Published in final edited form as:

Mol Cancer Ther. 2011 November ; 10(11): 2052–2061. doi:10.1158/1535-7163.MCT-11-0365.

Discovery and Evaluation of Inhibitors of Human Ceramidase

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Abstract

The ceramide/sphingosine-1-phosphate (S1P) rheostat has been hypothesized to play a critical role in regulating tumor cell fate, with elevated levels of ceramide inducing death and elevated levels of S1P leading to survival and proliferation. Ceramidases are key enzymes that control this rheostat by hydrolyzing ceramide to produce sphingosine, and may also confer resistance to drugs and radiation. Therefore, ceramidase inhibitors have excellent potential for development as new anticancer drugs. In this study, we identify a novel ceramidase inhibitor (Ceranib-1) by screening a small molecule library and describe the synthesis of a more potent analog (Ceranib-2). In a cell-based assay, both compounds were found to inhibit cellular ceramidase activity toward an exogenous ceramide analog, induce the accumulation of multiple ceramide species, decrease levels of sphingosine and S1P, inhibit the proliferation of cells alone and in combination with paclitaxel, and induce cell cycle arrest and cell death. *In vivo*, Ceranib-2 was found to delay tumor growth in a syngeneic tumor model without hematologic suppression or overt signs of toxicity. These data support the selection of ceramidases as suitable targets for anticancer drug development, and provide the first non-lipid inhibitors of human ceramidase activity.

Keywords

Ceramidase; Ceramide; Inhibitor; Sphingosine; Tumor

Introduction

The mechanisms and effects of the interconversion of sphingolipids have been the subject of a growing body of scientific investigation (reviewed in (1–7)). In the sphingolipid metabolic pathway, ceramide is produced by the hydrolysis of sphingomyelin in response to several growth stimulatory and/or inflammatory signals, and the accumulation of ceramide induces apoptosis in tumor cells without disrupting quiescent normal cells (8–11). In turn, ceramide can be hydrolyzed by the action of ceramidase enzymes to produce sphingosine, which is phosphorylated by sphingosine kinases (SK1 and SK2) to produce sphingosine-1-phosphate. It has been hypothesized that a critical balance, i.e. a ceramide/S1P rheostat, exists that determines the fate of the cell (12–13). When cells are exposed to mitogens, a rapid increase in intracellular levels of S1P is induced along with a depletion of ceramide. These conditions support cell survival and proliferation. In contrast, an accumulation of ceramide occurs with the activation of sphingomyelinase in the absence of ceramidase and/or SK activity resulting in an apoptotic response (reviewed in (14)). In addition, it has been demonstrated that tumor

cells can escape apoptosis by rapidly removing ceramide with ceramidases (reviewed in (15)). This makes the ceramidases interesting targets for anticancer drug development because inhibiting their activity leads to an accumulation of ceramide and tumor cell death (16–20). In addition, ceramide has been reported to enhance apoptosis in response to paclitaxel (21), etoposide (10) and gemcitabine (22–23). Therefore, inhibition of ceramidase activity could also increase tumor chemosensitivity.

It has been demonstrated that ceramidases are over-expressed in several forms of cancer including prostate, head and neck, and melanoma (24–28). In addition, over-expression of ceramidases has been shown to confer tumor cell resistance to tumor necrosis factor and radiation (29–31). Conversely, exposure to exogenous ceramides or sphingomyelinase, which generates ceramide, have been shown to induce apoptosis in pancreatic cancer and leukemia cell lines (32–33). Overall, these findings support ceramidases as viable targets for the development of novel anticancer agents.

In spite of the high level of basic interest in sphingolipid-mediated signaling, the pharmacology of ceramidase inhibition remains poorly characterized (34). A few compounds including N-oleoylethanolamine, B13, De-MAPP and D-NMAPPD have been characterized as inhibitors of ceramidase activity (18–19, 29, 35–37), and treatment of cancer cells with these inhibitors induces the accumulation of ceramide and promotes apoptosis in colon, prostate, glioma, and melanoma cell lines (18–19, 29, 37). However, these compounds have significant pharmaceutical shortcomings that will likely hinder their development as clinically viable therapeutics. Most importantly, the current ceramidase inhibitors are typically ceramide analogs containing long-chain alkyl moieties. Highly hydrophobic compounds such as these tend to have poor pharmacologic properties due to binding with albumin, resulting in poor bioavailability. Thus, there is a need to identify “drug-like” small molecule inhibitors of ceramidases that can be developed into anticancer agents.

