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A high-throughput sphingomyelinase assay using natural substrate

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Abstract

Sphingomyelinases are a group of hydrolases that cleave sphingomyelin, a common component of plasma membranes, to form ceramide and phosphocholine. Ceramide is a second messenger that is present in virtually all cell types and regulates a variety of cellular functions such as proliferation, differentiation, apoptosis, and inflammation response. Inhibition of sphingomyelinase activity to reduce ceramide concentrations has recently emerged as a potential therapeutic approach for several diseases including atherosclerosis, pathogen infections, inflammation, diabetes, and obesity. To effectively screen compound collections for the identification of new sphingomyelinase inhibitors, we have developed a high-throughput assay utilizing the natural substrate sphingomyelin in 1,536-well plate format. The assay has a signal-to-basal ratio of 6.1-fold in pH 5.0 buffer and 4.3-fold in pH 6.5 buffer, indicating a robust assay for compound library screening. A screen of ~300,000 compounds using this assay led to the identification of eight compounds as sphingomyelinase inhibitors (IC_{50} s=1.7 to 38.2 μ M) that exhibited different

activities between the natural substrate assay and profluorescence substrate assay. The results demonstrate the robustness and effectiveness of the natural substrate sphingomyelinase assay for screening sphingomyelinase inhibitors.

Keywords

Acid sphingomyelinase; ASM inhibitors; High-throughput screening; Natural enzyme substrate

Introduction

Sphingomyelinase (SMase), also named sphingomyelin phosphodiesterase (SMPD), hydrolyzes sphingomyelin to form phosphocholine and ceramide [1, 2]. Five forms of mammalian SMase have been identified, including two acid sphingomyelinases and three neutral sphingomyelinases, and are classified according to their pH dependence. Both lysosomal acid sphingomyelinase (L-ASM) and secreted acid sphingomyelinase (S-ASM) are encoded by a single *Smpd1* gene and undergo differential posttranslational modification [3, 4]. L-ASM is a lysosomal protein, and the genetic mutation of this gene causes Niemann Pick Disease types A and B with the characteristic of sphingomyelin accumulation in lysosomes [2]. S-ASM is a plasma protein secreted from cells, and its function is related to inflammation and is pathophysiologically linked to atherosclerosis [1, 3]. The acid sphingomyelinase (ASM) activity is dependent on Zn^{2+} which requires exogenous Zn^{2+} ions in the assay to retain full activity while L-ASM does not need additional Zn^{2+} ions in the assay buffer because Zn^{2+} tightly binds to the enzyme. Three neutral sphingomyelinases (nSMases), nSMase1, nSMase2, and nSMase3, are encoded by *Smpd2*, *Smpd3*, and *Smpd4* genes, respectively [5–7]. The nSMases are localized in the cytosol near the plasma membrane and play an important role in cell proliferation, differentiation, inflammation, and apoptosis [8–10].

Because SMases play an important role in a variety of cellular functions, they have emerged as a new drug target for the treatment of atherosclerosis [1, 11], ischemia/reperfusion injury [1], lung inflammation [12, 13], diabetes and obesity [14–16], as well as rare and neglected diseases such as pathogen infection (*Neisseria gonorrhoeae*) [17, 18] and Niemann Pick Disease types A and B [2]. Currently, there are no potent small molecule inhibitors of SMase available, although several weak inhibitors and indirect functional inhibitors have been reported [19, 20]. These available SMase inhibitors, however, are not suitable for use as therapeutic agents because of either low potency or toxicity, or lack of selectivity [1]. Therefore, lead discovery through compound library screening is essential for the identification of a new chemical series of SMase inhibitors.

Several SMase screening assays have been reported including fluorogenic, colorimetric, and radioactive assays [21–23]. These assays utilize either artificial substrates or radiolabeled substrates that are not ideal assays for the high-throughput screening (HTS) of large compound collections. A recent HTS using the artificial 6-hexadecanoylamino-4-methylumbelliferyl (HMU)-substrate failed to identify any valuable ASM inhibitors [24]. Although commercial SMase assay kits using the natural substrate sphingomyelin are

available in the 96-well plate format, the activity of those kits is only observed with bacterial SMase. We initially tried two commercial assay kits using the natural substrate for human ASM, but no sufficient assay signal was obtained. Here, we report the development and optimization of a new ASM assay, using the natural substrate sphingomyelin with human ASM as the enzyme source. The new SMase assay is optimized to work efficiently under acidic conditions and in the 1,536-well format for the high-throughput screening. This assay can be used in both pH 5.0 and 6.5 assay buffers and has been validated in a compound library screen with 1,536-well plates for the identification of ASM inhibitors.

