Biochemical and Biophysical Research Communications 433 (2013) 170–174

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A small molecule inhibitor of Bcl-2, HA14-1, also inhibits ceramide glucosyltransferase

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ARTICLE INFO

Article history:
Received 28 January 2013
Available online 26 February 2013

Keywords:
Glucosylceramide
Inhibitor
Bcl-2
Apoptosis

ABSTRACT

HA14-1 is a Bcl-2 inhibitor that is widely used for studies of apoptosis. In the course of searching for a ceramide glucosyltransferase inhibitor that catalyzes the first glycosylation step of glycosphingolipid synthesis, we unexpectedly found that HA-14-1 also has the ability to inhibit ceramide glucosyltransferase. The IC_{50} value of HA14-1 against ceramide glucosyltransferase is 4.5 μM, which is lower than that reported for Bcl-2 *in vitro*. Kinetic analyses revealed that HA14-1 is a competitive and mixed-type inhibitor with respect to C_6-NBD-ceramide and UDP-glucose, respectively.

1. Introduction

Ceramide glucosyltransferase (UDP-glucose: ceramide glucosyltransferase, GlcT-1. EC 2.4.1.80) catalyzes the first glycosylation step of GSL synthesis. The enzyme transfers glucose from UDP-glucose to ceramide, forming GlcCer [1,2]. The lipid substrate of GlcT-1, ceramide, serves as a second messenger of apoptosis [3], and GlcT-1 regulates intracellular ceramide content [4]. GlcT-1 has also been implicated in insulin resistance of lipid cells [5] and drug resistance of cancer cells [6]. GlcT-1 demonstrates its anti-apoptotic effect by metabolizing and inactivating ceramide, which direct apoptosis. In fact, certain types of drug resistant cancer cells have demonstrated enhanced GlcT-1 activity, and inhibition of the enzyme re-sensitized the cells against drugs [6]. Thus, inhibitors of GlcT-1 have potential for therapeutic use to treat these diseases.

Two lines of GlcT-1 inhibitors are widely used for basic studies. One line of inhibitor includes PDMP and its derivatives, which are analogs of ceramide and compete with it [7]. The other line of inhibitor includes an imino sugar *N*-butyldeoxynojirimycin (*N*B-DNJ), and its derivatives compete with UDP-glucose [8]. *N*B-DNJ has been used to treat Gaucher's disease, which is caused by accumulation of GlcCer due to a deficiency of the GlcCer-degrading enzyme glucocerebrosidase [9]. *N*B-DNJ decreases GlcCer levels by inhibiting *de novo* synthesis of GlcCer.

Bcl-2 is a protein factor known to suppress apoptosis by binding and inactivating pro-apoptotic proteins such as Bax and BH3-only family proteins. The formation of homodimers or heterodimers with other Bcl-2 family proteins is necessary for Bcl-2 to exert its anti-apoptotic activity. High levels of Bcl-2 expression are found in various cancers [10]. Similar to GlcT-1, overexpression of Bcl-2 confers cancer cell resistance against anti-cancer agents. HA-14 is a small molecule Bcl-2 inhibitor originally developed by Wang et al. [11]. HA14-1 mimics the BH3 domain required for homo- and heterodimer formation and thus inhibits the binding of these proteins to each other. Thus HA14-1 sensitizes the Bcl-2 over-expressing cancer cells.

Here, we report that the widely used Bcl-2 inhibitor HA14-1 also inhibits GlcT-1 activity.

2. Materials and methods

2.1. Materials

A fluorescent substrate for GlcT-1, 6-{((*N*-7-Nitrobenz-2-oxa-1,3-diazol-4yl)amino)caproyl}sphingosine (C_6-NBD-Cer) was purchased from Invitrogen (CA, USA). Ceramide from bovine brain was obtained from Sigma (Tokyo, Japan) Ethyl-2-amino-6-bromo-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14-1) was purchased from Wako (Tokyo, Japan) or prepared by

Abbreviations: GlcCer, glucosylceramide; LacCer, lactosylceramide; GSL, glycosphingolipid; SM, sphingomyelin; IPTG, isopropylthio-β-galactoside; HA14-1, ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate.

