Design, Synthesis, and Characterization of the Antitumor Activity of Novel Ceramide Analogues†

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Received June 8, 2001

A deficiency in apoptosis is one of the key events in the proliferation and resistance of malignant cells to antitumor agents; for these reasons, the search for apoptosis-inducing drugs represents a valuable approach for the development of novel anticancer therapies. In this study we report the first example of conformationally restrained analogues of ceramide (compounds **1**−**4**), where the polar portion of the molecule has been replaced by a thiouracil (**1**, **3**) or uracil (**2**, **4**) ring. The evaluation of their biologic activity on CCRF-CEM human leukemia cells demonstrated that the most active was compound **1** followed by compound **2** (mean 50% inhibition of cell proliferation [IC_{50}] 1.7 and 7.9 μM, respectively), while compounds **3** and **4** were inactive, as were uracil, thiouracil, and 5,6-dimethyluracil, the pyrimidine moieties of compounds **1**−**4**. For comparison, the IC_{50} of the reference substance, the cell-permeable C2-ceramide, was 31.6 μM. Compounds **1** and **2** and C2-ceramide were able to trigger apoptosis, as shown by the occurrence of DNA and nuclear fragmentation, and to release cytochrome *c* from treated cells. The treatment of female CD-1 nu/nu athymic mice bearing a WiDr human colon xenograft with the most active compound **1** at 2, 10, 50, and 200 mg/kg ip daily for 10 days resulted in an antitumor effect that was equivalent at 50 mg/kg or superior (200 mg/kg) to that of cyclophosphamide, 20 mg/kg ip daily, delivered on the same schedule, with markedly lower systemic toxicity. In conclusion, the present study demonstrates that the new ceramide analogues **1** and **2** are characterized by in vitro and in vivo antitumor activity and low toxicity.

Introduction

Within the past decade, sphingolipids, a family of biologically active components found ubiquitously in eukaryotic cell membranes, emerged as active participants in the regulation of key biologic functions, including cell growth, differentiation, transformation, and apoptosis.[1] The signaling pathway is initiated by the hydrolysis of membrane sphingomyelin (Figure 1) by sphingomyelinases in response to extracellular stimuli, including chemotherapeutic agents, cytokines, and ionizing radiations, to generate the second messenger ceramide.[1−3] Ceramide is also produced by de novo synthesis through ceramide synthase, and propagates the signal within the cell by the activation of stress-activated protein kinases (SAPKs), leading to apoptotic death through the interaction of ceramide with caspases and other apoptotic signaling cascades.[2,4]

Because natural ceramide is unable to cross the cell membrane, short-chain, cell-permeable compounds, such as the C2-ceramide, in which an acetyl group replaces the naturally occurring long-chain fatty acid, were used as a tool for investigating ceramide-mediated programmed death in cellular models, including leukemia cell lines.[5]

The complex biology of ceramide is underscored by the finding that platelet-derived growth factor triggers further hydrolysis of ceramide to sphingosine and activate sphingosine kinase to form the cell growth-promoting substance sphingosine 1-phosphate.[1] The dynamic balance among sphingolipid metabolites, ceramide, and sphingosine 1-phosphate, and the coordinated regulation of different family members of mitogen-activated protein kinases (MAPKs) and SAPK, is an important factor that determines the cell fate.[1,6]

The activation of the apoptosis program is the distinguishing characteristic of the response of cancer cells to chemotherapy;[7,8] ceramide participates in the process as an autonomous intracellular effector[7−9] and is capable of potentiating the cytotoxic activity of anticancer agents, including paclitaxel.[10] For these reasons, possible therapeutic targets of new pharmacological interventions directed at regulating abnormal tumor growth include the development of lipid analogues that mimic the effects of endogenous ceramide.

In the present study we report the synthesis and pharmacologic evaluation of compounds **1**−**4** in comparison with C2-ceramide in human leukemia cells, an in vitro model previously characterized for the biologic

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† The subject of this work was made the object of International Patent Application PCT/EP 00/07023.

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10.1021/jm010947r CCC: $20.00
Published on Web 10/03/2001

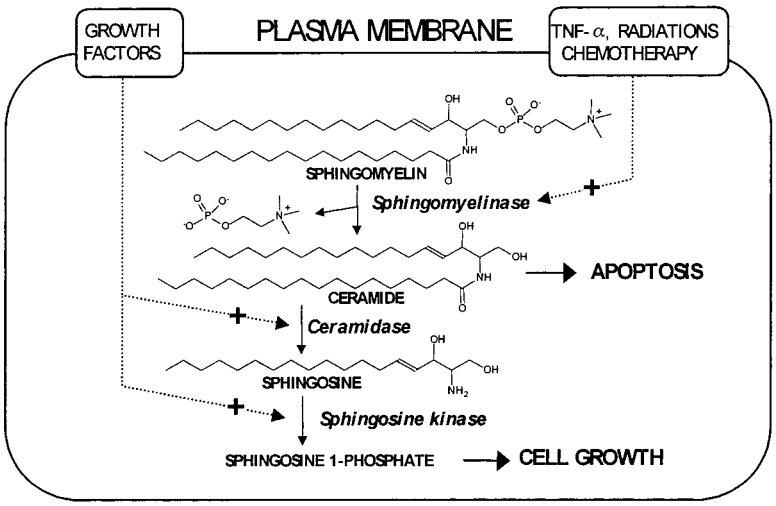
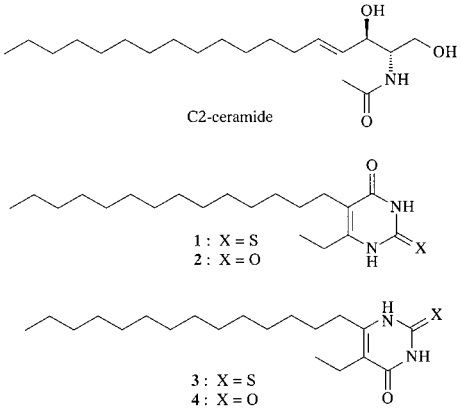


