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Original article

Identification of small molecule sphingomyelin synthase inhibitors

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ABSTRACT

Sphingomyelin synthase (SMS), which catalyzes ceramide as one of the substrates to produce sphingomyelin, is a critical factor in the sphingolipid biosynthesis pathway. Recent studies indicated that SMS could serve as a novel potential drug target for the treatment of various metabolic diseases such as insulin resistance and atherosclerosis. However, very few small-molecule inhibitors of SMS are known. In this study, we performed structure-based virtual screening in combination with chemical synthesis and bioassay and discovered a class of small-molecule SMS inhibitors. The most potent compound exhibited an IC_{50} value lower than 20 μM in an *in vitro* enzymatic assay. To the best of our knowledge, this is the first time that small-molecule SMS inhibitors with potency close to the micromolar range are publicly revealed. The structure–activity relationship demonstrated by this class of compounds provides insights into the structural features that are essential for effective SMS inhibition.

1. Introduction

Sphingomyelin synthase (SMS) is the last enzyme on the sphingomyelin (SM) biosynthesis pathway. As an integral membrane protein, SMS transfers the phosphorylcholine moiety from phosphatidylcholine onto the primary hydroxyl group on ceramide, producing SM and diglyceride. SMS plays various physiological and pathological roles [1, 2]. Recently, studies on mice indicated that SMS deficiency prevented the development of atherosclerosis [3], obesity [4], and insulin resistance [4], while SMS over-expression significantly increased atherosclerosis [5]. These studies suggest a connection between the activity of SMS and the change in SM level in plasma or plasma membrane [1,5–7]. Thus, SMS may serve as a valid target for developing novel therapies for metabolic diseases. Discovery of small-molecule inhibitors of SMS is a promising strategy for achieving this goal. Mammalian SMS is known to have two isoforms, i.e. SMS1 and SMS2. SM production is catalyzed by SMS1 in the Golgi and SMS2 on plasma membrane [5]. SMS1 and SMS2 share 57% sequence similarity and they have identical enzymatic activities [6]. Three-dimensional structures of SMS have not been resolved experimentally so far.

Before our work, the only publicly known small-molecule SMS inhibitor was tricyclo[5.2.1.0(2,6)]-decan-8-yl dithiocarbonate (D609, Fig. 1). It was originally identified as a cytotoxic anti-tumor and anti-virus reagent [7]. Unfortunately, it is not practical to use D609 as an effective SMS inhibitors due to its high instability ($t_{1/2} \approx 19.5$ min in saline solution at 24 °C) [6, 8] and weak inhibitory activity (*in vitro* $IC_{50} \approx 375$ μM, and cellular $EC_{50} \approx 90$ μM) [9]. Optimizations of D609 led to improved stability [7, 8]. An inhibitor of P-glycoprotein resistance protein-1, namely MS-209, was also referred as a SMS inhibitor, but there was no further specific experimental data to support this view [10, 11, 12]. We have synthesized MS-209, and the result of its SMS inhibitory activity assay (D609 was used as positive control) showed that the IC_{50} value was higher than 500 μM.

It is thus much desired to develop new small-molecule SMS inhibitors with higher potency and better stability. Such compounds may be developed into successful drug candidates or applied as effective chemical tools for probing the biological functions of SMS. In our study, we applied structure-based virtual screening to discover small-molecule compounds targeting at SMS.

Abbreviations: SMS, Sphingomyelin synthase; SM, Sphingomyelin; D609, potassium tricyclo[5.2.1.0(2,6)]-decan-8-yl dithiocarbonate; 3D-*h*SMS1, three-dimensional structure of human SMS1; *i.p.*, intraperitoneally; *i.v.*, intravenously; ICR mice, Institute of Cancer Research mice; HIS, histidine; Huh-7, human hepatoma cell; IC_{50}, 50% inhibiting concentration; CC_{50}, 50% cytotoxicity concentration.

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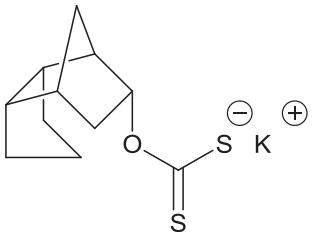


Fig. 1. The chemical structure of D609 (as potassium salt).

2. Results

2.1. Virtual screening

In a previous study [2], we reported a structural model of human SMS1 (*h*SMS1) which was derived basically from homology modeling and refined by molecular dynamics simulations. This structural model provided the structural basis for the virtual screenings conducted in this work.

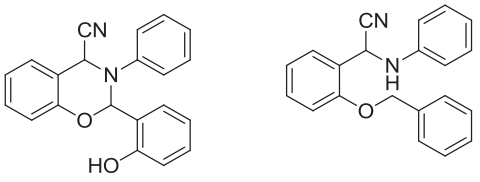
The SPECS library, which contained structural information of over 220,000 compounds, was considered in our study. A number of compounds were selected out of a multi-step structure-based virtual screening process. Samples of 93 compounds were purchased from SPECS and evaluated in SMS inhibitory experiments. Two compounds (**D1** and **D2** in Fig. 2) exhibited obvious inhibitions of SMS activity in our assay.

2.2. Structural modification of hit *D2*

By considering biological potency and synthetic feasibility, we selected **D2** as the lead compound for further structural modification. Three different sites (Ar^1, Ar^2, and X) were explored on the scaffold of **D2** (Table 1): Substituents at the Ar^1 site included phenyl and *o*-, *m*-, *p*-benzyloxy phenyl groups; substituents at the Ar^2 site included nucleophilic, electrophilic and hydrophobic group-substituted aromatic moieties and aromatic heterocyclic moieties; whereas X were hydrogen, cyano, carbamoyl and tetrazole groups. These chemical modifications were expected to explore the structure–activity relationship of this class of compounds. Synthetic methods were developed for preparation of the α-aminonitrile derivatives (Scheme 1). A total of 20 α-aminonitrile derivatives were synthesized (Table 1).