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http://dx.doi.org/10.1016/j.bbrc.2013.02.052

following an established procedure [12]. In short, 5-bromosalicylaldehyde was treated with ethyl cyanoacetate in the presence of MS3A in dry ethanol at room temperature to produce HA14-1 with a 55% yield. Synthesized HA14-1 had inhibitory activity comparable with commercial HA14-1. A SCADS inhibitor kit containing 325 small molecule bioactive compounds was obtained from the Screening Committee of Anticancer Drugs (Tokyo, Japan). All other reagents used were of analytical grade.

2.2. Cell line and culture conditions

Cell lines were obtained from RIKEN cell bank (Tsukuba, Japan). The cells were grown in humidified 5% CO_2 at 37 °C in DMEM medium containing 10% fetal bovine serum.

2.3. Enzyme assay

GlcT-1 activity was measured according to Lipsky and Pagano [13] with slight modifications [14]. To prepare cell lysates as an enzyme source, B16 or SK-Mel-28 cells were harvested at semi-confluency, washed with PBS, suspended in water, and lysed by freezing and thawing. The cell lysate was used as an enzyme source. For kinetic analysis with respect to UDP-glucose, the cell lysate was dialyzed against 50 mM Tris–HCl (pH 7.5) buffer containing 25 mM KCl, 25 μM amidinophenylmethanesulfonyl fluoride, 1 μg/ml leupeptin hemisulfate, and 1 μg/ml antipain hydrochloride. A reaction mixture (50 μL) composed of 10 mM Tris–HCl (pH 7.5), 500 μM UDP-glucose, 10 μL of liposomes (C_6-NBD-Cer (0.5 μg) and lecithin (5 μg) in water) and 30 μg of cell protein was incubated at 30 °C for 1 h. For kinetic analysis experiments, reaction mixtures contained NP-40 and C_6-NBD-Cer, instead of liposomes, to avoid C_6-NBD-sphingomyelin (C_6-NBD-SM) formation. After the incubation, lipids were extracted with $CHCl_3/CH_3OH$, 2:1 (vol/vol) and evaporated to dryness. The lipids were then redissolved in a small volume of $CHCl_3/CH_3OH$, 2:1 (vol/vol) and chromatographed on silica gel 60 TLC plates (Merck, Darmstadt, Germany) in $CHCl_3/CH_3OH/H_2O$, 65:25:4 (vol/vol/vol). NBD lipids were visualized by UV-B illumination. In some experiments, cell lysates from *Escherichia coli* (*E. coli*) expressing human GlcT-1 were used as described previously [14]. The LacCer synthase reaction was performed using the method of Nishie et al. with slight modifications [15]. A reaction mixture (50 μL) composed of 50 mM HEPES buffer (pH 7.5), 200 μM UDP-galactose, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μL liposomes (C_6-NBD-GlcCer (0.5 μg) and lecithin (5 μg) in water) and 60 μg cell protein was incubated at 30 °C for 12 h.

2.4. Inhibition of HA14-1 of intracellular GlcT-1 activity

Inhibition of intracellular GlcT-1 activity was examined as follows. B16 cells were plated on a 10-cm dish at a density of 1×10^6 cells/dish (10 ml) and cultured overnight. The next day, HA14-1 was added to a final concentration of 50 μM. Fifteen minutes after the addition, 50 μL of liposomes (C_6-NBD-Cer (0.5 μg) and lecithin (5 μg) in water) was added to the medium and incubated for another 30 min. After the incubation, cells were harvested. NBD lipids were extracted and analyzed as described in the "enzyme assay" section.