Figure 1. Schematic representation of the sphingomyelin cycle and effect of extracellular factors on ceramide generation and cell proliferation and death.

activities of ceramide,[10,11] as well as the in vivo efficacy and toxicity of the most active compound **1** in athymic mice xenografted with human WiDr colon cancer cells.



Compounds **1**−**4** were designed as ceramide analogues, in which we investigated the possibility of replacing the polar portion of the molecule by a potential mimic, identified as a thiouracil (**1**, **3**) or uracil (**2**, **4**) ring, for their similar steric and electronic properties.

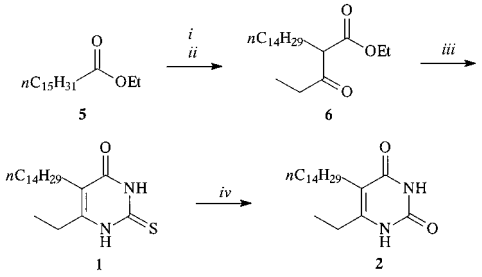
Compounds **1**−**4** represent the first examples of conformationally restrained analogues of ceramide; in fact, previously reported ceramide analogues were based on structural modification of the two side chains, such as shortening of the acyl side chain, as in the case of C2-ceramide or C6-ceramide, or the replacement of the alkenyl chain with a styryl or an allylic fluoride.[12,13] Furtermore, α-galactosylceramides possessing immunostimulatory properties were reported.[14]

In compounds **1**−**4**, the lipophilic portion of the C18 acyl moiety of ceramide has been replaced with an ethyl chain, to increase the intracellular penetration of these compounds in analogy with C2-ceramide; furthermore, they differ from each other only in the position of the two side chains linked to the heterocyclic nuclei.

Results

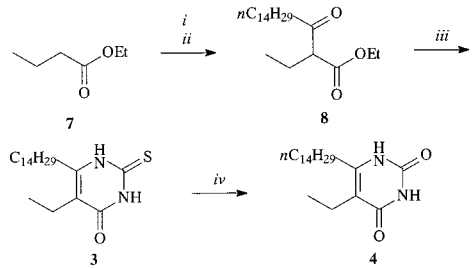
Chemistry. Compounds **1** and **2** were prepared following the synthetic pathway illustrated in Scheme 1. The lithium enolate obtained upon treatment of ethyl palmitate (**5**) with lithium diisopropylamide in THF was quenched with propionyl chloride. The resulting β-ketoester **6** was condensed with thiourea to give compound **1**.[15] Oxygen/sulfur exchange was brought about by treating **1** with a refluxing 10% aqueous solution of chloroacetic acid to produce compound **2**.[16,17] An analogous synthetic strategy was followed in the preparation of compounds **3** and **4**, as shown in Scheme 2. Ethyl butyrate (**7**) was deprotonated with lithium diisopropylamide in anhydrous THF under argon, and the resulting lithium enolate was treated with pentadecanoyl chloride to generate β-ketoester **8**. Reaction of **8** with thiourea in the presence of sodium ethoxide in hot ethanol afforded compound **3**, which was subsequently desulfurized to compound **4**, utilizing the conditions described above for compound **2**.

Scheme 1[a]



[a] Reagents and conditions: (i) LDA, 0 °C, THF, 30 min; (ii) propionyl chloride, room temperature, 12 h; (iii) thiourea, EtONa/EtOH, 90 °C, 1 h; (iv) chloroacetic acid (10%), reflux, 12 h.

Scheme 2[a]



[a] Reagents and conditions: (i) LDA, 0 °C, THF, 30 min; (ii) $nC_{14}H_{29}COCl$, room temperature, 12 h; (iii) thiourea, EtONa/EtOH, 90 °C, 1 h; (iv) chloroacetic acid (10%), reflux, 12 h.

