A lipid analogue that inhibits sphingomyelin hydrolysis and synthesis, increases ceramide, and leads to cell death

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Abstract We report the synthesis and characterization of a novel thiourea derivative of sphingomyelin (AD2765). In vitro assays using pure enzyme and/or cell extracts revealed that this compound inhibited the hydrolysis of BODIPY-conjugated or 14C-labeled sphingomyelin by acid sphingomyelinase and Mg2+-dependent neutral sphingomyelinase. Studies in normal human skin fibroblasts further revealed that AD2765 was taken up by cells and inhibited the hydrolysis of BODIPY-conjugated sphingomyelin in situ. In situ and in vitro studies also showed that this compound inhibited the synthesis of sphingomyelin from BODIPY-conjugated ceramide. The specificity of AD2765 for enzymes involved in sphingomyelin metabolism was demonstrated by the fact that it had no effect on the hydrolysis of BODIPY-conjugated ceramide by acid ceramidase or on the synthesis of BODIPY-conjugated glucosylceramide from BODIPY-conjugated ceramide. The overall effect of AD2765 on sphingomyelin metabolism was concentration-dependent, and treatment of normal human skin fibroblasts or cancer cells with this compound at concentrations > 10 μM led to an increase in cellular ceramide and cell death. Thus, AD2765 might be used to manipulate sphingomyelin metabolism in various ways, potentially to reduce substrate accumulation in cells resulting in the formation of ceramide and phosphocholine (2). ASM is found predominantly within lysosomes, but other enzyme forms, including a secreted, Zn2+-dependent form (3) and a plasma membrane form localized to raft structures (4, 5), have been identified. Each of these enzyme forms is derived from the same gene, located on the short arm of chromosome 11 and designated sphingomyelin phosphodiesterase-1 (SMPD-1) (6–9). The full-length cDNA encoding ASM has been isolated (10, 11), leading to the production of recombinant enzyme in overexpressing CHO cells (12) and insect cells (13). Other neutral and alkaline sphingomyelinase activities also exist in mammalian cells, but these are distinct enzymes derived from different genes (1).

In humans, inherited mutations in the SMPD-1 gene result in types A and B Niemann-Pick disease (NPD) (14). In addition, a recent report showed that deletion of the gene (Smpd3) encoding one of the neutral sphingomyelinases in mice results in osteogenesis and dentiogenesis imperfecta (15). As noted above, the reaction product of ASM activity, ceramide, is a potent lipid second messenger involved in diverse cellular functions, including cell growth, differentiation, and death (16, 17). Ceramide production has been implicated in the pathogenesis of diseases such as atherosclerosis and acquired immunodeficiency syndrome, as well as in liver damage and acute lung injury (18, 19).

The need for greater understanding of sphingomyelin and ceramide metabolism, as well as the potential clinical significance of modulating this pathway, has prompted the development of several classes of sphingomyelinase inhibitors. For example, various synthetic sphingomyelin analogues (20–22), as well as analogues of naturally occurring

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ring inhibitors, such as o-mangostin, a natural product derived from *Garcinia mangostana*, have been developed (23). In addition, the lipid phosphatidylinositol-3,5-bisphosphate has been shown to inhibit ASM activity (24). Such sphingomyelinase inhibitors may be useful for a wide range of applications. For example, sphingomyelinase inhibitors may potentially be used to alter the growth of cancer cells, to modulate the effects of inflammation, or, in the case of ASM inhibitors, as molecular chaperones for the treatment of types A and B NPD (25, 26). This latter approach was pioneered by the work of Fan and colleagues for the treatment of another lysosomal storage disorder, Fabry disease (27, 28).

Although many sphingomyelinase inhibitors are known, the broader effects these compounds have on sphingomyelin metabolism in living cells remain mostly unknown. Here, we have developed a new sphingomyelin analogue (designated AD2765) and extensively characterized it in vitro and in living cells. We reveal that this compound has broad effects on sphingomyelin metabolism, including the inhibition of ASM and Mg\(^{2+}\)-dependent neutral sphingomyelinase activities, as well as on the inhibition of sphingomyelin synthesis. There were no effects on ceramide hydrolysis or on the synthesis of glucosylceramide. In addition, treatment of cells with AD2765 at concentrations > 10 \(\mu\)M led to an increase in cellular ceramide and cell death. The potential therapeutic applications of this compound are discussed.