Materials and methods

Reagents and buffers

Sphingomyelinase from human placenta (catalog number: S5383) was obtained from Sigma-Aldrich (St. Louis, MO). Amplite™ Fluorimetric Acidic Sphingomyelinase Assay Kit containing pH 5.0 and pH 6.5 buffers (catalog number: 13622) was obtained from AAT Bioquest (Sunnyvale, CA). 6-Hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU-PC; catalog number: NPAB) was purchased from Moscerdam Substrates (2341 KS Oegstgeest, The Netherlands). Assay buffer was composed of 0.1 M sodium acetate, 10 μ M sodium taurocholate, and 0.01% Tween-20, pH 5.2. The stop solution consisted of 0.2 M glycine, 0.2 M NaOH, 0.2% SDS, and 2% Triton X-100, pH 10.7. The compound plates and black assay plates were purchased from Greiner Bio-one (Monroe, NC).

SMase assay with natural substrate sphingomyelin

The SMase assay was initially optimized in a 384-well plate format. The assay was performed according to the manufacturer's instruction from the assay kit. Briefly, the SMase reaction was initiated by adding 20 μ l/well substrate to 20 μ l/well enzyme (final concentration of 76 nM) and incubated for 5 h at 37 °C, followed by the addition of 20 μ l/well reporting enzyme mixture and 10 μ l/well profluorescence AmpliteRed dye. The mixture was incubated at room temperature for 2 h (unless stated otherwise). The assay plate was measured in a fluorescence plate reader (Tecan, Durham, NC) with excitation wavelength of 525 ($\pm$ 20)nm and emission wavelength of 598 ($\pm$ 20)nm (Table 1). No enzyme control was used for the calculation of basal signal in this assay.

The 1,536-well assay was performed similarly to above 384-well plate assay, with a proportional tenfold reduction in all assay reagents (Table 1). The order of reagent addition, incubation times for both the SMase enzyme reaction and reporting system, and readout format for the 1,536-well assay were identical to the optimized conditions of the 384-well assay format.

SMase assay using HMU-PC profluorescence substrate

This SMase assay uses HMU-PC, an artificial profluorescence substrate, as a replacement for the natural substrate. The assay protocol was slightly modified from a previously reported method [25] to make it more amenable to 1,536-well plate format. Briefly, the enzyme reaction is initiated by the addition of 2 μ l/well HMU-PC substrate (final concentration of 9 μ M) to 2 μ l/well enzyme (final concentration of 51 nM). After a 30-min

incubation at room temperature, 3 μ l/well stop solution was added, and the plate was measured in the fluorescence plate reader using an excitation wavelength of 385 ($\pm$ 20)nm and emission wavelength of 450 ($\pm$ 20)nm.

Compound library and instruments for liquid handling

A collection of 338,136 compounds from the NIH molecular Library collection (<http://mli.nih.gov/mli/compound-repository/mlsmr-compounds>) was used in the screen. All the compounds were dissolved in 100% dimethylsulfoxide (DMSO) as 10 mM stock solutions and dispensed into 1,536-well compound plates at 7 μ l/well using CyBi®-Well dispensing station with a 384-well head (Cybio, Woburn, MA). An automated dispensing station, BioRAPTR FRD (Beckman Coulter, Brea, CA) was used to dispense reagents into 1,536-well plates at 1 to 3 μ l/well. Compounds were transferred to 1,536-well assay plates at 23 nL/well using an automated pin-tool station (Kalypsys, San Diego, CA). A customized screening robot (Kalypsys) was used for the primary screen.

Data analyses

The primary screen data were analyzed using customized software developed internally. IC₅₀ values were calculated using the Prism software (Graphpad Software, Inc. San Diego, CA). Signal-to-background ratio was calculated as a comparison of signal in the presence or absence of enzyme. All values were expressed as the mean $\pm$ SD ($n=3$).

Results

Assay principle

Sphingomyelin is hydrolyzed in the presence of SMase to two products—ceramide and phosphocholine (Fig. 1). An enzyme reporting system is employed to quantitate the amount of phosphocholine formation. Three enzymes are used in this reporting system: alkaline phosphatase (ALP), choline oxidase, and horseradish peroxidase (HRP). The resulting phosphocholine is hydrolyzed by ALP to choline and phosphate, followed by a choline oxidase reaction to form H₂O₂. A profluorescence dye, AmplitudeRed, in the reaction mixture is then oxidized by HRP using newly formed H₂O₂ to yield a fluorescent resorufin analog that is detected at an excitation wavelength of 525 ($\pm$ 20) nm and emission wavelength at 598 ($\pm$ 20)nm (Fig. 1).

