MedChemComm

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/medchemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Discovery of novel Sphingosine Kinase 1 inhibitors via structure-based hierarchical virtual screening†

Xiaojian Wang,* ‡[a,b] Chenbin Sun, ‡[a] Liang Fang,[b] and Dali Yin*[a,b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Sphingosine kinase 1 (SphK1) is an oncogenic lipid kinase, emerging as a novel and promising target for cancer and other diseases. Herein, a hierarchical structure-based virtual screening against the sphingosine kinase 1(SphK1) binding pocket was performed. Consequently, three compounds have been identified as dual inhibitors of SphK1 and SphK2. Among them, compound **25** exhibited comparable SphK1 and SphK2 inhibitory activity to the positive control *N*, *N*-dimethylsphingosine (DMS) **1**, and exerted anti-proliferative effects on U937 cells. Furthermore, a molecule dynamic (MD) simulation has been conducted to provide a more detailed insight into the binding model between SphK1 and **25** in a state close to physiological conditions. **25** may serve as a potential novel scaffold for further development of SphK1 inhibitors.

Introduction

Sphingosine kinase (SphK) is an important lipid kinase that catalyzes the phosphorylation of D-erythro-sphingosine to produce sphingosine 1-phosphate which regulates cell survival, proliferation, neovascularization, and migration through five G-protein-coupled receptors ($S1PR_{1-5}$).[1-6] There are two isoforms of sphingosine kinase, SphK1 and SphK2, which are encoded by different genes and exhibit distinct biochemical properties, subcellular distribution, substrates and inhibitor sensitivities.[7] Overexpression of SphK1 is observed in a myriad of cancer cell lines and tissues. It is found that there is a correlation between SphK1 expression, severity of disease, drug resistance and reduced patient survival.[8] The inhibition or genetic ablation of SphK1 is observed to slow tumor growth as well as decrease the sensitivity of cancer cells to other chemotherapeutics.[9] Therefore, SphK1 represents a potential target for developing novel therapeutics for cancer and other diseases.[2, 10] The function of SphK2 appears to be less understood. In some instances, SphK2 may suppress cell growth and enhance apoptosis.[11] However, SphK2 plays an anti-apoptotic role in other cases.[12] Recent studies suggested that the role of SphK2 is influenced by its subcellular location.[13]

It is well acknowledged that SphK1 has been identified as a potential therapeutic target for broad cancer mitigation and chemotherapeutic sensitization due to its roles in regulating cell survival, growth, and migration of mammalian cells. However, the chemotypes of SphK1 inhibitors reported were limited. The first SphK inhibitors discovered were sphingosine analogues, such as N, N-Dimethylsphingosine (DMS 1), piperidine derivatives and water-soluble sphingosine analogue SK1-I **2** (**Fig 1.**).[14-17] French *et al.* reported a non-lipidic SphK dual inhibitor SK1-II **3**.[18] The amidine-based SphK1-selective inhibitor **4** was found to significantly lower endogenous S1P levels in human leukemia U937 cells.[19] Additionally, compounds from natural sources also have been recognized as SphK1 inhibitors.[20-23] It is desirable to identify inhibitors with novel chemical scaffolds for the study of the functions and roles of SphK1 in various diseases. Recently, high-resolution crystal structure of SphK1 has been reported, which offers an opportunity to discover novel chemotypes as SphK1 inhibitors.[24]

In this study, we presented the docking combined with pharmacophore based virtual screening of "lead-like" small molecules to discover new potential hits for SphK1 inhibitors. Three compounds with novel scaffolds were identified as dual inhibitors of SphK1 and SphK2. Among them, **25** showed comparable SphK1 and SphK2 inhibitory activity to DMS, and induced anti-proliferative effects toward U937 cells at a concentration dependent manner, which can be used as a starting point for the development of SphK1 inhibitors.

