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Inhibition of a Neutral Form of Sphingomyelinase by Alkylthioureido-1,3-propandiols, KY353X Series

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Abstract – Alkylthioureido-1,3-propandiols (KY353X series) were synthesized and evaluated as inhibitors for neutral sphingomyelinase (N-SMase). To examine whether KY353X series inhibit N-SMase, we purified the N-SMase from bovine brain. The N-SMase was partially purified by sequential chromatographies of DEAE-Cellulose anionic exchange and phenyl-5PW hydrophobic HPLC. These seqeuntial procedures for N-SMase resulted in a 67-fold purification and excluded other isoforms of SMase. Based on *in vitro* assay, KY353X series inhibited N-SMase activity in time, concentration-dependent manners and completely inactivated N-SMase at 50 μM. In particular, KY3535 and KY3536 inhibited more effectively than the others. To further determine the inhibitory pattern, a Dixon plot was constructed, to showing that the inhibition by KY3535 and KY3536 were competitive. The inhibition constant (K*i*) of KY3535 and KY3536 was 1.7 μM and 2.5 μM in 100 mM Tris-HCl buffer, pH 7.0, respectively.

Keywords □ Alkylthioureido-1,3-propandiols, Sphingomyelinase, inhibitor

INTRODUCTION

Sphingomyelinase (SMase), which catalyzes the hydrolysis of membrane sphingomyelin to generate ceramide and phosphocholine, are now recognized as important signal mediator in cell. It has been proposed that ceramide, the backbone of various sphingolipid, may play a crucial role in cell response such as cell differentiation (Okazaki *et al.*, 1990), cell cycle arrest (Jayadev *et al.*, 1995), cellular senescence (Venable *et al.*, 1995), and programmed cell death or apoptosis (Obid *et al.*, 1993).

To date, at least five types of SMases have been identified. An acidic sphingomyelinase (A-SMase) was first discovered and found to have an optimum pH at 5.0 (Spence, 1993). Although the acidic sphingomyelinase has been primarily found to reside in the lysosomes, it has also been detected as a soluble form in cytosol and extracellular media (Quintern *et al.*, 1987). This enzyme has been cloned, and it is deficient in Neimann-Pick cells. It is also suggested to play a role in radiation-induced apoptosis (Haimovitz-Friedman *et al.*, 1994) and has been described to be activated by Fas (Cifone *et al.*, 1994) and tumor necrosis factor-α (Schutze *et al.*, 1992). A neutral pH-optimum and magnesium-dependent SMase (N-SMase) has also been described (Chatterjee, 1993). This enzyme is associated with the plasma membrane. It is found to be activated in response to tumor necrosis factor-α, Fas, Ara-C, and serum deprivation, and its activation appears to be closely related to growth suppression and apoptosis (Strum *et al.*, 1994; Jayadev *et al.*, 1995; Tepper *et al.*, 1995). Recently, a cytosolic magnesium-independent neutral SMase was partially purified from HL-60 cells following treatment with vitamin D_3 (Okazaki *et al.*, 1994). In addition, a zinc-dependent acidic SMase was detected in sera from a variety of species (Spence *et al.*, 1989), and recent studies suggest that it is derived from the lysosomal A-SMase (Schiessel *et al.*, 1996). Finally, an alkaline pH-optimum SMase has been described in intestinal cells (Nyberg *et al.*, 1996).

Especially, N-SMase activity was strongly expressed in the central nervous system (Spence *et al.*, 1978). It is reported that the specific activity of N-SMase increases during the first 2 weeks of the neonatal period in the ontogenesis of rat brain. This leads to the possibility that ceramide generated from SM by N-SMase participates in the normal growth and maturation of neural cells. In recent years, studies have shown that the effects of NGF are mediated via generation of ceramide produced by N-SMase. These data strongly support a primary role

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for the p75^{NTR}-ceramide signaling pathway in mediating the effects of N-SMase on the growth of cultured hippocampal neurons.

However, direct links between brain N-SMase and specific signaling systems have not been established. To establish a clear picture of the metabolic links, the synthesis of brain N-SMase inhibitors is strongly required. Additionally, the brain N-SMase inhibitor is expected to have some clinical values, because ceramide generation following SM hydrolysis might be implicated in pathological states such as Alzheimer's disease and Parkinson's disease.