In this study, we sought to identify novel small molecule inhibitors of ceramidase activity with favorable pharmacologic properties that can be further developed into anticancer therapeutics. We herein demonstrate that our newly identified compounds inhibit the hydrolysis of an exogenous ceramidase substrate, and induce the accumulation of ceramide while decreasing intracellular levels of sphingosine and S1P. Additionally, these new ceramidase inhibitors are antiproliferative and proapoptotic, and have *in vivo* antitumor activity.

Materials and Methods

Materials

Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). The chemical library was purchased from ChemBridge Corporation (San Diego, CA) and compounds were provided as solutions at a concentration of 10 mM in DMSO. Additional samples of Ceranib-1 (3-(3-(4-methoxyphenyl)acryloyl)-6-methyl-4-phenylquinolin-2(1H)-one, Figure 1A) were purchased from ChemBridge Corporation (ID number 5849350).

Synthesis of Ceranib-2, (3-[3-(4-methoxyphenyl)acryloyl]-4-phenyl-1H-quinolin-2-one)

NMR spectra were obtained on Bruker 500 instrument in CDCl₃, and chemical shifts are quoted relative to tetramethylsilane for ¹H- and ¹³C-NMR spectra. MALDI-TOF MS spectra was obtained on a Voyager RP mass spectrometer. Solvents were dried and redistilled prior to use, and reactions requiring anhydrous conditions were conducted under an atmosphere of nitrogen. Ceranib-2 was prepared by a two-step synthesis (Figure 1B) as follows:

1. A solution of 1.97 g (0.01 mol) of *o*-aminobenzophenone (**1**) and 2.6 g (0.02 mol) of acetylacetone (**2**) was heated under reflux for 4 hr. The reaction mixture was cooled and poured slowly, with stirring, into an ice-cold solution of 40 ml of water. The resulting suspension was allowed to stand in an ice bath until the gummy precipitate had hardened, after which the crude product was collected, washed with water, and recrystallized from aqueous ethanol to give 1.82 g of 3-acetyl-4-phenyl-1H-quinolin-2-one (**3**, 70% yield) as off-white needles, with a melting point of 256–258°C. Recrystallization from aqueous ethanol raised the melting point to 260–261°C. Spectral data for **3**: ^1H NMR δ . 2.34 (s, 3H, COCH₃), 7.16–7.19 (t, J = 7.5 Hz, 1 H, Ar-H), 7.29–7.31 (d, J = 10 Hz, 1 H, Ar-H); 7.36–7.38 (d, J = 10 Hz, 2H, Ar-H), 7.48–7.53 (m, 4H, Ar-H), 7.56–7.59 (t, J = 7.5 Hz, 1H, Ar-H), 12.61 (s, 1H, NH); ^{13}C NMR δ 31.76, 116.45, 119.93, 123.0, 127.75, 128.61, 128.99, 129.06, 131.46, 132.88, 134.31, 138.23, 149.03, 161.43, 201.81; MS m/z (relative intensity) 264.41 (M^+ +1, 100), 265.41(30).
2. NaOH powder (12 mmol) was added to a 50 ml round-bottom flask containing a 20 ml EtOH plus 10 ml CH₂Cl₂ solution of **3** and 4-methoxybenzaldehyde (**4**, 4 mmol) under N₂ and stirred at 25 °C overnight. The reaction mixture was acidified to pH 3, washed three times with water (3 × 10 ml), and filtered to give the crude product 3-[3-(4-methoxyphenyl)acryloyl]-4-phenyl-1H-quinolin-2-one (**Ceranib-2**). Recrystallization from ethanol gave the target compound as off-white crystals in 89% yield with a melting point of 228–230 °C; Spectral data for Ceranib-2: ^1H NMR δ 3.84 (s, 3H, OCH₃), 6.67–6.70 (d, J = 15 Hz, 1H, HC=), 6.87–6.89 (d, J = 10 Hz, 2H, Ar-H), 7.14–7.17 (m, 1H, Ar-H), 7.33–7.50 (m, 10H, Ar-H), 12.61 (s, 1H, NH); ^{13}C NMR δ 55.40, 114.34, 116.78, 120.01, 122.95, 125.54, 127.16, 127.56, 128.41, 128.80, 129.17, 130.28, 131.04, 131.41, 134.41, 138.40, 145.66, 150.26, 161.77, 193.83; MS m/z (relative intensity) 382.55 (M^+ +1, 100), 383.54(60).