Table 1. Effects on Cell Growth (IC_{50}, Inhibitory Concentration at the 50% Effect Level) and Caspase-3 Activity of CCRF-CEM Human T-leukemia Cells by Compounds **1**−**4** and C2-ceramide

compd	IC_{50} (μM, mean ± SD)	caspase-3 activity (max % increase vs untreated cells, mean ± SD)	compd	IC_{50} (μM, mean ± SD)	caspase-3 activity (max % increase vs untreated cells, mean ± SD)
1	1.7 ± 0.2[a]	5.0 ± 1.8	**4**	not reached	1.7 ± 0.5
2	7.9 ± 0.9[a]	8.1 ± 3.2	C2-ceramide	31.6 ± 4.6	2.8 ± 0.7
3	not reached	1.2 ± 0.3			

[a] $P < 0.05$, ANOVA followed by the Tukey−Kramer post-test, vs C2-ceramide.

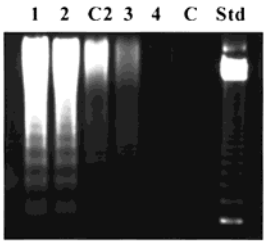


Figure 2. DNA fragmentation in CCRF-CEM human leukemia cells treated with ceramide analogues. The picture shows the results of a representative experiment on cells treated with ceramide analogues at their IC_{50} level (1, compound **1**; 2, compound **2**; C2, C2-ceramide; 3, compound **3**; 4, compound **4**; C, untreated controls). Std = 128 bp DNA ladder standard.

Cell Proliferation and Apoptosis in Vitro. The growth inhibitory effects of compounds **1**−**4** and C2-ceramide were assessed in the human T-cell acute leukemia cells CCRF-CEM. Exposure of the cells to ceramide analogues for 48 h resulted in a dose-dependent decrease in cell viability; the most active compounds were **1** and **2** (mean IC_{50} 1.7 and 7.9 μM, respectively, Table 1), while compounds **3** and **4** were inactive, since the IC_{50} was not reached at 50 μM (Table 1). Compounds **1** and **2** proved to be significantly more active than C2-ceramide with respect to inhibition of cell proliferation; indeed, the mean IC_{50} of C2-ceramide was 31.6 μM (Table 1), a value approximately 18.5-fold and 4-fold higher than those of compounds **1** and **2**. To ascertain whether the biologic activity of compounds **1** and **2** could be dependent, at least in part, on the presence of the pyrimidine ring, uracil, thiouracil, and 5,6-dimethyluracil were tested on CCRF-CEM cells at the same concentration and length of exposure of ceramide analogues; all the compounds did not affect the proliferation of CCRF-CEM cells (maximum 1.3%, 2.1%, and 1.8% inhibition of cell growth vs untreated control cells at 50 μM for uracil, thiouracil, and 5,6-dimethyluracil, respectively).

The exposure of CCRF-CEM cells to compounds **1**−**4** and C2-ceramide was indeed able to induce apoptosis; in particular, if compared at their IC_{50} levels, compounds **1** and **2** displayed similar abilities to induce DNA fragmentation after treatment. On the contrary, the amount of apoptotic DNA generated upon exposure to C2-ceramide was modest, while the extent of DNA fragmentation induced by compounds **3** and **4** was negligible (Figure 2). Apoptosis was also confirmed microscopically by measuring the apoptotic index; cells treated with compounds **1** and **2** showed apoptotic indexes of 27.9% and 9.4%, respectively, while treatment with C2-ceramide was associated with an apoptotic index of 2.9%. The number of apoptotic cells detected after treatment with compounds **3** and **4** at 50 μM was negligible (<1%).

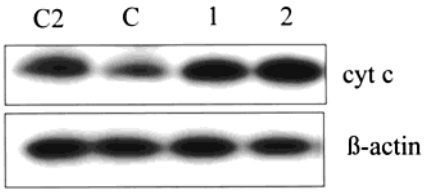


Figure 3. Immunoblot analysis of cytochrome *c* release from CCRF-CEM human leukemia cells treated with ceramide analogues. The picture shows the results of a representative experiment on cells treated with ceramide analogues at 50 μM for 12 h (1, compound **1**; 2, compound **2**; C2, C2-ceramide; C, untreated controls). β-Actin bands are visualized to show equal sample loading.

Caspase-3 Activation and Cytochrome *c* Release. Compounds **1**−**4** as well as C2-ceramide were unable to significantly increase the activity of caspase-3 over control values. The maximum changes were 5%, 8.1%, and 2.8% for compounds **1** and **2** and C2-ceramide, respectively, as compared to untreated cell cultures (Table 1). The activity of caspase-3 in cells exposed to compounds **3** and **4** was below 2% (Table 1). To explore in more detail the underlying mechanism of apoptosis induced by compounds **1** and **2**, the release of cytochrome *c* was investigated in CCRF-CEM cells. The cells were exposed to compounds **1** and **2** and C2-ceramide at 50 μM, and analyzed by immunoblotting. Compounds **1** and **2** and C2-ceramide induced cytochrome *c* release, before the onset of nuclear apoptosis, in a caspase-3-independent manner. Compound **1** was the most active, while C2-ceramide displayed the lowest effect (Figure 3).