2.3. In vitro SMS activity assay and SAR study

All obtained compounds were then evaluated in a HPLC-based assay for measuring SMS inhibition that was developed in our group [13]. As result, 17 compounds were observed to have higher SMS inhibitory activities than D609. Among them, five compounds exhibited SMS2 inhibition with IC_{50} values lower than 20 μM, which are 10-fold more potent than D609. To the best of our knowledge, these compounds are the first group of SMS inhibitors with potency close to the micromolar range that are publicly revealed.



D1 (IC_{50} =75 μM on SMS2) **D2** (IC_{50} =14 μM on SMS2)

Fig. 2. Chemical structures of two SMS inhibitors which were selected from structure-based virtual screening outcomes and were verified in biological assay.

The structure–activity relationship of this class of compounds indicates clearly that the cyano group in the α-aminonitrile moiety is the key factor for SMS inhibition. The binding mode of **D2**, which was given by molecular docking (Fig. 3), suggests that the cyano group points to the imidazole ring on the side chain of His285 residue, forming a possible favorable dipole–dipole interaction. In addition, a possible hydrogen bond is observed between the amino group connecting the Ar^2 moiety and His274, which seems to be another essential factor for SMS inhibition. The two aromatic branches (Ar^1, Ar^2) reside at two hydrophobic sub-pockets respectively. Our biological test results show that ortho-substituted aryl at the Ar^1 site is better than meta- or para-substituted counterparts. Hydrophobic substituted aromatic moiety in the ortho-position are preferred even more. As compared to **D2**, the derivative compounds listed in Table 1 did not really achieve a higher level of potency. However, they collectively confirmed the SMS inhibitory activities of this class of compounds. These preliminary structure–activity relationships are helpful for developing even more potent SMS inhibitors.

2.4. In vivo SMS activity assay

In order to explore the potential of **D2** as a promising lead compound for drug development, this compound was further tested *in vivo* for SMS inhibition. ICR mice were administrated with **D2** in dose of 6 mg/kg intraperitoneally (*i.p.*) or intravenously (*i.v.*). Our results showed that upon the treatment of **D2**, the total amount of ceramides was significantly increased (Fig. 4-A); while the total amount of SM was significantly decreased in mice plasma (Fig. 4-B). This observation is consistent with the expectation of SMS inhibition, which confirms that **D2** is an effective SMS inhibitor both *in vitro* and *in vivo*.

2.5. Cytotoxicity assay

Toxicity of **D2** was evaluated on Huh7 cells. The CC_{50} of **D2** was 245 μM, which suggested that **D2** has an efficient therapeutic window and further supported that **D2** could be used as a potential molecular tool for SMS bio-function studies.

3. Conclusion

We have discovered a class of small-molecule SMS inhibitors through structure-based virtual screening in combination with chemical synthesis and biological assays. Among them, compounds **D2**, **D24**, **D28**, **D29**, **D30** and **D31** have IC_{50} values lower than 20 μM in an *in vitro* SMS inhibition assay. They could be considered as lead compounds for obtaining even more potent SMS inhibitors and may be eventually developed into new therapies for treating various metabolic diseases.

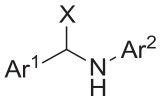
4. Experimental

4.1. Materials

4.1.1. Chemistry

The reagents (chemicals) were obtained from Alfa Aesar, Acros, or Shanghai Chemical Reagent Co. and were used without further purification. Low- and high-resolution mass spectra (LRMS and HRMS) were given with electrospray ionization (ESI) produced by a Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec 4.7 T. Nuclear magnetic resonance (NMR) spectroscopy was performed on Bruker AMX-500 and AMX-400 (tetramethylsilane (TMS) was used

Table 1
Biological Activity of the synthetic hits and hit modification.



Compound	X	Ar^1	Ar^2	IC_{50} (μM) (SMS2)[a,b]	IC_{50} (μM) (SMS)[a,b]
D2	Cyano-	2-Benzyloxyphenyl-	Phenyl-	13.5 ± 0.7	24.5 ± 2.4
D21	Cyano-	3-Benzyloxyphenyl-	Phenyl-	50.7 ± 5.2	ND[d]
D22	Cyano-	4-Benzyloxyphenyl-	Phenyl-	NA[c]	ND[d]
D23	Cyano-	Phenyl-	Phenyl-	NA[c]	ND[d]
D24	Cyano-	2-Benzyloxyphenyl-	2-Methoxyphenyl-	12.4 ± 1.6	35.8 ± 2.4
D25	Cyano-	2-Benzyloxyphenyl-	3-Methoxyphenyl-	NA[c]	ND[d]
D26	Cyano-	2-Benzyloxyphenyl-	4-Methoxyphenyl-	48.1 ± 4.3	ND[d]
D27	Cyano-	2-Benzyloxyphenyl-	2-Methyl-phenyl-	67.2 ± 9.0	ND[d]
D28	Cyano-	2-Benzyloxyphenyl-	4-Methyl-phenyl-	20.8 ± 3.4	ND[d]
D29	Cyano-	2-Benzyloxyphenyl-	2-Clorophenyl-	15.8 ± 2.6	90.8 ± 0.2
D30	Cyano-	2-Benzyloxyphenyl-	4-Clorophenyl-	22.5 ± 4.3	93.9 ± 0.2
D31	Cyano-	2-Benzyloxyphenyl-	2-Hydroxyphenyl-	19.0 ± 4.2	ND[d]
D32	Cyano-	2-Benzyloxyphenyl-	4-Hydroxyphenyl-	24.4 ± 1.6	ND[d]
D33	Cyano-	2-Benzyloxyphenyl-	2-Cyano-phenyl-	20.3 ± 0.6	ND[d]
D34	Cyano-	2-Benzyloxyphenyl-	4-Carbamoyl-phenyl-	80.5 ± 4.5	196.3 ± 3.6
D35	Cyano-	2-Benzyloxyphenyl-	2,6-Dimethyl-phenyl-	31.5 ± 8.4	ND[d]
D36	Cyano-	2-Benzyloxyphenyl-	2-Nitro-phenyl-	27.4 ± 1.6	ND[d]
D37	Cyano-	2-Benzyloxyphenyl-	3-Pyridyl-	35.0 ± 5.1	ND[d]
D38	Cyano-	2-Benzyloxyphenyl-	Furan-2-ylmethyl-	71.0 ± 5.4	ND[d]
D39	Hydrogen-	2-Benzyloxyphenyl-	Phenyl-	NA[c]	ND[d]
D40	Carbamoyl-	2-Benzyloxyphenyl-	Phenyl-	135.3 ± 4.9	ND[d]
D41	1*H*-tetrazol-5-yl-	2-Benzyloxyphenyl-	Phenyl-	NA[c]	ND[d]

