Published in final edited form as:

Bioorg Med Chem. 2009 February 15; 17(4): 1498–1505. doi:10.1016/j.bmc.2009.01.005.

Ceramides: Branched alkyl chains in the sphingolipid siblings of diacylglycerol improve biological potency

Ji-Hye Kang^a, Himanshu Garg^b, Dina M. Sigano^a, Nicholas Francella^b, Robert Blumenthal^{b,*}, Victor E. Marquez^{a,*}

^aLaboratory of Medicinal Chemistry, National Cancer Institute, National Institutes of Health, Bldg 376/104, Frederick, MD 21702, United States

^bNanobiology Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bldg 469/152, Frederick, MD 21702, United States

Abstract

The synthesis of a small number of ceramide analogues containing a combination of linear and highly branched alkyl chains on either the D-sphingosine or the *N*-acyl core of the molecule is reported. Regardless of location, the presence of the branched chain improves potency relative to the positive control, C2 ceramide; however, the most potent compound (**4**) has the branched side chain as part of the D-sphingosine core. The induction of apoptosis by **4** in terms of Annexin V binding and DiOC₆ labeling was superior to that achieved with C2 ceramide.

Keywords

Alkyl branched ceramides; D-Sphingosine; Apoptosis; SupT1 cells; Annexin V; DiOC₆

1. Introduction

The design of ceramide derivatives that mimic the activities of natural ceramide is an important area of research aimed at developing novel therapeutics for the treatment of cancer, allergy, and other diseases originating from cell regulation disorders.^{1–5}

For many years our laboratory has been engaged in the design of novel, more effective diacylglycerol (DAG) analogues as activators of protein kinase C (PKC). In converting the micromolar active, naturally occurring DAG analogues into more potent and pharmacologically effective compounds, we have pursued two independent strategies in modifying the structure of DAG. These involve (1) the conversion of the DAG backbone into a pentonolactone, more specifically a 5,5-bis(hydroxymethyl)-3,4,5-trihydrofuran-2-one and (2) the use of branched alkyl chains to replace the natural, linear aliphatic hydrocarbons.⁶

*Corresponding authors. Tel.: +1 301 846 5532; fax: +1 301 846 5598 (R. Blumenthal); tel.: +1 301 846 5954; fax: +1 301 846 6033. blumen@helix.nih.gov (R. Blumenthal), marquezv@mail.nih.gov (V.E. Marquez).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.005.

These changes, singly or in combination, have resulted in compounds with increased potencies, which have become useful probes and potential drug candidates.^{7,8}

Because ceramide can be considered to be the sphingolipid sibling of DAG,⁹ we reasoned that introducing similar structural changes in ceramide as those implemented with DAG might improve its biological potency. DAG is a second messenger derived from the phospholipase-C cleavage of phosphatidyl inositol 4,5-bisphosphate. Once released, DAG exerts its biological activity by binding to specific membrane targeting domains (C1 domains) present in PKC and other proteins.^{10,11} Ceramide is similarly generated through a phospholipase-C-type reaction by sphingomyelinases acting on the lipid precursor sphingomyelin (Fig. 1). The fate and function of the released ceramide, however, is less well understood and although its function as a second messenger has been challenged,¹² evidence for its role in apoptosis is experimentally sound.¹³ While the consensus is that ceramide indeed operates as a unique second messenger for the induction of apoptosis, as originally proposed by Hannun and co-workers,¹⁴ diverse effects on cell differentiation, senescence and proliferation are more complex and depend highly on the origin and cellular compartmentalization of ceramide.^{15,16} In addition to its production from the hydrolysis of sphingomyelin (Fig. 1), ceramide can also be generated by de novo synthesis in response to the cellular stress resulting from radiation and cancer chemotherapeutic agents.^{17–19}

Since natural ceramide is poorly soluble in physiologically acceptable solvents and does not enter cells, short chain ceramides, like C2 ceramide (**1**), have been used as surrogates. These compounds, albeit effective as apoptosis inducers, may not be ideal mimics of ceramide because of their different physicochemical properties.²⁰

Nevertheless, these lower molecular weight ceramides have been used extensively as biochemical tools to study the role of ceramide in apoptosis.²¹

2. Activity guided design of ceramide analogues

In a recent study Schultz and co-workers,³ described the synthesis and apoptotic activity of an extensive ceramide library, and concluded that one of the key structural elements required for activity was the sum of the carbon length in the two cores—the sphingosine and the *N*-acyl chain. The most potent analogue of such a library was a compound with a total length of 22 carbons containing a sphingosine unit of 12 carbons and an *N*-acyl chain 10 carbons long.³ Keeping this concept in mind, we decided to explore some structural changes on ceramides, similar to those implemented with DAG, beginning with the use of combined linear and branched alkyl chains on both the D-sphingosine (DES) and the *N*-acyl cores. To gauge the progress of our strategy, we measured cytotoxicity (IC₅₀) in cultured Sup T1 cells relative to C2 ceramide as a positive control (Table 1). Although C6 ceramide with the longer acyl chain had an IC₅₀ value similar to C2 (**1**) in this assay (48.01 μM and 41.44 μM, respectively), C2 ceramide (**1**) was chosen to be the positive control because of its extensive use as a reference. Both analogues have been reported in the literature as nearly equivalent.

C2 ceramide (**1**) has an unbalanced distribution of 20 carbons between the DES and *N*-acyl core (*18*DES-*N*-acyl2); so for our first target (**3**) with a matching log*P*,²³ we provided a more balanced distribution of 21 carbons (*15*DES-*N*-acyl6) with a branched chain as part of the DES core. This type of branched hydrocarbon chain was chosen because of the significant improvement in potency it produced compared to equivalent linear alkyl chains in DAG analogues.^{24–27} Because compound **3** was almost as cytotoxic as C2 ceramide (Table 1), we decided to increase the number of carbons on the *N*-acyl side to 11. The resulting compound (**4**) with a total distribution of 26 carbons (*15*DES-*N*-acyl11) showed an activity approaching 3 times the potency of C2 ceramide. We then synthesized **5**, an isomer of **4**, to test the importance of the location of the branched chain by swapping the components on each side while maintaining a similar *15*DES-*N*-acyl11 distribution. This arrangement proved to be less effective, and although the compound was about 1.5 times more potent than C2 ceramide, it displayed half the potency of **4**. Because the activity of ceramides is lost when the double bond is reduced, as confirmed by the inactivity of dihydro-C2 ceramide (**2**), the double bond in compound **5** was reduced to give compound **6** which was to be compared to **2**. Remarkably, despite a 3-fold loss in potency the compound still retained a significant level of cytotoxicity in the same range of C2 ceramide, showing only about a 2-fold decrease in potency. The cytotoxic activity for the dihydroceramide analogue of compound **4** (not shown) was also reduced by 3-fold.