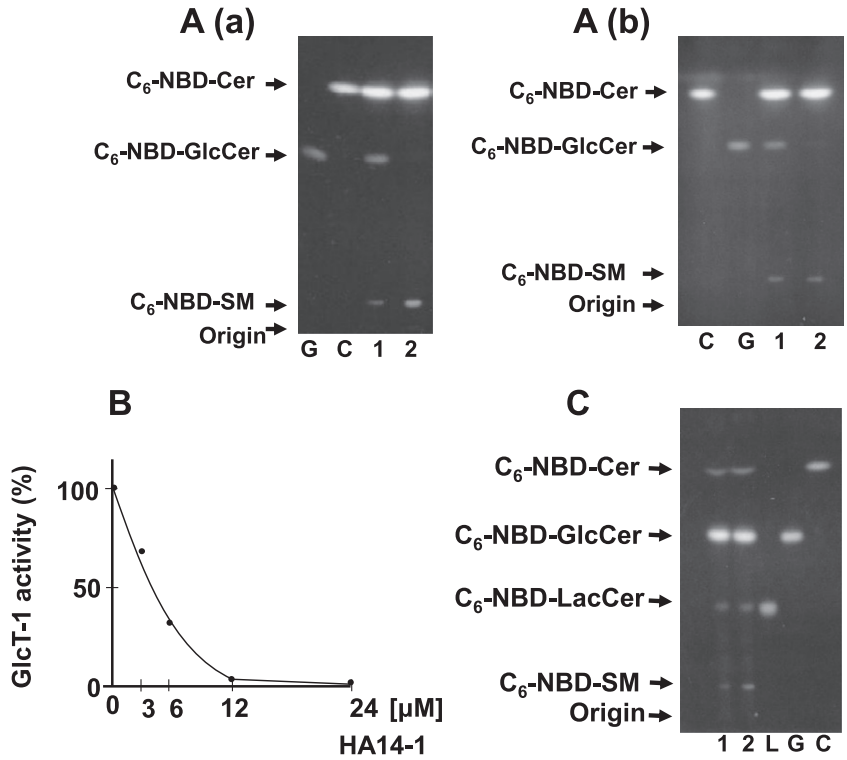

Fig. 1. Effect of HA14-1 on enzymatic activities. (A) Effect of HA14-1 on GlcT-1 and SM synthase activities. C_6-NBD-GlcCer and C_6-NBD-SM generated by GlcT-1 and SM synthase activities, respectively, in cell lysates were separated by TLC. Cell lysates (30 μg protein) from melanoma cells were used as the enzyme source. The enzyme reactions were performed in the absence or presence of HA14-1. A(a) B16 melanoma cells. Lane G, C_6-NBD-GlcCer standard; lane C, C_6-NBD-Cer standard; lane 1, untreated control; lane 2, HA14-1 treated cells. A(b) SK-Mel-28 melanoma cells. Lane G, C_6-NBD-GlcCer standard; lane C, C_6-NBD-Cer standard; lane 1, untreated control; lane 2, HA14-1 treated cells. (B) Dose dependent inhibition of HA14-1 on mouse GlcT-1 activity in B16 cell lysates. (C) HA14-1 had no effect on LacCer synthase activity. TLC analysis of the LacCer synthase product catalyzed by cell lysates from B16 cells. Cell lysate (60 μg protein) was used as the enzyme source. Lane L, C_6-NBD-LacCer standard, lane G, C_6-NBD-GlcCer standard; lane C, C_6-NBD-Cer standard; lane 1, untreated control; lane 2, HA14-1 treated cells. LacCer synthase activity is comprised of activities of β-1,4-galactosyltransferase V and β-1,4-galactosyltransferase VI.

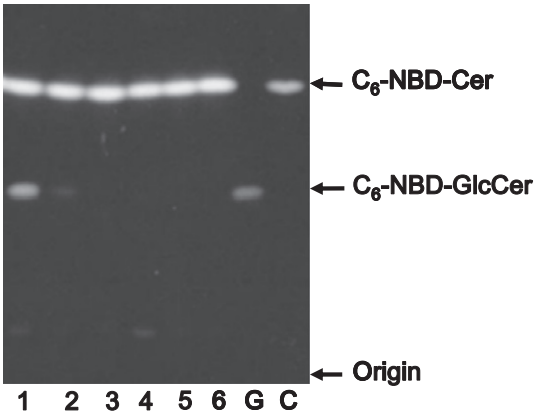

Fig. 2. Effect of HA14-1 on recombinant human GlcT-1 expressed in *E. coli*. pET-CG-1 plasmid carries human GlcT-1 cDNA in a pET3a *E. coli* expression vector. Human GlcT-1 can be induced by IPTG addition. The plasmid was transformed in *E. coli* BL21 (DE3). Lanes: 1, pET-CG-1/BL21 (DE3) with IPTG addition; 2, pET-CG-1/BL21 (DE3) without IPTG addition; 3, BL21 (DE3); 4, pET-CG-1/BL21 (DE3) with IPTG and HA14-1 additions; 5, pET-CG-1/BL21 (DE3) without IPTG addition and with HA14-1 addition; 6, BL21 (DE3) with HA14-1; G, C_6-NBD-GlcCer standard; C, C_6-NBD-Cer standard.

