A Novel Inhibitor of Ceramide Trafficking from the Endoplasmic Reticulum to the Site of Sphingomyelin Synthesis*

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Ceramide produced at the endoplasmic reticulum (ER) is transported to the lumen of the Golgi apparatus for conversion to sphingomyelin (SM). N-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide (HPA-12) is a novel analog of ceramide. Metabolic labeling experiments showed that HPA-12 inhibits conversion of ceramide to SM, but not to glucosylceramide, in Chinese hamster ovary cells. Cultivation of cells with HPA-12 significantly reduced the content of SM. HPA-12 did not inhibit the activity of SM synthase. The inhibition of SM formation by HPA-12 was abrogated when the Golgi apparatus was made to merge with the ER by brefeldin A. Moreover, HPA-12 inhibited redistribution of a fluorescent analog of ceramide, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)-N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-o-erythro-sphingosine (C6-DBMB-Cer), from intracellular membranes to the Golgi region. Among four stereoisomers of the drug, (1R,3R)-HPA-12, which resembles natural ceramide stereochemically, was found to be the most active, although (1R,3R)-HPA-12 did not affect ER-to-Golgi trafficking of protein. Interestingly, (1R,3R)-HPA-12 inhibited conversion of ceramide to SM little in mutant cells defective in an ATP- and cytosol-dependent pathway of ceramide transport. These results indicated that (1R,3R)-HPA-12 inhibits ceramide trafficking from the ER to the site of SM synthesis, possibly due to an antagonistic interaction with a ceramide-recognizing factor(s) involved in the ATP- and cytosol-dependent pathway.

Sphingolipids are ubiquitous constituents of membrane lipids in mammalian cells and play important roles in cell growth, differentiation, and apoptosis (1–3). Moreover, together with cholesterol, sphingolipids in plasma membrane constitute detergent-resistant microdomains termed lipid rafts, which are involved in membrane trafficking and cell signaling (4, 5).

De novo biosynthesis of sphingolipids in mammalian cells proceeds as follows (6). The first step is the condensation of L-serine and palmitoyl-CoA, a reaction catalyzed by serine palmitoyltransferase, to generate 3-ketodihydroporphosphine, which is reduced to dihydrosphingosine. Dihydrosphingosine undergoes N-acylation followed by desaturation to generate ceramide (Cer).1 These reactions to produce Cer occur at the cytosolic surface of the endoplasmic reticulum (ER) (7–9). Then, Cer is delivered to the luminal side of the Golgi apparatus and converted to sphingomyelin (SM) by SM synthase catalyzing the transfer of phosphocholine from phosphatidylcholine to Cer (10, 11). Cer is also converted to glucosylceramide (GlCer) by GlCer synthase catalyzing the transfer of glucose from UDP-glucose to Cer. Although the catalytic site of GlCer synthase appears to be oriented to the cytoplasm (12, 13), it is controversial whether GlCer synthase is localized to the Golgi apparatus or more broadly distributed to microsomes. After translocation to the luminal side of the Golgi apparatus, GlCer is further converted to more complex glycosphingolipids (14).

We have previously showed that there are two pathways for transport of de novo synthesized Cer from the ER to the site of SM synthesis in various types of cultured cells, including Chinese hamster ovary (CHO) cells, HeLa cells, and human skin fibroblasts (15). The main pathway of the two is ATP-dependent, and the minor pathway is ATP-independent (or less dependent). In contrast to the synthesis of SM, the access of Cer to the site of GlCer synthesis is largely independent of ATP. Analysis by in vitro assay of Cer transport in semi-intact CHO cells further revealed that the ATP-dependent transport of Cer, from the ER to the site of SM synthesis, requires cytosol but that cytosol is not required for transport of Cer to the site of GlCer synthesis (16). In addition, that a CHO cell mutant defective in the ATP-dependent pathway of Cer transport exhibits no defect in transport of proteins from the ER to the Golgi apparatus has suggested that the mechanism of Cer transport differs from that of ER-to-Golgi trafficking of proteins (15).

Inhibitors of de novo sphingolipid biosynthesis are useful tools to investigate the metabolism and functions of sphingolipids in cultured cells and in living animals. Various natural and chemically synthesized compounds have been found to be