[a] The IC_{50} of D609 as a positive control compound was 224 μM in SMS2 and 402 μM in SMS.
[b] The enzyme source of SMS was from ICR mice liver homogenate, and the enzyme source of SMS2 was from over-expression SMS2 cell lysis. IC_{50} value measurements using the SMS inhibition assay (as shown in experimental). Five concentrations of inhibitor were used to determine the IC_{50} values. The shown values were the means from triplicate assays. Statistical analysis were performed using Prism 5.02 (GraphPad Software, Inc.) and "Log[inhibitor] vs. response" was used as curve adjustment model for IC_{50} measurement. Values are means ± SD.
[c] NA: no activity at 100 μM.
[d] ND: not determined.

as the internal standard). Chemical shifts were reported in δ (ppm) downfield from TMS. Solvent residual peak, and peak multiplicity were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). The yield was not optimized.

4.1.2. Biochemistry

6-((*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)hexanoyl)-sphingosine (C6-NBD-Cer) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were obtained from Santa Cruz (USA). *N-(N-(7-nitro-2, 1, 3-benzoxadiazol-4-yl)-epsilon-aminohexanoyl) sphingosylphosphoryl choline* (C6-NBD-SM) was purchased from Sigma–Aldrich (USA). Potassium tricyclo [5.2.1.0(2, 6)]-decan-8-yl-dithiocarbonate (D609) was obtained from 3B Scientific Corporation (China). Laboratory-prepared water (Milli-Q) was used throughout the study. All other reagents were analytical grade available.

ICR (Institute of Cancer Research) mice were provided by Sino-British S1PPR/BK Lab. Animal Ltd. All experimental procedures were approved by the Institutional Review Board of Fudan University.

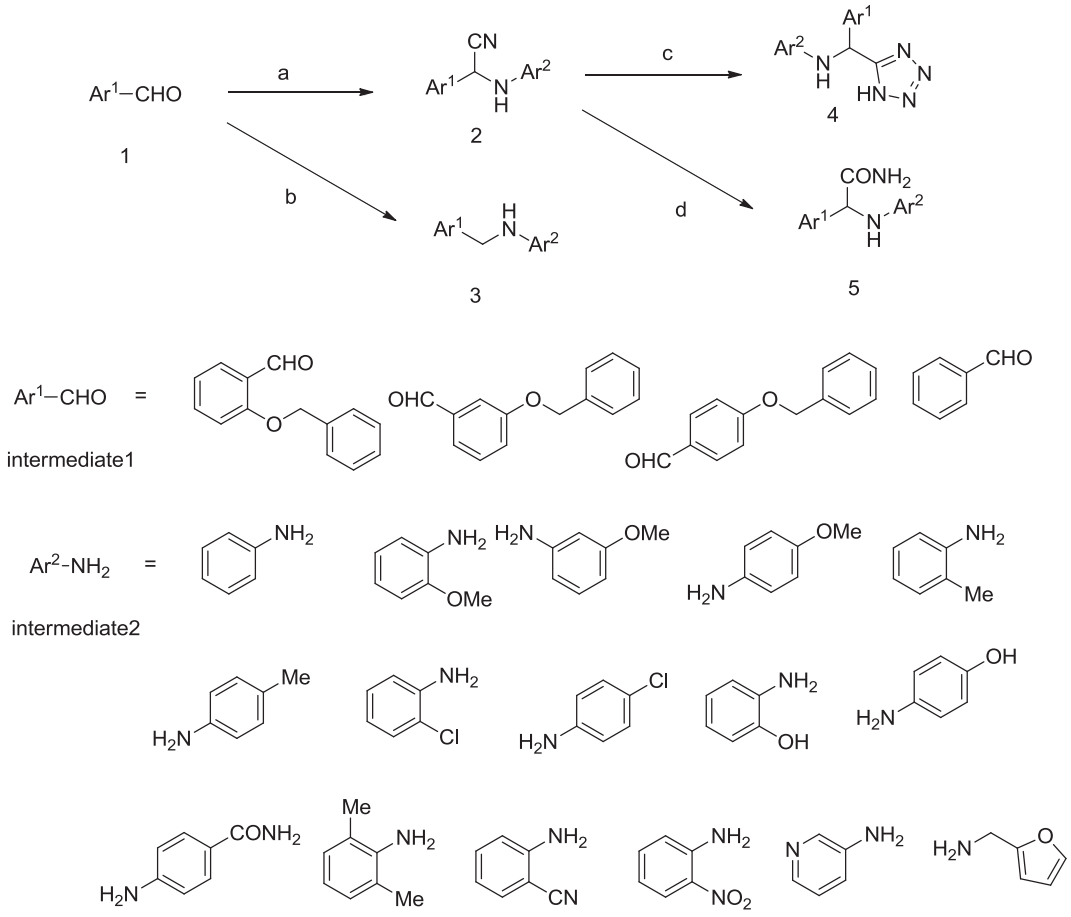
SMS2 was kindly provided by Dr. Yanhui Xu's lab, Institute of Bioscience, Fudan University. The enzyme was highly expressed in H5 insect cell. The cell pellet was weighed and then homogenized by Glass homogenizer with homogenate buffer 1 (0.25 M sucrose, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA) (50 mg/mL, weight of cell pellet/volume of buffer 1). The homogenates were subjected to centrifugation at 12,000 rpm for 10 min. The supernatant, as a raw SMS2 solution, was stored at −20 °C, or used directly.

An Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) HPLC system was used for our experiments, attached with a quaternary pump, a vacuum degasser and a FLD detector. The chromatographic separations were carried out on an Agilent C18 RP column (250 mm × 4.6 mm, 5 μm).

Buffer 1 for storage of SMS enzyme resource: 0.25 M sucrose, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA.

4.2. Virtual screening of the SPECS Library

A multi-step procedure was employed in our virtual screening to discover potential small-molecule inhibitors of *h*SMS1. Firstly, the entire SPECS library (about 220,000 compounds) was filtered by drug-likeness rules, which required molecular weight lower than 1000 Da, total number of hydrogen bond acceptors and donors between 2 and 10, number of non-hydrogen atoms between 9 and 50, and no more than 10 rotatable bonds. This step selected out approximately 80,000 compounds. Then, qualified molecules were docked into the active site of hSMS1 using the molecular docking program GOLD (version 3.2, released by CCDC). The structural model of *h*SMS1 was obtained through homology modeling, which was reported in one of our previous studies [2]. Key parameters were set as follows: Island number = 5; populations' size on each island = 100; total genetic algorithm operations = 30,000; mutation rate = 95%; crossover rate = 95%; migration rate = 10%; scoring function = ChemScore. Up to twenty binding poses were generated for each docked molecule. After this docking step, 20,000 molecules with the highest binding scores were selected out. These molecules were further screened with the Glide docking program implemented in the Schrödinger software (version 2008, released by the Schröodinger Inc.) at the "extra-precision" (XP) mode. The *h*SMS1 binding site was depicted by three key residues, i.e. H285, H328 and D332, in the catalytic site [2]. Default parameters were employed in molecular docking. The *h*SMS1 structure was kept fixed during this process. Then, the top-ranked 4000 molecules



Scheme 1. General synthesis route for SMS inhibitors. Reagents and conditions: a, TMSCN, I_2, CH_3CN, rt; b, $NaBH_4$, MeOH, rt; c, NaN_3, ethanol, reflux; d, K_2CO_3, H_2O_2, CH_2Cl_2, rt.

were subjected to similarity analysis to remove structurally redundant ones. Here, similarity matrix based on molecular fingerprint ECFP4 was generated by Pipeline Plot (released by Accelrys Inc.). A total of 500 clusters were obtained by using the Matlab software (released by MathWorks Inc.). The compound with the highest Glide binding score was selected from each cluster. These 500 compounds were inspected visually to examine their binding modes and likelihood of chemical modification. Finally, a total of 93 compounds were selected as candidates. The samples of these compounds were ordered from SPECS Inc. for subsequent biological evaluation.

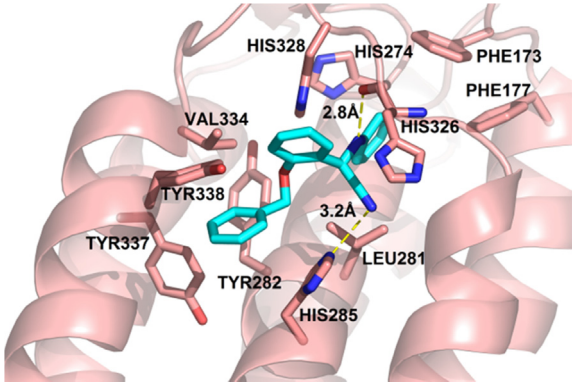


Fig. 3. Binding mode of **D2** at the active site of *h*SMS1 as predicted by molecular docking.

93 selected compounds were screened by the same method as shown in followed section 4.3.1, which ICR mice liver homogenate was used as enzyme source. 100 μM of compound was used as threshold concentration for initial screening. Compound **D1** and **D2** were discovered higher than 50% inhibitory under 100 μM concentration. So **D1** and **D2** were further determined to afford their IC_{50}. The result showed IC_{50} of **D1** and **D2** were 75 μM and 14 μM respectively (Fig. 2). And **D2** were selected as lead for further optimization.

4.3. Assessment of SMS inhibitory activities

4.3.1. SMS activity in vitro

The method of SMS activity was described by the previous report [13]. Briefly, ICR mice were sacrificed and livers were dissected, weighed and then homogenized with homogenate buffer 1 (0.25 M sucrose, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA) (300 mg/mL, weight of livers/volume of buffer 1). The homogenates were subjected to centrifugation at 12,000 rpm for 10 min. The supernatant, as a raw SMS solution, was stored at −20 °C, or used directly.

Inhibitory activity of SMS was assessed using a fluorescent substrate C6-NBD-Ceramide; λex/em = 475/525 nm. The assays were performed in Eppendorf tubes using 300 μL total reaction volumes. The samples were buffered in 10 mM HEPES (pH 7.4), 3 mM $MnCl_2$ and 0.3% BSA, SMS (equivalent to 2 mg total protein) was incubated for 30 min at 37 °C in the presence of inhibitor compounds and in the absence of substrates. The enzymatic reaction was started by the addition of 1.16 mM of C6-NBD-Cer and