Cellular ceramidase assay

Ceramidase activity in human ovarian adenocarcinoma (SKOV3) cells over 24 hr was measured using a previously described fluorogenic ceramidase substrate (38) according to our previously published protocol (39). Briefly, SKOV3 cells were grown to near confluence in 96-well plates, and then incubated with 16 μM N-((2S,3R)-1,3-dihydroxy-5-((2-oxo-2H-chromen-7-yl)oxy)pentan-2-yl)palmitamide (ceramidase substrate) for 24 hr at 37 °C. After incubation, the ceramidase cleavage product was converted to umbelliferone by adding 100 μl of 100% MeOH, 100 μl of NaIO₄ (10 mg/ml in 100 mM phosphate buffer pH 8.0) and 50 μl of BSA (2 mg/ml in 100 mM phosphate buffer pH 8.0) and incubating overnight at 37 °C. The following day the samples were analyzed using a SpectraMax M5 platereader (Molecular Devices) at excitation and emission wavelengths of 355 and 460 nm, respectively. The relative fluorescent units (RFUs) for wells that did not contain cells were considered negative controls, and their values were subtracted from the values obtained from each of the wells containing cells.

Cytotoxicity and cell proliferation assays

The effects of the test compounds on SKOV3 cells were determined in two different treatment scenarios. The first was designed to replicate the exposures used in the ceramidase assay described above. In this method, SKOV3 cells at near-confluence were treated with DMSO, Ceranib-1 or Ceranib-2 at varying concentrations for 24 hrs at 37 °C. Subsequently, cell viability was determined using the MTS assay according to a standard protocol. The second treatment scenario involved exposure of sparsely plated SKOV3 cells to Ceranib-1 or Ceranib-2 alone or in combination with paclitaxel for 72 hr, followed by cell

quantification using the sulforhodamine assay (40) to measure effects on cell proliferation. In either case, the percent cell survival was calculated as: % Survival = $(\text{Ab}_{\text{experimental compound}}/\text{Ab}_{\text{vehicle control}}) \times 100$, and IC₅₀ values were determined for each compound alone by non-linear regression analysis (GraphPad Prism 5.0). Combined drug effects were analyzed as isobolograms in which the line linking the individual drug IC₅₀s represents the line of additivity, with points below the line indicating synergy and points above the line indicating antagonism.

Sphingolipid analyses

Near-confluent SKOV3 cells were treated with DMSO, Ceranib-1 or Ceranib-2 at varying concentrations for 24 hr at 37 °C. The cells were harvested, washed 3 × with PBS and submitted to the Lipidomics Shared Facility Analytical Core of the Medical University of South Carolina for determination of sphingolipid and dihydrosphingolipid species by LC-MS/MS. The amounts of each lipid were normalized to phosphate levels in each biological sample, and the level of each sphingolipid was then expressed relative to levels in vehicle-treated cells, such that a value of 100% equals the level found in the controls.

Cell cycle analyses

SKOV3 cells in exponential growth were treated with DMSO, 0.75 μM, or 1.5 μM Ceranib-2 for 48 hr at 37 °C. The cells were then fixed in PBS:EtOH (1:3) at 4°C for 90 min, washed with PBS, and stained with PBS containing 20 μg/ml propidium iodide (Molecular Probes, P3566) and RNase A (Invitrogen, 12091-039) for 30 min at 37 °C. Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) at an excitation of 488 nm and emission of 585 nm. The number of cells emitting a given amount of fluorescence was plotted on histograms, and the percent of cells falling into each phase of the cell cycle was calculated. Histograms shown are representative of two experiments carried out in duplicate, and the values noted in the text represent the mean ± SEM of the percent of cells in each phase of the cell cycle.

In vivo antitumor assay

JC murine mammary adenocarcinoma cells (10⁶ cells in 100 μl PBS) were subcutaneously injected into the right flank of female Balb/c mice. Palpable tumors were apparent in 2 weeks, and the mice were randomized into three groups (n = 12–13) and treated with 0 (vehicle = PEG:DMSO (1:1)), 20 or 50 mg/kg of Ceranib-2. Treatments were administered by intraperitoneal injection daily for 5 days per week, and body weight and tumor size were measured twice per week. The volume of each tumor was calculated using the equation: Tumor Volume = (Tumor Length × Tumor Width²)/2, and was expressed relative to treatment Day 1 for each animal. Statistical significance was assessed by unpaired student's t-test, with p 0.05 considered to be significant.

