Supporting Information

Discovery of novel Sphingosine Kinase inhibitors via structure-based hierarchical virtual screening

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Experimental methods

Virtual screening

The structure-based hierarchical virtual screening was carried out using LibDock, LigandFit and structure-based pharmacophore modeling after preparing the SphK1 protein. All the screening procedures were performed with Discovery Studio, version 4.0. In the first selection step, LibDock program was employed to reduce library size. The binding site was formed from the position of co-crystallized ligand using From Current Selection protocol, and the sphere radius was set to 10 Å. All the parameters for the docking run were set to their default values, and Libscore was employed for ranking molecules. The top 20 000 molecules with highest libdock score were remained. Subsequently, LigandFit program was used as the secondary filter to prioritize the compounds. The binding site was constructed from the position of co-crystallized ligand. The grid resolution was set to 0.5 Å (default), and the ligand-accessible grid was defined such that the minimum distance between a grid point and the protein was 2.0 Å for hydrogen and 2.5 Å for heavy atoms. Docking was performed with all the parameters set to their default values based on characterization of the binding pockets. The top-ranked docking conformations were determined using the ligscore2 values. The 5000 top-ranked molecules were selected for further screening via structure-based pharmacophores. In a final filtering step, the receptor-ligand complex based pharmacophore model for SphK1 complex (PDB code: 4L02) was generated by using the Receptor−Ligand Pharmacophore Generation (RLPG) protocol, and was used as 3D query to screen the 5000 top-ranked molecules, leading to 500 molecules with highest fitvalue for visual inspection.

MD simulation

The MD simulation of compound **25** at the active site of the SphK1 was performed using GROMACS v.4.6.5 with the GROMOS43a1 force field.[1, 2] The initial orientation of the ligand was determined from the docking poses of LigandFit. The complexes were solvated using a cubic periodic box of filling with simple point charge (SPC) waters extending at least 10Å away from the boundary of any protein atoms. Na^+ and Cl^- ions were added at physiological concentration of 0.1 M to ensure the overall neutrality of the systems. Prior to the simulation, energy minimization was carried out for the full system until the value of Fmax converged to lower than 1000 kJ/mol/nm. Each simulation was coupled to a 300 K thermal bath at 1.0 atm pressure by applying the algorithm of Berendsen.[3] To ensure the dynamic stability of the MD trajectories, the RMSD values for the protein backbone atoms relative to the initial minimized structure through the phase of the simulation were calculated from the trajectories at 2 ps intervals (**Fig.1**), which showed that the RMSD changed a little after 2700 ps in the system. The representative structure was selected to display the closest conformation to the average structure during the last 1 ns of simulation.

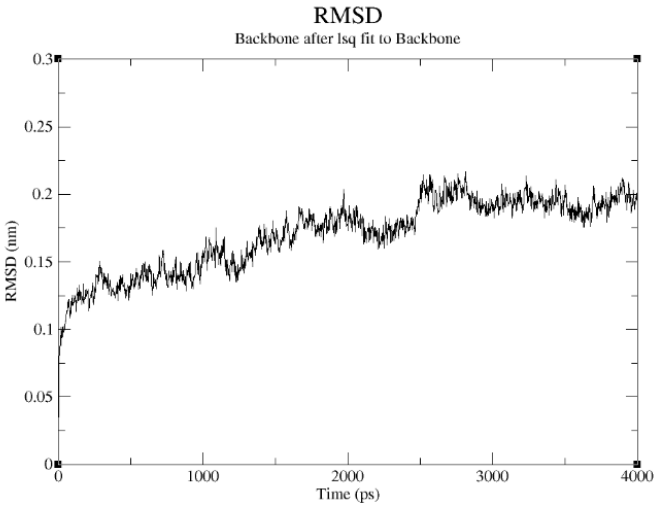

Fig. 1 The RMSD of the backbone atoms obtained during 4 ns of MD simulations.

