Discovery of an SphK1 inhibitor: A hybrid approach involving a receptor-ligand-complex-based pharmacophore and docking-based virtual screening

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Abstract
Sphingosine kinase is a lipid kinase that catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate. Sphingosine-1-phosphate is a bioactive lipid that regulates biological processes. The overexpression of sphingosine kinases is related to a variety of pathophysiological conditions. For example, SphK1 has been shown to be highly expressed in various cancer cells including ovarian, cervical, colon, stomach, lung, and brain cancer. Inhibition of sphingosine kinases is a promising way to treat diseases such as cancer. Through computer-aided drug design, we have discovered a new SphK1 inhibitor named Amb30572637 (SAMS10). In this report, we describe the discovery process and biological characteristics. In biochemical experiments, SAMS10 shows a prominent inhibitory effect on SphK1, with an IC50 value of 9.8 μM. Subsequent MTT experiments show that SAMS10 has anticancer effects toward A549, SKVO3, A375, and LOVO cell lines and has essentially no cytotoxicity against the healthy cell L929. SAMS10 has significant inhibitory activity against the A549 and LOVO cell lines, with IC50 values of 14.64 and 14.48 μM, respectively. It belongs to a moderately active SphK1 inhibitor with lower anticancer activity than the control compound cisplatin, but the effect of SAMS10 toward SphK1 and its anticancer activity indicate that it is a promising lead compound for the development of effective SphK1 anticancer inhibitors.

Keywords
anticancer, computer-aided drug design, SAMS10, sphingosine kinases, SphK1 inhibitor

Date received: 2 December 2021; accepted: 2 March 2022

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Introduction

Targeting the sphingosine-1-phosphate (S1P) signaling pathway is promising for the treatment of many diseases because elevated S1P levels is related to cardiovascular diseases, autoimmune/inflammatory diseases, cancers, and other pathophysiological conditions. Sphingosine kinases (SphKs) are key kinases in the S1P signaling pathway, which can regulate the balance between sphingosine (Sph), ceramide (Cer), and S1P, and regulate overall S1P levels. Inhibiting the activity of SphKs can inhibit Sph from being transformed into S1P that promotes cell proliferation, migration, differentiation, and survival. The SphK family includes two isoforms: SphK1 and SphK2. The SphK1 crystal structure and co-crystal structures of SphK1 with three inhibitors (SK-II, PF-543, Amgen 23; Figure 1) have been resolved yet. SphK1 contains two domains: N-terminal domain (NTD) and C-terminal domain (CTD). The ATP binding site is located in NTD, the Sph binding site is located in CTD, and the catalytic center of SphK1 is located between NTD and CTD. To date, the development of SphK1 inhibitors has mainly focused on compounds that bind to the Sph binding pocket and compete with Sph.

In this work, we also target the Sph binding site to develop novel sphingosine kinase 1 inhibitors. The shape of the Sph binding site resembles the letter J. SphK1 contains two domains: N-terminal domain (NTD) and C-terminal domain (CTD). The ATP binding site is located in NTD, the Sph binding site is located in CTD, and the catalytic center of SphK1 is located between NTD and CTD. To date, the development of SphK1 inhibitors has mainly focused on compounds that bind to the Sph binding pocket and compete with Sph. In this work, we also target the Sph binding site to develop novel sphingosine kinase 1 inhibitors. The shape of the Sph binding site resembles the letter J. The end near the NTD is called the hydrophilic tail and is surrounded by hydrophobic amino acids. Several sphingosine kinase inhibitors have been developed and many reviews have introduced them in detail. Several sphingosine kinase inhibitors have been developed and many reviews have introduced them in detail.

Results and discussion

Establishment and evaluation of the pharmacophore model

In this work, the computer-aided drug design software used was BIOVIA Discovery Studio2020 (DS). We built multiple pharmacophore models based on the co-crystal structures of SphK1 with PF-543 (PF-543 has the highest activity among the existing co-crystal structures) and selected the best pharmacophore model through evaluation (Figure 2). We used the method based on the receptor–ligand complex to construct the pharmacophore and constructed 50 pharmacophore models and then evaluated the pharmacophore models. We first used the pharmacophore and then used molecular docking to perform virtual screening of a compound database. A total of 120 compounds were selected as potential SphK1 inhibitors for in vitro kinase activity testing. Finally, we report a new SphK1 inhibitor, which has an IC50 value of 9.8 μM for SphK1 and demonstrates anticancer activity.