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1 The abbreviations used are: Cer, ceramide; ER, endoplasmic reticulum; SM, sphingomyelin; GlCer, glucosylceramide; CHO, Chinese hamster ovary; HPA, N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)alkanamide; C6-NBD-Cer, 6-[N-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)-amino]caproyl-o-erythro-sphingosine; C6-DMB-Cer, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-o-erythro-sphingosine; C6-Cer, N-palmitoyl-o-sphingosine; PBS, phosphate-buffered saline; BFA, brefeldin A; G418, N-acetylenauraminyl lacto-syleceramide; PLAP, human placental alkaline phosphatase; PLAP-HA, a chimera of human placental alkaline phosphatase with influenza hemagglutinin; Endo H, endoglycosidase H; SMase, sphingomyelinase; t-c-MAPP, (1S,2R)-o-erythro-2-(N-myristoylamino)-1-phenyl-1-propionol; BSA, bovine serum albumin; NBS, newborn bovine serum; PC, phosphatidylcholine; GPI, glycosylphosphatidylinositol; DMB, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene.
highly selective, even if not strictly specific, inhibitors of sphingolipid synthesis. For example, sphingofungins and ISP-1 (myrici) appear to be specific and potent inhibitors of serine palmitoyl transferase (17, 18). Fumonisins seem to inhibit (di- hydro)sphingosine-N-acyltransferase specifically (19). t-threo- 1-Phenyl-2-decanoylamino-3-morpholino-1-propanol and some of its derivatives selectively inhibit GlcCer synthase (20, 21).

However, no specific inhibitor for SM synthesis in mammalian cells has been found so far. In addition, no drug that inhibits intracellular trafficking of sphingolipids has been discovered, except for broad-spectrum inhibitors such as brefeldin A and energy poisons.

In the present study, we showed that N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide (HPA-12), a novel analog of Cer, inhibits ATP-dependent transport of Cer from the ER to the site of SM synthesis without inhibition of protein trafficking, thereby inhibiting conversion of Cer to SM, but not to GlcCer, in cells. Moreover, a stereoisomer having the 1R,3R configuration was found to be the most active among the four stereoisomers of HPA-12.

**EXPERIMENTAL PROCEDURES**

**Materials—** t-[(14)C]Serine (155 mCi/mmol) and (methyl-[(14)C]choline (55 mCi/mmol) were purchased from Amersham Pharmacia Biotech. [N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-D-[3H]Choline (C5-DMB-Cer) and N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-sindacene-3-pentanoyl)-tethyro-sphingosine (C6-NBD-Cer) were from Molecular Probes, Inc. t-erythro-[3H]Sphingosine (20 Ci/mmol), [palmitoyl-t-[3H]palmitoyl]-t-[14C]choline (55 mCi/mmol) and [choline- 14C]-phosphorylcholine (50 mCi/mmol) were from American Radiolabeled Chemicals, Inc. t-erythro-Sphingosine was from Matrana, Inc. Fumonisin B1, brefeldin A (BFA), and fatty acid-free bovine serum albumin were from Sigma Chemical Co. TLC and high performance TLC plates (Silica Gel 60) were from Merck. Dimethyl sulfoxide (MeSO) was from Wako Pure Chemical Industries, Inc (Osaka, Japan). (1S,2R)-t-erythro-2- (N-Myristoylaminio)-1-phenyl-1-propanol (t-MAPP) was from BIOMOL Research Laboratories, Inc.

N-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl)alkanamides (HPA-3, HPA-8, HPA-12, and HPA-16) were prepared based on three component Mannich-type reactions. For the synthesis of enaminites, catalytic asymmetric Mannich reactions were performed. Details will be described elsewhere.2

**Cells and Cell Culture—** The CHO cell line was obtained from the American Type Culture Cell Collection (ATCC CCL 61). Cells were routinely maintained in Ham’s F12 medium supplemented with 10% newborn bovine serum (NBS), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) at 33 °C. CHO cells were cultured in Ham’s F12 medium supplemented with 10% NBS and cultured at 33 °C overnight. After being washed twice with 10 ml of serum-free F12 medium, the cells were incubated in 20 ml of F12 medium containing 10% NBS and cultured at 33 °C. Each medium was replaced every 24 h. The cells were washed twice with 10 ml of PBS, harvested by scraping, and precipitated by centrifugation. Lipids were extracted from the harvested cells and resuspended in PBS (23). For phospholipids, extracted lipids were separated on TLC plates with a solvent of chloroform/methanol/acetate/H2O (25:15:4:2, v/v), and the phosphorous content of phospholipids was measured by the method of Rouser et al. (24). GlcCer and N-acetylneuraminyl lactosylceramide (GcNAc) contents were determined by densitometric analysis of lipids stained with Coomassie Brilliant Blue as described previously (25). The amounts of Cer were determined by using an sn-1,2-diaclyglycerol assay reagent system (Amersham Pharmacia Biotech).

**Enzyme Assays—** The membrane fractions prepared from CHO cells and the membrane brain were used as the enzyme sources. CHO cells cultured in spinner bottles were homogenized in buffer A (10 mm Tris-HCl buffer (pH 7.4) containing 0.25 m sucrose) with a stainless ball homogenizer as described previously (16). After the removal of nuclei and unbroken cells by centrifugation (900 × g for 10 min), the post nuclear supernatant of the cell homogenate was centrifuged (10,000 × g for 60 min). The precipitate as the membrane fraction was suspended in buffer A (7 μg of protein/ml), and stored at −80 °C until use. Bovine brain membranes were prepared as described previously (26).