Results and discussion

Aiming at discovering the promising candidates inhibiting SphK1 effectively, we performed structure-based hierarchical virtual

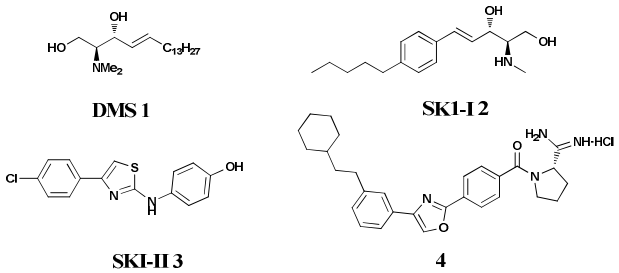


Fig. 1 Structures of selective sphingosine kinase inhibitors.

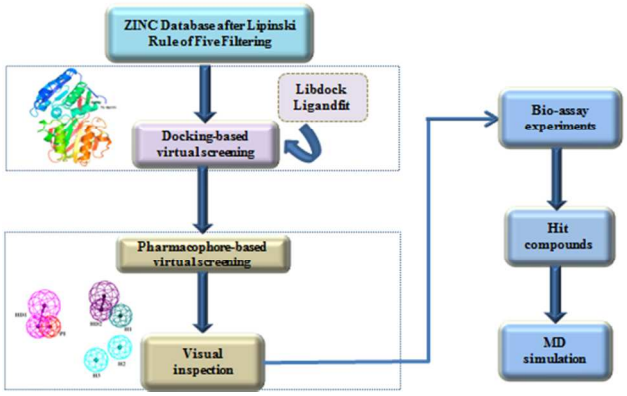


Fig. 2 Schematic representation of the hit discovery strategy.

screening of the ZINC database containing commercially available compounds (**Fig. 2**).[25] Briefly, our hierarchical procedure mainly consists of three steps: (1) reducing library size using a fast docking algorithm; (2) filtering compounds using a more accurate docking algorithm; (3) filtering further compounds via structure-based pharmacophores. In the virtual screening procedures, docking was performed prior to pharmacophore-based approach since it tends to select novel scaffolds.[26] However, the docking approach still faces some limitations, particularly, for example, lack of consideration of the receptor and/or ligand flexibilities and inaccurate scoring functions.[27, 28] Pharmacophore -based approach can be used to compensate for the limitation.[29] Therefore, the structure-based hierarchical virtual screening was developed to compensate for different computational approach.

Docking–based virtual screening

The crystal structure of SphK1 in a complex with its inhibitor **5** (PDB code: 4L02) was used for docking studies.[30] To prepare the enzyme, hydrogen atoms were added, and the protonation states were assigned, while the energy was minimized using the CHARMm force field. It was noted that crystallographic waters should be remained for the function of mediating the formation of hydrogen bond.[24] Two different docking programs, LibDock and LigandFit (Discovery Studio, version 4.0), were employed and validated by comparing the best docking pose obtained from the crystallized inhibitor **5** with its bound conformation (**Fig.3**).[31, 32] As a result, the root mean square deviations (RMSD) of 0.52 Å (LibDock) and 0.25 Å (Lignfit) were calculated, indicating that the docking procedure was reliable. Subsequently, various scoring functions (LigScore1, LigScore2, Jain, -PLP1, -PLP2 and -PMF)

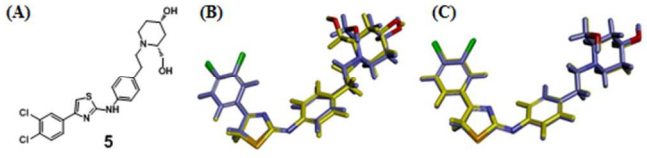


Fig. 3 The structrue of inhibitor **5** (**A**) and Overlays of crystallized inhibitor **5** with the docked poses from (**B**) LibDock (RMSD 0.52 Å) and (**C**) LigandFit (RMSD 0.25 Å); X-ray structure: Blue purple; docked structure: yellow.

were evaluated by docking a set of well-known SphK1 inhibitors into the active site using the LigandFit software, suggesting that LigScore2 was optimal for ranking molecules since it retrieved the most active compounds in the top 10% of screened ligands and thus performed better than the other scoring functions.