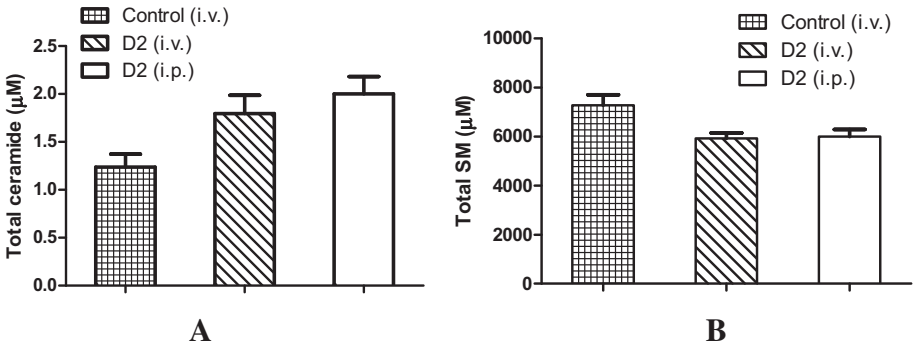


Fig. 4. The effect of **D2** *in vivo* on plasma ceramide (A) and SM (B). Mice as negative control were administered no **D2**. **D2** were administered *i.v.* or *i.p.* in the test set of mice in dose of 6 mg/kg. A: The amount of total ceramide in plasma of ICR mice were increased significantly in *i.v.* or *i.p.* groups. B: The amount of total SM in plasma of ICR mice were decreased significantly in *i.v.* or *i.p.* groups. Value are mean ± SD, $n = 5$. Student's *t*-test $P < 0.05$.

40 mM DMPC in 3 μL DMSO. The mixtures were incubated at 37 °C for an additional 2 h. The enzyme catalytic reactions were quenched by 600 μL of ethanol. After vortexing for 30 s, the reaction mixture was centrifuged and the supernatant was used for HPLC analysis. Extracts (20 μL) from the supernatant were analyzed for the formation of C6-NBD-SM by HPLC using the reversed-phase C18 column and an isocratic elution with methanol/water/trifluoroacetic acid (88:12:0.1 (v/v)). The flow rate of the mobile phase was 1.0 mL/min. Detection was accomplished with a fluorescence spectrophotometer ($\lambda_{excitation} = 475$ nm, $\lambda_{emission} = 525$ nm). Assays were performed at least in triplicate. Statistical analysis and nonlinear regression were performed using Prism 5.02 (GraphPad Software, Inc.).

4.3.2. SMS2 activity in vitro

Briefly, 300 μL of reaction mixture, which contained the samples buffered in 10 mM HEPES (pH 7.4), 3 mM $MnCl_2$, 0.3% BSA and SMS2 (equivalent to 2 μg total protein of cell homogenates of overexpressing SMS2 in buffer 1), was incubated for 30 min at 37 °C in the presence of inhibitor compounds and in the absence of substrates. The enzymatic reaction was started by the addition of 1.01 mM of C6-NBD-Cer and 40 mM DMPC in 3 μL DMSO. The mixtures were incubated at 37 °C for an additional 1 h. The resulting mixture was worked up and analyzed by the same procedure of SMS activity assay described above.

4.4. SMS activity in vivo

ICR mice (approximately 25 g) were maintained in restricted-access rooms with a controlled temperature (23 °C) and a 12 h light–dark cycle and were allowed free access to standard laboratory diet and tap water. All experimental procedures were approved by the Institutional Review Board of Fudan University.

Fifteen female ICR mice, which averagely weighed 25 g (25 ± 1 g), were randomly divided into 3 groups. Among them, 1–5 as control group were administered intravenously (mixture of 15 μL DMSO and 185 μL phosphate-buffer saline (PBS; pH 7.4)); 6–10 were administered intravenously (mixture of 150 μg in 15 μL DMSO and 185 μL PBS), 11–15 were administered intraperitoneally (mixture of 150 μg in 15 μL DMSO and 185 μL PBS). The drug dose of **D2** was equivalent to 6 mg/kg for each mouse. After 2 h absences or presence of drug treatment, blood was sampled from mice eyes and was directly frozen storage by liquid nitrogen for analysis. Plasma ceramide and sphingomyelin were measured as previously described via mass spectrometry (MS) [14, 15]. Differences between groups were tested by Student's *t*-test. Data are presented as mean ± S.D.

4.5. Toxicity of *D2* on Huh-7 cells

Huh-7 cells were incubated with **D2** in various concentrations for 17 h and then examined for cell viability by MTT assay. CC_{50} is the concentration of **D2** that causes 50% inhibition compared to the vehicle control (0.25% DMSO). Samples were run in quintuplicate, and the results are the mean of at least two independent tests.

4.6. Chemical synthesis

4.6.1. General procedure

The key transformation for synthesis of the α-aminonitrile derivatives was Strecker reaction[16, 17]. The synthetic route for the preparation of compounds **D2**, **D21**–**D38** was depicted in Scheme 1. The substituted benzaldehyde was treated with the substituted arylamine in CH_3CN solution under room temperature, followed with trimethylsilyl cyanide (TMSCN) and catalytic amount of I_2. The mixture was stirred at room temperature overnight to get the desired α-aminonitrile derivatives. The typical reaction procedure for preparation of α-aminonitrile derivatives was shown in synthesis detail of compound **D2**. Compound **D2** can be easily converted to compound **D41** by treating with NaN_3/ethanol [18], or to **D40** by potassium carbonate and hydrogen peroxide in water [19]. The de-cyano compound **D39** was obtained by following the reductive amination condition [20].