In Vivo Antitumor Activity and Toxicity. The administration of compound **1** at 2, 10, 50, and 200 mg/kg to seven animals per dose level xenografted with the WiDr human colon cancer cells resulted in a dose-dependent reduction of tumor growth (Figure 4). At the end of the fifth week of study, animals given 20 mg/kg cyclophosphamide showed a 24.6% tumor mass reduction as compared to controls (1.76 ± 0.61 cm^3 vs 2.33 ± 0.57 cm^3, cyclophosphamide vs control, $P < 0.05$). A significant decrease in tumor growth was detected in animals given compound **1** at 50 and 200 mg/m^2; the latter dose level was the most effective with a 42.7% reduction of tumor volume (1.33 ± 0.5 cm^3 vs 2.33 ± 0.57 cm^3, compound **1** vs control, $P < 0.05$) (Figure 4). Compound **1** was well tolerated; no toxic deaths or signs of toxicity were observed; the body weight of animals given 200 mg/m^2 was similar to that of controls at the end of the fifth week (Figure 4), while that of mice given cyclophosphamide was smaller (24.6 g vs 33 g, cyclophosphamide vs control, $P < 0.05$).

Discussion

Malignant cell proliferation and resistance to anticancer agents are mediated by a variety of regulatory

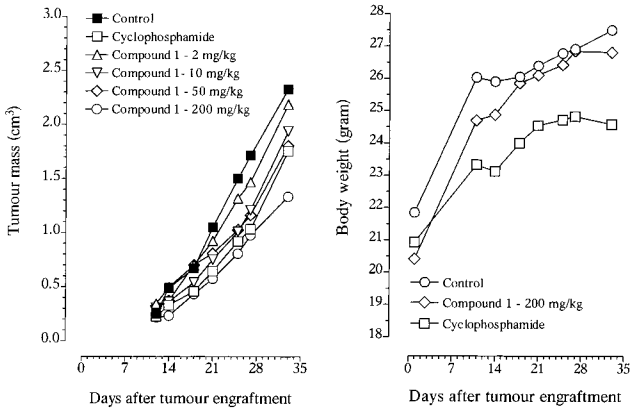


Figure 4. Increase in tumor mass (left) and changes in body weight (right) as a function of time in athymic mice xenografted with WiDr human colon cancer cells and treated with compound **1** (2, 10, 50, and 200 mg/kg), cyclophosphamide (20 mg/kg), and sterile water as a control vehicle. The symbols represent the mean values of seven animals per treatment.

pathways, including metabolites of sphingolipid biosynthesis and degradation.[1] A new wealth of biologic information on cancer underscores the important role of ceramide as an intracellular modulator of cell growth and differentiation,[1−4] and opens up strategies by which tumor cells can be targeted with anticancer agents that might selectively kill cells in a context that matches the molecular alterations responsible for tumor growth and progression. The advances in the biology of sphingolipids provide the rationale to design metabolically stable, cell-permeable analogues with enhanced ability to trigger death in tumor cells as compared to endogenous ceramide, alone or in combination with conventional anticancer agents, and to restore the sensitivity of cancer cells to regulatory mechanisms of growth that are lost, particularly in drug-resistant cancer cells.[18] This approach appears more feasible than the use of drugs that increase ceramide production or reduce its catabolism.

Previous experimental studies on the chemical synthesis and biologic characterization of ceramide analogues provided evidence of their wide spectrum of cellular activities, including stimulation of axonal growth of hippocampal neurons by analogues having either a *para*-substituted phenyl ring in the sphingoid moiety or an allylic fluoride,[12] or induction of apoptosis in Molt-4 and K-422 leukemia cells by fluorinated dihydroceramide analogues.[13] Additional work in this field demonstrated the immunostimulatory and immunosuppressive activities of α- and *β*-galactosylceramides, respectively,[14] as well as growth inhibition and cellular differentiating properties of *N*-acylphenylamino alcohol analogues of ceramide in HL-60 cells.[19] Finally, two synthetic ceramide analogues, *N*-thioacetylsphingosine and FS-5, induced apoptosis in Molt-4 cells through the cleavage of poly(ADP-ribose) polymerase (PARP), and the overexpression of bcl-2 oncogene antagonized the occurrence of DNA fragmentation.[20] This finding is in agreement with the experimental demonstration that the apoptosis inhibitory protein bcl-2 prevents ceramide-induced release of cytochrome *c*,[21,22] and underscores the key role of the mitochondrion in the death pathway initiated by anticancer agents.

In the present study we provide experimental evidence of inhibition of cell proliferation, induction of apoptosis and in vivo antitumor activity, and low toxicity of novel ceramide analogues designed to sensitize cancer cells to inhibition of growth by DNA fragmentation. In this work we present the experimental results of conformationally restrained ceramide analogues with enhanced growth-suppressor activity with respect to the reference compound C2-ceramide. The replacement of the polar portion of ceramide with a thiouracil or uracil ring, together with the reduction of its lipophilicity, leads to compounds **1** and **2**, which possess a significant cytotoxic activity and ability to induce ceramide-dependent cytochrome *c* release from mitochondria, at higher levels than observed with C2-ceramide. The position of the two side chains linked to the heterocyclic nuclei seems to be important for the antiproliferative effects, as demonstrated by the different profile shown by compounds **1** and **2** with respect to compounds **3** and **4**. In these two latter compounds, the presence of a uracil ring instead of a thiouracil one does not seem to have a significant influence on the activity. However, this modification was responsible for the different cytotoxicities observed between compounds **1** and **2**. The optimal lipophilic/hydrophilic ratio may explain, at least in part, the enhanced growth-suppressor activity of compounds **1** and **2** with respect to C2-ceramide, in terms of different cellular uptake and retention that may be increased in compounds **1** and **2** due to the presence of a pyrimidine ring in their structure instead of the polar headgroup, making these ceramide analogues likely to be resistant to the enzymes of the ceramide catabolic pathway. In agreement with these findings, C8-ceramine, a ceramide derivative in which the carbonyl group is replaced by a methylene group, is a more potent inducer of apoptosis than C8-ceramide in U937 human leukemia cells.[23]

