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Ceramide analogues in apoptosis: a new strategy for anticancer drug development

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Abstract

A survey on the role played by ceramide within the sphingolmyelin pathway is here reported, taking into account its importance as an intracellular effector molecule in apoptosis. Recently, several analogs of ceramide, able to pass the cell membrane and then to induce apoptosis, have been developed as a new potential approach in anticancer therapy.

Keywords: Ceramide analogues; Anticancer activity; Apoptosis; Cytochrome *c* release; Tumor xenograft

The levels of the various cell populations in multicellular organisms depend on a complex dynamic balance between the mechanisms which regulate cell proliferation and those involved in programmed cell death, commonly termed apoptosis [1]. Alterations and defects to these control mechanisms which determine whether a cell proliferates or dies, are the main pathogenesis of a variety of human diseases, including cancer, auto-immune disease and viral infections [2,3].

It has been demonstrated that tumor progression may depend not only on an uncontrolled cell proliferation but also on dysfunction in the physiological pathways of apoptosis [1] as an example, tumor development in mice occurs with a higher incidence in the presence of genetic alterations to apoptotic mediators, such as Bcl-2 overexpression or Fas ligand deletion [4]. For this reason, it is nowadays believed that the possibility of pharmacological interventions able to restore the physiological apoptotic death pathway represents a concrete expectation in an anticancer therapy with a selective toxicity toward malignant but not normal cells, whose apoptotic pathways are not altered [5–7].

Ceramide, a sphingosine-based lipid molecule, has recently attracted great attention due to its emerging role as an intracellular effector molecule in apoptosis; [8–10] in particular, ceramide represents the second messenger of the so-called 'sphingomyelin pathway' (Fig. 1) [11]. Membrane sphingomyelin is hydrolyzed by the action of a neutral or acidic sphingomyelinase, in response to extracellular stimuli, including chemotherapeutic agents, cytokines (TNF-α) and ionizing radiation,[10] to generate the second messenger ceramide. Ceramide, which is also generated by de novo synthesis controlled during apoptosis by serine palmitoyltransferase (Fig. 2), [12] then propagates the signal within the cell by the activation of stress-activated protein kinases (SAPK), [13] leading to apoptotic death through the interaction of ceramide with caspases and other apoptotic signaling cascades.

An intriguing aspect of this picture is that while ceramide has been shown to be an important stimulatory factor of apoptosis, on the other hand, growth factors, such as platelet-derived growth factors, trigger further hydrolysis of ceramide to sphingosine, mediated by ceramidase enzyme, and then activate sphingosine kinase to form sphingosine 1-phosphate with promotion of cell growth. For these reasons, the dynamic balance between levels of ceramide and sphingosine 1-phos-

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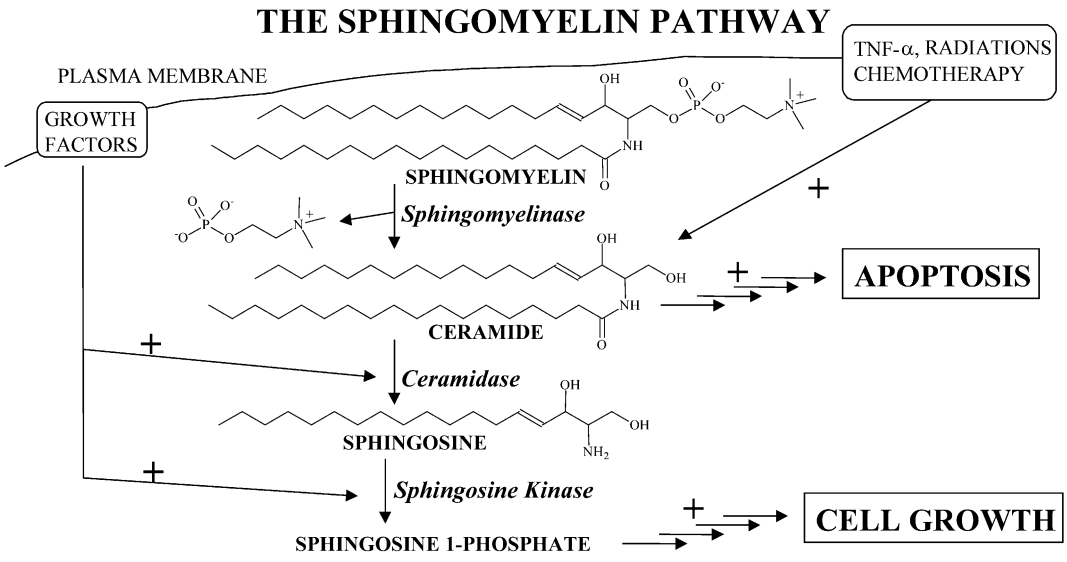