*4.6.1.1. General procedure for the preparation of 2-(2-(benzyloxy) phenyl)-2-(phenylamino) acetonitrile (**D2**).* A solution of 2-(benzyloxy)benzaldehyde (1.00 g, 4.71 mmol) and aniline (438.77 mg, 4.71 mmol) in acetonitrile (20 mL) was stirred at rt for 30 min. Then TMSCN (701.13 mg, 7.07 mmol) and I_2 (119.58 mg, 0.47 mmol) were added and the mixture was stirred overnight. The solvent was evaporated in vacuo and the residual was diluted with ethyl acetate/water. The organic layer was washed with saturated brine, dried over $MgSO_4$ and concentrated under vacuum. The residual was triturated with ethyl acetate/hexane (1/6), filtered to give the product, 1.35 g, 91% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.28 (br, 1H, NH), 5.15–5.21 (dd, J_1 = 11.78 Hz, J_2 = 15.4 Hz, 2H, OCH_2Ar), 5.62 (s, 1H, CH(CN)N), 6.72–6.73 (d, J = 7.79 Hz, 2H, H_{Ar}), 6.85–6.88 (m, 1H, H_{Ar}), 7.02–7.05 (m, 2H, H_{Ar}), 7.21–7.25 (m, 2H, H_{Ar}), 7.32–7.52 (m, 6H, H_{Ar}), 7.53–7.54 (d, J = 7.83 Hz, 1H, H_{Ar}). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 46.32, 70.59, 112.75, 114.48, 120.12, 121.48, 127.35, 128.24, 128.74, 128.89, 129.47, 130.99, 136.08, 155.94. ESI-MS *m/z* 315.2 ($M+H^+$). HRMS (ESI) m/z calcd for $M+Na^+$ 337.1311; found 337.1319. IR: 2227.59 cm^{-1} (CN).

*4.6.1.2. 2-(3-(benzyloxy) phenyl)-2-(phenylamino) acetonitrile (**D21**).* This compound was prepared by using the procedure as described for **D2**. Yield 219 mg (74%).

^{1}H NMR (400 MHz,$CDCl_3$): δ = 5.10 (s, 2H, OCH_2Ar), 5.41 (s, 1H, CH(CN)N), 6.77–6.79 (d, J = 7.8 Hz, 2H, H_{Ar}), 6.90–6.94 (m, 1H, H_{Ar}), 7.03–7.06 (m, 1H, H_{Ar}), 7.20–7.23 (m, 2H, H_{Ar}), 7.29–7.45 (m, 8H, H_{Ar}). ESI-MS *m/z* 315.2 ($M+H^+$). HRMS (ESI) m/z calcd for $M+H^+$ 315.1492; found 315.1490.

*4.6.1.3. 2-(4-(benzyloxy)phenyl)-2-(phenylamino) acetonitrile (**D22**).* This compound was prepared by using the procedure as described for **D2**. Yield 243 mg (82%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.10 (s, 2H, OCH_2Ar), 5.36 (s, 1H, CH(CN)N), 6.78 (d, J = 7.8 Hz, 2H, H_{Ar}), 6.89–6.92 (m, 1H, H_{Ar}), 7.02-7.05 (m, 2H, H_{Ar}), 7.28–7.35 (m, 2H, H_{Ar}), 7.36–7.46 (m, 5H, H_{Ar}), 7.49–7.52 (m, 2H, H_{Ar}). ESI-MS *m/z* 315.1 ($M+H^+$). HRMS (ESI) m/z calcd for $M+Na^+$ 337.1311; found 337.1318.

*4.6.1.4. 2-phenyl-2-(phenylamino) acetonitrile (**D23**).* This compound was prepared by using the procedure as described for **D2**. Yield 349 mg (89%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.03 (s, 1H, NH), 5.43 (s, 1H, CH(CN)N), 6.77–6.79 (d, J = 10 Hz, 2H, H_{Ar}), 6.89–6.92 (m, 1H, H_{Ar}), 7.25–7.29 (m, 2H, H_{Ar}), 7.42–7.48 (m, 3H, H_{Ar}), 7.60–7.61 (d, J = 5 Hz, 2H, H_{Ar}). ^{13}C NMR (500 MHz, CDCl3): δ = 50.26, 114.32, 118.16, 120.31, 127.25, 129.34, 129.54, 129.57, 133.98, 144.68. ESI-MS *m/z* 209.1 ($M+H^+$). HRMS (ESI) m/z calcd for $M+Na^+$ 231.0893; found 231.0896.

*4.6.1.5. 2-(2-(benzyloxy)phenyl)-2-((2-methoxyphenyl) amino) acetonitrile (**D24**).* This compound was prepared by using the procedure as described for **D2**. Yield 250 mg (77%). ^{1}H NMR (400 MHz, CDCl3): δ = 3.77 (s, 3H, CH3O), 5.21 (s, 2H, OCH2Ar), 5.69 (s, 1H, CH(CN)N), 6.96–6.80 (m, 4H, H_{Ar}), 7.06 (t, J = 7.8 Hz, 2H) , 7.48–7.31 (m, 6H, H_{Ar}), 7.58 (dd, J = 7.4, 1.5 Hz, 1H, H_{Ar}). ESI-MS *m/z* 345.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 345.1598; found 345.1597.

*4.6.1.6. 2-(2-(benzyloxy)phenyl)-2-((3-methoxyphenyl) amino) acetonitrile (**D25**).* This compound was prepared by using the procedure as described for **D2**. Yield 243 mg (75%). ^{1}H NMR (400 MHz, acetone-d6): δ = 3.73 (s, 3H, CH_3O) 5.15 (s, 2H, OCH_2Ar), 5.83–5.85 (d, J = 9.2 Hz, 1H, CH(CN)N), 6.37–6.39 (m, 1H, H_{Ar}), 6.49–6.51 (m, 2H, H_{Ar}), 7.07–7.50 (m, 10H, H_{Ar}). ESI-MS *m/z* 345.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 345.1598; found 345.1594.

*4.6.1.7. 2-(2-(benzyloxy)phenyl)-2-((4 methoxyphenyl) amino) acetonitrile (**D26**).* This compound was prepared by using the procedure as described for **D2**. Yield 263 mg (81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.77 (s, 3H, CH_3O), 5.18–5.19 (d, J = 3.5 Hz, 2H, OCH_2Ar), 5.50 (s, 1H, CH(CN)N), 6.70–6.82 (m, 4H, H_{Ar}), 7.02–7.06 (m, 2H, H_{Ar}), 7.34-7.52 (m, 7H, H_{Ar}). ESI-MS: *m/z* 345.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 345.1598; found 345.1599.