3. Chemistry

The branched chain substituents were derived from a common intermediate **7**, which could be converted to its acyl chloride (**8**) using previously published methodology.^{25–27} Alternatively, this same intermediate (**7**) could also be homologated to alkyne **9** using a one-pot procedure developed by Bestmann and co-workers by treating the aldehyde with dimethyl acetyldiazophosphonate under basic conditions (Scheme 1).²⁸

The ceramide analogues were synthesized as shown in Scheme 2. Commercially available *N*-Boc-L-serine methyl ester was protected as the oxazolidine derivative **10**. This compound was converted into Garner's aldehyde **11**^{29–31} according to the method of Williams et al.³¹ involving a tandem reduction–oxidation sequence. Nucleophilic addition by the deprotonated alkynes afforded **12a–b** which, following deprotection of the oxazolidine ring, gave **13a–b**. Partial reduction of the triple bond to the major trans double bond isomers (**14a–b**)^{32,33} was followed by removal of the Boc group and MgO-promoted acylation³⁴ to give the final targets (**3–5**). Further reduction of **5** gave **6**.

4. Biological results and discussion

As discussed above, preliminary cellular activity was measured in terms of cytotoxicity in cultured Sup T1 cells (Table 1) using a cell titer blue assay.³⁵ This was accomplished by measuring the ratio of fluorescence at 560/590 nm. In terms of cytotoxicity, compounds **4** and **5** displayed IC₅₀ values lower than C2 ceramide under similar conditions. Both compounds have the same molecular weight, logP and distribution of carbons between the two cores (*1,3*-DES-*N*-acyl). Therefore, their nearly 2-fold difference suggests that the role of the branched side chain is more effective when incorporated into the DES core (Table 1). The lower potency of compound **3**, which has a similar disposition of the branched chain as in **4**, could simply be due to its lower lipophilicity of 2logP units. The cytotoxicity of the dihydroceramide analogue **6** is interesting and consistent with the findings reported by Schultz and co-workers³ who showed that the lack of apoptotic activity of some dihydroceramide analogues could be restored when a bulky *N*-acyl tail was introduced. Although in such a study all of the synthesized ceramides had linear alkyl chains on the DES core, our results seem to indicate that the effect of branching the dihydroceramides is helpful regardless of whether the branched chain is located on the DES or *N*-acyl side of the molecule.

The fact that compound **4** was significantly more cytotoxic than C2 ceramide in SupT1 cells encouraged us to test the cytotoxicity of this compound in other cell lines. Since breast cancer remains a significant threat and the focus of intense research, we used breast cancer cell lines MCF-7, MDA MB231 and SKBR3 to test the potency of **4**. As seen in Table 2, the compound was significantly more cytotoxic than C2 ceramide in all of the cell lines with IC₅₀ values ranging from 10.15 μ M for SKBR-3 cells to 22.36 μ M for MDA-MB231 cells. The increase in activity was almost 5-fold for SKBR-3 although for other cell lines including SupT1 cells it was 2.7-fold. MDA-MB231 cells only showed a 1.6-fold increase in activity underscoring the difference between cell lines with regards to the ceramide signaling pathways. Cytotoxicity against non-cancerous cells, such as normal human dermal fibroblasts (NHDF), was also compared. The IC₅₀ for C2 ceramide was 66.5 μ M whereas for compound **4** it was 45.39 μ M (single determinations). The average IC₅₀ for C2 ceramide against the panel of cancer cell lines examined was 45.85 μ M with a selectivity index (SI) of just 1.5. On the other hand, the average IC₅₀ of compound **4** against the same panel was 16.06 μ M, thus providing a more favorable SI value of nearly 3-fold. Therefore, compound **4** represents a novel ceramide analog that shows significantly higher cytotoxicity and selectivity in a variety of cell lines by the induction of apoptosis (vide infra).

Compound **4** was selected for further studies in comparison with C2 ceramide beyond the simple determination of the IC₅₀ values in Sup T1 cells to determine the effect on apoptosis induction by two independent markers: (1) Annexin V as an early marker of apoptosis and (2) DiOC₆ to detect mitochondrial depolarization. Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA.³⁶ One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin

V, a phospholipid binding protein with a high affinity for PS.³⁷ The translocation of PS is an early event that normally precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. After the induction of apoptosis by C2 ceramide and compound **4**, cells were collected, washed, and stained with Annexin V conjugated to the fluorochrome FITC. Cells that stain brightly with Annexin V by flow cytometry are considered apoptotic and are marked by marker M1 in the histograms (Fig. 2 top).

Mitochondria are also critical regulators of apoptosis. The depolarization of the outer mitochondrial membrane releases cytochrome *c* into the cytoplasm. Cytoplasmic cytochrome *c* forms a complex with the apoptotic protease activating factor (apaf1) and caspase 9 to initiate the caspase cascade leading to apoptosis.³⁸ Short chain ceramides are known to induce apoptosis via the mitochondrial pathway.³⁹ It was thus important to look at mitochondrial depolarization induced by compound **4** and C2 ceramide in SupT1 cells. DiOC₆ is a dye that binds specifically to the mitochondrial membrane and the intensity of binding is proportional to the membrane potential. Hence, in the case of DiOC₆, less staining with DiOC₆ represents mitochondrial depolarization and an indicator of cells undergoing apoptosis as shown by marker M1 in the histograms (Fig. 2 bottom).