Fig. 1. Schematic representation of the Sphingomyelin pathway.

phate, is an important factor that determines whether a cell survives and proliferates or dies [12–14].

It has been demonstrated that malignant cells with low ceramide levels are resistant to apoptosis, as well as that tumor cell lines with a defect in ceramide generation are resistant to radiation-induced apoptosis [15–17]. Furthermore, ceramide is capable of potentiating the cytotoxic activity of anticancer agents, including paclitaxel [18].

On this basis, ceramide could be viewed as a possible therapeutic agent able to induce apoptosis in tumor cells. However, it is important to point out that natural ceramide is unable to cross the cell membrane and, therefore, cannot be used directly for therapeutic purposes; for this reason, ceramide analogs able to pass the cell membrane and then to mimic the effects of endogenous ceramide were developed in the past; [19] in particular, C2- and C6-ceramide, two ceramide cell-permeable analogs in which a short acyl group replaces the naturally occurring long-chain fatty acid, proved to mimic ceramide as apoptotic inducers in leukemia cell lines; [20,21] C2- and C6-ceramide inhibited tumor cell growth proliferation, but at a concentration too high to develop them as potential antiproliferative agents. For this reason, C2- and C6-ceramide can be viewed as tools for investigating ceramide-mediated programmed death in cellular models [22].

Other ceramide analogs possessing a wide spectrum of cellular activities have recently been reported: in particular, compounds having either a phenyl ring (**1**) in the sphingoid moiety or an allylic fluoride (**2**), [23] proved to stimulate axonal growth of hippocampal neurons, while fluorinated dihydroceramide derivative **3** showed to induce apoptosis in Molt-4 and K-422 leukemia cells [24].

Additional work in this field demonstrated the immunostimulatory and immunosuppressive activities of α- (**4**) and β-galactosylceramides (**5**), respectively [25]. Two other synthetic ceramide analogs, *N*-thioacetyl-sphingosine (**6**) and FS-5 (**7**), induced apoptosis in Molt-4 cells through the cleavage of poly-(ADP-ribose) polymerase (PARP), and the over-expression of the Bcl-2 oncogene antagonized the occurrence of DNA fragmentation [26].

Furthermore, ceramidase inhibitors (D-MAPP, **8**; B13, **9**), [27–29] able to block the conversion of ceramide into sphingosine, and consequently to increase the endogenous ceramide levels, were developed. B13, a potent ceramidase inhibitor able to increase the ceramide levels in malignant cells, and not in normal liver cells that were completely resistant, proved to be highly effective in inducing apoptosis and preventing growth of colon cancer [29].

Together with these studies, also in our laboratory, a series of metabolically stable ceramide analogues were developed: compounds **10**–**13** were designed by us as the first example of conformationally restrained analogs of ceramide, where the polar portion of ceramide has been replaced by a potential mimic, identified in thiouracil (**10**, **12**) or uracil rings (**11**, **13**), in view of their similar steric and electronic properties.[30] In compounds **10**–**13**, bearing in mind the structure of C2-ceramide, the lipophilic portion of the C18 acyl moiety of ceramide has been replaced with an ethyl chain, potentially capable of allowing a better intracellular penetration for these types of compounds. Compounds **10** and **12**, as well as **11** and **13**, differ from each other only in the position of the two side-chains linked to the heterocyclic nuclei.