While compounds **1** and **2** share important similarities with C2-ceramide, particularly the ability to induce cytochrome *c* release in conjunction with the onset of apoptosis, in a caspase-3-independent manner, as previously demonstrated for C2-ceramide in leukemia cells,[11] the profile of pharmacologic activity of compounds **1** and **2** was markedly different from that of pyrimidines, including uracil, thiouracil, and 5,6-dimethyluracil, which did not display a significant proliferation-suppressive capability in CCRF-CEM cells.

Finally, compounds **1** and **2** displayed higher therapeutic indexes, i.e., significant antitumor activity in vivo and low toxicity, with respect to cyclophosphamide, and may thus represent the lead of a new class of antitumor agents with enhanced activity on apoptosis-controlling pathways, as compared to the well-characterized C2-ceramide, and further research will be directed at improving the cellular uptake and potency of this class of antiproliferative agents.

Experimental Section

Materials and Methods. ^{1}H NMR spectra of all compounds were obtained with a Varian Gemini 200 instrument operating at 200 MHz in a ca. 2% solution of $CDCl_3$. IR spectra were taken as paraffin oil mulls or as liquid films on a Mattson 1000 Series FTIR spectrometer. Mass spectra were obtained with an HP-5988 A spectrometer. Analytical TLC was carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator (Macherey-Nagel Alugram SilG/UV254, art. no. 81813); spots were detected under UV light (254 nm) and by phosphomolybdic acid. All compounds were homogeneous by

TLC. Column chromatography was performed using 70−230 mesh silica gel (Macherey-Nagel silica gel 60, art. no. 81538). All chemicals and precursors were purchased from Sigma-Aldrich (Milwakee, WI), unless otherwise specified. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Evaporations were made in vacuo by a rotating evaporator.

Synthesis of Compound 1. A solution obtained by dissolving ethyl palmitate (**5**) (1.0 g, 3.5 mmol) in 3 mL of anhydrous THF was added dropwise, at 0 °C in an argon gas atmosphere, to 2.1 mL (4.2 mmol) of a 2 M solution of lithium diisopropylamide in anhydrous THF. After 30 min of stirring at 0 °C, the reaction mixture was added to a solution obtained by dissolving 0.39 g (4.2 mmol) of propionyl chloride in 5 mL of anhydrous THF. The resulting mixture was stirred at room temperature for 12 h and then added to a saturated solution of NH_4Cl. The organic phase was separated from the aqueous phase and then extracted with diethyl ether. The organic extracts were combined, washed with a saturated aqueous solution of NaCl, dried over anhydrous Na_2SO_4, and concentrated under vacuum to give a crude residue (1.31 g) composed almost exclusively of β-ketoester **6**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.79−0.92 (m, 6H), 1.11 (t, 3H, J = 7.6 Hz), 1.17−1.39 (m, 24H), 1.48−1.62 (m, 2H), 2.26 (q, 2H, J = 7.6 Hz), 3.36 (t, 1H, J = 7.3 Hz), 4.15 (q, 2H, J = 7.2 Hz). MS (EI, 70 eV): m/z 340 (M^+). The crude residue was dissolved, without further purification, in 20 mL of absolute ethanol and then added to 4.01 g of thiourea (52.7 mmol) and 7.18 g of sodium ethoxide (106 mmol). The mixture was stirred for 60 min at 90 °C. After being cooled to room temperature, the reaction mixture was filtered, and the filtrate was concentrated under vacuum. The residue thus obtained was then treated with a mixture of water and THF in a 10:1 ratio, until complete dissolution. The solution was then cooled to 0 °C and acidified to pH 2 with concentrated HCl; the precipitate that developed due to acidification was collected by filtration and washed with small portions of acetone, providing a crude residue which was purified by chromatography on silica gel (petroleum ether/EtOAc, 1:2), affording 310 mg (0.88 mmol; yield 25%, two steps) of pure product **1**. Mp: 100−102 °C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.88 (t, 3H, J = 6.4 Hz), 1.01 (t, 3H, J = 7.4 Hz), 1.18−1.38 (m, 24H), 2.35 (t, 2H, J = 7.4 Hz), 2.48 (q, 2H, J = 7.6 Hz), 9.08 (br, 1H, D_2O exchangeable), 9.73 (br, 1H, D_2O exchangeable). MS (EI, 70 eV): m/z 352 (M^+). Anal. ($C_{20}H_{36}N_2OS$) C, H, N, S.