As seen in Figure 2, both C2 ceramide and compound **4** induced apoptosis in SupT1 cells determined by Annexin V binding or DiOC₆ labeling. Compound **4** caused more apoptosis determined by Annexin V at 25 μ M (66.16%) than C2 ceramide at 50 μ M (42.33%) confirming our cytotoxicity assays. Similar results were seen for mitochondrial depolarization with C2 ceramide and compound **4** causing 56.14% and 75.15% apoptosis, respectively. Control cells did not show significant apoptosis in either assay.

Importantly, a dose-dependent effect was seen on apoptosis induction by both compounds with compound **4** being significantly more potent than C2 ceramide (Fig. 3 A and B). The induction of apoptosis by both compounds showing morphological features of apoptosis suggests that compound **4** acts via a mechanism similar to C2 ceramide albeit with a higher potency.

5. Conclusions

Herein we report for the first time the synthesis of ceramide analogues decked with a combination of linear and highly branched alkyl chains distributed on either side of the D-sphingosine (DES) and the *N*-acyl cores. We have determined that ceramide analogue **4**, with the branched chain on the DES core, is more potent than the corresponding isomer (**5**) that bears the branched alkyl chain on the *N*-acyl core. Both isomers **4** and **5** were more cytotoxic than C2 ceramide. A direct comparison between C2 ceramide and the most potent isomer **4** using Annexin V as an early marker of apoptosis and DiOC₆ to detect mitochondrial depolarization suggests that compound **4** is more potent than C2 ceramide.

6. Experimental

6.1. General procedures

All chemical reagents were commercially available. Column chromatography was performed on Silica Gel 60, 230–400 mesh(E. Merck) or by CombiFlash® instrument. ¹H and ¹³C NMR spectra were recorded on a Varian Unity Inova instrument at 400 and 100 MHz, respectively. Spectra are referenced to the solvent in which they were run (7.24 ppm for CDCl₃). Positive-ion, fast-atom bombardment mass spectra (FAB-MS) were obtained on a VG 7070E-HF double-focusing mass spectrometer operated at an accelerating voltage of 6 kV under the control of a MASPEC-II32 data system for Windows (Mass Spectrometry Services, Ltd). Either glycerol or 3-nitrobenzyl alcohol was used as the sample matrix, and ionization was effected by a beam of xenon atoms generated in a saddle-field ion gun at 8.0 ± 0.5 kV. Nominal mass spectra were obtained at a resolution of 1200, and matrix derived ions were background subtracted during data system processing. Optical rotations were recorded on a Jasco P-1010 polarimeter. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA.

6.2. Cells and reagents

SupT1 cells were maintained in RPMI media supplemented with 10% FBS and penicillin streptomycin (5000 U/mL). Breast cancer cell lines MCF-7 MDA-MB231 and SKBR3 cells were maintained in Dulbecco's modified Eagles Medium supplemented with 10% FBS and penicillin streptomycin (5000 U/mL). NHDF cells were obtained from Lonza (Walkersville, MD) and cultured in fibroblast cell medium as recommended by the manufacturer. Stock solutions of compounds were made in DMSO at a concentration of 50 mM. Compounds were diluted in media at 37 °C before addition to cells.

6.3. Cytotoxicity assay and IC₅₀ calculation

Cytotoxicity was determined on various cell lines by incubating with serial dilutions of the test compounds in media for 24 h. Cell viability was determined using the CellTiter-Blue® Cell Viability Assay (Promega Corp. Madison, WI) as per the manufacturer's instructions. The CellTiter-Blue® Cell Viability Assay is based on the ability of living cells to reduce resazurin to fluorescent resorufin. The formation of resorufin was measured using fluorescence plate reader (CytoFluor 4000, Applied Biosystems) at 560/590 excitation/emission. Data was normalized to no drug control and expressed as percent viability. Dose response curves obtained from serial dilution of the compounds were fit using sigma plot analysis software and IC₅₀ values for each experiment were calculated. Each experiment was repeated three times and the average IC₅₀ value was calculated along with its standard deviation.

6.4. Apoptosis detection

Sup T1 cells were incubated with different concentrations of the test compounds for 24 h. After 24 h incubation the cells were collected, washed with PBS and apoptosis was determined as phosphatidyl serine exposure by Annexin V FITC staining using ApoAlert® Annexin V Apoptosis Kit (Clontech) or as mitochondrial depolarization by staining with 10

μM DiOC₆ followed by flow cytometry on a BD FACScan flow cytometer (BD Bioscience). At least 10,000 events were collected and analyzed using CellQuest® software (BD Bioscience).

6.4.1. 6-Methyl-4-(2-methylpropyl)hept-1-yne (9)—According to a published procedure,²⁸ dimethyl acetyl diazophosphonate (5.9 g, 31 mmol) was added to a solution of 7^{25–27} (2.0 g, 12 mmol) and K₂CO₃ (5.8 g, 42 mmol) in MeOH (50 mL) and stirring continued until the reaction was complete as indicated by TLC. The reaction mixture was diluted with ether, washed with an aqueous solution of NaHCO₃, dried over MgSO₄ and concentrated in vacuo. Purification by Combiflash® column chromatography (hexanes) gave **9** (1.9 g, 92%) as a colorless oil which was used directly without further purification or characterization. ¹H NMR (400 MHz, CDCl₃) δ 2.14 (dd, J = 5.3, 2.7 Hz, 2H, CH₂C₂,C), 1.89 (t, J = 2.7 Hz, 1H, C₂,CH), 1.63 (m, 3H, CH), 1.25 (m, 2H, CH₂), 1.09 (m, 2H, CH₂), 0.86 (d, J = 6.6 Hz, 6H), 0.85 (d, J = 6.5 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 83.06, 69.07, 43.37, 31.86, 25.06, 23.12, 22.93, 22.49.

6.4.2. tert-Butyl (4S)-4-carbonyl-2,2-dimethyl-1,3-oxazolidine-3-carboxylate (11)—This compound, known as Garner's aldehyde^{29,30} was synthesized according to the method of Williams et al.³¹ from **10** involving a tandem reduction oxidation sequence.