After filtering compounds from ZINC database by Lipinski Rule of Five, the rest compounds were docked into the active site of SphK1 by a fast and feature-based LibDock program which is based on matching polar and apolar binding site features of the protein ligand complex. Later, the 20 000 molecules with highest libdock score were redocked with LigandFit, which provides a rapid accurate protocol for docking ligands into protein active sites by considering shape complimentarity between the ligand and the protein active site. LigScore2 was used to rank molecules. The 5000 top-ranked molecules were selected for further screening via structure-based pharmacophores.

Pharmacophore-based virtual screening

In this study, structure-based pharmacophore modeling had also been applied to screen the database due to its less biased towards existing ligand chemotypes than ligand-based pharmacophore modeling.[33] The receptor-ligand complex based pharmacophore model for SphK1 complex (PDB code: 4L02) was generated by using the Receptor−Ligand Pharmacophore Generation (RLPG) protocol, which contained one positive ionizable group (**PI**) pointed towards Asp178, two hydrogen bond donors (**HD**) pointed towards Asp81 (**HD1**) and Thr196 (**HD2**), and threee Hydrophobic groups (**H**) (**Fig. 4A**). A set of well-known inhibitors was prepared to validate the generated pharmacophore mode. From **Fig 4B**, it can been seen that **2** mapped well with four features except one **H** and one **HD** features, which suggested that a reliable pharmacophore model has been developed. Next, the pharmacophore model was used as 3D query to screen the 5000 top-ranked molecules, leading to 500 molecules with highest-fitvalue for visual inspection with following criteria: (a) chemotype diversity; (b) At least one polar interaction was formed between the small molecule and the key residues (Asp81 and Asp178), which were critical in the active site; [15] (c) limited rigidity; (d) commercial availability. Finally, 25 small molecules were selected and purchased for biological evaluation.

SphK Assay

The 25 small molecules were tested for inhibition of the SphK1 and SphK2 activity at 500 and 50 μM, while DMS was used as a positive control.[34] Six compounds (**7**, **8**, **12**, **21**, **25** and **29**)

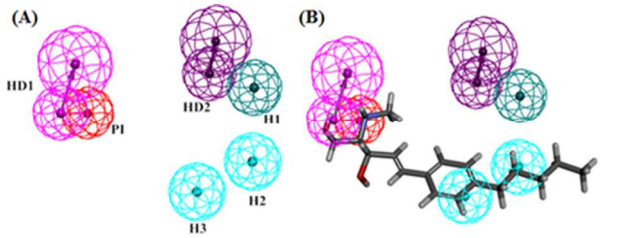


Fig. 4 The structure based pharmacophore model of SphK1. (**A**) Features of the pharmacophore model; (**B**) Mapping of selected compound **2** to the model.

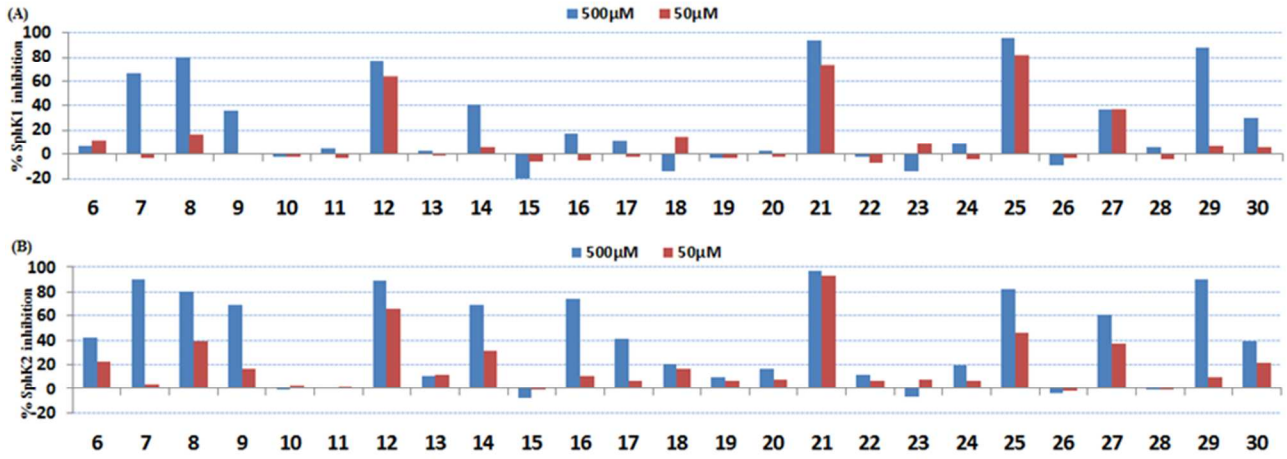
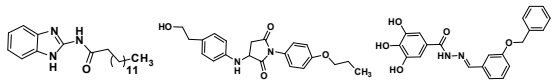