In this study, we have been interested in a novel structural class of brain N-SMase inhibitors. Alkylthioureido-1,3-propandiol analogues (KY353X series) were synthesised and evaluated *in vitro* assay using exogenous substrate and N-SMase partially purified from bovine brain.

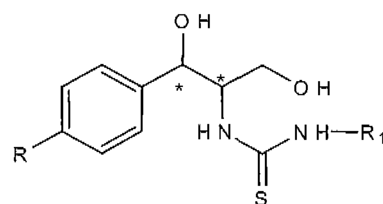
MATERIALS AND METHODS

Materials

[N-methyl-^{14}C]sphingomyelin ([N-methyl-^{14}C]SM, 47 mCi/mmol) were purchased from Amersham PharmaciaBiotech UK Ltd. (Buckinghamshire, England). KY353X series were synthesized and suspended at 5 mM in DMSO. DEAE-Cellulose anion exchange column and Phenyl-5PW hydrophobic HPLC columns were purchased from Tosoh Co. (Tokyo, Japan). Bovine brain tissues were obtained from a local slaughterhouse in Seoul and kept at -70°C. All other chemicals (Sigma Chemical Co.) were of the highest purity available from commercial sources.

Assay for N-SMase activity

The substrate, [N-methyl-^{14}C]SM (labeled with ^{14}C on the choline moiety), was dried under nitrogen stream and resuspended in ethanol. The standard incubation system (100 μl) for assay of N-SMase activity contained 10 mM $MgSO_4$, 50 mM [N-methyl-^{14}C]SM (approximately 60,000 cpm), 2 mM sodium deoxycholate (SDC), 100 mM Tris-HCl, pH 7.0. Reactions were carried out at 37°C for indicated times and stopped by adding 320 μl of chloroform/methanol (1:1, by volume) and 30 μl of 2N-HCl into the reaction mixture according to Bligh & Dyer's method (Bligh and Dyer, 1959). After vortex, the mixtures were microcentrifuged to separate the two phases. 200 μl of clear aqueous phase was removed into 2.5 ml of scintillation solution (Insta gel-XF, Packard Instrument Co., Meriden, CT, USA) and counted for radioactivity in Packard Tri-carb liquid-scintillation counter.



Alkylthioureido-1,3-propandiols

Compound	R	R1	Config
KY3533	NO_2	C_8H_{17}	1R, 2R
KY3534	NO_2	$C_{10}H_{21}$	1R, 2R
KY3535	NO_2	$C_{12}H_{25}$	1R, 2R
KY3536	NO_2	$C_{14}H_{29}$	1R, 2R

Fig. 1. Structure of N-SMase inhibitors.

Purification of N-SMase, from bovine brain

As described previously (Jung *et al.*, 2000), N-SMase, a salt-extractable form of the membrane-bound N-SMase, was purified as follows. First, to prepare the enzyme source for the purification of N-SMase, fresh bovine brain (2 kg) kept at -70°C was homogenized with 5 volumes (10 liters) of homogenizing buffer V (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 3 mM $MgCl_2$, 50 mM KCl, and 10 mM 2-mercaptoethanol) with a Polytron homogenizer (Model PT-MR 6000, Kinematica, Switzerland). The homogenate was centrifuged at 10,000×g for 10 min to remove the cell debris and nuclei. The resulting supernatants were again centrifuged at 10,000×g at 4°C for 1hr. The resulting 10,000×g pellets were resuspended with 1.2 liters of buffer V and centrifuged at 40,000×g at 4°C for 1hr. The resulting 40,000×g pellets were again resuspended with 1.2 liters of buffer V, adjusted to 0.5 M $(NH_4)_2SO_4$, and stirred at 4 for 1hr followed by centrifugation at 40,000×g at 4°C for 1hr. The resulting supernatants, termed "ammonium sulfate extracts", were collected and used as the enzyme source for the purification of N-SMase. The ammonium sulfate extracts (1.2 liters) were applied to a DEAE-Cellulose anion exchange column (bed volume of DE52 gel, 1.0 liter) pre-equilibrated with buffer D (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM 2-mercaptoethanol). The protein bound to the column was eluted at a flow rate of 20 ml/min with a stepwise application of buffer D containing 0.5 M $(NH_4)_2SO_4$ and 0.1% Triton X-100. An aliquot (10 μl) of each fraction (40 ml) was assayed for N-SMase activity. The active fractions were pooled, sonicated at 4°C with a cell disruptor (Sonics & Materials Inc., Danbury, CT, USA) six times for 3 sec with 5 sec intervals at an output setting of amplitude 70% and centrifuged at 100,000×g at 4°C for 1hr. An aliquot of stock solution of 4.0 M $(NH_4)_2SO_4$ was added to the resulting superna-

