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Research paper

Discovery and characterization of selective human sphingomyelin synthase 2 inhibitors

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ABSTRACT

Sphingomyelin synthase (SMS) is a membrane enzyme that catalyzes the synthesis of sphingomyelin, is required for the maintenance of plasma membrane microdomain fluidity, and has two isoforms: SMS1 and SMS2. Although these isoforms exhibit the same SMS activity, they are different enzymes with distinguishable subcellular localizations. It was reported that SMS2 KO mice displayed lower inflammatory responses and anti-atherosclerotic effects, suggesting that inhibition of SMS2 would be a potential therapeutic approach for controlling inflammatory responses and atherosclerosis.

This study aimed to discover a novel small-molecule compound that selectively inhibits SMS2 enzymatic activity. We developed a human SMS2 enzyme assay with a high-throughput mass spectrometry-based screening system. We characterized the enzymatic properties of SMS2 and established a high-throughput screening-compatible assay condition. To identify human SMS2 inhibitors, we conducted compound screening using the enzyme assay. We identified a 2-quinolone derivative as a SMS2 selective inhibitor with an IC_{50} of 950 nM and >100-fold selectivity for SMS2 over SMS1. The 2-quinolone exhibited efficacy in a cell-based engagement assay. We demonstrated that a more potent derivative directly bound to SMS2-expressing membrane fractions in an affinity selection mass spectrometry assay. Mutational analyses revealed that the interaction of the inhibitor with SMS2 required the presence of the amino acids S227 and H229, which are located in the catalytic domain of SMS2.

In conclusion, we discovered novel SMS2-selective inhibitors. 2-Quinolone SMS2 inhibitors are considered applicable for leading optimization studies. Further investigations using these SMS2 inhibitors would provide validation tools for SMS2-relevant pathways *in vitro* and *in vivo*.

1. Introduction

Sphingomyelin synthase 2 (SMS2) is a membrane protein that synthesizes sphingomyelin (SM) using phosphatidylcholine as a phosphorylcholine moiety donor and ceramide as an acceptor [1]. It was demonstrated that elevated plasma SM levels represent a risk factor for coronary artery diseases [2]. Therefore, reducing SM levels is considered a promising approach for pharmacological intervention in atherosclerosis. Growing evidences have illustrated the important roles of SMS2 in the regulation of SM levels [3]. Peritoneal macrophages from SMS2 knockout (KO) mice displayed increased cholesterol efflux [4]. SMS2 and apolipoprotein E double-KO mice exhibited reduced atherosclerosis accompanied by increased cholesterol efflux and decreased levels of plasma inflammatory cytokines [5]. In addition, SMS2 is associated with inflammatory responses and metabolic diseases. SMS2 deficiency attenuated NF-κB activation responses to inflammatory/immunologic stimuli, suggesting a pro-inflammatory role of SMS2 in pro-atherogenic processes [6]. SMS2 deficiency also protected mice against high-fat diet-induced obesity and insulin resistance [7]. These results suggested that inhibition of SMS2 ameliorates inflammatory responses and metabolic diseases and prevents the

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development of atherosclerosis.

SMS2 is the terminal enzyme of SM biosynthesis. Previously, the *de novo* sphingolipid biosynthesis pathway was targeted to inhibit sphingolipid biosynthesis for the development of anti-atherosclerotic drugs. Serine palmitoyltransferase (SPT) and dihydroceramide synthase 2 (CERS2, formerly called longevity assurance 2) have attracted attention as molecular targets for anti-atherosclerosis. The efficacy of SPT inhibition in inducing anti-atherosclerotic and anti-obesity effects was confirmed *in vivo* [8]. However, loss-of-function studies on *de novo* sphingolipid biosynthesis revealed accompanying abnormalities, as shown in SPT or CERS2 KO mice [7,9]. In contrast to these observations, SMS2 is responsible only for the synthesis of SM and it is not involved in the *de novo* biosynthesis of ceramide. Therefore, inhibition of SMS2 is expected to selectively decrease SM levels without inhibiting *de novo* sphingolipid biosynthesis. This could be an advantage of targeting SMS2 over other enzymes involved in sphingolipid metabolism.

Sequence analysis identified three SMS homologs in the human genome (i.e., SMS1, SMS2, and SMS-related protein) [10,11]. Among them, human SMS1 and SMS2 share 57% sequence identity and both possess SMS activity, whereas the function of SMS-related protein remains to be clarified [12–14]. SMS1 is located on Golgi, and SMS2 is found in plasma membranes [11]. It was reported that SMS1 and SMS2 are responsible for the bulk of SM synthase activity in HeLa cells, with SMS1 being responsible for most of the activity (60–80%), and SMS2 being responsible for a minor component (20–40%) [15]. In contrast to SMS2, SMS1 KO mice exhibited severe abnormalities in insulin secretion [16,17] or hearing impairments [17], which were not reported in SMS2 KO mice. Thus, the current pharmaceutical strategy is to develop specific SMS2 inhibitors with high selectivity against SMS1.