6.4.3. General procedure A—Alkylation—According to a literature procedure,³² the alkyne (1.2 equiv) was added slowly to a 70 °C stirring solution of THF (5 mL/mmol alkyne) and *n*-BuLi (1.6 M in hexanes, 1.5 equiv) under argon. A suspension of **11** (1 equiv) in THF (30 mL/mmol) was then added to the resultant acetylide and the mixture allowed to reach rt. After 1 h, the reaction mixture was poured into water and extracted with diethyl ether. The combined organic extracts were dried over MgSO₄, filtered and concentrated in vacuo. This reaction provides the alcohols as a mixture of *erythro* and *threo* isomers from which the desired, more abundant *erythro* isomer can be purified by Combiflash® column chromatography (hexanes/EtOAc, 4:1).^{32,33}

6.4.3.1. tert-Butyl 4-((1R)-1-hydroxytridec-2-ynyl) (4S)-2,2-dimethyloxazolidine-3-carboxylate (12a): According to general procedure A, **11** (800 mg, 3.4 mmol) in THF (10 mL) was added to a solution of *n*-BuLi (3.2 mL, 5.1 mmol) and 1-dodecyne (681 mg, 4.1 mmol) in THF (20 mL) to give **12a** (310 mg, 23%) as a colorless oil;

$[\alpha]_D^{22} = -37.07$ (c 3.85, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 4.14 (m, 5H, H₄, H₅ and CHOH), 2.16 (td, J = 7.1, 1.8 Hz, 2H, C₂,CCH₂), 1.55 (br s, 4H, CH₂ and OH), 1.48 (s, 6H, CH₃), 1.47 (s, 9H, CH₃), 1.23 (m containing s at 1.23, 14H, CH₂), 0.85 (t, J = 6.9 Hz, 3H, CH₃); ¹³C NMR (100 MHz, CDCl₃) δ 154.04, 94.87, 86.59, 81.14, 77.85, 65.03, 64.07, 62.78, 31.84, 29.53, 29.46, 29.26, 29.08, 28.83, 28.52, 28.33, 25.71, 25.38, 22.62, 18.70, 14.05.; FABMS m/z (relative intensity) 396 (MH⁺, 19), 57 (100); Anal. Calcd for C₂₃H₄₁NO₄ 0.2H₂O: C, 69.20; H, 10.45; N, 3.51. Found: C, 69.18; H, 10.26; N, 3.60.

6.4.3.2. tert-Butyl 4-[(1R)-1-hydroxy-7-methyl-5-(2-methylpropyl)oct-2-ynyl](4S)-2,2-dimethyl-1,3-oxazolidine-3-carboxylate (12b): According to general procedure A, **11** (900 mg, 3.9 mmol) in THF (10 mL) was added to a solution of *n*-BuLi (3.7 mL, 5.9 mmol)

and **9** (778 mg, 4.7 mmol) in THF (20 mL) to give **12b** (320 mg, 20%) as a colorless oil; $[\alpha]_D^{22} = -34.61$ (c 0.81, CH_2Cl_2); ^1H NMR (400 MHz, CDCl_3) δ 4.60 (m, 1H, CHOH), 4.25 (br s, 1H, CHOH), 4.05 (m, 2H, H_5), 3.93 (br s, 1H, H_4), 2.13 (dd, $J = 5.4, 1.9$ Hz, 2H, C, CCH_2), 1.58 (m, 3H, CH), 1.46 (s, 6H, $\text{C}(\text{CH}_3)_2$), 1.45 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.19 (m, 2H, CH_2), 1.04 (m, 2H, CH_2), 0.83 (d, $J = 6.5$ Hz, 6H, $\text{CH}(\text{CH}_3)_2$), 0.82 (d, $J = 6.6$ Hz, 6H, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (100 MHz, CDCl_3) δ 153.78, 94.83, 84.99, 81.04, 79.02, 64.83, 63.71, 62.59, 43.49, 32.00, 28.34, 25.89, 25.02, 23.25, 23.10, 22.44; FABMS m/z (relative intensity) 396 (MH^+ , 13), 57 (100); Anal. Calcd for $\text{C}_{23}\text{H}_{41}\text{NO}_4$: C, 69.83; H, 10.45; N, 3.54. Found: C, 69.60; H, 10.53; N, 3.64.

6.4.4. General procedure B. Deprotection of the oxazolidine—According to the literature procedure,^{32,33} a catalytic amount of *p*-toluenesulfonic acid was added to solution of **12a–b** in anhydrous MeOH and stirred at rt until the reaction was complete by TLC analysis. The crude mixture was concentrated in vacuo and purified by Combiflash® column chromatography (hexane/EtOAc, 2:1) to give **13a–b**.

6.4.4.1. *N*-[(1*S*,2*R*)-2-Hydroxy-1-(hydroxymethyl)tetradec-3-ynyl](*tert*-butoxy)carboxamide (13a**).:** According to general procedure B, *p*-toluenesulfonic acid (30 mg) was combined with **12a** (300 mg, 0.76 mmol) in anhydrous MeOH (15 mL) and stirred for 27 h to give **13a** (155 mg, 58%) as a colorless oil; $[\alpha]_D^{22} = -13.50$ (c 0.33, CH_2Cl_2); ^1H NMR (400 MHz, CDCl_3) δ 5.36 d (d, $J = 8.3$ Hz, 1H, NH), 4.55 (s, 1H, CHOH), 4.01 (d, $J = 7.6$ Hz, 1H, CHHOH), 3.65 (m, 3H, CHN , CHHOH), 3.57 (s, 1H, OH), 3.11 (s, 1H, OH), 2.16 (t, $J = 7.1$ Hz, 2H, C, CCH_2), 1.46 (m, 11H, CH_2 and $\text{C}(\text{CH}_3)_3$), 1.29 (m, 14H, CH_2), 0.83 (t, $J = 6.5$ Hz, 3H, CH_3); ^{13}C NMR (100 MHz, CDCl_3) δ 156.22, 87.92, 79.98, 77.80, 64.36, 62.64, 55.81, 31.83, 29.53, 29.46, 29.25, 29.07, 28.87, 28.49, 28.30, 22.61, 18.65, 14.04; FABMS m/z (relative intensity) 356 (MH^+ , 39), 57 (100); Anal. Calcd for $\text{C}_{20}\text{H}_{37}\text{NO}_4 \cdot 0.5\text{H}_2\text{O}$: C, 65.90; H, 10.51; N, 3.84. Found: C, 66.00; H, 10.42; N, 3.92.