Fig. 5 A quantitative ADP based assay to evaluate the inhibition of *in vitro* SphKs activity by virtual screening hits. Both SphK1 and SphK2 activity were measured using 5μM Sph and 1μM ATP. (**A**) Effect of the 25 small molecules for inhibition of SphK1 activity at 500 and 50μM; (**B**) Effect of the 25 small molecules for inhibition of SphK2 activity at 500 and 50μM.

Table 1 IC_{50} Values of DMS and Hit Compounds on Human SphK1 and SphK2[a]



12 **21** **25**

Entry	SphK1 IC_{50} (μM)	SphK2 IC_{50} (μM)
DMS	35.79 ± 5.88	16.30 ± 6.03
12	40.52 ± 5.08	31.30 ± 9.62
21	61.60 ± 16.85	62.60 ± 8.37
25	23.07 ± 4.59	17.10 ± 5.13

[a]A range of concentrations of the indicated compounds was tested in the SphK1, SphK2 enzyme assay measuring sphingosine-dependent formation of ADP (Adapta™ Universal Kinase Assay). Compounds were tested at 0.2-500 μM. IC_{50} = Means ± SD (n = 3). For details see the Materials and Methods section.

inhibited at least 60% of SphK1 activity at the concentration of 500 μM. Among them, **12**, **21** and **25** inhibit more than 60% of the SphK1activity at a lower concentration of 50 μM (**Fig. 5A**). It was also found that **12**, **21** and **25** exerted inhibitory activity toward SphK2 at both 500 and 50μM (**Fig. 5B**). Further IC_{50} determination identified these three compounds as dual inhibitors of SphK1 and SphK2 at micromolar levels (**Table 1**). In particular, **25** exhibited comparable SphK1 and SphK2 activities to DMS. Among these three compounds, **12**, **21** and **25** possessed benzimidazole, 3-(phenylamino) pyrrolidine-2,5-dione and [4-(benzyloxy)benzylidene]-trihydroxybenzohydrazide scaffolds respectively, which represented interesting chemical classes for further exploration.

MTS Assays

Compound 25 was also screened in the MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfoph-enyl) -2H- tetrazolium/PMS (phenazine methosulfate)} assay

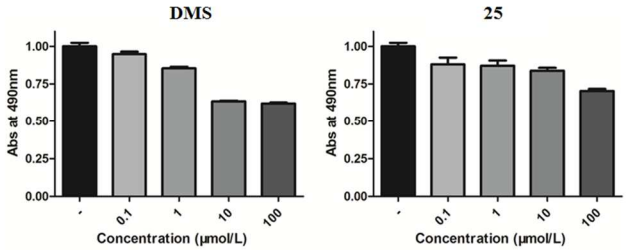


Fig. 6 MTS assay result at 72h using U937 cell line. Compounds were added at 0.1; 1; 10; 100 μM concentration.

using U937 cells, a human leukemia cell line, in order to determine their cytotoxicity to cancer cells.[35] After incubation at 0.1, 1, 10, 100 μM for 72 h, **25** showed comparable anti-proliferative activities against U937 cells to DMS with a concentration dependent manner (**Fig.6**).