**MATERIALS AND METHODS**

**Cell culture**

Human skin fibroblasts were maintained in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, and L-glutamine (Invitrogen, Carlsbad, CA). Human lymphoblasts and Jurkat lymphoma cells were maintained in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin (Invitrogen). M609I Glioma cells (American Tissue Culture Collection) were maintained in DMEM/Ham’s F12 (1:1, v/v) supplemented with 10% FBS, 15 mM HEPES, and 1% penicillin-streptomycin (Invitrogen). HL-60 cells were grown in DMEM supplemented with 10% FBS, 15 mM HEPES, and 1% penicillin-streptomycin (Invitrogen).

**Synthesis of AD2765**

Twenty milligrams of sphingosylphosphorylcholine was dissolved in 5.0 ml of 0.5 N sodium bicarbonate in a 10 ml Erlenmeyer flask. To the stirred solution was added 20 mg of tert-butyl isothiocyanate. The solution was left to stir for 48 h and then filtered, and the upper aqueous phase containing the \[^{14}C\]phosphocholine reaction product was then removed for scintillation counting with an ISODATA \(\gamma\) counter (Polymedico, Inc.).

**Neutral sphingomyelin synthesis.** The activity of Mg\(^{2+}\)-dependent neutral sphingomyelinase was assessed in vitro using the fluorescence-based HPLC method described above for the ASM assay except that the assay buffer contained 200 mM Tris-\(\mathrm{HCl}\) (pH 7.4), 5 mM magnesium chloride, and 0.6% Triton X-100.

**Acid ceramidase activity.** Acid ceramidase activity was assessed in vitro essentially as described previously (30). Briefly, the standard 10 \(\mu\)l reaction mixture consisted of 5 \(\mu\)l of enzyme source (prepared by three cycles of freeze-thaw in 0.25 M sucrose) plus 5 \(\mu\)l of 0.2 M citrate/ phosphate buffer (pH 4.5) containing 200 \(\mu\)M B12CER, 300 mM NaCl, 0.1% BSA, and 0.2% Igepal CA-630. The reaction mixtures were then incubated at 37°C for 60 min. The reaction was terminated by the addition of ethanol, and the fluorescent fatty acid product was detected and quantified by chromatographic analysis using a reversed-phase column (BetaBasic-18, 4.6 x 30 mm; Keystone Scientific, Inc., Bellefonte, PA).

**Sphingomyelin and glucosylceramide synthase activities.** Jurkat lymphoma or HL-60 cells (2 x 10\(^6\)) were dispersed in 1 ml of the following homogenization buffer: 50 mM Tris-\(\mathrm{HCl}\), pH 7.4, 50 mM NaCl, and 5 mM EDTA. The tubes were kept in liquid nitrogen for 4 min and then in a 37°C water bath for 3 min. This freezing and thawing was repeated two more times. For experiments testing the effects of AD2765, varying concentrations of the compound (dissolved in 0.1% DMSO) were added, and the tubes were preincubated for 5 min at 37°C. BODIPY-C5 or -C12 ceramide (as a solution in 0.1% DMSO) was then added, and incubation at 37°C was continued for 30 min. Two milliliters of dichloromethane-methanol (1:1) was added to stop the reactions, the tubes were vortexed and centrifuged, and the upper phase was removed. The dichloromethane was evaporated under a stream of air, and the residue was dissolved in 50 \(\mu\)l of dichloromethane-methanol (1:1) and applied to a thin-layer chromatography plate. Standards of BODIPY-C5 or -C12 sphingomyelin and BODIPY-C5 or -C12 glucosylceramide were also applied. The plate was developed in dichloromethane-methanol-water (90:10:1), and the fluorescence of the respective sphingomyelin and glucosylceramide products was quantified using a Fuji FLA-2000 fluorometer.