Enzyme assays of SM synthase activity were carried out by the modifications of a previously described method (27). In brief, CHO cell membranes (400 μg of protein) were incubated in 800 μl of 10 mM Hepes-NaOH buffer (pH 7.5) containing 2 mM EDTA, and 1 μg of C5-DMB-Cer or C6-NBD-Cer complexed with BSA at 33 °C for 10 min. Because exogenous [14C]C5-Cer is a poor substrate for SM synthase due to its water insolubility, 0.1% Triton X-100 was added to the reaction buffer and the reaction period was prolonged to 1 h when [14C]C5-Cer was used as the substrate. The radioactivity of [14C]SM that formed after TLA of extracted lipids. Assays of acid and neutral sphingomyelase (SMase) activities were performed as described previously (28). Briefly, for the acid SMase activity, CHO cell membranes (5 μg of protein) were incubated in 50 μl of 0.1 mM sodium acetate buffer (pH 4.8) containing 10 μM [3H]S1M, 0.2% β-oc tylgycoside, and 0.1% Triton X-100 at 37 °C for 30 min. For the assays of neutral SMase activity, bovine brain membranes (5 μg of protein) were incubated in 50 μl of 50 mM Hepes-NaOH (pH 7.5) containing 10 μM [3H]S1M, 0.2% β-oc tylgycoside, and 0.1% Triton X-100, 10 mM MgCl2 and

2 Ueno, M., Kitagawa, H., Ishitani, H., Yasuda, S., Hanada, K., and Kobayashi, S., Tetrahedron Lett. in press.
3.0 mutants expressing PLAP or PLAP-HA (30) were seeded at a density of 10^5 cells per 35-mm dish in 1 ml of F12 medium containing 10% NBS and cultured at 33 °C overnight. The cell monolayers were incubated in 500 μl of F12 medium supplemented with 10% NBS and cultured at 33 °C overnight. The cell monolayers were incubated in 500 μl of F12 medium containing various concentrations of HPA-12 at 33 °C overnight. The viability of cells was determined by using 4-(3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide as described previously (31).

**Determination of Protein Concentration**—Protein concentrations were determined with the Pierce BCA assay reagent kit, using BSA as the standard.
RESULTS

Effects of N-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl)alkanamides on de Novo SM Synthesis in CHO Cells—A series of N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)alkanamides (HPAs), novel analogs of Cer, were chemically synthesized (Fig. 1A). To examine the effects of these drugs on the de novo biosynthesis of sphingolipids in mammalian cells, CHO cells were incubated with [14C]serine in the presence or absence of 2.5 μM of each drug for 2 h at 33 °C. HPAs inhibited the de novo synthesis of sphingolipids, depending on the length of the N-acyl chains of the drugs. HPA-12, the dodecanoyl derivative, inhibited [14C]SM formation to ~10% of the drug-free control level (Fig. 1B). Other HPAs, in which the carbon atom numbers of the acyl chain is 3, 8, and 16, had far less of an inhibitory effect than HPA-12 (Fig. 1B).

At 2.5 μM, these drugs did not affect the viability of cells (data not shown, see also below).

HPA-12 significantly inhibits the formation of [14C]SM even at 0.1 μM, and the inhibitory effect reaches a plateau around 1 μM (Fig. 2A). HPA-12 at more than 10 μM was highly toxic to CHO cells (data not shown), not allowing us to carry out metabolic labeling experiments at more than 10 μM of this drug.

The time course of metabolic labeling up to 5 h showed that 1 μM HPA-12 inhibited formation of [14C]SM to <30% of the drug-free control level throughout the incubation period (Fig. 2B). HPA-12 did not affect the formation of [14C]phosphatidylserine (PS) or [14C]phosphatidylethanolamine (PE), both of which are metabolically labeled with [14C]serine via a pathway distinct from sphingolipid synthesis (Figs. 1B and 2B), indicat-
ing that the inhibition of sphingolipid synthesis by HPA-12 was not due to a nonspecific dysfunction of lipid metabolism. HPA-12 moderately inhibited the formation of \[^{14}\text{C}]\text{Cer}\text{ and } [^{14}\text{C}]\text{GlcCer}, although its inhibitory effect on GlcCer and Cer synthesis was weaker than that on SM synthesis (Figs. 1B and 2B).