MD simulation

In an attempt to obtain more precise ligand-receptor model and reveal key residues for inhibitory potency in the state close to natural conditions, MD simulation of compound **25** at the active site of the SphK1 was performed. The result showed that the hydrogen bond interaction presented by LigandFit between the carbonyl oxygen of Asp81 and hydroxyl hydrogen of the 1,2,3-trihydroxybenzene moiety was disappeared and was compensated by a new hydrogen bond between the carbonyl oxygen of Glu189 and hydroxyl hydrogen of the 1,2,3-trihydroxybenzene moiety (**Fig. 7**). The other hydrogen bond was observed between the carbonyl oxygen of **25** and residue -OH of Thr196**.** In addition, the MD simulation suggested that the hydrophobic and aromatic interactions were formed between the receptor and the compound **25**. The benzyloxy substituted benzene moiety was tracked tightly into the hydrophobic pocket formed by Met306; Phe288; Phe303; IIe174; Leu200; Leu259. Although the binding mode analysis revealed that the orientation and conformation of **25** were different from the docking pose of LigandFit, it was reasonable that the interactions between SphK1 and **25** would be

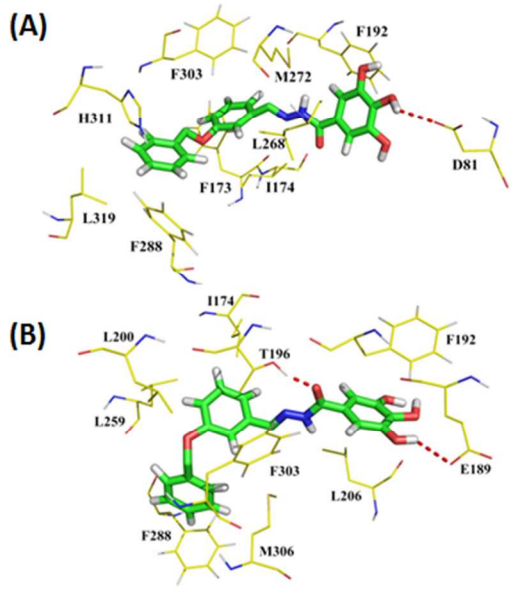


Fig. 7 (A) Binding mode of compound **25** and SphK1 obtained from LigandFit; (B) Binding mode of compound **25** and SphK1 obtained from MD simulation; the hydrogen bonds are illustrated as red lines;

altered during MD simulation by adding water molecules and salt ions in a physiologically setting.

Conclusions

In summary, a structure-based virtual screening of 300 000 compounds from the ZINC database against SphK1 was performed. After a hierarchical virtual screening protocol including docking-based and pharmacophore-based virtual screening, 25 molecules were selected and tested *in vitro* against SphK1 and SphK2, which led to the identification of three micromolar sphingosine kinase inhibitors with new chemotypes. Importantly, compound **12**, **21** and **25** were identified as dual SphK1 and SphK2 inhibitors. Furthermore, a cell bases MTS assay indicated that **25** showed comparable anti-proliferative activities to DMS against U937 cells in a concentration dependent manner. In addition, MD simulation studies of the binding mode of **25** suggested that the compound **25** was hydrogen-bonded to Glu189 and Thr196 and exhibited hydrophobic and aromatic interactions with Met306; Phe288; Phe303; IIe174; Leu200; Leu259. The hits discovered in this work will provide novel scaffolds for further optimization and lay the foundation for further development of potent SphK1 inhibitors.

Acknowledgements

The authors are grateful to National Major Scientific and Technological Special Project (No. 2009ZX09301-003), and the Fundamental Research Funds for the Central Universities.

Notes and references

[a] *State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, P.R. China. E-mail: wangxiaojian@imm.ac.cn; yindali@imm.ac.cn; Fax: +8610 63165248; Tel: +86 10 63165248.*

[b] *Department of Medicinal Chemistry, Beijing Key Laboratory of Active Substances Discovery and Drugability Evaluation, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, P.R. China. E-mail: wangxiaojian@imm.ac.cn; yindali@imm.ac.cn; Fax: +8610 63165248; Tel: +86 10 63165248.*

† Electronic Supplementary Information (ESI) available: Pharmacological methods; a full list of screened compounds. See DOI: 10.1039/b000000x/ .

‡ These authors contributed equally to this work.