Sphingosine Kinase Assay

We adapted and validated an ADP quantification methodology based on Adapta™ Universal Kinase Assay to establish a homogeneous high-throughput SphK enzyme assay. The kinase buffer A was purchased from Invitrogen (catalogue number PV3189). In a SphK kinase reaction, the 5× kinase buffer A stock should be diluted with deionized water to make a 1× solution of kinase reaction buffer. A typical reaction contained 1×kinase buffer A, 5 μM sphingosine kinase substrate (Invitrogen, catalogue number PV5372), 1 μM ATP and 0.025 ng/μl SphK1 (Invitrogen, catalogue number PV5214). The reaction for SphK2 (Invitrogen, catalogue number PV5216) was the same as SphK1, except kinase concentration which is 0.8 ng/μl. Compounds were tested at 0.2, 0.7, 2.1, 6.2, 18.5, 55.6, 166.7 and 500.0 μM in the presence of 1% DMSO with a 5 min pre-incubation of SphK1 (or SphK2) and compounds. All reactions were started by the addition of substrates, incubated at room temperature for 60 min and quenched with the stop buffer containing 10 mM EDTA, 2 nM Adapta Eu-anti-ADP Antibody and 1.8 nM Alexa Fluor 647 ADP Tracer. After 60 min incubation, the fluorescence at 665 nm and 620 nm was measured with PHERAstar FS plate reader (BMG LABTECH) using a time delay of 100 μs. The ATP, EDTA, Adapta Eu-anti-ADP Antibody and Alexa Fluor 647 ADP Tracer were provided in Adapta™ Universal Kinase Assay kit (Invitrogen, catalogue number PV5099).

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Inhibition and IC_{50} calculation

The data for dose responses were plotted as percent inhibition calculated with the data reduction formula $100 \times [1 - (U1 - C2) / (C1 - C2)]$ versus concentration of compound, where U is the emission ratio of 665 nm and 620 nm of test sample, C1 is the average value obtained for no reaction control (no kinase sample), and C2 is the average value obtained for solvent control (1% DMSO). Inhibition curves were generated by plotting percentage control activity versus log10 of the concentration of each kinase. The IC_{50} values were calculated by nonlinear regression with Graphpad Prism 5 and were determined from three independent experiments. The dose response curves of DMS and Hits were depicted in Figure 2.