tant to adjust to 0.5 M $(NH_4)_2SO_4$. The sample was applied to a DEAE-Cellulose column previously equilibrated with buffer D. The protein bound to the column was eluted at a flow rate of 5 ml/min with a 200 ml-linear gradient of buffer D containing 0.5 M $(NH_4)_2SO_4$ and 0.1% Triton X-100 as an elution buffer. An aliquot (3 μl) of each fraction (5 ml) was assayed for N-SMase activity. The active fractions were pooled and applied to a Phenyl-5PW HPLC column (21.5 mm×15 cm, Tosoh Co., Tokyo, Japan) previously equilibrated with buffer D containing 0.2 M $(NH_4)_2SO_4$. The protein bound to the column was eluted at a flow rate of 5 ml/min with a 100 ml-gradient elution of distilled water. Immunization was performed by injecting 200 g of recommbinat protein in Freund's complete adjuvant (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA) intraperitoneally into S/D rats, and 50 g of neutral SMase into BALB/c mice. Two additional injections with same amount of antigens (all emulsified in Freund's incomplete adjuvant) were given at an interval of 3 weeks. A final injection of same amount of antigens (without adjuvant) were administered 3 days prior to the fusion followed by collecting blood. Antiserum was obtained by centrifuging the blood at 10,000×g for 30 min.

Inhibitory Effect of KY353X series on N-SMase

Partially purified bovine brain N-SMase were preincubated in different concentrations of KY353X series inhibitors at 37°C for 10 min in 100 ml of reaction buffer (100 mM Tris-HCl, pH 7.0, 10 mM $MgSO_4$, 2 mM sodium deoxycholate (SDC)), respectively, and then the mixture was assayed for SMase activity.

RESULTS AND DISCUSSION

Preparation of N-SMase from bovine brain

To examine whether KY353X series inhibit N-SMase, we obtained N-SMase from bovine brain, which was partially purified by sequential chromatographies of DEAE-Cellulose column and phenyl-5PW as described in methods. The supernatants from centrifugation at 40,000×g, showed a specific activity of 140 pmol/min/mg. $(NH_4)_2SO_4$ extract was loaded on DEAE-Cellulose anionic exchange column. The activity of a portion (~30%) was pass-through from the anion exchange column, but the majority (~70%) bound to the column and eluted by buffer D containing 0.5M $(NH_4)_2SO_4$ and 0.1% Triton X-100 with a yield of 30.8%. The turbid activity pool of DEAE-Cellulose column was clarified by ultracentrifugation, and the majority (63%) was recovered in 100,000×g supernatant with a 4.3-fold purification increase. Next, supernatants by ultracentrifugation were applied to the phenyl-5PW hydrophobic column and eluted as 72% yield. These seqeuntial procedures for brain N-SMase resulted in a 67-fold purification and excluded isoforms of SMase. The supernatant was separated in 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with N-SMase antibody as described in methods. The bands were visualized by the NBT/BCIP reagent. Among several bands, the intensity of N-SMase band paralleled each relative activity of the active fractions (Fig. 2).