**Protein assay.** Protein levels were quantified with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.
In situ assays

Inhibition of B12SM hydrolysis. Pulse/chase labeling of human skin fibroblasts was accomplished as described previously (31). Briefly, B12SM was incorporated into liposomes by mixing with dioleoyl phosphatidylcholine (DOPC; Sigma, St. Louis, MO) at a molar ratio of 1:4. The solvent was evaporated and the lipid mixture was resuspended in PBS, followed by sonication. Human skin fibroblasts were incubated with B12SM/DOPC liposomes (final concentration of 2-3 nmol/ml) at 37°C for 5 h. Labeling was terminated by removal and replacement of the medium with fresh growth medium lacking B12SM/DOPC liposomes. The cells were then incubated (i.e., “chased”) for a further 24–48 h before processing.

After pulse/chase labeling, the fibroblasts were harvested by trypsinization and centrifuged at 2,000 g, and the pellets were resuspended in phosphate-buffered saline. Aliquots of cells were taken for protein determination, and the remaining sample was processed as follows. Cells were centrifuged at 2,000 g, and the cell pellet was resuspended in 150 μl of chloroform-methanol (1:2, v/v). The resulting cell suspension was then sonicated for 5 min, followed by incubation at 55°C for 10 min and a further 2 min sonication. The solvent was evaporated, and the dried lipid extract was resuspended in 50 μl of ethanol. The hydrolytic product, B12CER, was then detected and quantified by HPLC analysis using a reversed-phase column (Aquasil C-18; Keystone Scientific, Inc.) as described above for the ASM activity assays.

Inhibition of sphingomyelin and glucosylceramide synthesis. To assess sphingomyelin and glucosylceramide synthesis in situ, the methods described by Dagan et al. (32) were used. A 2 ml suspension of Jurkat lymphoma cells (0.75 × 10⁶/ml) was incubated with or without AD2765 for 1 h in medium lacking fetal calf serum. B12CER (2.5 μM) was then added, and the incubation continued for an additional 3 h. Cells were sedimented by centrifugation and washed twice with PBS. To the cell pellet was added 1 ml of dichloromethane-ethanol (1:2), and after vortexing and centrifugation, the liquid supernatant was collected. One milliliter of dichloromethane-ethanol (1:1) was added to the residual pellet, which was then vortexed and recentrifuged, and the solvent was evaporated under a stream of air. The residue was dissolved in dichloromethane-methanol (1:1) and applied to a thin-layer chromatography silica gel plate. The latter was developed in dichloromethane-methanol-water (85:15:1.5), and the fluorescence of the B12SM and B12 glucosylceramide products was identified by comigration with standards and quantified using a Fuji FLA-2000 scanner.

Ceramide quantification

The total ceramide content in cells was determined by a previously published method (33). Briefly, a total lipid extract was prepared using organic solvents, air-dried, and resuspended in the nonionic detergent 0.2% Igepal CA-630. The ceramide present in these lipid extracts was then fully hydrolyzed into sphingosine using recombinant acid ceramidase, and the sphingosine was derivatized with naphthalene-2,3-dialdehyde and quantified by reverse-phase high-performance liquid chromatography.

Fluorescence microscopy

A fluorescent analogue of AD2765, FITC-AD2765, was added to the medium of normal skin fibroblasts grown on glass chamber slides at a concentration of 0.25 μM for 2 h. Cells were then fixed in 4% paraformaldehyde for 25 min at 4°C before three 5 min washes with PBS. Cells were then either immediately mounted in Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and analyzed or processed further for lysosomal-associated membrane protein-2 (LAMP-2) colocalization. To minimize the nonspecific reactivity of the anti-LAMP-2 antibodies, the fixed cells were blocked for 2 h at room temperature in PBS containing 10% normal goat serum and 0.1% Tween 20. After a brief rinse with PBS, cells were incubated with LAMP-2-specific primary antibody (purified mouse anti-human CD107B monoclonal antibody; BD Biosciences Pharmining, San Diego, CA) for 2 h at room temperature at a concentration of 1.25 μg/ml in PBS containing 5% normal goat serum and 0.1% Tween 20. The slides were then washed twice for 5 min each in PBS containing 0.1% Tween 20, followed by two 5 min washes in PBS, before incubation for 1 h at room temperature with secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes) at a concentration of 10 μg/ml. Slides were then washed three times for 5 min each in PBS containing 0.1% Tween 20, followed by two 5 min washes in PBS, before mounting as described above and analyzed.