Virtual screening of databases

The compound library we used was Ambinter (http://www.ambinter.com/). We randomly selected 200,000 commercial ligands with drug-like properties from Ambinter for this screening. These ligands were optimized by the Prepare Ligand Procedure module in the DS software. We used the pharmacophore and then used molecular docking to perform virtual screening of the compound database (Figure 3). The 200,000 commercial ligands were matched with optimal pharmacophore model 38. After this preliminary screening, the top 20,000 of molecules were selected according to the ranking of fit vlues and were used for subsequent molecular docking screening. When using molecular docking for screening, we chose the CDOCKER module in the DS software. After the docking was complete, sorted by -CDOCKERInteraction energy (The negative of the receptor–ligand interaction energy is reported as -CDOCKERInteraction energy), and then the top 2000 of small molecules were manually screened. We selected compounds of suitable length that matched the shape of the substrate binding pocket and based on their interactions with the key amino acid residues Asp167 and Asp264, we selected 12 compounds as potential lead compounds for subsequent in vitro kinase activity testing (Figure 4). The Supporting Information provides the -CDOCKERInteraction energy data for the 12 selected compounds and the detailed process of the molecular docking.

Kinase inhibitory activities

These 12 compounds (Figure 4) were tested for their inhibition activity against SphK1 at a concentration of 10 μM. The inhibitory activity of these compounds against SphK1 is shown in Table 1. At 10 μM, most of the compounds had no inhibitory effects against SphK1. However, to our delight, compound SAMS10 (SAMS10 used in the experiments is a racemate), is excellent. Inhibition of SphK1 is at 73%.

We further evaluated the IC50 value of SAMS10 (SAMS10 used in the experiments is a racemate) against SphK1 and SphK2. As shown in Table 2, SAMS10 has strong inhibitory activity against SphK1, with an IC50 value of 9.8 μM, and only exhibits a weak inhibitory effect against SphK2. This indicates that SAMS10 is a highly selective SphK1 inhibitor.
Figure 1. Structures of SphK inhibitors.

| Compound | 
|----------|--------------------|
| Amgen 23 | SphK1 IC$_{50}$ = 0.02 μM  
SphK2 IC$_{50}$ = 1.6 μM |
| PF-543  | SphK1 IC$_{50}$ = 2.7 nM  
SphK2 IC$_{50}$ = 356 nM |
| SKI-11  | SphK1 $K_i$ = 16 μM  
SphK2 $K_i$ = 8 μM |

Figure 2. (a) ROC curve (The ROC curve graph is a curve reflecting the relationship between sensitivity and specificity) for pharmacophore 38. (b) Schematic diagram of pharmacophore 38 superimposed into the co-crystal structure of SphK1 and PF-543. The gray balls represent the excluded volume, the magenta balls are the hydrogen bond acceptor, and the blue balls represent hydrophobic characteristics.

Figure 3. Schematic diagram of the virtual screening process.

Cytotoxic activity

Cells with high SphK1 expression are distributed in liver cancer, prostate cancer, melanoma, lung cancer, and gliomas. Thus, it is necessary to test the cytotoxic activity of SAMS10 (SAMS10 used in the experiments is a racemate). For this purpose, we used four cell lines, A549, SKVO3, A375, and LOVO to test the cytotoxic activity of SAMS10 (Table 3). SAMS10 does not exhibit strong anticancer effects compared with cisplatin, but it had certain anticancer effects toward the SKVO3, A549, LOVO, and A375 cell lines. Among them, SAMS10 has a prominent inhibitory activity against the A549 and LOVO cell line, with an IC$_{50}$ value of 14.64 and 14.48 μM. In healthy cells L929,
SAMS10 is basically noncytotoxic and its IC$_{50}$ value is 74.28 μM, which is beneficial to its future development.