Pharmacokinetic assays

Female Balb/c mice (6–8 weeks old) were administered a dose of 50 mg/kg Ceranib-2 by intraperitoneal injection, and blood was harvested into EDTA-containing syringes by cardiac puncture at 0.5, 2 or 6 hr (n = 5/group). Plasma samples were prepared by centrifugation (1500 × g for 10 min at 4 °C), and 0.1 ml of plasma was extracted twice with 1 ml of ethyl acetate. The combined organic extracts were dried under nitrogen at 35 °C and dissolved in 65 μl of Solvent A (0.1% formic acid in MeOH). The samples were fractionated by reverse-phase HPLC on a Supelco Discovery C18 column (20× 2.1 mm) using a linear gradient beginning with 30% Solvent A and 70% Solvent B (5% acetonitrile and 0.1% formic acid in water) and ending with 100% Solvent B over 9 min at a flow rate of 0.4 ml/min. Ceranib-2

eluted at approximately 10.2 min, and was quantified by measuring its absorbance at 341 nm using a calibration curve of pure Ceranib-2.

Results

Screen for inhibitors of human ceramidase activity

A ceramide analog that generates a fluorescent product following cleavage by ceramidase (38) was used to screen a ChemBridge DIVERset library consisting of approximately 50,000 drug-like compounds. SKOV3 cells were exposed to pools of 10 compounds (each at a final concentration of 30 μ M) and incubated with the fluorogenic ceramide overnight. Ceramidase activity was measured as the increase in fluorescence as previously described (39). This assay was found to have an average Z'-factor of 0.71, indicating that it is suitable for screening for ceramidase inhibitors. Compound pools that inhibited ceramidase activity were deconvoluted to identify individual active compounds, which were defined as those that reduced ceramidase activity by at least 75% at 100 μ M. This criterion was satisfied by only 0.03% of the screened compounds, and the 3-phenylacryloyl-4-phenyl-1H-quinolin-2-one scaffold was identified in multiple screening hits. The most active compound in the screening set was designated as Ceranib-1 (Figure 1A), and chemical optimization was initiated (to be reported in detail elsewhere) which provided Ceranib-2 (Figure 1B).

Inhibition of ceramidase activity by Ceranib-1 and Ceranib-2

To determine the potencies of Ceranib-1 and Ceranib-2 for inhibition of cellular ceramidase activity, SKOV3 cells at near-confluence were treated with varying concentrations of each for 24 hr. Subsequently, increased fluorescence produced by the hydrolysis of a fluorogenic ceramidase substrate by cellular ceramidase enzymes was measured. As shown in Figure 2A, both compounds produce a dose-dependent decrease in ceramidase activity, with 50% inhibition at 55 and 28 μ M for Ceranib-1 and Ceranib-2, respectively. To ensure that the decreases in ceramidase activity were not caused by toxicity to the cells in this timeframe, SKOV3 cells in identical culture conditions were treated with Ceranib-1 or Ceranib-2 for 24 hr and the metabolic viability of the cells was determined using the MTS assay. As shown in Figure 2B, neither compound caused significant cytotoxicity at concentrations up to at least those used in the ceramidase assay, indicating that the reduction in activity observed is not due to cell death. To compare the potency of our new compounds to previously described ceramidase inhibitors, we treated SKOV3 cells with DMAPP or N-oleoylethanolamine. DMAPP had no effect on ceramidase activity or cell survival up to at least 300 μ M, whereas N-oleoylethanolamine had no effect on ceramidase activity in this assay but did induce cell death with an IC_{50} of 50 ± 16 μ M. Therefore, the Ceranibs are considerably more potent than these lipid analogs for inhibiting ceramidase activity in an intact cell.

To determine if the compounds are able to inhibit the hydrolysis of endogenous ceramides, SKOV3 cells at near-confluence were treated with Ceranib-1 or Ceranib-2 for 24 hr and the levels of intracellular sphingolipid and dihydrosphingolipid species were quantified by HPLC-mass spectrometry. As indicated in Figure 3A, treatment with 12.5 μ M Ceranib-1 induced an accumulation of ceramide species including C_{14} , C_{24} , C_{26} and $C_{26:1}$, with an overall increase of approximately 32% in total ceramide levels compared with vehicle-treated cells. In addition, Ceranib-1 induced a dose-dependent decrease in the intracellular levels of both sphingosine and S1P to 10 and 34% of vehicle-treated cells, respectively. Concerning the dihydrosphingosine species, Figure 3C demonstrates that Ceranib-1 had no effect on the levels of these lipids until a concentration of 50 μ M, at which point the levels of dh C_{16} -Cer and dhS1P increased to approximately 650 and 3000% of that observed in vehicle-treated cells, respectively. In contrast, as shown in Figure 3B, Ceranib-2 induced an increase in the levels of every ceramide species measured, except C_{22} ceramide, with an