Cell counting and trypan blue staining

Cell proliferation and viability were assessed through cell counting and trypan blue staining. Briefly, a small sample of a cell suspension was diluted 1:5 in 0.4% (w/v) trypan blue. The resulting suspension was applied to a hemocytometer, and cells were counted according to the manufacturer’s instructions. Cell viability was assessed as the percentage of cells incorporating the trypan blue dye.

RESULTS

Inhibition of ASM activity by AD2765

A novel thiourea derivative of sphingomyelin (Fig. 1B) was synthesized, and its ability to inhibit the hydrolysis of B12SM by recombinant human ASM was tested in vitro. As illustrated by the Dixon plots in Fig. 2A, this compound, designated AD2765, inhibited this reaction with an apparent inhibition constant (Ki) of ~70 μM. These plots also

![Fig. 1. Structures of sphingomyelin, AD2765, and FITC-AD2765. A: Structure of sphingomyelin. R represents the portion of sphingomyelin altered to produce AD2765. B: Structure of R in AD2765. C: Structure of R in FITC-AD2765.](image-url)
suggested that under these nonphysiological conditions, the inhibition of ASM was noncompetitive. Studies performed using \(^{14}\)C-labeled sphingomyelin as the substrate confirmed the inhibitory effect of AD2765 on the activity of pure ASM in vitro (data not shown).

We next incubated normal human skin fibroblasts with varying amounts of AD2765 for 48 h, washed the cells extensively, and then used cell extracts to determine ASM activity. As shown in Fig. 2B, significant inhibition of ASM activity was observed in the cell extracts, revealing that AD2765 had entered the cells and was present in the extracts. Because cells incubated with only 2 \(\mu\)M AD2765 for 48 h had a 50% inhibition of ASM activity, and the apparent \(K_i\) of this compound using the pure enzyme was \(\sim 70\ \mu M\) (Fig. 2A), this suggested that AD2765 was concentrating in the cells over the 48 h incubation period. Similar experiments also were performed to determine the effects of AD2765 on the activities of Mg\(^{2+}\)-dependent neutral sphingomyelinase and acid ceramidase. As shown in Fig. 3, the activity of Mg\(^{2+}\)-dependent neutral sphingomyelinase (Fig. 3A), but not acid ceramidase (Fig. 3B), was significantly inhibited by this compound.

**In situ inhibition of B12SM hydrolysis and epifluorescence microscopy**

We next carried out experiments to evaluate the ability of AD2765 to inhibit the hydrolysis of B12SM in situ. A pulse/chase experiment was conducted whereby the cells were pulsed with B12SM to allow the accumulation of the

![Fig. 2. A: Dixon plots showing the inhibition of human recombinant acid sphingomyelinase (ASM) activity by AD2765. All reactions contained 0.256 ng of purified ASM and varying concentrations of AD2765. Curves were generated at three different substrate concentrations ([BODIPY®-labeled C12-sphingomyelin (B12SM)]) concentrations (ranging from 0.01 to 0.1 mM). In vitro assays were performed as described in Materials and Methods. B: Inhibition of ASM activity in cell extracts. Normal human skin fibroblasts were incubated in the presence of AD2765 at the indicated concentrations for 48 h before harvesting and measurement of ASM activity in vitro. All reactions contained 1.5–3 \(\mu\)g of human fibroblast cell extract protein and 100 \(\mu M\) substrate. Data are presented as means ± SD (n = 3). *P < 0.05 by Student’s t-test. \(K_i\) inhibition constant.](https://www.jlr.org/content/jlr/46/9/2318/F2.large.jpg)