Docking of SAMS10 and SphK1

We performed molecular docking analysis on SAMS10 and the co-crystal structures of SphK1 with PF-543 (4V24). According to the docking results (for the docking results of SAMS10, we chose the R configuration that matches the protein better), SAMS10 occupies in the “J-shaped” cavity of SphK1, and its hydroxyl group can form hydrogen bond with the key amino acid residues Asp264 with a bond length of 1.64 Å and a bond angle of 165.28°. The oxygen at the head of this compound forms a hydrogen bond with Asn200 with a bond length of 3.06 Å and a bond angle of 100.43°. In addition, the head of this compound forms unconventional hydrogen bonds with Asp167, Leu354, Glu275, and Gly428. The middles and tails of small molecules form a wide range of hydrophobic interactions with proteins, with the middle of SAMS10 forming interactions with Val263, Met358, Met392, and Ile260, while the tails form interactions with Leu345, Val376, Leu405, and Leu388 (Figure 5). SAMS10 is active against SphK1.

Synthesis of SAMS10

SAMS10 has a prominent role in inhibiting SphK1 and a variety of cell lines. The synthetic process is show in Figure 4.

Table 1. *SphK1 inhibitory activity of the 12 selected compounds at a concentration of 10 μM.

| Compound | Inhibition (%) | Compound | Inhibition (%) |
|----------|---------------|----------|---------------|
| SAMS1    | NA            | SAMS7    | NA            |
| SAMS2    | NA            | SAMS8    | NA            |
| SAMS3    | NA            | SAMS9    | NA            |
| SAMS4    | NA            | SAMS10   | 73            |
| SAMS5    | NA            | SAMS11   | NA            |
| SAMS6    | NA            | SAMS12   | NA            |
| PF-543   | 99            |          |               |

NA: no activity. *The value is the percentage of inhibition against SphK1 (average of two independent experiments) with a standard deviation of ±5%.

Table 2. The inhibitory effects of Amb30572637 (SAMS10) against SphK1 and SphK2.

| Compound | IC$_{50}$ (μM) | Selectivity (SphK2/SphK1) |
|----------|----------------|--------------------------|
| SAMS10   | 9.8 ± 0.4  | >100                     |
| PF-543   | 0.052 ± 0.007 | 6.1               |

IC$_{50}$ of PF-543 varies with assay conditions.13
Scheme 1. We first obtained compound 4 through a three-step reaction, and then obtained compound 6 through a one-step reaction, and finally compound 4 reacted with 6 to produce SAMS10 (Compound 6 and SAMS10 are both racemates). The nuclear magnetic resonance (NMR) data for SAMS10 and compounds 2, 3, 4, 6 was provided in the Supporting Information.

Conclusion

In sum, through virtual screening methods and biological verification, we have discovered a new SphK1 inhibitor. In vitro enzyme activity experiments revealed that the compound Amb30572637 (SAMS10) has the potential to inhibit the activity of SphK1. SAMS10 proved to be a selective SphK1 inhibitor (IC$_{50}$ 9.8 μM). To our delight, SAMS10 showed anticancer effects toward the A549, SKVO3, A375, and LOVO cell lines. Thus, SAMS10 represents a promising lead compound for SphKs inhibition for future development.

Experimental

Materials

2-Ethoxyphenol, 4-hydroxy-3-methoxybenzaldehyde, 2-bromoethanol, 4-methylbenzenesulfonyl chloride, triethylamine, DMAP, 2-(bromomethyl)oxirane, potassium carbonate, tetrabutylammonium iodide, epibromohydrin, acetonitrile, pyridine, isopropanol, 4-methylpiperidine, tetrabutylammonium iodide, ethyl acetate, methylamine hydrochloride, sodium borohydride, fetal bovine serum, SphK1 (Signalchem, Canada), SphK2 (Signalchem, Canada), and other materials were purchased from

Table 3. Anti-proliferative effects of SAMS10 and cisplatin as a reference.

| Compound | IC$_{50}$ (μM) |
|----------|----------------|
|          | A549 | SKVO3 | A375 | LOVO | L929  |
| SAMS10   | 14.64 ± 0.06 | 21.07 ± 0.18 | 27.11 ± 0.66 | 14.48 ± 0.07 | 74.28 ± 6.98 |
| Cisplatin| 4.21 ± 1.31  | 1.91 ± 0.43  | 5.91 ± 1.64  | 2.64 ± 1.12  | 7.616 ± 1.05  |

Figure 5. Schematic diagram of the interaction between SAMS7-10 and SphK. The green lines represent the hydrogen bonding interactions. The pink lines indicate hydrophobic interactions. (a) Three-dimensional schematic diagram of the interaction between SAMS10 and SphK. (b) Two-dimensional schematic diagram of the interaction between SAMS10 and SphK. (c) Two-dimensional schematic diagram of the interaction between SAMS7 and SphK. (d) Two-dimensional schematic diagram of the interaction between SAMS9 and SphK. (e) Two-dimensional schematic diagram of the interaction between SAMS8 and SphK.
commercial sources (Aladdin, Macklin, Innochem) and were used without further purification.