6.4.4.2. *N*-[(1*S*,2*R*)-2-Hydroxy-1-(hydroxymethyl)-8-methyl-6-(2-methoxypropyl)non-3-ynyl](*tert*-butoxy)carboxamide (13b**).:** According to general procedure B, *p*-toluenesulfonic acid (30 mg) was combined with **12b** (310 mg, 0.78 mmol) in anhydrous MeOH (10 mL) and stirred for 3 h to give **13b** (131 mg, 47%) as a colorless oil; $[\alpha]_D^{22} = -12.96$ (c 0.28, CH_2Cl_2); ^1H NMR (400 MHz, CDCl_3) δ 5.33 (br d, $J = 8.2$ Hz, 1H, NH), 4.58 (br s, 1H, CHOH), 4.04 (br d, $J = 7.7$ Hz, 1H, CHHOH), 3.72 (m, 2H, CHHOH , CHNH), 3.32 (br s, 1H, OH), 2.90 (br s, 1H, OH), 2.16 (dd, $J = 5.3, 2.0$ Hz, 2H, C, CCH_2), 1.59 (m, 3H, CH), 1.42 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.18 (m, 2H, CH_2), 1.06 (m, 2H, CH_2), 0.84 (m, 12H, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (100 MHz, CDCl_3) δ 156.25, 86.45, 80.01, 78.84, 64.59, 62.85, 55.87, 43.55, 31.97, 28.31, 25.05, 23.16, 23.13, 22.46, 22.44.; FABMS m/z (relative intensity) 356 (MH^+ , 16), 57 (100); Anal. Calcd for $\text{C}_{20}\text{H}_{37}\text{NO}_4 \cdot 0.3\text{H}_2\text{O}$: C, 66.56; H, 10.50; N, 3.88. Found: C, 66.37; H, 10.34; N, 3.87.

6.4.5. General procedure C: Reduction of alkyne to alkene—According to the literature procedure,³² Red-Al (65% w in toluene) was added to solution of alkyne (150 mg, 0.42 mmol) in anhydrous THF (20 mL). Stirring at rt continued until TLC analysis showed

the reaction was complete. The mixture was quenched with water and extracted with diethyl ether. The combined organic extracts were dried (MgSO₄), filtered and concentrated in vacuo. Purification by Combiflash® column chromatography (hexane/EtOAc, 1:1) gave the trans-double bond alkene.

6.4.5.1. *N*-[(3*E*)-(1*S*,2*R*)-2-Hydroxy-1-(hydroxymethyl)tetradec-3-enyl](*tert*-butoxy)carboxamide (14a**).:** According to general procedure C, Red-Al (0.26 mL, 0.84 mmol) was combined with **13a** (150 mg, 0.42 mmol) in anhydrous THF (20 mL) and stirred for 18 h to give **14a** (100 mg, 67%) as a white amorphous solid;

$[\alpha]_D^{22} = -4.13$ (*c* 1.485, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 5.75 (irr dt, 1H, C=CH), 5.50 (ddd, *J* = 15.4, 6.4, 1.2 Hz, 1H, C=CH), 5.28 (br d, *J* = 5.9 Hz, 1H, NH), 4.28 (s, 1H, CHOH), 3.90 (dd, *J* = 11.3, 3.4 Hz, 1H, CHHOH), 3.68 (br d, *J* = 12.1 Hz, 1H, CHHOH), 3.57 (s, 1H, CHNH), 2.66 (s, 2H, OH), 2.03 (q, *J* = 7.0 Hz, 2H, C=CHCH₂), 1.43 (s, 9H, CCH₃), 1.34 (m, 2H, CH₂), 1.23 (s, 14H, CH₂), 0.85 (t, *J* = 6.5 Hz, 3H, CH₃); ¹³C NMR (100 MHz, CDCl₃) δ 165.38, 134.09, 128.92, 79.75, 74.82, 65.83, 55.39, 32.27, 31.88, 29.61, 29.59, 29.46, 29.31, 29.18, 29.09, 28.35, 22.66, 15.23, 14.09; FABMS *m/z* (relative intensity) 358 (MH⁺, 35), 284 (100); Anal. Calcd for C₂₀H₃₉NO₄ 0.1H₂O: C, 66.85; H, 11.00; N, 3.90. Found: C, 66.95; H, 10.89; N, 3.90.

6.4.5.2. *N*-[(3*E*)-(1*S*,2*R*)-2-Hydroxy-1-(hydroxymethyl)-8-methyl-6-(2-methylpropyl)non-3-enyl](*tert*-butoxy)carboxamide (14b**).:** According to general procedure C, Red-Al (0.68 mmol, 0.2 mL) was combined with **13b** (120 mg, 0.34 mmol) in anhydrous THF (10 mL) and stirred for 16 h to give **14b** (80 mg, 65%) as a colorless oil;

$[\alpha]_D^{22} = -2.91$ (*c* 2.425, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 5.71 (m, 1H, CH=CH), 5.50 (dd, *J* = 15.4, 6.0 Hz, 1H, CH=CH), 5.30 (br d, *J* = 4.7 Hz, 1H, NH), 4.32 (br s, 1H, CHOH), 3.91 (br d, *J* = 9.4 Hz, 1H, CHHOH), 3.68 (m, 1H, CHHOH), 3.58 (br s, 1H, CHNH), 2.71 (br s, 2H, OH), 2.00 (t, *J* = 6.1 Hz, 2H, CH₂), 1.61 (sept, *J* = 6.3 Hz, 2H), 1.49 (m, 1H, CH), 1.43 (s, 9H, C(CH₃)₃), 1.03 (m, 4H, CH₂), 0.83 (d, *J* = 6.4 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 158.94, 131.93, 130.46, 79.81, 74.72, 62.68, 55.47, 43.68, 36.58, 32.58, 28.37, 25.08, 23.09, 22.65; FABMS *m/z* (relative intensity) 358 (MH⁺, 21), 284 (100); Anal. Calcd for C₂₀H₃₉NO₄ 0.3H₂O: C, 66.19; H, 11.00; N, 3.86. Found: C, 66.19; H, 10.56; N, 3.67.