![Fig. 3. Inhibition of Mg\(^{2+}\)-dependent neutral sphingomyelinase and acid ceramidase activities in cell extracts. Normal human skin fibroblasts were incubated in the presence of AD2765 at the indicated concentrations for 48 h before harvesting and measurement of Mg\(^{2+}\)-dependent neutral sphingomyelinase (A) and acid ceramidase (B) activities in vitro. In vitro assays were performed as described in Materials and Methods. All reactions contained 1.5–3 \(\mu\)g of human fibroblast cell extract protein and 100 \(\mu M\) substrate. Data are presented as means ± SD (n = 3). *P < 0.05, **P < 0.01 by Student’s t-test.](https://www.jlr.org/content/jlr/46/9/2318/F3.large.jpg)

![Fig. 4. Inhibition of B12SM hydrolysis as measured by pulse/chase in situ labeling. Normal human skin fibroblasts were incubated with B12SM/dioleoyl phosphatidylcholine (DOPC) liposomes in growth medium for 5 h (pulse) before the addition of fresh medium lacking B12SM/DOPC liposomes. The cells were then incubated for a further 48 h (chase) in medium with or without AD2765 (10 \(\mu M\)). Total lipids were then extracted, and BODIPY®-labeled C12-ceramide formation was detected as described in Materials and Methods. Data are presented as means ± SD (n = 3). *P < 0.05 by Student’s t-test.](https://www.jlr.org/content/jlr/46/9/2318/F4.large.jpg)
fluorescent substrate within the cells, followed by a 48 h chase period in which the medium was changed to medium lacking B12SM. Quantification of the product (B12CER) formed in situ was determined by extraction of total lipids and analysis using the HPLC-based detection method described (33). As shown in Fig. 4, when 10 μM AD2765 was included in the chase medium, the amount of B12CER formed was reduced substantially, confirming the ability of this compound to enter cells and inhibit sphingomyelinase activity in situ.

Because the experiment described above monitored the hydrolysis of B12SM only, and previous studies have indicated that this fluorescent lipid is trafficked principally to lysosomes (34), the fact that AD2765 could inhibit this reaction suggested that this compound was delivered, at least in part, to this compartment. To evaluate this further, a FITC derivative of sphingomyelin was prepared, and its uptake and localization in cells were examined by fluorescence microscopy (Fig. 5). Although the FITC derivative was not chemically identical to AD2765 (Fig. 1), the results revealed the rapid uptake of this compound and delivery to LAMP-2-positive compartments (i.e., lysosomes and/or late endosomes), consistent with the cell experiments described above. It is important to note that because of the high fluorescence of the FITC compound, low concentrations (0.25 μM) were used for the microscopy experiments. It is possible that at higher concentrations the compound might be delivered to additional compartments, but this could not be confirmed.

**Inhibition of sphingomyelin synthesis**

To further investigate the effects of AD2765, we next performed an experiment in which normal skin fibroblasts (data not shown) and Jurkat lymphoma cells were incubated with BODIPY-conjugated C3 ceramide, and the ability to form BODIPY-C3 sphingomyelin was evaluated after 4 h. Surprisingly, as shown in Fig. 6A, this synthetic reaction also was inhibited when 10 μM AD2765 was included in the chase medium. In fact, even low concentrations (2 μM) of AD2765 inhibited the formation of sphingomyelin.

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**Fig. 5.** Localization of FITC-AD2765 in normal human skin fibroblasts. Normal human skin fibroblasts were incubated in the presence of 0.25 μM FITC-AD2765 for 2 h. Cells were then either fixed and mounted in Vectashield® mounting medium plus 4',6-diamidino-2-phenylindole (DAPI) and immediately analyzed by epifluorescence microscopy or also incubated with anti-lysosomal-associated membrane protein-2 (LAMP-2) antibodies (α-LAMP-2) before analysis. Colocalization is indicated by yellow in the merged image of the green (FITC-AD2765) and red (α-LAMP-2). Two different fields are shown at two different magnifications. Negative controls lacked either FITC-AD2765 or primary anti-LAMP-2 antibodies.
by ~70% (data not shown). Within this time frame (4 h), no effects on cell growth were observed when AD2765 was included in the culture medium. Notably, the same inhibitory effects on sphingomyelin synthesis were seen when BODIPY-C12 ceramide was used instead of BODIPY-C3 ceramide (data not shown).