Synthesis of Compound 2. Compound **1** (160 mg, 0.45 mmol) was added to 11 mL of a 10% aqueous solution of chloroacetic acid, and the mixture thus obtained was heated to reflux for 12 h. The resulting precipitate was then collected by filtration and washed with absolute ethanol and diethyl ether to obtain a crude residue which, after purification by chromatography on silica gel (hexane/EtOAc, 2:1), afforded 57 mg (0.17 mmol; yield 38%) of compound **2**. Mp: 110−112 °C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.89 (t, 3H, J = 6.4 Hz), 1.02 (t, 3H, J = 7.4 Hz), 1.12−1.42 (m, 24H), 2.34 (t, 2H, J = 7.2 Hz), 2.49 (q, 2H, J = 7.6 Hz), 9.15 (br, 1H, D_2O exchangeable), 9.53 (br, 1H, D_2O exchangeable). MS (EI, 70 eV): m/z 336 (M^+). Anal. ($C_{20}H_{36}N_2O_2$) C, H, N.

Synthesis of Compound 3. A solution prepared by dissolving 0.37 g (3.2 mmol) of ethyl butyrate (**7**) in 2 mL of anhydrous THF was added dropwise, at 0 °C and in an argon gas atmosphere, to 1.9 mL (3.8 mmol) of a 2 M solution of lithium diisopropylamide in anhydrous THF. After 30 min of stirring at 0 °C, the reaction mixture was added to a solution obtained by dissolving 1.0 g of pentadecanoyl chloride (3.8 mmol) in 5 mL of anhydrous THF, previously cooled to 0 °C. The resulting mixture was stirred at room temperature for 12 h and then added to a saturated solution of NH_4Cl. The organic phase was separated from the aqueous phase and then extracted with diethyl ether. The organic extracts were combined, washed with a saturated aqueous solution of NaCl, dried with anhydrous Na_2SO_4, and then concentrated under vacuum to provide a crude residue (1.20 g) composed almost exclusively of β-ketoester **8**. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.83−0.94 (m, 6H), 1.07 (t, 3H, J = 7.4 Hz), 1.15−1.36 (m, 24H), 1.81−2.02 (m, 2H), 2.11−2.57 (m, 2H), 3.34 (t, 1H, J = 7.3 Hz), 4.15 (q, 2H, J = 7.3 Hz). MS (EI, 70 eV): m/z 340 (M^+). The crude residue obtained was dissolved, without further purification, in 20 mL of absolute ethanol and then treated with 3.61 g of thiourea (47.4 mmol) and 6.47 g of sodium ethoxide (95.1 mmol). The mixture was stirred for 60 min at 90 °C. After being cooled to room temperature, the reaction mixture was filtered, and the filtrate was concentrated under vacuum. The residue thus obtained was then restored with a mixture of water and THF in a 10:1 ratio, until it became completely soluble. The solution, cooled to 0 °C, was acidified to pH 2 with concentrated HCl; the precipitate was collected by filtration and washed with small quantities of acetone, affording a crude residue which was purified by chromatography on silica gel (petroleum ether/EtOAc, 1:2) to give 290 mg (0.82 mmol; yield 26%, two steps) of the required compound **3**. Mp: 167−169 °C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.89 (t, 3H, J = 6.2 Hz), 1.09 (t, 3H, J = 7.4 Hz), 1.17−1.36 (m, 24H), 2.34−2.49 (m, 4H), 8.88 (br, 1H, D_2O exchangeable), 9.81 (br, 1H, D_2O exchangeable). MS (EI, 70 eV): m/z 352 (M^+). Anal. ($C_{20}H_{36}N_2OS$) C, H, N, S.

Synthesis of Compound 4. Compound **3** (160 mg, 0.45 mmol) was added to a 10% aqueous solution of chloroacetic acid (11 mL), and the mixture thus obtained was heated to reflux for 12 h. The resulting precipitate was then collected by filtration and washed with absolute ethanol and diethyl ether to obtain a crude residue that, after purification by chromatography on silica gel (hexane/EtOAc, 2:1), afforded 48 mg (0.14 mmol; yield 32%) of pure compound **4**. Mp: 132−134 °C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.87 (t, 3H, J = 6.2 Hz), 1.06 (t, 3H, J = 7.4 Hz), 1.15−1.36 (m, 24H), 2.31−2.49 (m, 4H), 9.06 (br, 1H, D_2O exchangeable), 9.89 (br, 1H, D_2O exchangeable). MS (EI, 70 eV): m/z 336 (M^+). Anal. ($C_{20}H_{36}N_2O_2$) C, H, N.