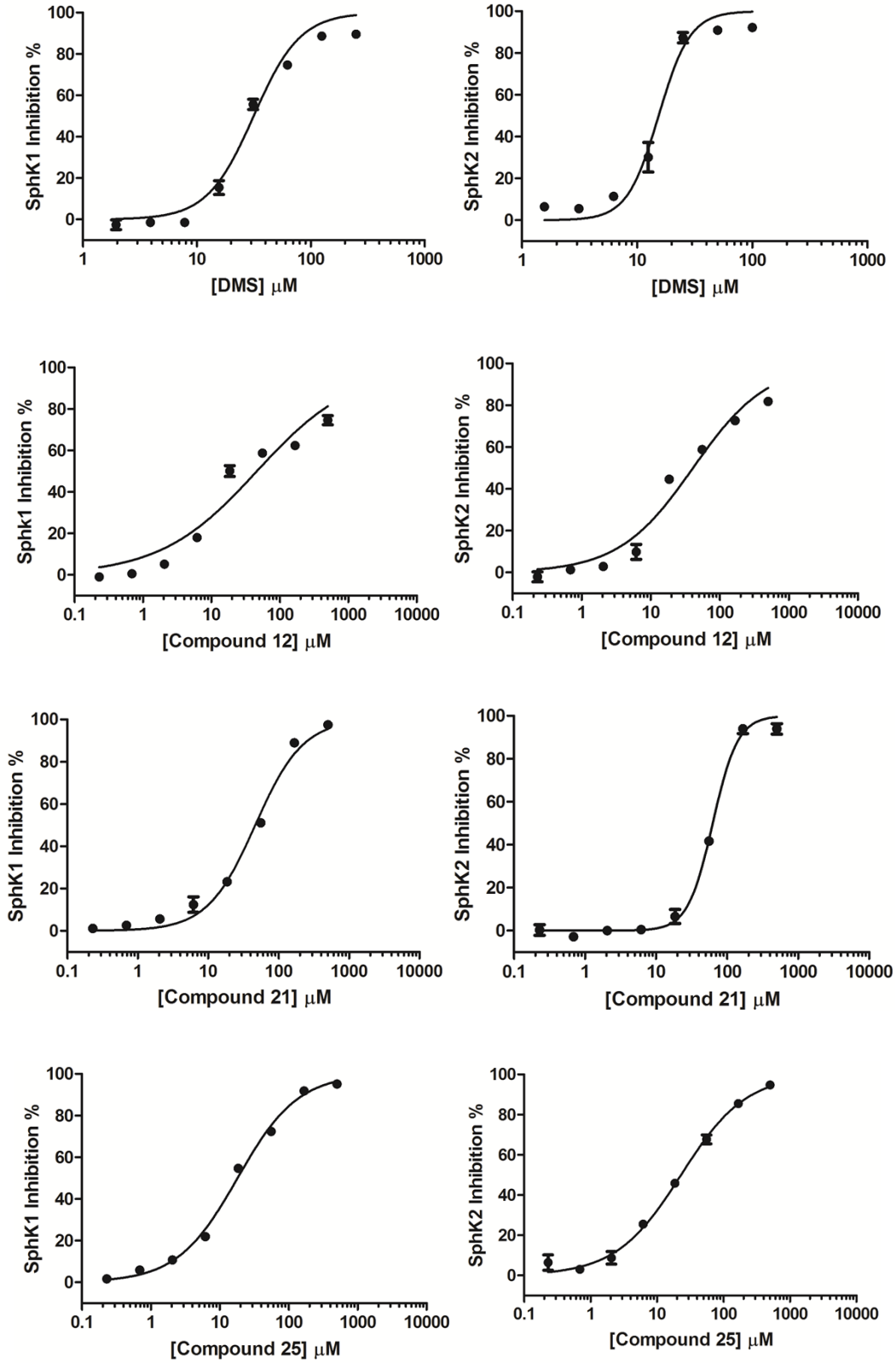


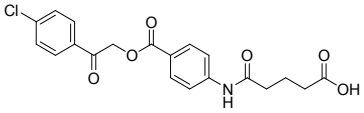
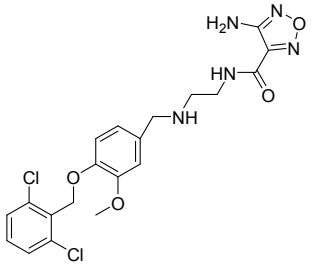
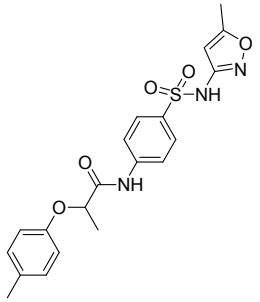
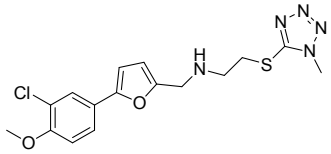
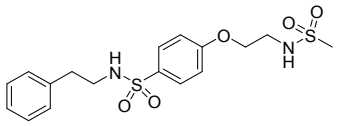
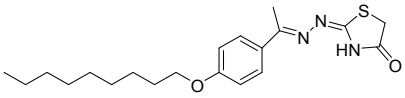
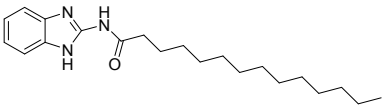
Fig2. The dose response curves of DMS and Hit Compounds on Human SphK1 and SphK2.

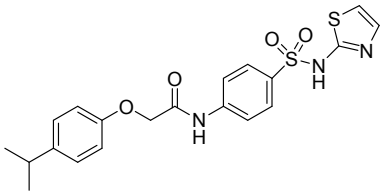
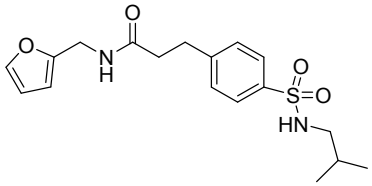
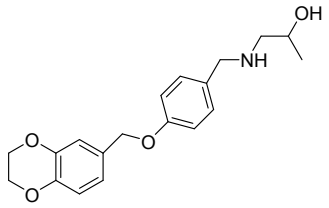
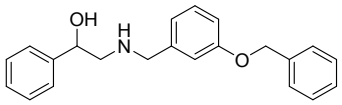
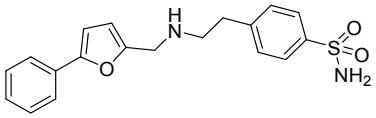
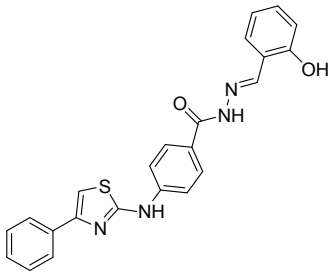
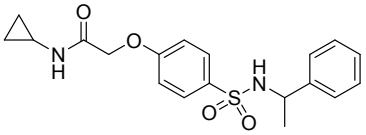
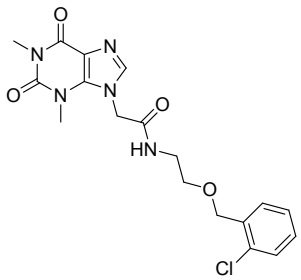
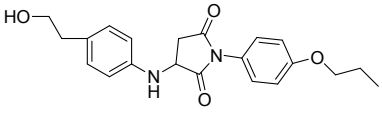
MTS assay