To confirm these in situ observations, the ability of AD2765 to inhibit the synthesis of BODIPY-C3 sphingomyelin from BODIPY-C3 ceramide in vitro was tested using extracts prepared from Jurkat cells. As shown in Fig. 6B, these results also showed that sphingomyelin synthesis was inhibited by AD2765. Of note, HL-60 cell extracts similarly were used to confirm the in vitro effects on sphingomyelin synthesis, as well as to test the effects on the synthesis of glucosylceramide. As shown in Fig. 7, AD2765 inhibited BODIPY-C3 sphingomyelin synthesis from BODIPY-C3 ceramide in HL-60 cell extracts, but not the synthesis of BODIPY-C3 glucosylceramide.

**Effect of AD2765 on ceramide levels and cytotoxicity**

Incubation of normal human skin fibroblasts with 20 μM AD2765 for 24 h led to a significant increase in total ceramide, whereas only modest increases were observed using 10 μM AD2765 (Fig. 8). One potential source of this ceramide is inhibition of sphingomyelin synthesis, as described above, but it also was possible that AD2765 could inhibit ceramidase activity, leading to a similar increase. However, using pure acid ceramidase (data not shown) or cell extracts (Fig. 3B), we observed no inhibition of ceramidase activity by AD2765, further revealing the specificity of its effects for enzymes involved in sphingomyelin metabolism.

Fig. 6. Inhibition of sphingomyelin synthesis by AD2765. A: In situ analysis. Jurkat lymphoma cells were incubated in the presence of 10 μM AD2765 for 4 h before harvesting and measurement of BODIPY-C3 ceramide conversion to BODIPY-C3 sphingomyelin (SM) as described in Materials and Methods. Data are presented as means ± SD (n = 3). * P < 0.01 by Student’s t-test. B: In vitro analysis. Jurkat cells were homogenized, and cell extracts were prepared and used to test the inhibitory effect of 10 μM AD2765 on the conversion of BODIPY-C3 ceramide to sphingomyelin in vitro. Data are presented as means ± SD (n = 3). * P < 0.01 by Student’s t-test.

Fig. 7. Inhibition of sphingomyelin and glucosylceramide synthesis in HL-60 cells by AD2765. Varying concentrations of AD2765 were added to the HL-60 cell extracts, which were then incubated in the presence of BODIPY-conjugated C3 ceramide. The conversion to BODIPY-C3 sphingomyelin (squares) and BODIPY-C3 glucosylceramide (diamonds) was then determined after 4 h. Data are presented as means ± SD (n = 3).

Next, normal human skin fibroblasts, human skin fibroblasts from a patient with ASM-deficient NPD, and a human malignant Glioma cell line (M059J) were incubated with AD2765 and the effects on cell viability were examined at 24 and 48 h (Fig. 9). As can be seen, this compound inhibited cell growth and led to cell death in each of the three cell lines tested in a concentration-dependent manner. Of note, 10 μM AD2765 did not cause significant cell death in these cells (although 20 μM did), consistent with a much lower production of cellular ceramide (Fig. 8). Because AD2765 caused cell death in ASM-deficient NPD cells as well as in cells expressing ASM, the cell death phenotype was not dependent on the stimulation of an ASM-dependent signaling pathway. Overall, these results...
suggested that when cells were grown in the presence of >10 μM AD2765 for 24 h or longer, the principal in situ effect was likely to be an increase of cellular ceramide and cell death.