**Apparatus**

High-resolution mass spectrometry (HRMS) was performed on an AB SCIEX X500R Accurate Mass Q-TOF by using electrospray ionization (ESI). The NMR spectra were recorded on a Bruker AVANCE-400 spectrometer with tetramethylsilane as the internal standard.

**Synthesis**

4-(2-Hydroxyethoxy)-3-methoxybenzaldehyde (2). To a 500 mL reaction flask equipped with a stir bar was added compound 1 (5.0 g, 32.9 mmol, 1.0 equiv), 2-bromoethanol (5.8 mL reaction flask equipped with a stir bar was added compound 1 (5.0 g, 32.9 mmol, 1.0 equiv), 2-bromoethanol (5.8 g, 82.2 mmol, 2.5 equiv), CH3CN (260.0 mL), followed by the addition of K2CO3 (6.8 g, 49.3 mmol, 1.5 equiv). The reaction mixture was allowed to stir at room temperature for 24 h, sodium borohydride (253.5 mg, 6.7 mmol, 1.5 equiv) was slowly added in an ice bath. The reaction mixture was quenched with saturated sodium bicarbonate after 30 min. The mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na2SO4. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (CH2Cl2/MeOH 20:1) to give the desired product 3. The Supporting Information contains the 1H and 13C spectrum of compound 3.

2-(4-Formyl-2-methoxyphenoxy)ethyl 4-methylbenzenesulfonate (3). Yield: 10.3 g, (90%); white solid. 1H NMR (600 MHz, CDCl3): δ = 9.86 (s, 1H), 7.84 – 7.79 (m, 2H), 7.41 (d, J = 7.4 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 6.92 (d, J = 8.3 Hz, 1H), 4.45 – 4.40 (m, 2H), 4.32 (dd, J = 5.7, 4.2 Hz, 2H), 3.90 (s, 3H), 2.44 (s, 3H). 13C NMR (150 MHz, CDCl3): δ = 190.84, 152.90, 150.04, 145.05, 132.70, 130.91, 129.87 (2C), 128.01 (2C), 126.38, 112.52, 109.71, 67.58, 66.56, 56.01, 21.68. HRMS (ESI): m/z calcd for C17H19O6S [M + H]+: 351.0902. found: 351.0824.

1-(3-Methoxy-4-{[2-(4-methylpiperidin-1-yl)ethoxy]phenyl}-N-methylmethanamine (4). To a 250 mL reaction flask equipped with a stir bar was added compound 3 (1.56 g, 4.45 mmol, 1.0 equiv), 4-methylpiperidine (662.5 mg, 6.68 mmol, 1.5 equiv), tetrabutylammonium iodide (328.74 mg, 0.89 mmol, 0.2 equiv), and CH3CN (135 mL). The reaction mixture was allowed to stir at 50℃ for 24 h under nitrogen protection. After cooling to room temperature, the mixture was filtered and concentrated under reduced pressure. Next, to the mixture was added MeOH (200 mL) followed by the addition of methylamine hydrochloride (1.5 eq) under the protection of nitrogen. After the reaction was stirred at room temperature for 24 h, sodium borohydride (253.5 mg, 6.7 mmol, 1.5 equiv) was slowly added in an ice bath. The reaction mixture was quenched with saturated sodium bicarbonate after 30 min. The mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na2SO4. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (CH2Cl2/MeOH 20:1) to give the desired product 3. The Supporting Information contains the 1H and 13C spectrum of compound 3.

2-(4-Formyl-2-methoxyphenoxy)ethyl 4-methylbenzenesulfonate (3). To a 500 mL reaction flask equipped with a stir bar was added compound 2 (6.4 g, 32.6 mmol, 1.0 equiv), 4-methylbenzenesulfonyl chloride (18.7 g, 97.9 mmol, 3 equiv), CH2Cl2 (280.0 mL), triethylamine (22.6 mL, 163.1 mmol, 5 equiv) and DMAP (0.8 g, 6.524 mmol, 0.2 equiv). The reaction mixture was allowed to stir at room temperature for 24 h. After cooling to room temperature, the mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na2SO4. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (CH2Cl2/MeOH 20:1) to give the desired product 3. The Supporting Information contains the 1H and 13C spectrum of compound 3.