Assay of Cell Proliferation. The human T-cell leukemia cell line CCRF-CEM (American Type Culture Collection, Manassas, VA) was used to test the inhibitory effect on cell proliferation of compounds **1**−**4**, C2-ceramide, uracil, thiouracil, and 5,6-dimethyluracil. C2-ceramide was used as the reference compound, while uracil, thiouracil, and 5,6-dimethyluracil were tested to assess whether the two moieties used to replace the polar portion of the ceramide molecule were endowed with antitumor activity in vitro. CCRF-CEM cells were maintained in RPMI 1640 medium supplemented with fetal calf serum (10%), glutamine (2 mM), penicillin (50 IU/mL), and streptomicin (50 μg/mL) (HyClone, Cramlington, U.K.), in an atmosphere of 5% CO_2 and 95% air at 37 °C. Cell proliferation was assessed by CellTiter 96 (Promega, Madison, MA) on the basis of the cellular metabolism of the tetrazolium compound XTT. Compounds **1**−**4**, C2-ceramide (Sigma, St. Louis, MO), and 5,6-dimethyluracil (Aldrich) were dissolved in sterile dimethyl sulfoxide (DMSO) at 10 mM, while uracil (Aldrich) and thiouracil (Aldrich) were dissolved in sterile water at 10 mM and stored at −20 °C; the drugs were further diluted in sterile culture medium immediately before their use. CCRF-CEM cells were seeded at 5000 cells/200 μL of culture medium containing compounds **1**−**4**, C2-ceramide, uracil, thiouracil, and 5,6-dimethyluracil at 0.01−50 μM into each well of a 96-well microtiter plate, and incubated at 37 °C with 5% CO_2 for 48 h. Control cultures received vehicle only (DMSO or sterile water). At the end of drug exposure, 50 μL of medium containing 50 μg of XTT and 0.38 μg of phenazine methosulfate was added; the cells were incubated for an additional 3−4 h, and the absorbance was measured at 450 nm with a microplate reader (MicroReader, Bio-Rad, Manassas, VA). Inhibition of proliferation was assessed as the percentage reduction of the UV absorbance of treated cells vs control cultures, and the 50% inhibitory concentration of cell growth (IC_{50}) was calculated by nonlinear least-squares curve fitting.[15]

Assay of Apoptosis by DNA Fragmentation and Nuclear Staining. To demonstrate whether ceramide analogues are able to trigger apoptosis, CCRF-CEM cells were plated in 60 mm sterile dishes and treated with compounds **1**−**4** and C2-ceramide at their IC_{50} levels (1.7, 7.9, 50, and 31.6 μM; see Table 1) for 48 h. Apoptosis was documented by the occurrence of DNA internucleosomal fragmentation, as previously described.[24] At the end of treatment, the cells were

washed twice with PBS, pH 7.4, and centrifuged at 1000*g* for 15 min, and 3×10^6 cells for each drug concentration were solubilized in 1 mM EDTA, 10 mM Tris base, pH 7.5, and 0.2% (v/v) Triton X-100 for 90 min at 4 °C. The cell lysates were centrifuged at 10000*g* for 1 h at 4 °C, and the clear supernatants containing fragmented chromatin were incubated at 42 °C for 30 min with proteinase K (200 μg/mL) and then diluted 1:1 in phenol/chloroform/isoamyl alcohol, shaken for 30 s, and centrifuged at 10000*g* for 10 min. The supernatants were again collected and mixed with 100 μL of NaCl (5 M), 1 mL of cold ethanol, and 1 μL of glycogen. DNA fragments were precipitated at −20 °C overnight and then centrifuged at 10000*g* for 30 min; the supernatants were discarded, and the pellets were washed with 70% ethanol and dried under air flow. Each sample was resuspended in 1 mM EDTA and 10 mM Tris base, pH 7.5, containing 1 mg/mL boiled bovine pancreatic RNAse A, incubated at 40 °C for 60 min, and mixed with DNA sample buffer (15 mM EDTA, pH 8.0, 0.1% SDS, 0.025% xylene cyanole, 0.025% bromophenol blue, and 0.5% glycerol). Separation of DNA fragments was obtained by electrophoresis in 1% agarose gel, Tris base (32 mM), 1% glacial acetic acid, and 1 mM EDTA; the bands were visualized by ethidium bromide staining under UV light, compared against a 180 bp DNA ladder (GIBCO, Gaithersburg, MD) for fragment size identification, and the gels were photographed with a Polaroid MP4 Land camera (Polaroid, Cambridge, MA). The reagents for apoptosis assay were from Sigma, unless otherwise indicated.

Compounds **1**−**4**, C2-ceramide, and untreated controls were also characterized by their ability to induce nuclear condensation and fragmentation in CCRF-CEM cells, as detected by bisbenzimide staining.[25] Briefly, the cells were treated as described above for DNA fragmentation analysis; at the end of incubation, the cells were washed twice in phosphate-buffered saline (PBS; pH 7.4), fixed in 3% paraformaldehyde, and incubated at room temperature for 10 min. Then the cells were resuspended in a solution containing 8 μg/mL bisbenzimide trihydrochloride (Sigma), and incubated for 15 min at room temperature. The cell suspensions were spotted on sylanized microscope glass slides and examined for the presence of morphologic features of apoptosis, i.e., chromatin condensation and nuclear fragmentation, by fluorescence microscopy (Leica, Germany). Two hundred cells from randomly chosen microscopic fields were counted, and the apoptotic index was calculated as the percentage ratio between the number of cells displaying apoptotic features and the total number of counted cells.