increase in total ceramide levels approximately 109% greater than vehicle-treated cells at 3.125 μ M Ceranib-2. Like Ceranib-1, Ceranib-2 caused a dose-dependent decrease in the intracellular levels of sphingosine and S1P to 16 and 30% of vehicle-treated cells, respectively. Also, as seen in Figure 3D, Ceranib-2 caused an increase in the levels of dhC₁₆-Cer at every concentration tested, reaching a peak of approximately 800% of that found in vehicle-treated cells at 50 μ M Ceranib-2. In contrast, this compound did not cause a large elevation in the levels of dhS1P, and induced a decrease of intracellular dhSph. Overall, these data suggest that the compounds prevent the hydrolysis of endogenous ceramide species and reduce intracellular sphingosine and S1P.

Antiproliferative effects of Ceranib-1 and Ceranib-2

To examine the effects of these compounds on cell proliferation, SKOV3 cells in exponential growth were treated with a ceramidase inhibitor for 72 hr and then the cell number was quantified. As shown in Figure 4A, cell proliferation and/or survival were inhibited with IC₅₀ values of 3.9 ± 0.3 and 0.73 ± 0.03 μ M for Ceranib-1 and Ceranib-2, respectively. In contrast, DMAPP did not inhibit cell proliferation to doses of at least 100 μ M, and N-oleoylethanolamine demonstrated an IC₅₀ of approximately 60 μ M (data not shown). Therefore, the increased potencies of the Ceranibs for inhibition of cell proliferation is consistent with their increased potencies for inhibiting cellular ceramidase activity.

Because SKOV3 cells are an ovarian adenocarcinoma cell line, we sought to determine if these ceramidase inhibitors could be effectively combined with paclitaxel, a standard drug for ovarian cancer. As shown in Figures 4B and 4C, paclitaxel inhibited SKOV3 proliferation with an IC₅₀ of 2.60 ± 0.24 nM, whereas single-agent Ceranib-1 and Ceranib-2 demonstrated IC₅₀s of 4.10 ± 0.42 and 0.71 ± 0.08 μ M, respectively. When used in combination, paclitaxel and Ceranib-1 or Ceranib-2 had additive effects on SKOV3 proliferation.

To determine the effects of Ceranib-2 on the cell cycle, SKOV3 cells in exponential growth were treated with DMSO, 0.75 μ M or 1.5 μ M Ceranib-2 for 48 hr. The cells were fixed, stained with PI, and the DNA content of each cell was measured by flow cytometry. As indicated in Figure 5A, 45.52 ± 1.67 , 28.58 ± 1.02 , 25.90 ± 2.68 and 0.25 ± 0.07 % of vehicle-treated cells were found to be in G1, S, G2 and sub-G1, respectively. In contrast, Figure 5B demonstrates that incubation of SKOV3 cells with 0.75 μ M Ceranib-2 changed the distribution of cells to 25.42 ± 0.68 , 41.36 ± 3.54 , 33.23 ± 4.22 and 11.38 ± 3.37 % in G1, S, G2 and sub-G1, respectively. A higher dose of Ceranib-2 (1.5 μ M, Figure 5C) further altered the cell cycle distribution to 22.53 ± 0.17 , 29.90 ± 2.75 , 47.57 ± 2.58 and 15.82 ± 3.30 % in G1, S, G2 and sub-G1. Overall, these data indicate that Ceranib-2 causes accumulation of cells in the sub-G1 (apoptosis), G2 and S (0.75 μ M only) phases of the cell cycle, concomitant with reductions in the number of cells in G1 phase.