**DISCUSSION**

The hydrolysis of sphingomyelin by cellular sphingomyelinases leads to the production of ceramide, a potent lipid second messenger that mediates apoptosis through the modulation of lipid raft structures (16, 17, 35). Ceramide is also the precursor of most major sphingolipids, including many other important second messenger molecules such as sphingosine, sphingosine 1-phosphate, and ceramide 1-phosphate (for review, see 36). Therefore, the regulation of ceramide levels is critical to cell survival and the prevention of disease, and this importance has led to the production of agents capable of modulating these levels, such as inhibitors of sphingomyelinases and ceramidases.

The syntheses of several sphingomyelinase inhibitors have been reported previously (20, 21, 23). Such inhibitors could potentially be used to alter the growth of cancer cells, modulate inflammation, or, in the case of ASM inhibitors, as molecular chaperones for the treatment of...
NPD. Here, we describe the synthesis and characterization of a novel sphingomyelin analogue (AD2765) and show that it can inhibit the hydrolysis of B12SM in vitro and in living cells. Moreover, in vitro and in situ studies also revealed that AD2765 could inhibit sphingomyelin synthesis as well. Importantly, these effects were specific for enzymes involved in sphingomyelin metabolism, because AD2765 had no effect on acid ceramidase activity or the synthesis of glucosylceramide.

Initial characterization of the inhibitory effects of AD2765 on sphingomyelin hydrolysis involved kinetic analysis using purified, recombinant human ASM. This analysis showed that the $K_i$ of AD2765 was $\approx 70 \mu M$ and suggested that the inhibition under these in vitro conditions was noncompetitive. We also investigated the ability of AD2765 to inhibit the hydrolysis of B12SM in situ using a pulse/chase experiment. These results confirmed the inhibitory effect of this compound on sphingomyelin hydrolysis. We also performed studies to assess the specificity of this reaction. As shown in Fig. 2A, AD2765 inhibited not only the activity of ASM in cell extracts, but also the activity of Mg$^{2+}$-dependent neutral sphingomyelinase. Notably, there was no effect on the activity of acid ceramidase using either fibroblast extracts or pure enzyme. Thus, AD2765 appeared to have a broad effect on sphingomyelin hydrolysis in vitro and in situ but no effect on ceramide hydrolysis. Although the in situ studies do not reveal which sphingomyelinase(s) were inhibited in living cells, because cultured skin fibroblasts contain low levels of neutral sphingomyelinase activity compared with ASM and it has been shown previously that B12SM primarily traffics to lysosomes (31, 34), we predicted that most of this in situ effect was attributable to the inhibition of ASM.

These observations were confirmed by fluorescence microscopy using a FITC derivative of AD2765. Although, based on its structure (Fig. 1), the FITC derivative is likely to have somewhat different properties compared with AD2765, we found that, like AD2765, it also inhibited the activity of recombinant human ASM in vitro (whereas FITC alone did not; data not shown) and was primarily trafficked to lysosomes (Fig. 5). It is important to note that because of the high fluorescence of this compound, it was used at very low concentrations for the microscopic analyses. Thus, we cannot conclude from these studies that the only site of delivery is the lysosome, because it is possible that when higher amounts are added to cells it can be trafficked to additional compartments. Indeed, the experiments assessing the effects on sphingomyelin synthesis presented in Figs. 6, 7 (and discussed below) confirm this hypothesis.

In addition to the sphingomyelinase inhibitory activity, it also was noted that high concentrations (e.g., $>10 \mu M$) of AD2765 inhibited cell growth and induced cell death in cultured fibroblasts and human M059J Glioma cells. Reduction of serum levels was found to exacerbate this cytotoxic effect (data not shown). These results were surprising in that we predicted that a sphingomyelinase inhibitor such as this would lead to reduced ceramide production, thereby removing a potential apoptotic and growth inhibitory signal. In contrast, cells treated with 20 $\mu M$ AD2765 had an overall increase in ceramide levels (Fig. 8). We considered three possible mechanisms to explain the observed effects. First, it is possible that AD2765 was hydrolyzed by ASM within the lysosomal compartment, leading to the production of a ceramide analogue that could activate ceramide-mediated apoptotic pathways. However, using pure, recombinant ASM in our standard in vitro assay system, we did not find any evidence of AD2765 hydrolysis (data not shown). A second possible mechanism underlying the cytotoxicity stems from evidence suggesting an important role for lysosomal proteases in the induction of apoptosis (for review, see 37). For example, a recent report demonstrated the induction of apoptosis through the release of lysosomal, papain-like cysteine proteases into the cytoplasm after disruption of the lysosome (38). Thus, it is possible that AD2765 led to the disruption of lysosomal membranes and the release of cytotoxic compounds into the cytoplasm. Finally, because AD2765 was a sphingomyelin analogue, it was possible that this compound inhibited sphingomyelin synthesis as well as degradation, thereby leading to an overall increase in ceramide levels.