2.5. Lipid analyses

Total lipids were extracted from the cells with $CHCl_3/CH_3OH$, 2:1 (vol/vol) and evaporated to dryness. The lipids were then redissolved in a small volume of $CHCl_3/CH_3OH$, 2:1 (vol/vol), and chromatographed on silica gel 60 TLC plates (Merck, Darmstadt, Germany). Glucosylceramide and ceramide were analyzed as described previously [16].

2.6. Protein assay

Total protein was assayed using the BCA protein assay reagent kit according to the manufacturer's instructions (PIERCE, Rockford, IL USA).

3. Results

3.1. Inhibitory effect of HA14-1 against GlcT-1

A screen of 325 bioactive small-molecules identified HA14-1 as an inhibitor of GlcT-1. HA14-1 is a compound originally developed as an inhibitor of Bcl-2 and is widely used in basic research of apoptosis (11). We used a cell lysate from mouse melanoma B16 cells for the GlcT-1 assay because of its high enzymatic activity. As shown in Fig. 1A (a), GlcT-1 activity was inhibited by HA14-1. These studies also indicated dose-dependent inhibitory activity of HA14-1 against GlcT-1, with an IC_{50} value of 4.5 μM (Fig. 1B). Since mouse GlcT-1 was used for the assay, we next examined the effect of HA14-1 against human GlcT-1. A cell lysate from a human melanoma cell line SK-Mel-28 was used as a source of GlcT-1, and the effect of HA14-1 was examined. Fig. 1A (b) shows the inhibitory effect of HA14-1 on human GlcT-1 activity, which was similar to that found using the mouse enzyme. Since crude cell lysates were used as enzyme sources, there was a possibility that the inhibitory effect of HA14-1 was not directly due to GlcT-1 itself, but was the result of indirect effects on proteins that regulated GlcT-1 activity. For instance, if Bcl-2 binds to GlcT-1 and enhances GlcT-1 activity, HA14-1 might inhibit GlcT-1 activity by interfering with the binding of GlcT-1 to Bcl-2. It is also possible that modified derivatives of HA14-1 generated by enzymes in the cell lysate can inhibit GlcT-1 activity. Although it would be desirable to use purified GlcT-1 enzyme for assays, it is a membrane protein and is thus difficult to purify. To eliminate the first alternative possibility, we used a cell lysate from *E. coli* that expressed human GlcT-1. *E. coli* lysates would not be expected to contain GlcT-1 regulatory proteins. In addition, any enzymes that might modify HA14-1 would be different from those in animal cells. Human GlcT-1 expressed in *E. coli* was inhibited by HA14-1 to the same extent as in B16 and SK-Mel-28 cell lysates, indicating that HA14-1 had a direct effect on GlcT-1 (Fig. 2). The second alternative possibility was ruled out by time course experiments, in which no increase of inhibitory activity was observed even after prolonged incubation times (data not shown).

3.2. Selectivity of H14-1 Inhibition

HA14-1 might have a broad specificity and inhibit many enzymes and proteins other than GlcT-1 and Bcl-2. However, the inhibitory effect of HA14-1 appeared to be rather selective. In our GlcT-1 assay system, C_6-NBD-Cer was supplied as a liposome formulation containing phosphatidylcholine. In this system, SM synthase in cell lysates transfers phosphocholine from phosphatidylcholine to C_6-NBD-Cer, forming C_6-NBD-SM (Fig. 1A (a) and A (b)). As shown in the figures, SM synthase was not inhibited by addition of HA14-1. We also examined the effect of H14-1 on another glycosyltransferase, LacCer synthase, which transfers galactose from UDP-galactose to GlcCer, forming LacCer. Again, H14-1 had no effect on LacCer synthase activity (Fig. 1C).

3.3. Kinetic analyses

The kinetics of inhibition of GlcT-1 by HA14-1 have been investigated using Lineweaver–Burk plots. The results revealed that HA14-1 was a competitive and mixed type inhibitor with respect to C_6-NBD-Cer and UDP-glucose, respectively (Fig. 3). Interestingly, HA-14 does not possess structural homology with the substrates UDP-glucose and ceramide.