To examine effects of HPA-12 on the turnover of de novo synthesized SM, we pulse-labeled CHO cells with \[^{14}\text{C}]\text{serine}\ for 2 h at 33 °C, and chased for 24 h at 33 °C in the presence of 10 mM non-radioactive serine with or without 1 μM HPA-12. The level of cell-associated \[^{14}\text{C}]\text{SM}\ was not affected by HPA-12 throughout the chase (Fig. 3A). In addition, HPA-12 did not affect the activities of acid or neutral sphingomyelinases in vitro at 20 μM (Fig. 3, B and C). These results eliminated the possibility that HPA-12 accelerated degradation of SM.

**HPA-12 Inhibits Conversion of Cer to SM in Intact Cells**—When conversion of Cer to SM is inhibited, de novo synthesis of sphingoid bases seems to be suppressed, as suggested previously (15). Thus, the moderate inhibition of the formation of \[^{14}\text{C}]\text{serine-derived Cer and GlcCer by HPA-12 might be due to a secondary effect of this drug on inhibition of Cer-to-SM conversion.** To examine effects of HPA-12 on the step of Cer-to-SM conversion specifically, we employed another protocol for metabolic labeling. When CHO cells are incubated with \[^{3}\text{H}]\text{sphingosine}\ at 15 °C, \[^{3}\text{H}]\text{sphingosine is efficiently N-acylated to generate }[^{3}\text{H}]\text{Cer without further conversion of }[^{3}\text{H}]\text{Cer to }[^{3}\text{H}]\text{SM in intact cells (16). After pulse labeling with }[^{3}\text{H}]\text{sphingosine at 15 °C for 30 min, cells were treated with 1 μM HPA-12 at 4 °C for 15 min and further incubated at 33 °C for up to 1 h for chase in the presence of fumonisin B1, an inhibitor of sphingosine N-acyltransferase. Under these pulse and chase conditions, 1 μM HPA-12 inhibited the formation of }[^{3}\text{H}]\text{SM by }\sim\text{50%}, whereas it did not inhibit but rather slightly increased the formation of }[^{3}\text{H}]\text{GlcCer (Fig. 4). The level of }[^{3}\text{H}]\text{Cer gradually decreased concomitantly with the formation of }[^{3}\text{H}]\text{SM and }[^{3}\text{H}]\text{GlcCer during chase, and the rate of decrease was slower in HPA-12-treated cells than in drug-free control cells, although the initial levels of }[^{3}\text{H}]\text{Cer at the start of the chase were almost identical between the two (Fig. 4). These results indicated that HPA-12 inhibits conversion of Cer to SM, but not to GlcCer, in intact cells.**

SM is synthesized by the transfer of phosphocholine from phosphatidylcholine (PC) to Cer, a reaction catalyzed by SM synthase (19). Metabolic labeling experiments with \[^{14}\text{C}]\text{choline}\ showed that treatment of cells with 1 μM HPA-12 did not inhibit formation of \[^{14}\text{C}]\text{PC}\ but that the level of \[^{14}\text{C}]\text{SM}\ in drug-treated cells was about half of the control level (Fig. 5). Therefore, inhibition of Cer-to-SM conversion by HPA-12 was not due to inhibition of PC synthesis. HPA-12 itself was unlikely to be converted to choline-containing metabolites, because HPA-12-dependent generation of radioactive lipids was not detected in TLC patterns of \[^{14}\text{C}]\text{choline-labeled lipids of one-dimensional (Fig. 5) or two-dimensional (data not shown) analysis.**

The Content of SM, but Not Of Glycosphingolipids, in Cells Is Reduced by Cultivation with HPA-12—We determined chemical amounts of various lipids in cells cultured in a sphingolipid-free medium in the presence or absence of 2.5 μM HPA-12 at 33 °C for 2 days (Table I). HPA-12 did not affect levels of total phospholipids: The contents of total phospholipids in untreated and drug-treated cells were 288 ± 3 and 315 ± 2 nmol/mg of protein, respectively. However, the content of SM in HPA-12-treated cells was lower by ~30% than the drug-free control level, and the reduction in SM in HPA-12-treated cells seemed to be compensated by an increase in the amount of PC (Table I). HPA-12 did not influence contents of other major glycerophospholipid types. In contrast, HPA-12 treatment increased the

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**Table I**

Effects of HPA-12 on phospholipid composition and sphingolipid contents in CHO cells

CHO cells were cultured in 20 ml of Nutridoma medium for 2 days in the absence or presence of 2.5 μM HPA-12, and the medium was replaced every 24 h. Total lipids of cells were extracted and separated by TLC, and the amounts of the indicated phospholipids, \(G_{\text{MAX}}\text{ GlcCer, and Cer, were determined as described under "Experimental Procedures." Values are expressed as means ± S.D. from three experiments.**

| HPA-12 | Phospholipid composition\(^{a}\) | % of total phospholipids recovered | \(G_{\text{MAX}}\) | GlcCer | Cer |
|--------|---------------------------------|----------------------------------|----------------|-------|-----|
| None   | SM 12.0 ± 0.1                   | 43.3 ± 0.1                       | 31.5 ± 0.2     | 0.9 ± 0.1 | 0.45 ± 0.04 |
| 2.5 μM | SM 8.4 ± 0.1                    | 47.5 ± 0.2                       | 30.6 ± 0.3     | 8.5 ± 0.8 | 1.4 ± 0.1 |