Antitumor activity of Ceranib-2

To determine if the novel ceramidase inhibitor has antitumor activity, a syngeneic tumor model consisting of JC mammary adenocarcinoma cells growing subcutaneously in immunocompetent Balb/c mice was used. When palpable tumors were observed, mice were randomized into groups (n = 12–13) receiving 0, 20 or 50 mg/kg Ceranib-2 by intraperitoneal injection on 5 days per week. As indicated in Figure 6A, the average normalized size of tumors in each treatment group increased over time. However, by Day 11 both Ceranib-2-treated groups had significantly lower tumor volumes than did the vehicle-treated controls. By Day 21, the average normalized tumor volumes for the control, 20 mg/kg and 50 mg/kg Ceranib-2 groups were 1400 ± 370 , 940 ± 290 , and 710 ± 170 %, respectively. Administration of Ceranib-2 did not affect the total body weight of the animals

(insert in Figure 6A). Also, blood samples from three representative mice of each group were assessed for blood chemistry and complete blood cell counts, and all groups were statistically equivalent (data not shown). Finally, in preliminary pharmacokinetic evaluations (Figure 6B), intraperitoneal administration of 50 mg/kg Ceranib-2 resulted in progressive increases in its circulating levels, reaching a peak plasma concentration of approximately 40 μ M at the 2 hr time point. This concentration of Ceranib-2 is well-above that required to inhibit cell proliferation; however, the compound appears to be cleared with a half-life of less than 2 hr.

Discussion

To identify inhibitors of ceramidase activity with more favorable “drug-like” properties than existing compounds, a high-throughput screen was developed in SKOV3 cells using a previously described fluorogenic ceramidase assay (39), and approximately 50,000 compounds from the ChemBridge DIVERset Library were screened. Ceranib-1 emerged from the screen as a lead compound, and several analogs were synthesized to begin the assessment of structure-activity relationships. Ceranib-2 was found to be the most potent inhibitor in the cell-based assay with an IC_{50} of 28 μ M compared to 55 μ M for Ceranib-1.

While this data provided a strong indication that the compounds inhibit intracellular ceramidase activity in SKOV3 cells, it was necessary to rule out the potential that these results represented a false positive due to cell death induced by the compounds. To study this, SKOV3 cells under the same conditions as those in the high-throughput screen were assessed for metabolic activity using the MTS assay to determine cell viability. Neither compound induced significant cell death under the same conditions as the high-throughput screen, indicating that the inhibition of ceramidase activity observed upon treatment with the compounds was not simply due to cell death. In contrast, DMAPP did not decrease cellular ceramidase activity nor cell viability at up to at least 300 μ M. While N-oleoylethanolamine did decrease cellular viability with an IC_{50} of 50 μ M, it did not affect cellular ceramidase activity below 300 μ M, suggesting that it can induce cell death by a mechanism other than ceramidase inhibition. This is not surprising since N-oleoylethanolamine is a highly hydrophobic analog of ceramide that may have a variety of membrane-disruptive effects. In any case, the data demonstrate that our experimental compounds Ceranib-1 and -2 are more potent inhibitors of ceramidase activity than the commonly used ceramidase inhibitors DMAPP and N-oleoylethanolamine. Furthermore, these compounds meet all of the Lipinski “Rule of Five” requirements for a drug-like compound, making them more amenable to further development.

To determine if the compounds inhibit the activity of the ceramidase enzymes toward endogenous ceramide species, SKOV3 cells were treated with the compounds under similar conditions to those in the high-throughput screen, and levels of intracellular sphingolipid and dihydrosphingolipid species were determined. Both compounds caused an accumulation of various ceramides and decreases in sphingosine and S1P. These data suggest that the compounds are inhibiting the activity of one or more ceramidase enzymes, causing an accumulation of ceramide species and decreasing the levels of sphingosine, the product of ceramidase activity. While both compounds demonstrate a roughly similar ability to decrease the intracellular levels of sphingosine and S1P, Ceranib-2 caused a greater accumulation of total ceramides than did Ceranib-1. This observation supports the dose-response data indicating that Ceranib-2 is a more potent inhibitor of ceramidase activity than is Ceranib-1.

To determine the effect of these compounds on replicating cancer cells, SKOV3 cells in exponential growth were treated with the compounds and incubated for 72 hr. Both

compounds produced a substantial decrease in the number of cells; however, Ceranib-2 was approximately 5 times more potent than Ceranib-1 with IC₅₀ values of 0.73 and 3.92 μ M, respectively. There are two potential explanations for this difference in potency compared with the cell-based ceramidase activity assay. First, the ceramide/S1P rheostat model dictates that as the balance shifts in the direction of greater total ceramides and lower S1P cells undergo apoptosis. Thus, because lower concentrations of Ceranib-2 cause a greater accumulation of total ceramides than Ceranib-1, Ceranib-2 causes a shift in the rheostat at lower concentrations leading to cell death. Second, Ceranib-1 greatly increases the levels of intracellular dhS1P, whereas Ceranib-2 does not. DhS1P is known to activate the ERK signaling pathway, which stimulates survival and proliferation (41). It should also be noted that both compounds induce an accumulation of the anti-apoptotic species dhC₁₆-Cer, which has been shown to inhibit ceramide channel formation in the mitochondria, decreasing permeabilization of the membrane, blocking apoptosis (42). However, the increase in this ceramide species may simply be a survival response to the increase in pro-apoptotic ceramide species.