6.4.6. General procedure D: Deprotection/N-acylation—Trifluoroacetic acid was added to a solution of **14a–b** in CH₂Cl₂ and stirred for 30 min to 1 h at rt. The crude reaction was then concentrated in vacuo and purified by Combiflash® chromatography (CH₂Cl₂/MeOH, 6:1 or 4:1) to give the free amine as a colorless oil which was used immediately without further characterization. According to a literature procedure,³⁴ magnesium oxide was added to a solution of the sphingosine in THF/H₂O (4:1) and stirred vigorously for 30 min to 1 h at rt. The corresponding acid chloride was then added and stirring continued until the reaction was complete by TLC analysis. The reaction mixture was then filtered through a bed of Celite® and the filtrate was concentrated in vacuo. Purification by Combiflash® chromatography (100% EtOAc or hexane/EtOAc, 1:1) gave **3–5**.

6.4.6.1. *N*-[(3E)(1S,2R)-2-Hydroxy-1-(hydroxymethyl)-8-methyl-6-(2-methylpropyl)non-3-enyl]hexanamide (3): According to general procedure D, trifluoroacetic acid (1 mL) was combined with **14b** (40 mg, 0.11 mmol) in CH₂Cl₂ (1 mL) to give a colorless oil which was dissolved in THF/H₂O (4 mL) and treated with magnesium oxide (11 mg, 6.3 mmol) and hexanoyl chloride (41 mg, 0.2 mmol) to give **3** (24.2 mg, 62%) as a colorless oil; $[\alpha]_D^{22} = -7.50$ (c 0.875, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 6.28 (d, *J* = 7.3 Hz, 1H, *NH*), 5.72 (m, 1H, *CH=CH*), 5.50 (ddt, *J* = 15.4, 6.2, 1.1 Hz, 1H, *CH=CH*), 4.31 (irr br t, 1H, *CHOH*), 3.90 (m, 2H, *CHHOH*, *CHNH*), 3.68 (dd, *J* = 10.9, 3.0 Hz, 1H, *CHHOH*), 2.94 (v br s, 2H, *OH*), 2.21 (t, *J* $\approx$ 7.7 Hz, 2H, (CO)*CH*₂), 2.00 (t, *J* = 6.3 Hz, 2H, *CH*₂), 1.61 (m, 4H, *CH*₂ and *CH*), 1.52 (m, 1H, *CH*), 1.30 (m, 4H, *CH*₂), 1.03 (m, 4H, *CH*₂), 0.87 (t, *J* = 7.0 Hz, 3H, *CH*₃), 0.84 (d, *J* = 1.9 Hz, 6H, *CH*₃), 0.83 (d, *J* = 1.9, 6H, *CH*₃); ¹³C NMR (100 MHz, CDCl₃) δ 173.96, 132.04, 130.33, 112.53, 74.53, 62.47, 54.52, 43.67, 36.78, 36.59, 32.58, 31.40, 25.41, 25.08, 23.08, 22.66, 22.38, 13.92; FABMS *m/z* (relative intensity) 356 (MH⁺, 56), 338 (100); Anal. Calcd for C₂₁H₄₁NO₃: C, 70.94; H, 11.62; N, 3.94. Found: C, 70.67; H, 11.67; N, 3.94.

6.4.6.2. *N*-[(3E)(1S,2R)-2-Hydroxy-1-(hydroxymethyl)-8-methyl-6-(2-methylpropyl)non-3-enyl]undecanamide (4): According to general procedure D, trifluoroacetic acid (2 mL) was combined with **14b** (70 mg, 0.19 mmol) in CH₂Cl₂ (2 mL) to give a colorless oil which was dissolved in THF/H₂O (5 mL) and treated with magnesium oxide (18 mg, 0.43 mmol) and undecanoyl chloride (41 mg, 0.2 mmol) to give **4** (39 mg, 48%) as a colorless oil. $[\alpha]_D^{22} = -7.63$ (c 0.06, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ (d, *J* = 7.1 Hz, 1H, *NH*), 5.71 (m, 1H, *CH=CH*), 5.49 (ddd, *J* = 15.4, 6.1, 1.1 Hz, 1H, *CH=CH*), 4.30 (br s, 1H, *CHOH*), 3.89 (m, 2H, *CHHOH*, *CHNH*), 3.67 (br d, *J* = 9.2 Hz, 1H, *CHHOH*), 2.91 (v br s, 2H, *OH*), 2.20 (t, *J* = 7.1 Hz, 2H, =*CHCH*₂), 2.00 (t, *J* = 6.3 Hz, 2H, C(O)*CH*₂), 1.61 (m, 3H, *CH*), 1.50 (m, 2H, *CH*₂), 1.25 (m, 14H, *CH*₂), 1.02 (m, 4H, *CH*₂), 0.84 (m, 15H, *CH*₃); ¹³C NMR (100 MHz, CDCl₃) δ 173.99, 131.99, 130.33, 74.46, 62.44, 54.55, 43.67, 36.83, 36.60, 32.57, 31.87, 29.56, 29.49, 29.35, 29.28, 25.75, 25.08, 23.08, 22.66, 14.09; FABMS *m/z* (relative intensity) 426 (MH⁺, 26), 408 (100); Anal. Calcd for C₂₆H₅₁NO₃ 0.2H₂O: C, 72.74; H, 12.07; N, 3.26. Found: C, 72.60; H, 12.06; N, 3.24.