\(^{a}\) PC, phosphatidylcholine; PI/PS, phosphatidylinositol and phosphatidylserine; PE, phosphatidylethanolamine.
content of GlcCer and GM₃ to ~150% and ~120%, respectively, of the untreated control level (Table I). There was no significant difference in the content of Cer between the two groups (Table I). These results were another line of evidence that the primary target of HPA-12 is the step of conversion of Cer to SM.

HPA-12 Is Not an Inhibitor of SM Synthase—We determined whether HPA-12 inhibited the activity of SM synthase *in vitro* by using fluorescent short-chain analogs of Cer (C₆-NBD-Cer and C₅-DMB-Cer) and a radioactive long-chain Cer ([¹⁴C]C₁₆-Cer) as enzyme substrates. HPA-12 did not inhibit conversion of these substrates to their SM metabolites in isolated membranes even at a high concentration (20 µM) (Table II). In addition, 20 µM HPA-12 did not affect the activity of serine palmitoyl transferase, sphingosine-N-acyltransferase, or GlcCer synthase (data not shown). Therefore, HPA-12 is not an inhibitor of the key enzymes involved in *de novo* sphingolipid synthesis.

**HPA-12 Does Not Inhibit SM Synthase in BFA-treated Cells**—We next examined the possibility that HPA-12 affected transport of Cer from the site of its synthesis to the site of SM synthesis. If this is the case, fusion of the ER (where Cer synthase exists) with the Golgi apparatus (where SM synthase exists) should suppress the inhibitory effect of HPA-12 on Cer-to-SM conversion in intact cells. Actually, when CHO cells were pretreated with BFA, which merges the ER and the Golgi apparatus (32), 1 µM HPA-12 no longer inhibited the formation of [¹⁴C]serine-derived SM (Fig. 6). In BFA-treated cells, 1 µM HPA-12 did not affect the levels of labeled Cer, GlcCer, [¹⁴C]phosphatidylserine, or [¹⁴C]phosphatidylethanolamine either (Fig. 6). The abrogation of HPA-12-mediated inhibition of SM synthesis in BFA-treated cells was also observed in semi-intact cells, whose plasma membrane was perforated. When conversion of [³H]Cer to [³H]SM was analyzed by using a semi-intact CHO cell system (16), 2.5 µM HPA-12 was found not to inhibit Cer-to-SM conversion in BFA-treated perforated cells, whereas the drug inhibited the conversion by ~60% in control perforated cells untreated with BFA.

**Effects of HPA-12 on Redistribution and Metabolism of C₅-DMB-Cer in Cells**—The activity to transport natural Cer from the ER to the Golgi apparatus can be qualitatively assessed from analysis of the redistribution of C₅-DMB-Cer, a fluorescent Cer analog, from the ER to the Golgi apparatus (15, 33). To obtain another line of evidence that HPA-12 inhibited Cer transport, we examined the effects of HPA-12 on the behavior of C₅-DMB-Cer in intact CHO cells (Fig. 7, A–D). For pulse labeling of various intracellular membranes, including the ER, cells were incubated with C₅-DMB-Cer at 4 °C for 30 min and incubated in the presence or absence of 2.5 µM HPA-12 at 4 °C for a further 15 min. Then the pulse-labeled cells were chased at 33 °C for 15 min for redistribution of C₅-DMB-Cer. Intracellular DMB fluorescence before chase was distributed throughout intracellular membranes, and this pattern was quite similar between HPA-12-treated and untreated cells (Fig. 7, A and B). In contrast, clearly less DMB fluorescence was accumulated in the perinuclear Golgi region after chase in HPA-12-treated cells than in untreated control cells (Fig. 7, C and D). Being consistent with the inhibition of the redistribution of C₅-DMB-Cer, 2.5 µM HPA-12 inhibited conversion of C₅-DMB-Cer to C₅-DMB-SM by ~50% in intact cells (Fig. 8C). In contrast, when cells were labeled with C₅-NBD-Cer, which spontaneously moves between and across membranes even without physiological mechanisms for intracellular ceramide trafficking (15, 34), there was no appreciable difference in fluorescence

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** HPA-12 does not inhibit synthesis of SM from [¹⁴C]serine in BFA-treated cells. CHO cells were preincubated with 1 µg/ml BFA at 33 °C for 30 min, treated with *closed circles* or without *open circles* 1 µM HPA-12 at 4 °C for 15 min. After addition of [¹⁴C]serine, the cells were further incubated at 33 °C for the indicated time in the presence of 1 µg/ml BFA. Lipids of cells were extracted, and the radioactivity of each lipid was determined.