3.4. Inhibitory effect of HA14-1 on intracellular GlcT-1 activity

B16 cells were next used to examine whether HA14-1 could inhibit intracellular GlcT-1 activity. C_6-NBD-Cer, which can penetrate into cells, was added to the medium as a liposome formulation in the presence of HA14-1. Thirteen minutes afterwards, the cells were collected and the lipids were analyzed. The amount of C_6-NBD-GlcCer synthesized in this system was decreased compared to control experiments (Fig. 4A). The authentic sphingolipid contents were further examined in B16 cells treated with 50 μM HA14-1. Rounding of the cells began 4 h after addition of HA14-1 and most of the cells died within 24 h. The cells were thus harvested 4 h after addition of HA14-1 and their lipid contents were analyzed. The GlcCer content was not altered (Fig. 4B and C (b)) at this point. In contrast, an increase in ceramide content (Fig. 4B and C (a)) was observed.

4. Discussion

Cell death along apoptotic pathways is regulated by various signaling events. HA14-1 is widely used as a Bcl-2 inhibitor in order to analyze apoptosis. Bcl-2 family proteins are anti-apoptotic or pro-apoptotic mitochondrial proteins. Among them, Bcl-2 has an anti-apoptotic effect and inhibition of this protein induces apoptosis in various cell lines, especially cancer cells that overexpress Bcl-2.

In the present study, we identified HA14-1 as a novel GlcT-1 inhibitor with a new structure. It should be noted that HA14-1 has two asymmetric carbon atoms in its structure, indicating the presence of four stereoisomers. It is desirable to isolate and test

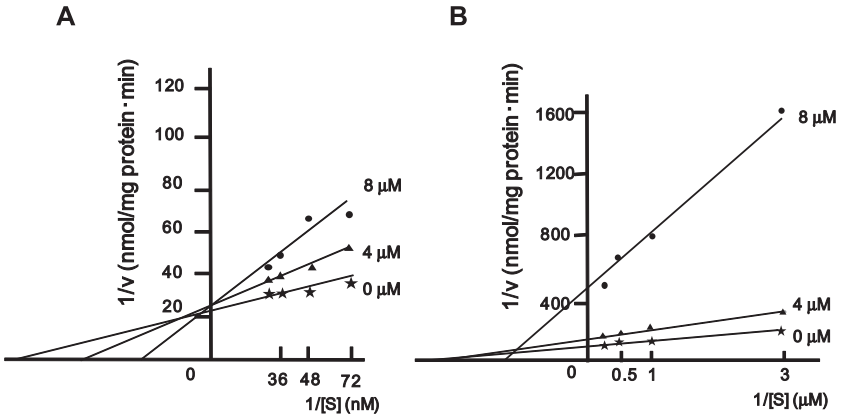

Fig. 3. Kinetics of inhibition of GlcT-1 by HA14-1. GlcT-1 activities were measured in the presence of 0, 4, or 8 μM HA14-1. (A) Lineweaver–Burk plot of ceramide in the absence and presence of HA14-1. (B) Lineweaver–Burk plot of UDP-glucose in the absence and presence of HA14-1.