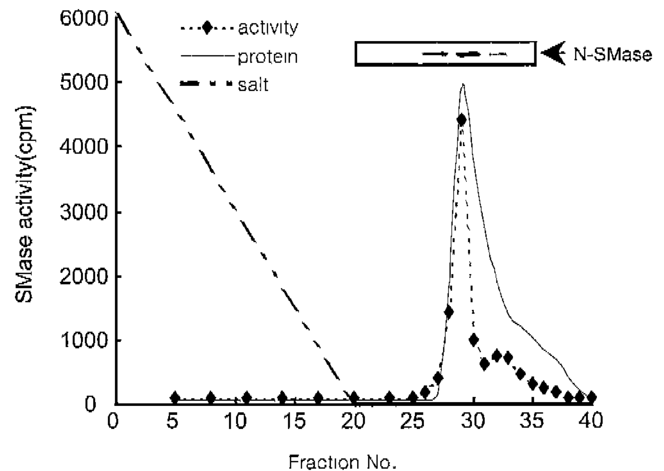


Fig. 2. Detection of N-SMase in bovine brain by phenyl-5PW HPLC and western blot analysis. The DEAE 5PW ion exchange column eluent pool was applied to a phenyl-5PW hydrophobic column pre-equilibrated with 25 mM Tris (pH 7.5) buffer containing 1 mM EDTA, 0.5M $(NH_4)_2SO_4$ at a flow rate of 5 ml/min. The column was eluted at a flow rate of 5 ml/min with 100 ml of the same buffer in a linear gradient of 0.5-0 M $(NH_4)_2SO_4$. An aliquot (3 μl) of each fraction was assayed for N-SMase activity and western blotting analysis was performed. The insert shows western blotting analysis; each active fraction (26-31) was taken.

Effect of KY353X series on activity of brain N-SMase

The inhibitory effect of KY353X series on brain N-SMase activity was examined in a concentration-dependent manner. The enzyme was preincubated with 10, 25, and 50 μM of inhibitors for 10 min in 37°C, before the addition of substrate. As shown in Fig. 3, the enzyme activity was inhibited in a concentration-dependent manner and inactivated completely at 50 μM. Especially, KY3535 and KY3536 inhibited more effectively than the others. Thus, next experiments were performed with KY3535 and KY3536. As shown in Fig. 4, the inhibitory effect of KY3535 and KY3536 on N-SMase activity increased in a time-dependent manner. When the N-SMase incubated with 10, 25, and 50 μM of inhibitors for 60 min in 37°C, KY3535 inhibited

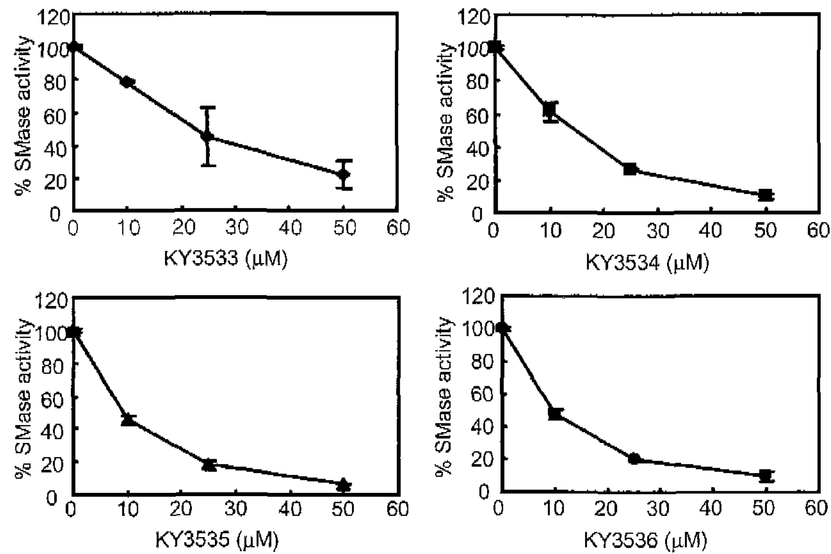


Fig. 3. Concentration-dependent inhibition of bovine brain N-SMase by KY353X series. N-SMase activity was assayed in the presence of increasing concentrations of inhibitors as described under Methods. Inhibitors were preincubated with N-SMase (2,500 cpm) for 10 min at 37°C. Each data point represents the mean ± SEM of three independent experiments.