1. N. J. Pyne and S. Pyne, *Nat. Rev. Cancer*, 2010, **10**, 489-503.
2. J. W. Antoon and B. S. Beckman, *Cancer Biol. Ther.*, 2011, **11**, 647-650.
3. N. C. Hait, C. A. Oskeritzian, S. W. Paugh, S. Milstien and S. Spiegel, *Biochim. Biophys. Acta*, 2006, **1758**, 2016-2026.
4. H. Rosen, P. J. Gonzalez-Cabrera, M. G. Sanna and S. Brown, *Annu. Rev. Biochem.*, 2009, **78**, 743-768.
5. S. Spiegel and S. Milstien, *Nat. Rev. Mol. Cell Biol.*, 2003, **4**, 397-407.
6. P. Xia, J. R. Gamble, L. Wang, S. M. Pitson, P. A. B. Moretti, B. W. Wattenberg, R. J. D'Andrea and M. A. Vadas, *Curr. Biol.*, 2010, **10**, 1527-1530.
7. S. M. Pitson, *Trends Biochem. Sci.*, 2011, **36**, 97-107.
8. D. Kapitonov, J. C. Allegood, C. Mitchell, N. C. Hait, J. A. Almenara, J. K. Adams, R. E. Zipkin, P. Dent, T. Kordula, S. Milstien and S. Spiegel, *Cancer Res.*, 2009, **69**, 6915-6923.
9. H. Alshaker, L. Sauer, D. Monteil, S. Ottaviani, S. Srivats, T. Böhler and D. Pchejetski, *Adv. Cancer Res.*, 2013, **117**, 143-200.
10. D. Plano, S. Amin and A. K. Sharma, *J. Med. Chem.*, 2014, **57**, 5509-5524.
11. H. Liu, R. E. Toman, S. K. Goparaju, M. Maceyka, V. E. Nava, H. Sankala, S. G. Payne, M. Bektas, I. Ishii, J. Chun, S. Milstien and S. Spiegel, *J. Biol. Chem.*, 2003, **278**, 40330-40336.
12. N. C. Hait, J. Allegood, M. Maceyka, G. M. Strub, K. B. Harikumar, S. K. Singh, C. Luo, R. Marmorstein, T. Kordula, S. Milstien and S. Spiegel, *Science*, 2009, **325**, 1254-1257.
13. H. A. Neubauer and S. M. Pitson, *FEBS J.*, 2013, **280**, 5317-5336.
14. Y. Yatomi, F. Ruan, T. Megidish, T. Toyokuni, S.-i. Hakomori and Y. Igarashi, *Biochem.*, 1996, **35**, 626-633.
15. D. J. Baek, N. MacRitchie, N. G. Anthony, S. P. Mackay, S. Pyne, N. J. Pyne and R. Bittman, *J. Med. Chem.*, 2013, **56**, 9310-9327.
16. D. J. Baek, N. MacRitchie, N. J. Pyne, S. Pyne and R. Bittman, *Chem. Commun.*, 2013, **49**, 2136-2138.
17. S. W. Paugh, B. S. Paugh, M. Rahmani, D. Kapitonov, J. A. Almenara, T. Kordula, S. Milstien, J. K. Adams, R. E. Zipkin, S. Grant and S. Spiegel, *Blood*, 2008, **112**, 1382-1391.
18. K. J. French, R. S. Schrecengost, B. D. Lee, Y. Zhuang, S. N. Smith, J. L. Eberly, J. K. Yun and C. D. Smith, *Cancer Res.*, 2003, **63**, 5962-5969.
19. A. J. Kennedy, T. P. Mathews, Y. Kharel, S. D. Field, M. L. Moyer, J. E. East, J. D. Houck, K. R. Lynch and T. L. Macdonald, *J. Med. Chem.*, 2011, **54**, 3524-3548.
20. K. Kono, M. Tanaka, Y. Ono, T. Hosoya, T. Ogita and T. Kohama, *J. Antibiot.*, 2001, **54**, 415-420.
21. K. Kono, M. Tanaka, T. Ogita and T. Kohama, *J. Antibiot.*, 2000, **53**, 759-764.
22. K. Kono, M. Tanaka, T. Ogita, T. Hosoya and T. Kohama, *J. Antibiot.*, 2000, **53**, 459-466.
23. K. Kono, M. Tanaka, T. Mizuno, K. Kodama, T. Ogita and T. Kohama, *J. Antibiot.*, 2000, **53**, 753-758.
24. Z. Wang, X. Min, S.-H. Xiao, S. Johnstone, W. Romanow, D. Meininger, H. Xu, J. Liu, J. Dai, S. An, S. Thibault and N. Walker, *Struct.*, 2013, **21**, 798-809.
25. J. J. Irwin and B. K. Shoichet, *J. Chem. Inf. Model.*, 2004, **45**, 177-182.
26. J.-X. Ren, L.-L. Li, R.-L. Zheng, H.-Z. Xie, Z.-X. Cao, S. Feng, Y.-L. Pan, X. Chen, Y.-Q. Wei and S.-Y. Yang, *J. Chem. Inf. Model.*, 2011, **51**, 1364-1375.
27. G. L. Warren, C. W. Andrews, A.-M. Capelli, B. Clarke, J. LaLonde, M. H. Lambert, M. Lindvall, N. Nevins, S. F. Semus, S. Senger, G.