6.4.6.3. *N*-[(3E)(1S,2R)-2-Hydroxy-1-(hydroxymethyl)tetradec-3-enyl]-5-methyl-3-(2-methylpropyl)hexanamide (5): According to general procedure D, trifluoroacetic acid (3 mL) was combined with **14a** (95 mg, 0.27 mmol) in CH₂Cl₂ (3 mL) to give a colorless oil which was dissolved in THF/H₂O (5 mL) and treated with magnesium oxide (27 mg, 0.68 mmol) and 3-isobutyl-5-methyl-hexanoyl chloride (**8**)²⁵⁻²⁷ (66 mg, 0.32 mmol) to give **5** (44 mg, 37%) as a colorless oil; $[\alpha]_D^{22} = -6.05$ (c 0.305, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 6.25 (d, *J* = 6.9 Hz, 1H, *NH*), 5.75 (m, 1H, *CH=CH*), 5.49 (dd, *J* = 15.4, 6.3 Hz, 1H, *CH=CH*), 4.27 (s, 1H, *CHOH*), 3.88 (m, 2H, *CH*₂OH), 3.65 (d, *J* = 8.1 Hz, 1H, *CHNH*), 3.15 (br s, 2H, *OH*), 2.09 (d, *J* = 6.6 Hz, 2H, C(O)*CH*₂), 2.03 (m, 2H, *CH=CHCH*₂), 1.94 (m, *J* = 6.7 Hz, 1H, *CH*), 1.61 (sept, *J* = 6.6 Hz, 2H, *CH*(CH₃)₂), 1.31 (m, 16H, *CH*₂), 1.10 (m, 4H, *CH*₂), 0.85 (m, 15H, *CH*₃); ¹³C NMR (100 MHz, CDCl₃) δ 173.65, 134.12, 128.78, 74.41, 62.44, 54.69, 44.19, 42.35, 32.29, 31.88, 31.00, 29.61, 29.46, 29.31, 29.22, 29.12, 25.16, 22.87, 22.78, 22.76, 22.65, 14.08; FABMS *m/z* (relative intensity) 426 (MH⁺, 39), 408

(100); Anal. Calcd for $C_{26}H_{51}NO_3$: C, 73.36; H, 12.08; N, 3.29. Found: C, 73.35; H, 12.16; N, 3.32.

6.4.7. N-[(1S,2R)-2-Hydroxy-1-(hydroxymethyl)tetradecyl]-5-methyl-3-(2-methyl-propyl)hexanamide (6)—The double bond in compound **5** was reduced in MeOH with 5% Pd/C under and H_2 atmosphere at rt after 30 min. The crude reaction was then concentrated in vacuo to give **6** as a colorless oil; 1H NMR (400 MHz, $CDCl_3$) δ 6.26 (d, $J = 7.7$ Hz, 1H, NH), 3.99 (dd, $J = 11.3, 3.5$ Hz, 1H, $CHOH$), 3.82 (m, 1H, $CHHOH$), 3.75 (m, 2H, $CHHOH$, $CHNH$), 2.53 (s, 1H, OH), 2.10 (d, $J = 6.7$ Hz, 2H, $(CO)CH_2$), 1.95 (p, $J = 6.7$ Hz, 1H, CH), 1.58 (m, 7H, CH , OH and CH_2), 1.24 (s, 21H, CH_2), 1.11 (m, 4H, CH_2), 0.87 (d, $J = 1.9$ Hz, 6H, $CH(CH_3)_2$), 0.85 (m, 9H, CH_2CH_3 and $CH(CH_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.14, 74.26, 62.58, 53.84, 44.25, 42.42, 34.57, 31.91, 31.06, 29.66, 29.63, 29.57, 29.55, 29.35, 25.96, 25.19, 22.91, 22.80, 22.69, 14.12; FABMS m/z (relative intensity) 428 (MH^+ , 100); HRMS (FAB) Calcd for $C_{26}H_{53}NO_3$: (MH^+) 428.4104; found 428.4107; ($M+K^+$) 466.3662, found 466.3687.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

References and notes

- Nussbaumer P ChemMedChem 2008, 3, 543. [PubMed: 18061920]
- Reynolds CP; Maurer BJ; Kolesnick RN Cancer Lett. 2004, 206, 169. [PubMed: 15013522]
- Chang YT; Choi J; Ding S; Prieschl EE; Baumruker T; Lee JM; Chung SK; Schultz PG J. Am. Chem. Soc 2002, 124, 1856. [PubMed: 11866590]
- Bieberich E; Hu B; Silva J; MacKinnon S; Yu RK; Fillmore H; Broaddus WC; Ottenbrite RM Cancer Lett. 2002, 181, 55. [PubMed: 12430179]
- Delgado A; Casas J; Llebaria A; Abad JL; Fabrias G ChemMedChem 2007, 2, 580. [PubMed: 17252619]
- Marquez VE; Blumberg PM Acc. Chem. Res 2003, 36, 434. [PubMed: 12809530]
- Caloca MJ; Garcia-Bermejo ML; Blumberg PM; Lewin NE; Kremmer E; Mischak H; Wang SM; Nacro K; Bienfait B; Marquez VE; Kazanietz MG Proc. Natl. Acad. Sci. U.S.A 1999, 96, 11854. [PubMed: 10518540]
- Garcia-Bermejo ML; Leskow FC; Fujii T; Wang Q; Blumberg PM; Ohba M; Kuroki T; Han KC; Lee J; Marquez VE; Kazanietz MG J. Biol. Chem 2002, 277, 645 [Published erratum appears in J. Biol. Chem. 2004, 279, 23846]. [PubMed: 11584014]
- Hannun YA; Obeid LM Trends Biochem. Sci 1995, 20, 73. [PubMed: 7701566]
- Newton AC Chem. Rev 2001, 101, 2353. [PubMed: 11749377]
- Brose N; Rosenmund CJ Cell Sci. 2002, 115, 4399.
- Hofmann K; Dixit VM Trends Biochem. Sci 1998, 23, 374. [PubMed: 9810222]
- Levade T; Malagarie-Cazenave S; Gouaze V; Segui B; Tardy C; Betito S; Andrieu-Abadie N; Cuvillier O Neurochem. Res 2002, 27, 601. [PubMed: 12374195]
- Obeid LM; Linardic CM; Karolak LA; Hannun YA Science 1993, 259, 1769. [PubMed: 8456305]
- Okazaki T; Kondo T; Kitano T; Tashima M Cell. Signal 1998, 10, 685. [PubMed: 9884019]