The absence of a robust assay system for SMS2 has hindered the development of potent and specific inhibitors. SMS2 belongs to the lipid phosphate phosphatase superfamily [1]. The reported assays for this target class is limited to less sensitive and low-throughput methods [18]. Concerning SMS enzymes, an HPLC assay was reported for activity measurements using fluorescence-labeled ceramide in a cellular context [19]. However, the method is not applicable to large-scale compound screening. Recently, a quantitative biochemical and cellular SMS assay was reported [20]. Thus far, D609 has been reported as a SMS inhibitor [21,22] although its potency is weak. Recently, small molecule SMS inhibitors, D2 and D24, have been identified through structure-based virtual screening [23,24]. However, their inhibitory activities are still weak with IC_{50}s of 10–20 μM and possess α-aminonitrile group. A series of 2-(4-(*N*-phenethylsulfamoyl)phenoxy)acetamide (SAPA) compounds has been investigated and reported to be SMS1 inhibitors with the most potent IC_{50} of 2.1 μM [25]. Specific inhibitors have not been reported for SMS2. In the present study, we developed a novel high-throughput screening system using mass spectrometry (MS) and performed compound screening to identify specific SMS2 inhibitors from large-scale compound libraries. Furthermore, we pharmacologically characterized the SMS2 inhibitors with biochemical and cell-based analyses.

2. Experimental protocols

2.1. Chemistry

2.1.1. General

^{1}H NMR spectra were recorded on Bruker AVANCE III (300 MHz), Agilent 400-MR (400 MHz), Agilent Mercury 300 (300 MHz) or Agilent VNMR System (300 MHz) spectrometer. Chemical shifts for ^{1}H NMR are given in parts per million (ppm) downfield from tetramethylsilane (δ) as the internal standard in deuterated solvent and coupling constants (*J*) are in Hertz (Hz). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, brs = broad singlet), and coupling constants. All solvents and reagents were obtained from commercial suppliers and used without further purification. Thin-layer chromatography (TLC) was performed on Merck TLC silica gel plates $60F_{254}$ or Fuji Silysia Chromatorex TLC plates NH. Column chromatography was performed on silica gel 60 (0.063–0.200 or 0.040–0.063 mm, Merck), basic silica gel (Chromatorex NH, 100–200 mesh, Fuji Silysia Chemical Ltd.) or UNIVERSAL™ (Si or NH, Yamazen corporation). LC-MS analysis was performed on a Waters, Agilent, or Shimadzu Liquid Chromatography-Mass Spectrometer System, operating in APCI (+ or −) or ESI (+ or −) ionization mode. Analytes were eluted using a linear gradient of 0.05% TFA containing water/acetonitrile, 0.1% TFA containing water/acetonitrile or 5 mM ammonium acetate containing water/acetonitrile mobile phase. The purities of compounds submitted for biological evaluation were determined by HPLC analysis. Analytical HPLC was performed with Corona Charged Aerosol Detector (CAD) or photo diode array detector. The column was a L-column 2 ODS (30 mm × 2.0 mm I.D., CERI, Japan) with a temperature of 50 °C and a flow rate of 0.5 ml/min. Mobile phase A and B under a neutral condition were a mixture of 50 mmol/L ammonium acetate, water, and acetonitrile (1:8:1, v/v/v) and a mixture of 50 mmol/L ammonium acetate and acetonitrile (1:9, v/v), respectively. The ratio of mobile phase B was increased linearly from 5% to 95% over 3 min, 95% over the next 1 min.

2.1.2. *N*-(3,5-bis(trifluoromethyl)benzyl)-4-hydroxy-*N*,1-dimethyl-2-oxo-1,2-dihydroquinoline-3-carboxamide (**6**)

To a mixture of **4** (1.0 g, 4.0 mmol) in toluene (20 ml) was added commercially available **5** (1.0 g, 4.0 mmol) at rt. The mixture was stirred at 120 °C for 12 h. The mixture was neutralized with aqueous 1 M HCl at 0 °C and extracted with AcOEt. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 30%–100% AcOEt in hexane). The residue was crystallized from AcOEt–IPE to give **6** (1.4 g, 76%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.05 (3H, s), 3.68 (3H, s), 4.86 (2H, brs), 7.27–7.37 (2H, m), 7.58–7.73 (1H, m), 7.77–7.95 (3H, m), 8.05–8.19 (1H, m), 12.11 (1H, brs). LCMS (ESI^+) *m/z* 459.2.

2.1.3. *N*-(3,5-bis(trifluoromethyl)benzyl)-4-chloro-*N*,1-dimethyl-2-oxo-1,2-dihydroquinoline-3-carboxamide (**7**)

To a mixture of **6** (2.0 g, 4.4 mmol) in MeCN (10 ml) was added $POCl_3$ (10 ml, 110 mmol). The mixture was stirred at 90 °C for 3 h. The mixture was poured into iced water. The precipitation was filtered and washed with aqueous $NaHCO_3$ and water to afford **7** (1.8 g, 85%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.87–2.96 (3H, m), 3.63–3.72 (3H, m), 4.54–5.12 (2H, m), 7.39–7.50 (1H, m), 7.65–7.86 (2H, m), 7.98–8.18 (4H, m). LCMS (ESI^+) *m/z* 477.2.

2.1.4. *N*-(3,5-bis(trifluoromethyl)benzyl)-*N*,1-dimethyl-2-oxo-4-(pyrrolidin-1-yl)-1,2-dihydroquinoline-3-carboxamide (**1**)

To a mixture of **7** (300 mg, 0.63 mmol) and Et_3N (0.18 ml, 1.3 mmol) in DMSO (5.0 ml) was added pyrrolidine (0.10 ml, 1.3 mmol). The mixture was stirred at 80 °C overnight. The mixture was quenched with aqueous 1 M HCl and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 30%–80% AcOEt in hexane) to afford **1** (290 mg, 90%) as a colorless solid. ^{1}H NMR