Supporting Information contains the 1H and 13C spectrum of compound 4.
layers were washed with brine and dried over Na₂SO₄. The then extracted into ethyl acetate. The combined organic to room temperature, the mixture was poured into water then extracted into ethyl acetate. The combined organic layers were allowed to stir at 50°C dropwise under the protection of nitrogen. The reaction mixture was allowed to stir at 100°C for 6 h. After cooling to room temperature, the mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na₂SO₄. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (PE/EtOAc 20:1) to give the desired product 6. The Supporting Information contains the 1H and 13C spectrum of compound 6.

2-[(2-ethoxyphenoxy)methyl]oxirane (6). To a 25 mL glass tube equipped with a stir bar was added 2-ethoxyphenol (414.3 mg, 3.0 mmol, 1.0 equiv), K₂CO₃ (1.2 g, 9 mmol, 3.0 equiv), tetrabutylammonium iodide (221.6 mg, 0.2 mmol, 0.6 equiv) and CH₂CN (16 mL), followed by the addition of epibromohydrin (1.0 mL, 12.0 mmol, 4.0 equiv) dropwise under the protection of nitrogen. The reaction mixture was allowed to stir at 50°C for 36 h. After cooling to room temperature, the mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na₂SO₄. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (PE/EtOAc 20:1) to give the desired product 6. The Supporting Information contains the 1H and 13C spectrum of compound 6.

1-(2-ethoxyphenoxy)-3-[(3-methoxy-4-[2-(4-methylpiperidin-1-yl)ethoxy]benzyl]methyl)amino)propan-2-ol (SAMS10). To a 25 mL glass tube equipped with a stir bar was added compound 6 (194.2 mg, 1.0 mmol, 1.0 equiv), 4 (350.9 mg, 1.2 mmol, 1.2 equiv) and isopropanol (15 mL) followed by the addition of a catalytic amount of pyridine for 6 h. After cooling to room temperature, the mixture was poured into water then extracted into ethyl acetate. The combined organic layers were washed with brine and dried over Na₂SO₄. The organic layer was filtered and concentrated under reduced pressure to provide the crude product, which was purified by silica gel chromatography (CH₂Cl₂/MeOH 20:1) to give the desired product 6. The Supporting Information contains the 1H and 13C spectrum of compound 6.

The in vitro kinase test
The SphKs assays were carried out as described previously.13 We used the Kinase-Glo Plus Luminescent Kinase Assay Kit purchased from Promega to measure the kinase activity by quantifying the amount of ATP remaining in the solution after the kinase reaction. The reaction volume is 50µM. PF-543 purchased from Selleck was used as a positive control. SAMS10 was dissolved in pure DMSO to prepare 10 mM stock solutions and diluted with kinase buffer (pH = 7.4, composition: 40 mM/L Tris, 10 mM/L MgCl₂, 0.1 g/L BSA, 1 mM/L DTT, 10 µM/L ATP). SphK1 or SphK2 was added to 96-well plates and treated with the appropriate concentration of SAMS10 (0.01, 0.1, 1, 10 or 100 µM) at 30°C for 40 min. ATP test solution was added and the mixture was incubated at room temperature for 5 min. The luminescence was immediately measured using a microplate spectrophotometer (AD 340, Beckman, USA).

MTT cell viability assay
Here we used cisplatin as a positive control and used the MTT method to test the effect of compound SAMS10 on the survival of five cell lines A549, SKVO3, A375, LOVO and L929 (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China). First, five cell lines were seeded at a density of 6 × 10⁴ per well in 96-well plates and cultured for 24 h. Compounds were dissolved in pure DMSO and diluted into 10 mM stock solution with the culture medium. Then different concentrations of SAMS10 (0.01, 0.1, 1, 10, 100 µM) at 30°C for 40 min. The OD value of each well was measured at 570 nm in a microplate reader (Bio-Rad Laboratories). Finally, the IC₅₀ values were calculated.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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Supplemental material
Supplemental material for this article is available online.

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