### Table II

| HPA-12 | C₆-NBD-SM | C₅-DMB-SM | [¹⁴C]C₁₆-SM |
|--------|-----------|-----------|-------------|
| None   | 171.5 ± 6.9 | 81.1 ± 7.7 | 16.8 ± 2.1  |
| 20 µM  | 168.1 ± 1.7 | 81.9 ± 6.1 | 16.9 ± 1.4  |

\(^{3}\) S. Yasuda and K. Hanada, unpublished observations.
accumulation in the Golgi apparatus between HPA-treated and control cells (Fig. 7, E and F), eliminating the possibility that HPA-12 disrupted the Golgi apparatus. Therefore, the inhibition of DMB fluorescence redistribution to the Golgi apparatus in HPA-treated cells was most likely due to inhibition of ER-to-Golgi transport of ceramide.

Identification of a Specific Stereoisomer of HPA-12 as an Inhibitor of Cer Transport—HPA-12 has two chiral carbon atoms at the 1- and 3-positions, and therefore, four stereoisomers: (1S,3R), (1R,3S), (1R,3R), and (1S,3S)-types (Fig. 8A). Nuclear magnetic resonance analysis showed that HPA-12 contains (1R,3S), (1R,3S), (1R,3R), and (1S,3S)-types at a ratio of 23:23:27:27 (data not shown). We determined whether the stereoisomers of HPA-12 at 4°C for 15 min and, after addition of [14C]serine, incubated at 33°C for 2 h. Lipids extracted from cells were separated, and the radioactivity of SM was determined. The data are shown as means ± S.D. from three experiments. C, CHO cells were labeled with 1 μM C5-DMB-Cer at 4°C for 30 min, treated with or without 2.5 μM stereoisomers of HPA-12 at 4°C for 15 min and warmed at 33°C for 15 min. Lipids extracted from cells were separated, and the amounts of C5-DMB-SM were determined. Each bar shows the mean from duplicate experiments, and each point represents the actual value for individual experiments. D, HeLa cells were treated with or without 1 μM (1R,3R)-HPA-12 at 4°C for 15 min and, after addition of [14C]serine, incubated at 33°C for 2 h (batched columns). Lipids extracted from cells were separated, and radioactivities of SM were determined. HeLa cells, prelabeled with 1 μM C5-DMB-Cer, were treated with or without 2.5 μM (1R,3R)-HPA-12 at 4°C for 15 min and then incubated at 33°C for 15 min (open columns). Lipids extracted from cells were separated, and amounts of C5-DMB-SM were determined. The data are shown as means ± S.D. from three experiments.

(1R,3R)-HPA-12 was also effective against HeLa cells, a human cervical carcinoma cell line (Fig. 8D), and HuH7 cells, a human hepatoma cell line.3

(1R,3R)-HPA-12 Predominantly Inhibits an ATP-dependent Pathway—Mammalian cells have two pathways for transport...
of de novo synthesized Cer from the ER to the site of SM synthesis: The main pathway of the two is ATP-dependent, and the minor pathway is ATP-independent (or less dependent) (15, 16). The ATP-dependent pathway of Cer transport is almost completely impaired in LY-A, a CHO mutant cell line (15, 16). We next addressed the question of whether (1R,3R)-HPA-12 selectively inhibited the ATP-dependent pathway by using LY-A cells. Under drug-free conditions, conversion of [3H]Cer to [3H]SM in LY-A cells was ~30% of the wild-type level, although both cell types produced similar levels of [3H]Cer during pulse (Fig. 9), consistent with our previous studies (15, 16). Interestingly, 1 μM (1R,3R)-HPA-12 did not significantly inhibit conversion of [3H]Cer to [3H]SM in LY-A cells, whereas this drug inhibited the conversion by ~60% in wild-type CHO cells (Fig. 9). Conversion of [3H]Cer to [3H]GlcCer was not affected by (1R,3R)-HPA-12 in wild-type or LY-A cells (Fig. 9). These results indicated that (1R,3R)-HPA-12 predominantly inhibits the ATP-dependent pathway of Cer transport. Note that treatment of CHO cells with HPA-12 did not affect intracellular ATP levels.1