Assay optimization

The assay optimization was performed in a 384 well plate format (Table 1). The enzyme concentration-response was first titrated. The SMase activity linearly increased with the increasing concentration of enzyme up to 120 nM (Fig. 2a). Nonlinear enzyme activity was observed above this enzyme concentration. Therefore, the enzyme concentration was kept under 120 nM in subsequent experiments. The time course of the enzyme reporting system was determined for all three pH conditions. We found that the increase in assay signal was linear from 30 to 120 min for all three buffers tested (Fig. 2b, c, and d). The signal-to-basal (S/B) ratios at pH 5.0, pH 6.5, and pH 7.0 were 4.4-, 3.9-, and 2.5-fold, respectively, after a 60-min incubation period, whereas they were 5.8-, 5.4-, and 3.1-fold, respectively, after a

120-min incubation period. Therefore, the 120 min incubation time was selected for subsequent experiments.

Assay miniaturization and DMSO plate test in 1,536-well plates

To miniaturize this assay to 1,536-well plate, the volume of all reagents used in the 384-well assay were proportionally reduced (Table 1). The miniaturized SMase assay is a homogeneous assay that consists of four independent reagent addition steps, one compound dispensing and two plate incubation steps. A DMSO plate was used to determine the assay performance in the miniaturized assay. The S/B ratio was 6.1-fold and Z' factor was 0.81 in the pH 5.0 buffer (Fig. 3a), while the S/B ratio and Z' 's factor in the pH 6.5 buffer were 4.3 and 0.73, respectively (Fig 3b). Together, the results demonstrated a robust screen assay that is suitable for high-throughput screening.

High-throughput screening and SMase inhibitor identification

A high-throughput screen using the SMase assay in a pH 6.5 buffer was carried out against 338,136 compounds at 12 μ M of final compound concentration. A total of 1,420 compounds showed greater than 50% inhibition in the primary screen with a hit rate of 0.42%. Among the 1,344 cherry-picked compounds for confirmation, 74 of them exhibited IC_{50} values under 10 μ M in both the pH 6.5 and pH 5.0 buffer (all the data from the primary and confirmation screens are deposited in the Pubchem database). Data mining and running comparison analysis of several hydrolase screens including glucocerebrosidase, beta-glucosidase, and alpha-galactosidase resulted in the selection of ten compounds for further evaluation (Fig. 4). The newly ordered powder samples of the ten compounds were used for confirmation in the SMase assays against the natural and fluorescence substrate.

The SMase inhibitory activity against the natural substrate was confirmed in eight out of ten compounds (Fig. 4). Among the confirmed compounds, MLS000737788, MLS001164567, and MLS000097294 exhibited complete inhibition on SMase in the pH 6.5 buffer with IC_{50} values of 1.7, 3.2, and 38.2 μ M, respectively. Interestingly, the activity of MLS000737788 reduced 5.6-fold against the natural substrate assay in the pH 5.0 buffer, while the activities of MLS001164567 and MLS000097294 remained the same. Five of the ten compounds, MLS000574110, MLS000707545, MLS000574110, MLS000950682, and MLS000720877, were partial inhibitors.

The ten compounds were further characterized in the SMase assay against the artificial profluorescence substrate, HMU-PC. Their activities did not correlate with the activity observed using the natural substrate (Table 2). Two previous active compounds, MLS001164567 and MLS000097140, lost their activities, while two previously inactive compounds, MLS002248315 and MLS001164606, became partial inhibitors in the artificial substrate assay. In addition, the inhibitory activities of MLS000737788 and MLS000720877 in the HMU-PC substrate assay increased 35.3- and 55.6-fold, respectively, compared with the natural substrate assay at the same pH 5.0 buffer. The results indicate that the inhibitory activities of these compounds are substrate-dependent.