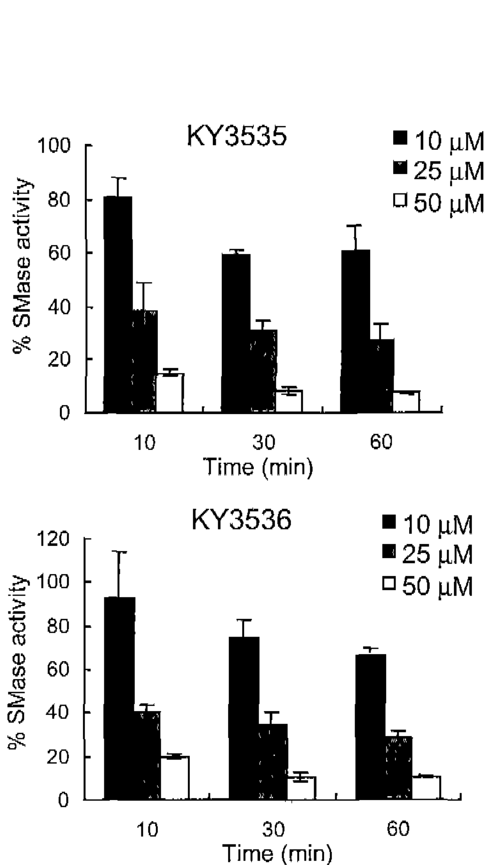


Fig. 4. Time-dependent inhibition of brain N-SMase by KY3535 and KY3536 inhibitors. Purified bovine brain N-SMase (5,000 cpm) was incubated with different concentrations of inhibitors at 37°C for the indicated times, and then the activity was measured as descriebed under Methods. Each data point represents the mean ± SEM of three independent experiment.

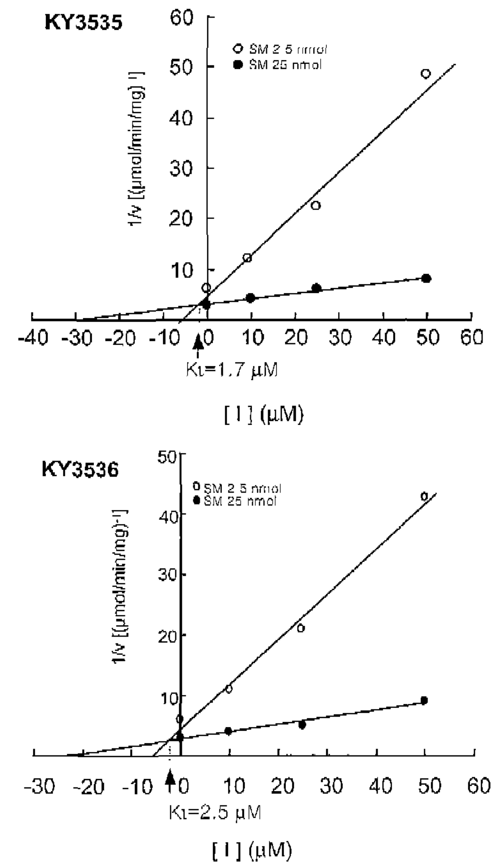


Fig. 5. Determination of the inhibitory pattern on brain N-SMase by KY3535 and KY3536. The N-SMase activity was assayed for 20 min at 37°C in the presence of the indicated concentrations of inhibitors and 25 μM (○) and 250 μM (●) of SM as described under Methods. Shown are values from one experiment representative of three independent experiments producing

39, 73, and 93% of control activity, respectively and KY3536 also inhibited in a similar pattern.

Evaluation for inhibition of KY3535 and KY3536 on brain N-SMase activity

To determine the inhibitory pattern on N-SMase by KY3535 and KY3536, Dixon plots were constructed from the hydrolysis rates of the substrate ([N-methyl-^{14}C]SM of 25 and 250 μM) by N-SMase at various KY3535 and KY3536 concentrations. Fig. 5 illustrates that the apparent Kis of KY3535 and KY3536 were 1.7 μM and 2.5 μM respectively. Thus, both of inhibitior were apparently competitive and this result supports a direct binding of KY3535 and KY3536 to the enzyme.

Together, we report the inhibitory effect of KY353X series on brain N-SMase *in vitro*. During a high throughput screening with a preparation of brain N-SMase, we identified KY353X series, a molecule that inhibited effectively the enzyme activity. In further study, these inhibitors will be evaluated whether these block the ceramide generation from insuits-stimulated cells and show specifically the inhibitory effect on the 60 kDa N-SMase compared with other isoforms of N-SMase isolated from the brain tissue.

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