The fluorogenic ceramide analog used on our studies is a direct analog of saturated dhC₁₆-Cer. Therefore, the cellular ceramidase assay is specifically measuring the activity of ceramidases that hydrolyze dhC₁₆-Cer. Previous studies have shown that acid and neutral ceramidase have activity toward this substrate (43–44). In contrast, the alkaline ceramidases have not been reported to have activity toward this specific substrate (45–46). Therefore, it appears likely that the fluorogenic cellular assay is measuring the activity of acid and neutral ceramidases; however, we cannot conclude that our compounds only inhibit these two enzymes. In addition, while the ceramidase isozymes demonstrate substrate specificity, there is considerable overlap among the isozymes. In particular, alkaline ceramidase 2 has a very broad range of substrate specificity, with activity toward ceramide species from C₂ to C₂₄ (47). Therefore, we cannot attribute the observed accumulation of ceramide species to the inhibition of any one particular ceramidase enzyme. Further studies are in progress to determine if Ceranib-1 and Ceranib-2 are selective or pan-inhibitory toward the ceramidases.

Ceramides have been shown to regulate the sensitivity of tumor cells to anticancer drugs (48–49), including paclitaxel (21). Our data show that co-administration of Ceranib-1 or Ceranib-2 with paclitaxel leads to additive inhibition of proliferation of SKOV3 cells. While synergy was not observed under these conditions, we will continue to assess the combined effects of new Ceranib analogs with established and experimental anticancer drugs. Exposure of cells to Ceranib-2 caused an accumulation in the G2 phase of the cell cycle and promoted apoptosis. This is consistent with a previous report of quinolinones with similar structures to the Ceranibs which are reported to induce caspase activation (50). The ability of Ceranib-2 to induce apoptosis and cause arrest in the G2/M phase likely contributes to the additivity seen with the microtubule stabilizer paclitaxel, which also causes arrest in the G2/M phase. Overall, these data demonstrate that Ceranib-2 is both antiproliferative and cytotoxic to ovarian cancer cells.

To determine if Ceranib-2 has *in vivo* effects on tumor growth, Balb/c mice were subcutaneously injected with JC cells, allowed to develop palpable tumors, and treated intraperitoneally with 0, 20 or 50 mg/kg Ceranib-2. This model was utilized to assess the effects of the compound in animals with a functional immune system. Ceranib-2 produced a dose-dependent suppression in tumor growth over time, with statistically significant differences in tumor volume occurring by Day 11 of the study. By termination of the study on Day 21, mice treated with 50 mg/kg Ceranib-2 had only half of the increase in tumor size observed in vehicle-treated mice, indicating that Ceranib-2 is able to suppress tumor growth *in vivo*. Total body weight of mice treated with Ceranib-2 did not change over time, and no

significant difference was observed in the blood chemistry or blood cell counts among any group, suggesting low toxicity with repeated administration of the compound. The plasma concentration of Ceranib-2 peaked at approximately 40 μM 2 hr after administration, and remained above the IC_{50} of the *in vitro* antiproliferative assays for approximately 6 hr. However, more robust studies will need to be undertaken to determine other pharmacokinetic parameters such as volume of distribution, clearance and half-life of the compound.

In conclusion, these studies characterize Ceranib-1 and Ceranib-2 as novel inhibitors of human ceramidase activity that may serve as a new chemotype for the development of anticancer therapeutics. These compounds are able to inhibit cellular ceramidase activity causing an accumulation of ceramides and a reduction in cellular sphingosine and S1P. These compounds were also found to inhibit cell proliferation and induce cell death in a human ovarian cancer cell line, both as single agents and in an additive manner with paclitaxel. In addition, administration of Ceranib-2 to tumor-bearing mice reduced tumor growth with no overt signs of toxicity with repeated treatment. Overall, these studies suggest that Ceranib-2 can serve as a lead compound in the development of a new class of anticancer therapeutics. In future studies, we will focus on identifying the specific ceramidase isozyme(s) affected, characterizing the signaling pathways disrupted by administration, and broaden the scope of the *in vivo* data to include multiple cancer models with pharmacokinetic and toxicology profiles.