Assay of Caspase-3 Activity. To assess whether caspase-3, an enzyme involved in the effector phase of apoptosis, is a target of ceramide analogues, enzyme activity was assessed by the caspase-3 assay kit (Calbiochem, Oxford, U.K.). Briefly, CCRF-CEM cells were plated in 24-well plates (1×10^6 cells/well in 1 mL of culture medium) and treated for 48 h with compounds **1**−**4** and C2-ceramide at concentrations ranging from 0.01 to 50 μM. At the end of incubation, the cells were washed twice with PBS (pH 7.4), pelleted by centrifugation (1000*g* for 10 min at 4 °C), and resuspended in 50 μL of lysis buffer [HEPES (50 mM), DTT (1 mM), EDTA (0.1 mM), CHAPS (0.1%), pH 7.4, and Nonidet P-40 (0.1%)] at 4 °C for 5 min. The cell lysate was centrifuged at 10000*g* for 10 min at 4 °C, and the supernatants, representing cytoplasmic extracts, were transferred to a microtiter plate and mixed with assay buffer [HEPES (50 mM), DTT (10 mM), glycerol (10%), and CHAPS (0.1%), pH 7.4] added to each well. Reaction was started by adding 10 μL of caspase-3 colorimetric substrate (Ac-DEVD-pNA); controls were obtained with the caspase-3 inhibitor Ac-DEVD-CHO, to measure nonspecific hydrolysis of the substrate, and with the human recombinant caspase-3, to compare the activity of a known amount of enzyme with the caspase-3 activity in CCRF-CEM extracts. The plates were then incubated at 37 °C for 10 min, after which the absorbance was read at 405 nm in a microplate reader and the data were recorded at 10 min intervals for 120 min to construct a plot of absorbance increase (corresponding to the release of chromogenic substrate by the activity of caspase-3) as a function of time.

Immunoblot Analysis of Cytochrome *c* Release. Evidence has been provided for the release of mitochondrial cytochrome *c* as a consequence of ceramide activity.[21,26] Cells treated with compounds **1** and **2** and C2-ceramide at 50 μM for 12 h and untreated controls were washed twice with PBS (pH 7.4), and solubilized at 4 °C for 45 min in lysis buffer [Tris base (50 mM), pH 7.6, EDTA (2 mM), NaCl (100 mM), Nonidet-P40 (1%, v/v), phenylmethylsulfonyl fluoride (PMSF; 1 mM), aprotinin, pepstatin, and antipain (2 μg/mL each)]. The cell lysates were then centrifuged at 15000 rpm for 20 min, and aliquots of the supernatants (50 μg of cytoplasmic proteins) were boiled for 5 min in SDS sample buffer (50 mM Tris base, pH 6.8, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% glycerol, and 0.025% β-mercaptoethanol) and separated with 15% SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The blots were probed with rabbit anti-cytochrome *c* and goat anti-β-actin polyclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA; dilution 1:500, v/v) to demonstrate equal sample loading, and detected with the use of ^{125}I-labeled secondary antibody (dilution 1:2500).

Assessment of Antitumor Activity and Toxicity in Vivo. The efficacy and toxicity of the most active ceramide analogue, compound **1**, were tested in CD-1 nu/nu female athymic mice (25 g mean body weight; Charles River, Italy) bearing a solid subcutaneous tumor induced by engraftment of WiDr human colon adenocarcinoma cells (American Type Culture Collection). The in vivo study was performed at the RBM Research Institute (Torino, Italy). WiDr cells were cultured in Eagle's MEM with nonessential amino acids (90% Eagle's BSS and 10% fetal bovine serum) and maintained in exponential growth phase. On day 0, the cells were harvested with EDTA (2 mM), and $10 \times 10^6 \pm 5\%$ cells/mouse were inoculated subcutaneously between the scapulae in 0.2 mL of culture medium without FBS per mouse using an insulin syringe with a 25G needle. On day 1, compound **1** was dissolved in olive oil and administered intraperitoneally (ip) with an insulin syringe with a 25G needle at 2, 10, 50, and 200 mg/kg in 0.2 mL of vehicle. A control group receiving 0.2 mL of vehicle and a positive control group given 20 mg/kg ip cyclophosphamide dissolved in 0.2 mL of sterile saline per mouse and administered on the same schedule as compound **1** were also included in the study. The treatment was continued for 9 consecutive days; the animals were observed daily until the fifth week of study for tumor growth, toxicity, and body weight changes. Mortality was recorded daily; the mice found to be in preagonal conditions were killed with an ip overdose of pentobarbital (0.2 mL of a 5% solution per mouse). The anticancer efficacy of compound **1** and cyclophosphamide was assessed on the basis of tumor mass (cm^3) measured in two dimensions, according to the following formula:

$$V_{tm} = (\text{greatest diameter} \times \text{smallest diameter})^2/2$$

Data Analysis. Experimental data are expressed as mean values ± standard deviation, and statistical comparisons are obtained by analysis of variance (ANOVA) followed by the Tukey−Kramer post-test to compare pairs of group means.

Acknowledgment. This work was supported in part by Bracco Imaging, Milano, Italy. We gratefully acknowledge Dr. D. Barone, Istituto di Ricerche Biomediche "Antoine Marxer" RBM SpA, Colleretto Giacosa, Torino, Italy.

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JM010947R