*4.6.1.8. 2-(2-(benzyloxy)phenyl)-2-(o-tolylamino)acetonitrile (**D27**).* This compound was prepared by using the procedure as described for **D2**. Yield 260 mg (84%).. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.98 (s, 3H, CH_3), 5.15–5.22 (m, 2H, OCH_2Ar), 5.58 (s, s, 1H, CH(CN)N), 6.78–6.81 (m, 2H, H_{Ar}), 7.02–7.07 (m, 3H, H_{Ar}), 7.14–7.18 (m, 1H, H_{Ar}), 7.31–7.40 (m, 7H, H_{Ar}). ESI-MS: *m/z* 329.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 329.1648; found 329.1651.

*4.6.1.9. 2-(2-(benzyloxy) phenyl)-2-(p-tolylamino)acetonitrile (**D28**).* This compound was prepared by using the procedure as described for **D2**. Yield 251 mg (81%). ^{1}H NMR (400 MHz,$CDCl_3$): δ = 2.28 (s, 3H, CH_3), 5.09 (s, 2H, OCH_2Ar), 5.37 (s, 1H, CH(CN)N), 6.68–6.70 (d, J = 8 Hz, 2H, H_{Ar}), 7.02–7.04 (dd, J_1 = 8.2 Hz, J_2 = 2.4 Hz, 1H, H_{Ar}), 7.07–7.09 (d, J = 7.8 Hz, 2H, H_{Ar}), 7.19–7.22 (m, 2H, H_{Ar}), 7.34–7.45 (m, 6H, H_{Ar}). ESI-MS: *m/z* 329.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 329.1648; found 329.1643.

*4.6.1.10. 2-(2-(benzyloxy)phenyl)-2-((2-chlorophenyl) amino)acetonitrile (**D29**).* This compound was prepared by using the procedure as described for **D2**. Yield 240 mg (73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.74 (br, 1H, NH), 5.10 (s, 2H, OCH_2Ar), 5.45 (s, 1H, CH(CN)N), 6.82–6.87(m, 2H, H_{Ar}), 7.05–7.07(m, 1H, H_{Ar}), 7.20–7.23 (m, 3H, H_{Ar}), 7.33–7.45 (m, 7H, H_{Ar}). ESI-MS *m/z* 349.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 371.0922; found 371.0934.

*4.6.1.11. 2-(2-(benzyloxy)phenyl)-2-((4-chlorophenyl) amino) acetonitrile (**D30**).* This compound was prepared by using the procedure as described for **D2**. Yield 250 mg (76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.14–5.17 (m, 2H, OCH_2Ar), 5.56 (s, 1H, CH(CN)N), 6.62–6.64 (d, J = 8.8 Hz, 2H, H_{Ar}), 7.03–7.06 (m, 2H, H_{Ar}), 7.16–7.18 (d, J = 8.8 Hz, 2H, H_{Ar}), 7.34–7.42 (m, 6H, H_{Ar}), 7.50–7.52 (m, 1H, H_{Ar}). ^{13}C NMR (400 MHz, $CDCl_3$): 46.50, 70.65, 112.77, 115.65, 118.29, 121.52, 122.47, 124.92, 127.39, 128.34, 128.76, 128.84, 129.36, 131.16, 135.95, 143.67, 155.91. ESI-MS: *m/z* 349.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 371.0922; found 371.0910.

*4.6.1.12. 2-(2-(benzyloxy) phenyl)-2-((2-hydroxyphenyl) amino) acetonitrile (**D31**).* This compound was prepared by using the procedure as described for **D2**. Yield 224 mg (72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.50 (s, 1H), 5.24 (s, 2H, OCH_2Ar), 5.47 (s, d, CH(CN)N), 6.81–7.07 (m, 6H, H_{Ar}), 7.34–7.57 (m, 7H, H_{Ar}). ESI-MS *m/z* 331.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 353.1260; found 353.1254.

*4.6.1.13. 2-(2-(benzyloxy) phenyl)-2-((4-hydroxyphenyl)amino) acetonitrile (**D32**).* This compound was prepared by using the procedure as described for **D2**. Yield 240 mg (77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.09 (s, 2H, OCH_2Ar), 5.30 (s, 1H, CH(CN)N), 6.68–6.79 (m, 4H, H_{Ar}), 7.01–7.04 (m, 1H, H_{Ar}), 7.18–7.22 (m, 2H, H_{Ar}), 7.32–7.45 (m, 6H, H_{Ar}). ESI-MS: *m/z* 331.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 353.1260; found 353.1260.

*4.6.1.14. 2-(((2-(benzyloxy)phenyl)(cyano)methyl)amino)benzonitrile (**D33**).* This compound was prepared by using the procedure as described for **D2**. Yield 243 mg (76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.12-5.14 (d, J = 7.8 Hz, NH), 5.20 (s, 2H, OCH_2Ar), 5.70–5.72 (d, J = 7.8 Hz, CH(CN)N), 6.86–7.10 (m, 4H, H_{Ar}), 7.31–7.58 (m, 9H, H_{Ar}). ESI-MS *m/z* 340.1 ($M+H^+$), 362.0 ($M+Na^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 362.1264; found 362.1264.

*4.6.1.15. 2-(2-(benzyloxy) phenyl)-2-((2, 6-dimethylphenyl) amino) acetonitrile (**D35**).* This compound was prepared by using the procedure as described for **D2**. Yield 232 mg (72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.35 (s, 6H, 2× CH_3) 5.10 (s, 1H, CH(CN)N), 5.11 (s, 2H, OCH_2Ar), 6.96–7.09 (m, 4H, H_{Ar}), 7.24–7.29 (m, 2H, H_{Ar}), 7.35–7.47 (m, 6H, H_{Ar}). ESI-MS: *m/z* 343.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 343.1805; found 343.1810.