To further clarify the mechanism underlying the cytotoxicity of AD2765, human fibroblasts derived from an ASM-deficient NPD patient were incubated with increasing concentrations of the compound, and it was found that it caused growth inhibition and cell death in a similar manner to that observed in the normal skin fibroblasts. This revealed that the cytotoxic effects were not ASM-dependent. We next examined the ability of AD2765 to inhibit sphingomyelin synthesis using an in situ assay measuring the conversion of BODIPY-conjugated C3 and C12 ceramide into BODIPY sphingomyelin. Indeed, within 4 h, these reactions were markedly inhibited in the presence of AD2765, suggesting that this may be a likely explanation for the observed effects, alone or perhaps in combination with the disruption of lysosomal membranes. These in situ results were confirmed in vitro using cell extracts, suggesting a direct inhibition on the enzymes involved in sphingomyelin synthesis, rather than an effect on ceramide trafficking. Also, despite a profound effect on sphingomyelin synthesis, there was no effect of AD2765 on the synthesis of glucosylceramide from BODIPY-conjugated ceramide, further revealing the specificity of this compound for enzymes involved in sphingomyelin metabolism.

Clearly, further work is required to determine the exact mechanisms involved in the observed effects on cell proliferation and death. An important factor is likely the subcellular site(s) to which this compound gains access. Our in situ results on the inhibition of sphingomyelin hydrolysis and immunofluorescence localization studies show that the compound is delivered, at least in part, to lysosomes. However, it is again important to note that these studies do not exclude the possibility of delivery to other compartments. Therefore, we hypothesize that after administration to cells, a pool of compound can gain access to sites of sphingomyelin synthesis, as well as degradation, and it is presumably this former pool that leads to ceramide accumulation and cell death. The amount of compound that
gains access to these alternative sites is likely dependent on the amount of compound the cells are exposed to, as well as on how the compound is formulated and presented.

Of note, treatment of cells with 10 µM AD2765 did not lead to increased ceramide or cause significant cell death (Fig. 9), despite the fact that this amount of compound inhibited sphingomyelin synthesis profoundly. One potential hypothesis to explain these apparently contradictory observations is that the majority of ceramide produced by 10 µM AD2765 is reused in other synthetic pathways. In contrast, the amount of ceramide produced by 20 µM AD2765 may saturate these “reutilization” pathways, leading to an overall increase in cellular ceramide and cell death. Also of note, the cytotoxic effect was exacerbated by reduced serum, again suggesting that how the compound is presented to cells (e.g., in complex with serum lipoproteins) might affect where it is delivered and what effect(s) it has. Indeed, ample evidence exists in the literature showing that how sphingolipids or their analogues are presented to cells may affect their localization and metabolism in cells (39, 40).

In summary, this study describes the synthesis and characterization of a novel sphingomyelin analogue that had multiple effects on sphingomyelin metabolism in situ. The specificity of this compound for enzymes involved in sphingomyelin metabolism was demonstrated by the lack of effect on acid ceramidase activity and the synthesis of glucosylceramide. Moreover, treatment of cells with >10 µM AD2765 led to an increase in ceramide and cell death through a mechanism that was independent of ASM activity. Therefore, AD2765 may represent a suitable compound for future studies aimed at modulating sphingomyelin and ceramide levels in living cells and animals, and may have differential effects dependent on how the compound is presented to cells and to which intracellular compartment(s) it gains access.}

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