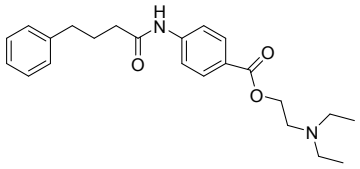
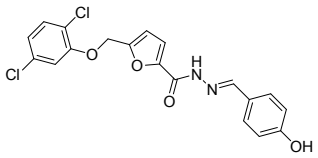
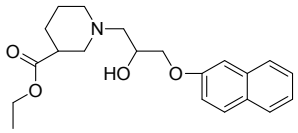
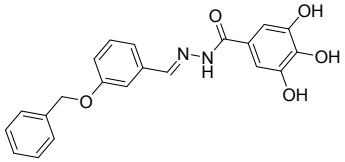
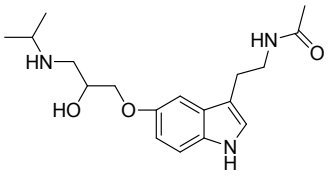
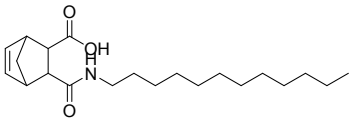
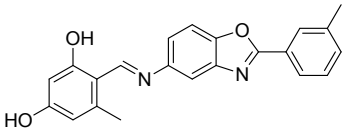
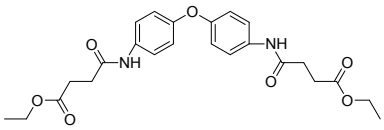
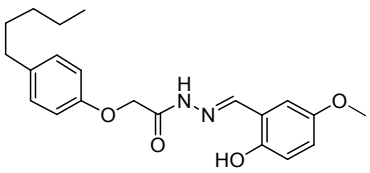
U937 cells (5×10^3) were seeded into 96-well plates with RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) and treated with tested compounds for 72 hours before the MTS assay. MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/PMS (phenazine methosulfate)} is a tetrazolium dye that is converted to a formazan product by dehydrogenase enzymes of metabolically active cells, which is widely used to access the cell viability. According to the manufacturer's protocol, assays were performed by adding 20uL MTS reagent (Promega, Madison, USA) directly into culture wells, incubating for 4 hour and then recording the absorbance at 490nm with a 96-well plate reader. All experiments were performed in triplicate.

Compound List

Table 1S. Chemical structures and the inhibitory activities of SphK1 and SphK2 *in vitro* of selected compounds from virtual screening

Compound (Specs Number)	Structure	SphK1 inhibitory %		SphK2 inhibitory%	
		500μM	50μM	500μM	50μM
6 AK-918/42829288		7.09	11.90	41.92	22.41
7 AP-970/42897773		66.41	-2.33	90.16	4.31
8 AN-329/42868371		79.95	16.78	80.53	38.76
9 AN-153/43310919		35.58	0.28	69.65	16.76
10 AO-080/43378300		-1.73	-1.33	-1.21	2.70
11 AJ-292/41424155		4.62	-2.53	0.48	1.93
12 AN-652/41793133		76.97	64.05	88.87	65.79

13 AN-329/42138493		2.83	-0.04	10.42	11.53
14 AH-487/42191677		40.74	6.60	69.49	30.99
15 AN-465/43411374		-21.87	-5.82	-8.09	-1.35
16 AN-465/42887815		17.49	-4.62	73.71	11.14
17 AN-465/43369277		11.61	-1.89	41.13	7.25
18 AO-081/15137002		-13.37	14.46	20.20	16.44
19 AH-487/41976462		-3.13	-2.98	9.83	6.75
20 AN-465/41992649		3.01	-1.26	16.36	7.68
21 AG-205/14552015		93.78	72.18	96.40	93.16

22 AN-652/43024630		-2.02	-6.89	11.78	6.60
23 AK-968/40642194		-13.40	8.98	-6.82	7.33
24 AP-263/42466841		8.34	-3.17	19.94	6.41
25 AK-968/40385937		95.60	81.50	82.04	45.73
26 AI-981/36563016		-9.36	-3.00	-4.22	-2.14
27 AK-968/37129416		36.99	37.12	60.28	36.81
28 AG-205/33148008		6.07	-3.34	-0.36	-0.97
29 AN-329/40688266		87.40	7.38	89.80	9.28
30 AG-205/07681036		30.61	6.04	38.56	21.05

Reference

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2. H. J. C. Berendsen, D. van der Spoel and R. van Drunen, *Comput. Phys. Commun.*, 1995, **91**, 43-56.
3. D. D. Humphreys, R. A. Friesner and B. J. Berne, *J. Phys. Chem.*, 1994, **98**, 6885-6892.