*4.6.1.16. 2-(2-(benzyloxy)phenyl)-2-((2-nitrophenyl) amino) acetonitrile (**D36**).* This compound was prepared by using the procedure as described for **D2**. Yield 274 mg (81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.10 (s, 2H, OCH_2Ar), 5.55–5.57 (d, J = 6.8 Hz, 1H, CH(CN)N), 6.89–6.93 (m, 1H, H_{Ar}), 6.99–7.01 (d, J = 8.6 Hz, 1H, H_{Ar}), 7.06–7.08 (m, 1H, H_{Ar}), 7.18–7.20 (m, 2H, H_{Ar}), 7.34–7.44 (m, 5H, H_{Ar}), 7.56–7.60 (m, 1H, H_{Ar}), 8.22–8.27 (m, 2H, H_{Ar}). ESI-MS: *m/z* 360.3

($M+H^+$), 382.1 ($M+Na^+$). HRMS (ESI) *m/z* calcd for $M+Na^+$ 382.1162; found 382.1153.

*4.6.1.17. 2-(2-(benzyloxy) phenyl)-2-(pyridin-3-ylamino) acetonitrile (**D37**).* This compound was prepared by using the procedure as described for **D2**. Yield 217 mg (73%). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.54–4.56$ (d, J = 9 Hz, 1H, NH), 5.17 (s, 2H, OCH_2Ar), 5.59–5.61 (d, J = 8.6 Hz, CH(CN)N), 7.01–7.16 (m, 4H, HAr), 7.33–7.53 (m, 7H, H_{Ar}), 8.08–8.12 (m, 2H, H_{Ar}). ESI-MS *m/z* 316.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 316.1444; found 316.1443.

*4.6.1.18. 2-(2-(benzyloxy)phenyl)-2-((furan-2-ylmethyl) amino) acetonitrile (**D38**).* This compound was prepared by using the procedure as described for **D2**. Yield 234 mg (78%). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.90–4.02$ (m, 2H, OCH_2Ar), 4.93 (s, 1H, CH(CN)N), 5.10–5.18 (m, 2H, NCH_2Furan), 6.22–6.28 (m, 2H, H_{Ar}), 6.97–7.02 (m, 2H, H_{Ar}), 7.31–7.44 (m, 8H, H_{Ar}). ESI-MS *m/z* 319.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 319.1441; found 319.1439.

*4.6.1.19. N-(2-(benzyloxy)benzyl) aniline (**D39**).* A solution of 2-(benzyloxy)benzaldehyde (200.00 mg, 0.942 mmol) and aniline (87.75 mg, 0.942 mmol) in methanol (5 mL) was stirred at rt for 2 h. $NaBH_4$ (178.25 mg, 4.71 mmol) was added to the reaction mixture by portionwise, meanwhile, acetic acid was used for adjusting pH 5–6. After 2 h, the solvent was evaporated in vacuo; the residue was diluted with ethyl acetate/water. The organic layer was washed with saturated brine, dried over anhydrous $MgSO_4$ and concentrated under vacuum. The residual was triturated with ethyl acetate/hexane (1/5), filtered to give the required product, 84 mg, 31% yield. ^1H NMR (400 MHz, DMSO-d6): $\delta = 4.64$ (s, 2H, OCH_2Ar), 5.17 (s, 2H, NCH_2Ar), 6.51–6.57 (m, 2H, H_{Ar}), 6.85–6.89 (m, 1H, H_{Ar}), 7.03–7.46 (m, 11H, H_{Ar}). ESI-MS *m/z* 290.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 290.1539; found 290.1545.

*4.6.1.20. 2-(2-(benzyloxy)phenyl)-2-(phenylamino) acetamide (**D40**).* To a suspension of **D2** (200 mg, 0.636 mmol), potassium carbonate (264 mg, 1.91 mmol) in water (5 mL), 30% hydrogen peroxide (36 μL, 0.318 mmol) was added dropwise at 0 °C. The reaction mixture was warmed to rt and stirred continuously for 2 h. The precipitate was filtered and washed with water, dried under vacuum; the crude product was recrystallized with ethyl acetate/hexane to give the required product, 91 mg, 43% yield. ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.23–5.30$ (m, 4H, OCH_2Ar and NH_2CO), 5.40 (s, 1H, CH(CN)N), 6.49–6.51(d, J = 8 Hz, 2H, H_{Ar}), 6.64 (s, 1H, NH(CH)(CN)), 6.68–6.72 (m, 1H, H_{Ar}), 6.98–7.01 (m, 1H, H_{Ar}), 7.10–7.14(m, 3H, H_{Ar}), 7.28–7.33 (m, 1H, H_{Ar}), 7.37–7.54 (m, 6H, H_{Ar}). ESI-MS *m/z* 333.2 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 333.1598; found 333.1602.

*4.6.1.21. N-((2-(benzyloxy)phenyl) (1H-tetrazol-5-yl) methyl) aniline (**D41**).* A solution of **D2** (200 mg, 0.636 mmol) and sodium azide (207 mg, 3.18 mmol) in ethanol (5 mL) was stirred and refluxed for 2 h. The solvent was evaporated in reduced pressure; the residue was triturated, washed with water, filtered, and dried under vacuum. The crude product was recrystallized with ethyl acetate/hexane to give the required product, 61 mg, 27% yield. ^1H NMR (400 MHz, DMSO-d6): $\delta = 5.10–5.18$ (m, 2H, OCH_2Ar), 6.29 (s, 1H, PhCHN), 6.55–6.59 (m, 3H, H_{Ar}), 6.92–7.38 (m, 10H, H_{Ar}). ESI-MS *m/z* 358.1 ($M+H^+$). HRMS (ESI) *m/z* calcd for $M+H^+$ 358.1662; found 358.1659.

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