(1R,3R)-HPA-12 Does Not Inhibit Protein Transport from the ER to the Golgi Apparatus—To examine effects of (1R,3R)-HPA-12 on protein transport from the ER to the Golgi apparatus in cells, we employed CHO-K1 cells expressing GPI-anchored alkaline phosphatase (PLAP) or a chimeric alkaline phosphatase having a single membrane-spanning region in place of the GPI anchor (PLAP-HA) and examined the rate at which de novo synthesized PLAP and PLAP-HA were processed to the Endo H-resistant forms. Cells were pulse-labeled with [35S]methionine/cysteine, incubated at 4 °C for 15 min in the absence or presence of 2.5 μM (1R,3R)-HPA-12, and then incubated for various periods of time at 33 °C for chase. In both CHO transfectants, the acquisition rates (t1/2 = ~30 min) of the Endo H-resistant forms were virtually identical between the control and (1R,3R)-HPA-12 treated cells (Fig. 10). Thus, (1R,3R)-HPA-12 was unlikely to inhibit the main transport route(s) of membrane-spanning and GPI-anchored glycoproteins from the ER to the Golgi apparatus.

DISCUSSION

In this study, we showed that (1R,3R)-HPA-12, a novel analog of Cer, inhibits ATP-dependent transport of Cer from the ER to the site of SM synthesis without appreciable inhibition of the main pathway of ER-to-Golgi trafficking of glycoproteins. As far as we know, (1R,3R)-HPA-12 is the first pharmacological tool that can selectively inhibit the ATP-dependent pathway of intracellular Cer transport.

HPA-12 inhibited conversion of [3H]Cer to [3H]SM, but not to [3H]GlcCer, in CHO cells (Fig. 4). Furthermore, incubation of cells with HPA-12 for a prolonged period (2 days) reduced SM contents by ~30% but not glycosphingolipid contents in cells (Table I). HPA-12 did not affect the turnover of de novo synthesized SM in intact cells and inhibited neither activities of acid nor neutral SMase in vitro (Fig. 3). In addition, HPA-12 did not inhibit synthesis of PC, which is the donor of the phosphocholine moiety in SM synthesis (Fig. 5). These results indicated that HPA-12 inhibits the step of conversion of Cer to SM. Nevertheless, HPA-12 is not an inhibitor of SM synthase in vitro or in vivo (Table II and Fig. 6). It has previously been shown that transport of natural Cer from the ER to the Golgi compartment where SM synthesis occurs is mainly ATP-dependent in various types of cells, including CHO cells, HeLa cells, and human skin fibroblasts and that intracellular movement of C17-DMB-Cer mimics the movement of natural Cer via the ATP-dependent pathway (15). HPA-12 inhibited the redistribution of C17-DMB-Cer from intracellular membranes to the...
Golgi region and partially inhibited the conversion of C8-DMB-Cer to C2-DMB-SM in intact cells (Figs. 7, A-D, and 8C). Moreover, when cells were preincubated with BFA, HPA-12 no longer inhibited de novo synthesis of SM (Figs. 2B and 6). Our interpretation of the BFA effect is that HPA-12 inhibits intermembrane transport of Cer from the ER to the Golgi compartment for SM synthesis. However, because catalytic sites of SM synthase and GlcCer synthase have been suggested to exist in the luminal and cytoplasmic sides, respectively, of the Golgi complex, there is the alternative interpretation that HPA-12 might inhibit transport of Cer across the Golgi membrane, but that HPA-12-resistant transbilayer movement of Cer might occur in the merged organelle formed by BFA treatment. We could not eliminate this possibility clearly, because no method for assay of transmembrane movement of long-chain ceramide has been established yet. Thus, we carefully interpret these results to indicate that HPA-12 inhibits intermembrane or intramembrane transport of Cer from the ER to the site for SM synthesis.

Among the four stereoisomers of HPA-12, only (1R,3R)-HPA-12 efficiently inhibits conversion of Cer to SM (Fig. 8). Interestingly, (1R,3R)-HPA-12 did not affect Cer-to-SM conversion in the CHO mutant LY-A cells that are defective in the ATP-dependent pathway of Cer transport from the ER to the site of SM synthesis (Fig. 9). Moreover, (1R,3R)-HPA-12 did not inhibit transport of GPI-anchored PLAP and membrane-spanning PLAP-HA from the ER to the Golgi apparatus in wild-type CHO cells (Fig. 10). These results together with the observation that the vital stain of the Golgi apparatus with C6-NBD-Cer was not affected by HPA-12 (Fig. 7, E and F), ruled out the possibility of disintegration of the Golgi apparatus by this drug. Collectively, we conclude that HPA-12 selectively, if not specifically, inhibits the ATP-dependent pathway of Cer transport from the ER to the site of SM synthesis, although HPA-12 might affect one or more still undefined minor pathways for ER-to-Golgi transport of proteins. The inability of HPA-12 to inhibit GlcCer formation (Fig. 4 and Table I) is accounted for by the notion that the main pathway for the access of Cer to the site of de novo GlcCer synthesis differs from the ATP-dependent pathway (15, 16).

