efficacy was modest, at best, this new compound was found to be specific for SphK1. The importance of this finding offsets the modest increase in efficacy by providing a new SphK1 specific lead compound platform for further drug development including QSAR studies, which are currently being performed in our laboratory. Using this new lead compound as a model for future studies will allow us to simultaneously assess whether a modification of the lead compound affects SphK1 inhibition efficacy and/or SphK1 specificity. Thus we will be able to identify determinants of inhibitory activity and isozyme specificity. Identifying the determinants of SphK1 specificity should enable us to rationally design improved general SphK inhibitors as well as SphK2 specific inhibitors for future testing in hyperproliferative disease models.

There is conflicting evidence that suggests that either SphK1 or SphK2 would be the preferred targets for disease intervention. In this regard, a study of glioblastoma patients reported a significant correlation between poor patient survival and elevated SphK1 levels, but found no correlation with SphK2 or S1P receptor expression levels.[31] These findings are consistent with a clinical study of breast tumor samples which demonstrated that SphK1 is significantly over-expressed in both hormone-independent (i.e., ER^-) and -dependent tumors, and concluded that high SphK1 expression is a functional prognostic marker for decreased metastasis-free survival and the overall poor prognosis of patients.[32] Interestingly, a recent study also reported that high SphK1 expression levels are a good indicator of daunorubicin resistance of leukemia cells.[33]

Conversely, a recent study of SphK2 deficient breast cancer cells indicated that SphK2 catalyzed S1P production was required for xenograft proliferation and that decreased S1P production attenuated tumor-associated macrophage polarization.[34] Separately, Hait et al.[35] identified a role for SphK2 in migration of breast cancer cells toward EGF. Thus, these conflicting results indicate the need for specific inhibitors of SphK1 and/or SphK2 to determine whether strategies targeting SphK1 or SphK2 alone or the SphKs in combination offer the best therapeutic effect. The development of SphK isoform-specific inhibitors also offer the ability to dissect the complex signaling pathways associated with S1P production and to assign those effects to specific SphK isoforms.

There is ample evidence to suggest that SphK derived S1P production has a vital role in tumor development, progression of tumors to highly aggressive and metastatic phenotypes (tumorigenesis) and the development of multi-drug resistance (MDR). However, it remains unclear whether strategies that target SphK1 or SphK2 specifically or SphKs in general are more efficacious for the prevention/treatment of hyperproliferative diseases including cancer. Thus with the identification of SphK1 specific small-molecule non-lipid like inhibitors as reported herein, we may now be able to address this central question of SphK pathophysiology.

Acknowledgements

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21. Melting points were recorded on a Fischer–Johns melting point apparatus and are uncorrected. NMR spectra were recorded using a Bruker Avance 500 MHz spectrometer. Chemical shifts (δ) were reported in parts per million downfield from the internal standard. The signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet). High-resolution MS (EI) were determined at the Chemistry Instrumentation Center, State University of New York at Buffalo, NY. Thin-layer chromatography (TLC) was developed on aluminum-supported precoated silica gel plates (EM industries, Gibbstown, NJ). Column chromatography was conducted on silica gel (60–200 mesh).
2-Hydroxy-4-(4-methoxyphenyl)-4-oxomethylbutenoate **(22)**. A solution sodium (0.92 g, 39.9 mmol) in methanol (10 mL) was added to a mixture containing 4-methoxyacetophenone (**21**) (5.0 g, 33.33 mmol) and dimethyl oxalate (4.33 g, 36.66 mmol) in benzene (150 mL) dropwise at 0 °C. After the addition was complete, the mixture was allowed to warm to room temperature, stirred overnight, quenched with 1 N HCl solution, and filtered. The residue was purified through silica gel column chromatography using a mixture of methylene chloride/hexanes (7:3) as an eluent to yield 6.0 g (87%) of **22** as a pale-yellow solid; mp 97–98 °C; ^{1}H NMR ($CDCl_3$): δ 8.01 (2H, d, J = 7.0 Hz), 7.06 (1H, s), 7.01 (2H, d, J = 7.0 Hz), 3.96 (3H, s), 3.92 (3H, s).
5-(4-Methoxyphenyl)-2H-pyrazole-3-carboxylic acid hydrazide (**23**). To a solution of methyl ester **22** (5.0 g, 24.0 mmol) in EtOH (150 mL) was added anhydrous hydrazine (3.1 g, 96.0 mmol) and the reaction mixture was refluxed for 6 h under nitrogen. The reaction mixture was cooled to room temperature, the precipitated white solid was filtered and washed with a mixture of ethanol/hexanes (1:9) to yield 5.0 g (90%) of **23** as a white solid; mp 231–232 °C; ^{1}H NMR (DMSO-d_6): δ 13.5 (1H, s), 7.7 (2H, d, J = 8.5 Hz), 7.02 (3H, m), 4.49–4.45 (2H, m), 3.8 (3H, s); MS (ESI) 233 (M^++1).
N′-[1-(3,4-Dimethoxyphenyl)ethylidene]-3-(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide **(20).** To a solution of hydrazide **23** (1.0 g, 4.3 mmol) in DMSO (50 mL) was added 3′,4′-dimethoxyacetophenone (0.85 g, 4.74 mmol) in DMSO (10 mL) followed by a catalytic amount of acetic acid. The reaction mixture was stirred overnight at room temperature and then poured into water (100 mL). The solid thus formed was filtered and washed with CH_2Cl_2/hexanes (7:3) to yield the crude product which was purified by column chromatography using a mixture of CH_2Cl_2/MeOH (9:1) as an eluant to afford 1.1 g (65%) of **20** as a white solid; mp 210–211 °C; ^{1}H NMR (DMSO-d_6): δ 7.77 (2H, d, J = 8.5 Hz), 7.48 (1H, d, J = 2.0 Hz), 7.42 (1H, s), 7.06–7.01 (4H, m), 3.81 (3H, s), 3.82 (3H, s), 3.18 (3H, s), 2.35 (3H, s). HRMS (ESI) calcd for $C_{21}H_{22}N_4O_4H$, 395.1714; found, 395.1717.
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