Discussion

Enzyme assays are commonly used for characterizing enzyme property, disease diagnosis, as well as compound screening for identifying and characterizing enzyme activators and inhibitors. Enzyme assay used in the screening of large compound libraries requires a simple assay format with high assay sensitivity. In addition, assays should be amenable to miniaturization into 384-well and 1,536-well high density plates to save on reagents and to increase screening throughput. Thus, a homogenous assay format that eliminates plate wash steps is most commonly employed for this purpose. Fluorogenic enzyme assays using a profluorescence substrate are frequently used because of their homogenous assay format and assay sensitivity. The profluorescence substrate is artificially engineered by conjugating a fluorophore to a chemical moiety that quenches the fluorescence from adjacent fluorophore. Once the quenching group on the profluorescence substrate is removed by an enzyme, the released fluorophore becomes fluorescent and its signal is proportional to the enzyme activity. The advantages of these profluorescence substrates are (1) enabling homogenous assay format for high-throughput screening, (2) high assay sensitivity due to the significantly enhanced fluorescence signal, and (3) low reagent cost. In addition, the recent effort to replace blue and green fluorophores with a red fluorophore in profluorescence substrates improves the screening data quality by reducing interference caused by fluorescence compounds, residual UV fluorescence signal from the plastic plate, or/and lint/dust [26, 27].

Although fluorogenic enzyme assays that utilize artificial substrates are useful for disease diagnosis, it has been increasingly observed that the use of these substrates in compound screens frequently results in false-positives and false-negatives. An artificial substrate may not have the same active site on the enzyme as the natural substrate. The function of an enzyme inhibitor or activator is dependent on its binding site on the enzyme. The binding of an enzyme inhibitor or activator to the enzyme modulates the affinity of substrate to the enzyme or the enzyme's catalytic ability. Thus, a potent enzyme inhibitor in an artificial substrate assay could be less potent or inactive in a natural substrate assay, and vice-versa. We have previously found that the activities of some glucocerebrosidase inhibitors, identified in enzyme assays using artificial substrate and recombinant enzyme, dramatically reduced in the natural substrate assay and cell-based assay [28]. An alternative compound screen using native enzyme extracted from patient tissue significantly improved the quality of glucocerebrosidase inhibitors discovered, and an improved correlation of compound activities between the enzyme assay and cell based assay was observed [28, 29].

In the SMase assay described in this study, the natural substrate sphingomyelin is used with a purified SMase preparation that is a human L-ASM purified from placenta as previously described [30] and has the optimal activity at lower pH (4.5–5.0). The assay condition more closely mimics physiological condition in mammalian cells, thus rendering it more suitable for screening of compound collections to identify high quality SMase inhibitors. Among three of ten compounds selected for further confirmation, MLS000737788, MLS001164567, and MLS000097294 are full SMase inhibitors determined in the natural substrate assay, but their activities were very different in the artificial substrate assay. The potency of MLS000737788 reduced 35-fold and the potency of MLS000097294 increased 5-fold,

whereas MLS001164567 was inactive in the artificial substrate assay. In contrast, MLS002248315 and MLS001164606 were inactive in the natural substrate assay but became SMase “inhibitors” in the artificial substrate assay. MLS00720877, MLS000737788, and MLS000097294 were full inhibitors in the artificial substrate assay, though MLS00720877 was a partial inhibitor in the natural substrate assay. No correlation was observed for compound activities between the natural substrate assay and artificial substrate assay. Only two compounds, MLS000737788 and MLS000097294, showed full SMase inhibitory activities in both assays, with MLS000737788 being consistently the most potent. Although MLS000737788 (2-arsenobenzoic acid) is a FDA-approved antineoplastic agent, the arsenate functional group decreases the chemo-attractiveness of the molecule as lead for further SAR studies. Replacement of this group by a phosphate moiety may be a good starting point for chemical modifications. MLS000097294 is structurally a more appealing compound, but its IC₅₀ value is high. However, the molecule has a low molecular weight with room for chemical optimization and improvement. MLS001164567 has a low molecular weight, and it has low micromolar activity in the natural substrate assay, which renders this molecule particularly attractive for further optimization. Together, the results demonstrated a robust SMase screening assay using natural substrate that can be used in both HTS and lead compound optimization for the development of SMase inhibitors.

In conclusion, a SMase assay utilizing a natural substrate and purified SMase from human placenta has been developed and optimized, and this assay employs an enzyme-coupled fluorescence reporting system for detection. The signal-to-basal ratio of this assay in 1,536-well plate was 4.3- to 6.1-fold, and Z’ factor was 0.73 to 0.81, indicative of a robust high-throughput assay for lead discovery. A screen of ~300,000 compounds using this assay led to identification of eight ASM inhibitors. We also found that the activities of these SMase inhibitors determined in the natural substrate assay did not correlate with those measured in the artificial substrate assay. Therefore, the novel SMase assay using a natural substrate should be a useful tool for the screening of compound library, as well as for assaying compounds to support lead optimization.