When HPA-12 stereoisomers and ω-erythro-Cer are aligned in terms of their aminoalcohol backbone, the “R” configuration of the aminoacyl group at the C1-position of HPA-12 corresponds to the “S” configuration of that at the C2-position of natural ω-erythro-Cer (Fig. 8A). Thus, the same relative configuration of the aminoacyl group of HPA-12 as the natural Cer type is crucial for the activity. Interestingly, the configuration of the hydroxy group at the C3-position of HPA-12 appears also to be important for inhibiting ATP-dependent transport of Cer (Fig. 8, A and B), although ω-erythro-Cer does not have a hydroxy group at the corresponding C4-position, and phytoce-ramide (4-hydroxy-ω-erythro-Cer) has an hydroxy group at the C4-position with a relative configuration opposite to the hydroxy group of (1R,3R)-HPA-12. These structure and activity relationships among HPA-12 isomers suggest that (1R,3R)-HPA-12 interacts antagonistically with a Cer-recognizing factor(s) involved in ATP-dependent trafficking of Cer, although the factor(s) remains unidentified.

Because no radioactive form of HPA-12 is available, it is currently impossible to determine the metabolism of HPA-12 in cells. However, it seems unlikely that modifications of reactive groups of HPA-12 are prerequisite for the effect of the drug, because HPA-12 exerts its activity soon after co-culturing with cells even at 4°C (Figs. 4, 8, and 9).

d-e-MAPP, an inhibitor of alkaline ceramidase (35), has structural similarity to HPA-12. However, 5 μM d-e-MAPP did not appreciably inhibit labeling of SM with [14C]serine in CHO cells (data not shown). It has previously been shown that 5 μM d-e-MAPP inhibits alkaline ceramidase activity in vitro by ~50% and increases markedly the Cer content in HL60 cells (35), whereas HPA-12 decreased the amount of SM without any effect on Cer content in CHO cells (Table 1). Therefore, the effects of HPA-12 on the sphingolipid metabolism are quite distinct from the effects of d-e-MAPP.

Because (1R,3R)-HPA-12 is effective not only in CHO cells but also in HeLa (Fig. 8, B–D) and human hepatoma HuH7 cells, this drug would be a useful tool with which to investigate the ATP-dependent transport of Cer in various types of cells. When the Cer produced catalytically modulates various cellular events (2, 3), it might be transported from the sites of its formation to different sites for exertion of signaling in cells. If so, various undefined pathways of intracellular Cer trafficking would also exist. (1R,3R)-HPA-12 may represent an invaluable prodrug for development of a family of inhibitors of Cer trafficking.

REFERENCES

1. Hanada, K., Nishijima, M., and Hanada, K. (1999) J. Cell. Biol. **144**, 673–685
2. Spiegel, S., and Merrill, A. H., Jr. (1996) J. Lipid Res. **37**, 911–917
3. Michel, C., and van Echten-Deckert, G. (1997) FEBS Lett. **416**, 153–155
4. Parcells, J. M., and Flaherty, J. M. (1993) J. Biol. Chem. **268**, 155–156
A Novel Inhibitor of Ceramide Trafficking from the Endoplasmic Reticulum to the Site of Sphingomyelin Synthesis
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A novel inhibitor of ceramide trafficking from endoplasmic reticulum to the site of sphingomyelin synthesis.

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In the work indicated above, we depicted the stereochemistry of active HPA-12 to be 1R,3R. However, Duriš et al. (Duriš, A., Wiesenganger, T., Moravčíková, D., Baran, P., Kožišek, J., Daïch, A., and Berkeš, D. (2011) Expedient and practical synthesis of CERT-dependent ceramide trafficking inhibitor HPA-12 and its analogues. Org Lett. 13, 1642–1645) recently proposed the stereochemistry of active HPA-12 to be 1R,3S. In response to this proposal, we have developed a gram-scale preparation method of HPA-12 and confirmed the (1R,3S) stereochemistry of active HPA-12 by x-ray crystallographic analysis (Ueno, M., Huang, Y.Y., Yamano, A., and Kobayashi, S. (2013) Revised stereochemistry of ceramide-trafficking inhibitor HPA-12 by x-ray crystallography analysis. Org Lett. 15, 2869–2871). The initial incorrect stereochemical assignment is probably because of an unexpected epimerization at the benzylic position occurred in an intermediate under harsh conditions in the synthetic route that we used previously (Ueno et al.). Accordingly, the stereochemistry of HPA-12 in this work should be corrected: (1R,3R), (1S,3S), (1R,3S), and (1S,3R) should be read as (1R,3S), (1S,3R), (1R,3R), and (1S,3S), respectively.

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.