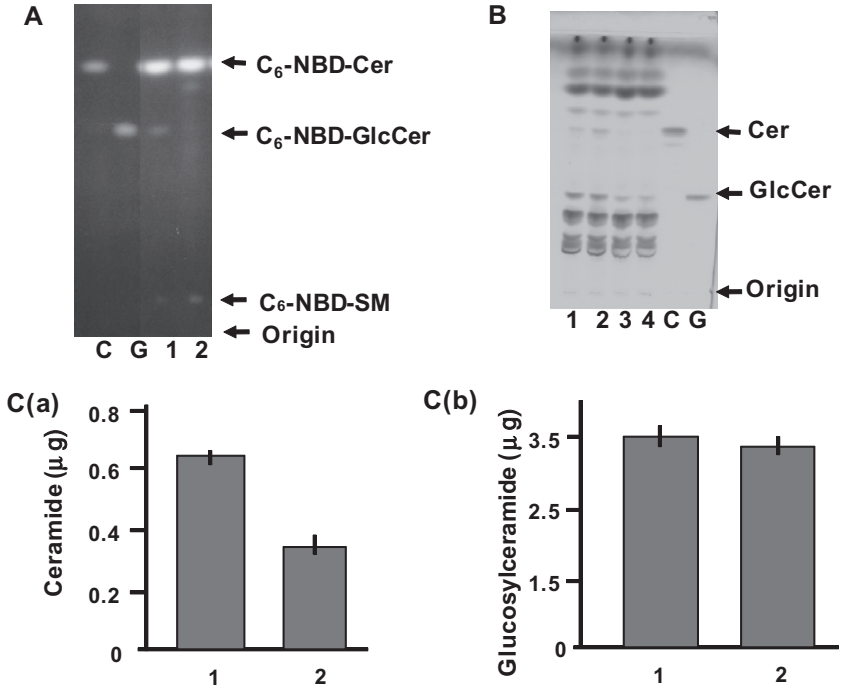

Fig. 4. Effect of HA14-1 on GlcT-1 activity and sphingolipid contents in HA14-1 treated B16 cells. (A) Effect on intracellular GlcT-1 activity. C_6-NBD-Cer, a cell permeable substrate, was added to the medium as a liposome formulation. Incorporated C_6-NBD-Cer was metabolized to C_6-NBD-GlcCer. The generation of C_6-NBD-GlcCer was inhibited in the presence of 50 μM HA14-1. Lane C, C_6-NBD-Cer standard; lane G, C_6-NBD-GlcCer standard; lane 1, untreated control; lane 2, HA14-1 treated cells. Details are described in Section 2. (B) Effect of HA14-1 on ceramide and GlcCer contents: Total lipid from 2×10^6 cells was used for the analysis. The cells were treated with 50 μM HA14-1 for 4 h. After separation by TLC, lipids were visualized with cupric acetate reagent. Lane C, ceramide standard; lane G, GlcCer standard; lane 1, untreated control; lane 2, HA14-1 treated cells; lane 3 ISP-1 (50 μM) treated cells; lane 4, HA14-1 and ISP-1 (50 μM) treated cells. ISP-1 is an inhibitor of serine palmitoyltransferase that catalyzes the first step in GSL synthesis. Thus, GlcCer and ceramide bands are very weak in lanes 3 and 4. C(a) Ceramide was quantitated after TLC separation. Column 1, HA14-1 treated cells; column 2, untreated control. Data represent means from four experiments and bars indicate SD. C(b) GlcCer was quantitated after TLC separation. Column 1, HA14-1 treated cells; column 2, untreated control. Data represent means from four experiments and bars indicate SD.

each isomer, since a distinct isomer might possess the inhibitory activity on GlcT-1 or/and Bcl-2. However, analyses with HPLC and NMR revealed that these isomers are unstable and rapidly interconvert with each other (data not shown).

Although no amino acid sequence homology between GlcT-1 and Bcl-2 family proteins was detected, these two proteins might have similar three-dimensional structures. Competitive and mixed-type inhibition of substrates suggested a structural homology between the GlcT-1 active site and the Bcl-2 BH3 domain, where HA14-1 binds. The inhibitory activities also suggested possible binding of GlcT-1 to Bcl-2, since the BH3 domain is responsible for dimerization of Bcl-2 family proteins.

Inhibition of intracellular GlcT-1 activity estimated by C_6-NBD-GlcCer formation indicates that HA14-1 can permeate cells (Fig. 4A). Unexpectedly, the content of authentic GlcCer was not decreased in HA14-1 treated cells (Fig. 4B and C (b)). This is presumably due to the slow degradation rate of the GSLs in B16 cells. On the other hand, an elevated level of ceramide as a result of blocking GlcCer synthesis was detected (Fig. 4B and C (a)).

Separovic et al. have demonstrated that the microsome fraction from HA14-1 (20 μM) treated Jurkat cells had decreased GlcT-1 as well as SM synthase activity [17]. However, they presumably observed different phenomena from those described herein. In their experiments, the decreased GlcT-1 activity was presumably not

due to a direct effect on the enzyme, because HA14-1 is a reversible inhibitor and must be diluted to under the effective concentration during preparation of microsome fractions. In addition, HA14-1 did not inhibit SM synthase under our experimental conditions.

Our results also indicate that the use of HA14-1 as a Bcl-2 inhibitor in studies of apoptosis might produce misleading results, because HA14-1 could also affect a ceramide-mediated cell death pathway by inhibiting GlcT-1. From a different point of view, however, derivatives of HA14-1 could provide unique lead compounds for cancer treatment, because this compound can induce death in cancer cells, especially multi-drug resistant cells, via Bcl-2 inactivation and ceramide accumulation.

Acknowledgments

We thank the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan for providing us SCADS inhibitor kit.

This study was supported by a Grant from the Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sport, Science, and Technology, Japan, 2010-2012 (S1001030).

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