16. Pettus BJ; Chalfant CE; Hannun YA *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 2002, 1585, 114.
17. Zheng W; Kollmeyer J; Symolon H; Momin A; Munter E; Wang E; Kelly S; Allegood JC; Liu Y; Peng Q; Ramaraju H; Sullards MC; Cabot M; Merrill AH *BBA. Biomembranes* 2006, 1758, 1864. [PubMed: 17052686]
18. Wedeking A; van Echten-Deckert G *Curr. Org. Chem* 2007, 11, 579.
19. Morales A; Lee H; Goni FM; Kolesnick R; Fernandez-Checa JC *Apoptosis* 2007, 12, 923. [PubMed: 17294080]
20. Sot J; Goni FM; Alonso A *BBA. Biomembranes* 2005, 1711, 12. [PubMed: 15904658]
21. Verheij M; Bose R; Lin XH; Yao B; Jarvis WD; Grant S; Birrer MJ; Szabo E; Zon LI; Kyriakis JM; HaimovitzFriedman A; Fuks Z; Kolesnick RN *Nature* 1996, 380, 75. [PubMed: 8598911]
22. Phillips DC; Allen K; Griffiths HR *Arch. Biochem. Biophys* 2002, 407, 15. [PubMed: 12392710]
23. Wildman SA; Crippen GM J. *Chem. Inf. Comput. Sci* 1999, 39, 868.
24. Nacro K; Bienfait B; Lewin NE; Blumberg PM; Marquez VE *Bioorg. Med. Chem. Lett* 2000, 10, 653. [PubMed: 10762046]
25. Nacro K; Bienfait B; Lee J; Han KC; Kang JH; Benzaria S; Lewin NE; Bhattacharyya DK; Blumberg PM; Marquez VE J. *Med. Chem* 2000, 43, 921. [PubMed: 10715158]
26. Lee J; Han KC; Kang JH; Pearce LL; Lewin NE; Yan S; Benzaria S; Nicklaus MC; Blumberg PM; Marquez VE J. *Med. Chem* 2001, 44, 4309 [Erratum published in J. Med. Chem. 2003, 46 (13): 2794].
27. Choi Y; Kang JH; Lewin NE; Blumberg PM; Lee J; Marquez VE J. *Med. Chem* 2003, 46, 2790. [PubMed: 12801241]
28. Müller S; Liepold B; Roth GJ; Bestmann HJ *Synlett* 1996, 521.
29. Garner P; Park JM J. *Org. Chem* 1987, 52, 2361.
30. Garner P; Park JM *Org. Syn* 1992, 70, 18.
31. Williams L; Zhang ZD; Shao F; Carroll PJ; Joullie MM *Tetrahedron* 1996, 52, 11673.
32. Suzuki H; Mori M; Shibakami M *Synlett* 2003, 2163.
33. Nimkar S; Menaldino D; Merrill AH; Liotta D *Tetrahedron Lett.* 1988, 29, 3037.
34. Kim DH; Rho HS; You JW; Lee JC *Tetrahedron Lett.* 2002, 43, 277.
35. O'Brien J; Wilson I; Orton T *Eur. J. Biochem* 2000, 267, 5421. [PubMed: 10951200]
36. Hail N; Carter BZ; Konopleva M; Andreeff M *Apoptosis* 2006, 11, 889. [PubMed: 16547589]
37. Reutelingsperger CPM; vanHeerde WL *Cell. Mol. Life Sci.* 1997, 53, 527. [PubMed: 9230931]
38. Kroemer G; Galluzzi L; Brenner C *Physiol. Rev* 2007, 87, 99. [PubMed: 17237344]
39. Hearps AC; Burrows J; Connor CE; Woods GM; Lowenthal RM; Ragg SJ *Apoptosis* 2002, 7, 387. [PubMed: 12207171]

Figure 1. Generation of DAG and ceramide by the hydrolysis of phosphatidylinositol-4,5-bisphosphate and sphingomyelin, respectively.

Figure 2. Apoptosis induced by compound **4** (25 μM) relative to C2 ceramide (50 μM) and control.

Figure 3. Apoptotic dose-responses for compound 4 (●) and C2 ceramide (○) in terms of Annexin V binding (A) and DiOC₆ labeling (B).

Scheme 1.

Reagents and conditions: (a) see Refs. 25–27; (b) dimethyl acetyldiazophosphonate, K_2CO_3 , MeOH.

Scheme 2.

Reagents and conditions: (a) 2,2-dimethoxypropane, TsOH H_2O , C_6H_6 , $110\text{ }^\circ\text{C}$; (b) (i) $NaBH_4$, MeOH/THF, (ii) $(COCl)_2$, DMSO, Et_3N , CH_2Cl_2 ; (c) $nBuLi$, $RC\equiv CH$, THF, $-78\text{ }^\circ\text{C}$; (d) $pTSA$, MeOH, rt; (e) Red-Al, THF, rt; (f) (i) TFA, CH_2Cl_2 , rt, (ii) MgO , $R'\text{'COCl}$, THF/ H_2O , rt; (g) H_2 , 5% Pd/C, MeOH, rt.

Table 1Ceramide analogues with mixed linear and branched alkyl chains and cytotoxicity (IC₅₀) in SupT1 cells

Compound #	Ceramides	IC ₅₀ (μM)	Formula	S log P ²³
1 C2 ceramide		41.44 ± 5.04	C ₂₀ H ₃₉ NO ₃	4.1016
2 Dihydro-C2 ceramide		>200	C ₂₀ H ₄₁ NO ₃	4.3256
3		52.89 ± 7.92	C ₂₁ H ₄₁ NO ₃	4.0594
4		15.45 ± 6.22	C ₂₆ H ₅₁ NO ₃	6.0099
5		29.08 ± 3.1	C ₂₆ H ₅₁ NO ₃	6.0099
6		76.30 ± 13.78	C ₂₆ H ₅₃ NO ₃	6.2339

Table 2IC₅₀ of compound **4** in various tumor cell lines

	C2 Ceramide IC ₅₀ μ M	Compound 4 IC ₅₀ μ M	Fold increase in activity
MCF-7	45.41 $\pm$ 7.24	16.30 $\pm$ 7.55	2.78
MDA-MB-231	35.37 $\pm$ 1.81	22.36 $\pm$ 8.19	1.58
SKBR-3	49.20 $\pm$ 1.7	10.15 $\pm$ 3.32	4.84
SupT1	41.44 $\pm$ 5.04	15.45 $\pm$ 6.22	2.68