Acknowledgments

These studies utilized several shared resources funded in part by the Hollings Cancer Center, including the Drug Discovery Core, the Lipidomics Shared Facility Analytical Core, and the Flow Cytometry Core Facility.

Grant Support

Financial support was provided by NIH grant 1 R01 CA122226 (C. D. Smith)

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Figure 1. Ceranib-1 and synthesis of Ceranib-2
(A) Structure of Ceranib-1. (B) Synthetic route to Ceranib-2.

Figure 2. Inhibition of cellular ceramidase activity and acute cytotoxicity of ceramidase inhibitors

(A) Near-confluent SKOV3 cells were incubated in media containing 16 μ M fluorogenic ceramidase substrate and DMSO (vehicle control), Ceranib-1 (●) or Ceranib-2 (■) at the indicated concentrations for 24 hr at 37°C. Hydrolysis of the ceramidase substrate was analyzed on a SpectraMax platereader by measuring the increase in fluorescence at Excitation = 355 nm, Emission = 460 nm. (B) SKOV3 cells at near-confluence were treated with DMSO, Ceranib-1 (●) or Ceranib-2 (■) at the indicated concentrations for 24 hrs at 37°C. Cell viability was determined by the MTS assay. The values represent the mean $\pm$ SEM of three experiments carried out in triplicate.

Near-confluent SKOV3 cells were treated with Ceranib-1 (A, C) or Ceranib-2 (B, D) at final concentrations of 0.78 (open bars), 3.125 (striped bars), 12.5 (checkered bars), or 50 μ M (solid bars) for 24 hr at 37°C, and sphingolipid and dihydrosphingolipid species was quantified by HPLC-MS/MS. These values were normalized to the lipid levels in vehicle-treated cells, such that a value of 100% equals the level found in these controls. The values represent the mean $\pm$ SEM of two experiments carried out in duplicate.

Figure 4. Cytotoxicities of Ceranibs alone and in combination with paclitaxel

(A) SKOV3 cells in exponential growth were treated with varying concentrations of Ceranib-1 (●) or Ceranib-2 (■) for 72 hr at 37°C, and cell numbers were quantified using the SRB assay. The values represent the mean $\pm$ SEM of three experiments carried out in triplicate. (B) SKOV3 cells in exponential growth were treated with paclitaxel alone, Ceranib-1 alone or paclitaxel in combination with Ceranib-1 for 72 hr at 37°C. (C) SKOV3 cells in exponential growth were treated with paclitaxel alone, Ceranib-2 or paclitaxel in combination with Ceranib-2 for 72 hr at 37°C. Cell survival was determined by SRB assay. In Panels B and C, isobolograms were constructed to show the combined effects of paclitaxel and Ceranib-1 (B) or Ceranib-2 (C). The line of additivity connects the single-agent IC₅₀s for the test compounds, and values represent the mean $\pm$ SEM of six experiments carried out in triplicate.

Figure 5. Cell cycle effects of Ceranib-2

SKOV3 cells in exponential growth were treated with (A) DMSO, (B) 0.75 μM Ceranib-2 or (C) 1.5 μM Ceranib-2 for 48 hr at 37°C. The DNA content of each cell was determined by flow cytometry after staining with propidium iodide. Chromatograms are representative of two experiments carried out in duplicate.

Figure 6. *In vivo* antitumor activity and pharmacokinetics of Ceranib-2

(A) Female Balb/c mice with subcutaneous JC cell tumors were treated intraperitoneally with 0 (n=13, ●), 20 (n=12, ■) or 50 mg/kg Ceranib-2 (n=12, ▲) daily for 5 days per week. The volume of each tumor was normalized to Day 1 for each individual mouse, and the group average and standard deviations were calculated. Statistical significance was determined by unpaired student's t-test with $p < 0.05$, 0.01 and 0.001 represented by *, **, and ***, respectively. (B) Female Balb/c mice were administered a dose of 50 mg/kg Ceranib-2 by intraperitoneal injection, and blood was harvested at 0.5, 2 and 6 hr. Plasma levels of Ceranib-2 were quantified by HPLC analyses. Values represent the mean $\pm$ SD (n = 5).