Tedesco, I. D. Wall, J. M. Woolven, C. E. Peishoff and M. S. Head, *J. Med. Chem.*, 2005, **49**, 5912-5931.
28. E. Perola, *Proteins: Structure, Function, and Bioinformatics*, 2006, **64**, 422-435.
29. S.-Y. Yang, *Drug Discov. Today*, 2010, **15**, 444-450.
30. D. J. Gustin, Y. Li, M. L. Brown, X. Min, M. J. Schmitt, M. Wanska, X. Wang, R. Connors, S. Johnstone, M. Cardozo, A. C. Cheng, S. Jeffries, B. Franks, S. Li, S. Shen, M. Wong, H. Wesche, G. Xu, T. J. Carlson, M. Plant, K. Morgenstern, K. Rex, J. Schmitt, A. Coxon, N. Walker, F. Kayser and Z. Wang, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 4608-4616.
31. S. N. Rao, M. S. Head, A. Kulkarni and J. M. LaLonde, *J. Chem. Inf. Model.*, 2007, **47**, 2159-2171.
32. C. M. Venkatachalam, X. Jiang, T. Oldfield and M. Waldman, *J. Mol. Graphics Modell.*, 2003, **21**, 289-307.
33. M. P. A. Sanders, R. McGuire, L. Roumen, I. J. P. de Esch, J. de Vlieg, J. P. G. Klomp and C. de Graaf, *Med. Chem. Comm.*, 2012, **3**, 28-38.
34. M. E. Schnute, M. D. McReynolds, T. Kasten, M. Yates, G. Jerome, J. W. Rains, T. Hall, J. Chrencik, M. Kraus, C. N. Cronin, M. Saabye, M. K. Highkin, R. Broadus, S. Ogawa, K. Cukyne, L. E. Zawadzke, V. Peterkin, K. Iyanar, J. A. Scholten, J. Wendling, H. Fujiwara, O. Nemirovskiy, A. J. Wittwer and M. M. Nagiec, *Biochem. J.*, 2012, **444**, 79-88.
35. S. A. O'Toole, B. L. Sheppard, E. P. J. McGuinness, N. C. Gleeson, M. Yoneda and J. Bonnar, *Cancer Detect. Prev.*, 2003, **27**, 47-54.

Graphic Abstract

Discovery of novel Sphingosine Kinase 1 inhibitors via structure-based hierarchical virtual screening

Xiaojian Wang,* ‡[a,b] Chenbin Sun,‡[a] Liang Fang,[b] and Dali Yin*[a,b]

[a]*State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, P.R. China*

[b]*Department of Medicinal Chemistry, Beijing Key Laboratory of Active Substances Discovery and Drugability Evaluation, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, P.R. China*

E-mail: wangxiaojian@imm.ac.cn; yindali@imm.ac.cn; Fax: +8610 63165248; Tel: +86 10 63165248.

A hierarchical structure-based virtual screening against sphingosine kinase 1(SphK1) binding pocket was performed. 25 compounds from ZINC database were selected for biological evaluation. Compound **25** exhibited comparable SphK1 and SphK2 inhibitory activity and anti-proliferative effects on U937 cells to the positive control *N*, *N*-Dimethylsphingosine (DMS) **1**. Further molecule dynamic (MD) simulation revealed the binding mode between SphK1 and **25**.