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Fig. 1.

Schematic illustration of sphingomyelinase (*SMase*) assay using natural substrate. The natural substrate sphingomyelin is hydro-lyzed by *SMase* to yield ceramide and phosphocholine. The resulting product phosphocholine is detected by an enzyme coupled reporting system. Phosphocholine is hydrolyzed by alkaline phosphatase (*ALP*) to yield choline, which is then oxidized by choline oxidase to produce betaine and H₂O₂. Amplitude-Red, a profluorescence dye, is oxidized by horseradish peroxidase (*HRP*) in the presence of H₂O₂ to generate red-fluorescent resorufin analog. The fluorescence intensity generated through this enzyme coupled reporting system is proportional to the *SMase* enzyme activity

Fig. 2.

SMase assay optimization in 384-well plates. **A** Enzyme concentration titration in a pH 7.0 buffer. The SMase activity linearly increased with the SMase up to 120 nM. The time course of enzyme coupled reporting system was determined in the pH 5.0 buffer (**B**), pH 6.5 buffer (**C**), and pH 7.0 buffer (**D**). The enzyme activity linearly increased from 30 min incubation to 120 min incubation. The signal-to-basal ratios were 5.8-, 5.4-, and 3.1-fold after 120 min incubation, respectively

Fig. 3.

Scatter plot of a DMSO plate test for the SMase assay using natural substrate in 1,536-well plates. A DMSO plate was tested in the pH 5.0 buffer (**A**) and pH 6.5 buffer (**B**). The signal-to-basal (S/B) ratios in pH 5.0 and 6.5 buffers were 6.1- and 4.3-fold, and the Z'-factors were 0.81 and 0.73, respectively

Fig. 4.

Structures and concentration response curves of ten compounds determined in the confirmation assays. The compounds were titrated in the SMase natural substrate assay in the pH 6.5 buffer (A) and pH 5.0 buffer (B). The activities of eight out of ten compounds were confirmed though five of them were partial SMase inhibitors. The inhibitory activities of ten compounds determined in the SMase assay (C) using the artificial substrate, HMU-PC, were not correlated with these measured in the SMase natural substrate assay

Table 1

SMase natural substrate assay protocol (pH 6.5)

Step	Parameter	Value		Description
		384-well plate	1,536-well plate	
1	Enzyme solution	20 μ l/well	2 μ l/well	153 nM SMase in buffer
2	Compound	0.2 μ l/well	0.02 μ l/well	In DMSO solution
3	Substrate solution	20 μ l/well	2 μ l/well	100 μ M sphingomyelin in buffer
4	Incubation	5 h	5 h	37 °C
5	Detection mixture	20 μ l/well	2 μ l/well	From the assay kit
6	Amplite-Red dye	10 μ l/well	1 μ l/well	From the assay kit
7	Incubation	120 min	120 min	Room temperature
8	Plate reading	Ex0540 nm Em0590 nm	Ex0540 nm Em0590 nm	Fluorescence intensity

Table 2Summary of IC₅₀ values and maximal inhibitions of SMase inhibitors

Compound ID	Natural substrate (pH 6.5)		Natural substrate (pH 5.0)		HMU-PC substrate (pH 5.0)	
	IC ₅₀ (μ M)	Maximal resp.	IC ₅₀ (μ M)	Maximal resp.	IC ₅₀ (μ M)	Maximal resp.
MLS000737788	1.7	104.2	9.5	94.3	0.27	101.9
MLS000574110	2.4	84.8	1.7	79.1	15.1	82.1
MLS000707545	2.9	77.2	2.3	72.0	17.1	93.4
MLS001164567	3.2	100.7	4.2	97.8	n.a.	15.8
MLS000097140	3.3	82.7	5.0	81.7	n.a.	6.2
MLS000720877	5.6	61.6	5.7	31.1	0.10	99.2
MLS000950682	33.1	71.2	6.4	43.4	3.5	79.0
MLS000097294	38.2	104.4	25.1	103.2	5.3	99.1
MLS002248315	n.a.	8.6	n.a.	0.8	0.99	31.4
MLS001164606	n.a.	26.8	n.a.	9.1	1.5	51.8

Resp. response, *n.a.* not active (maximal response is under 30%)