Compounds **10** and **11** were prepared as shown in Scheme 1. Treatment of propionyl chloride with the

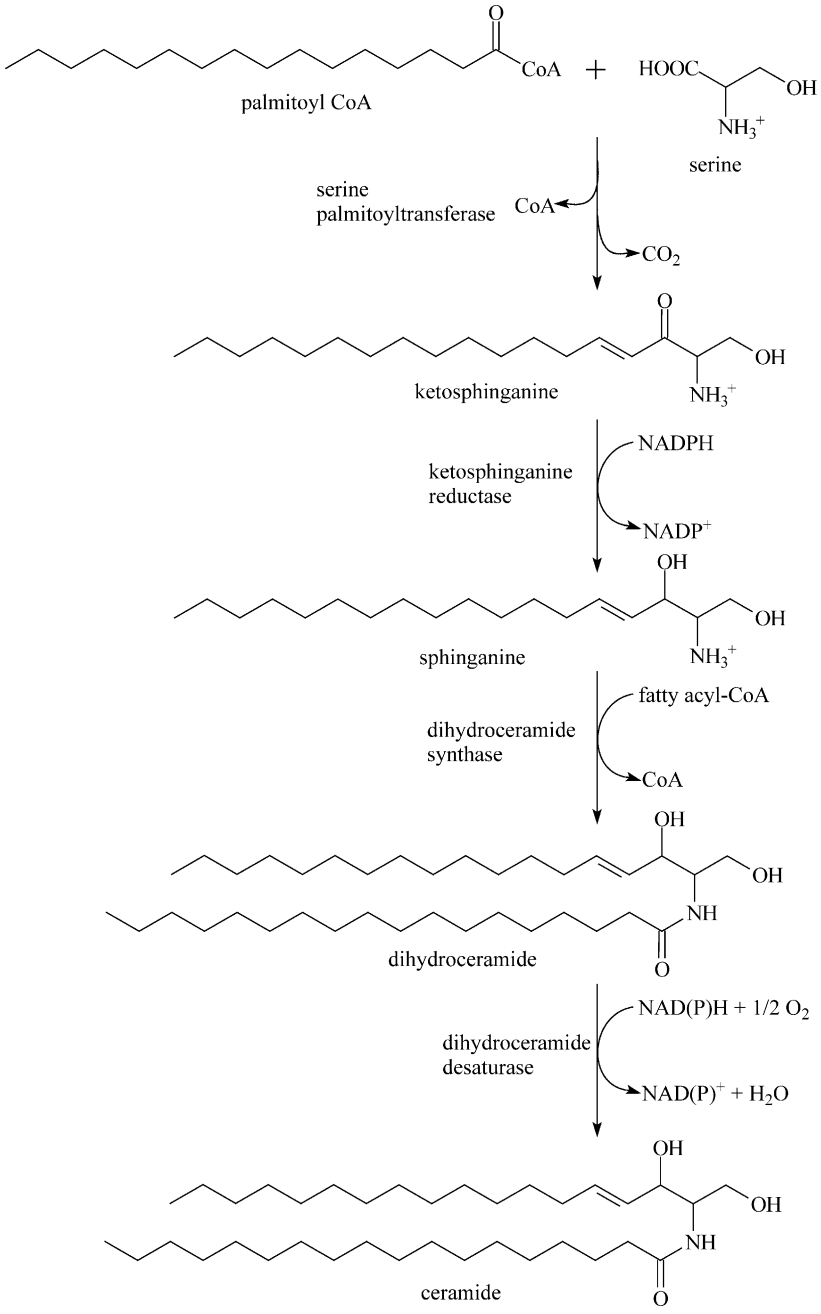
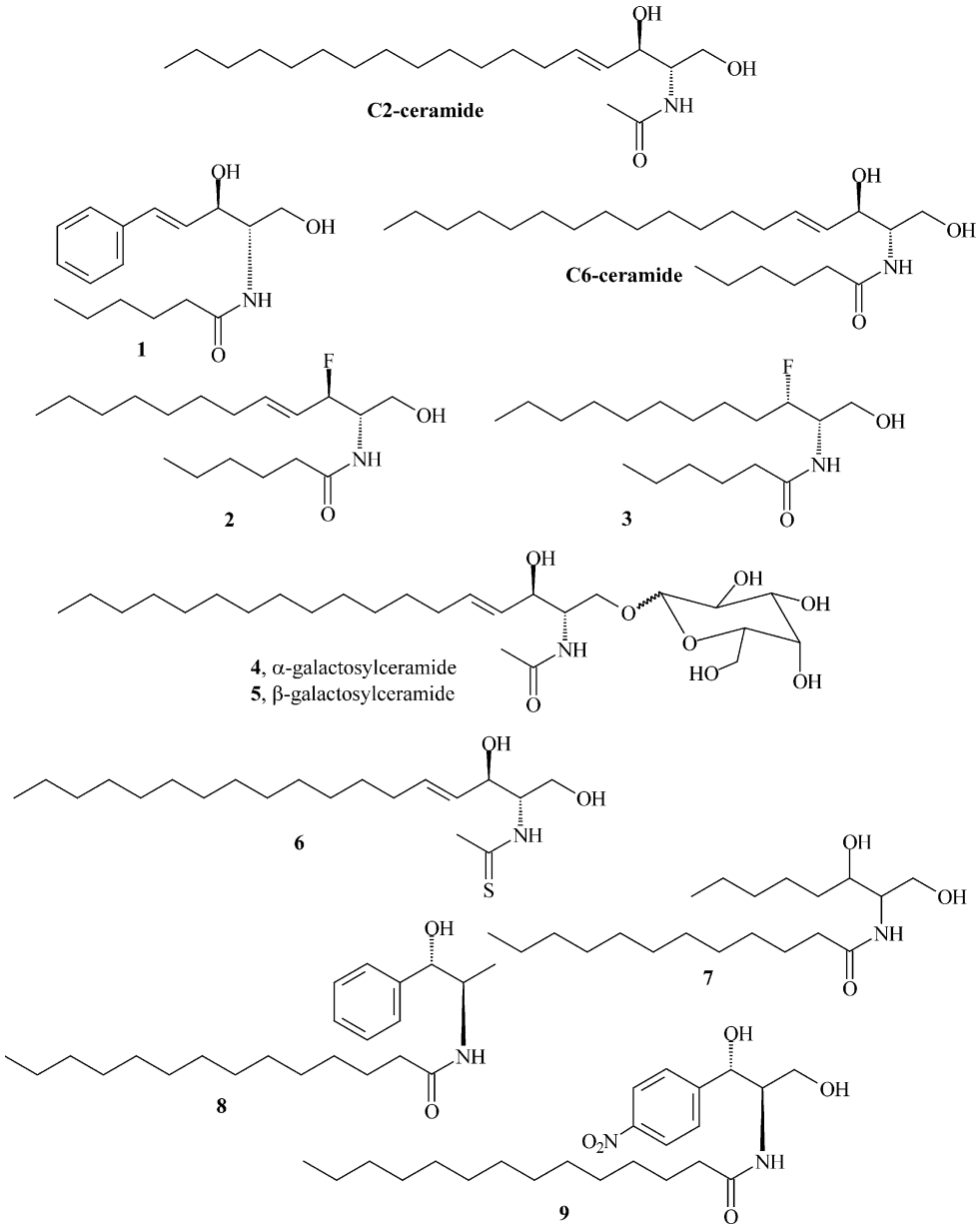


Fig. 2. The de novo pathway of ceramide biosynthesis.

lithium enolate obtained upon treatment of ethyl palmitate (**14**) with lithium diisopropylamide in THF furnished the β-ketoester **15**. Condensation of **15** with thiourea in the presence of sodium ethoxide in hot ethanol afforded compound **10**, which was then converted to compound **11** by oxygen/sulfur exchange by treatment with a refluxing 10% aqueous solution of chloroacetic acid. An analogous synthetic strategy was followed in the preparation of compounds **12** and **13**, as illustrated in Scheme 2, starting with ethyl butyrate (**16**) which was deprotonated to lithium enolate and then treated with pentadecanoyl chloride to generate β-ketoester **17**. Reaction of **17** with thiourea afforded compound **12**, which was subsequently desulfurized to compound **13**, under the same conditions described above for compounds **10** and **11** [30].

Compounds **10** and **11**, when tested for their growth inhibitory effects on the human T-cell acute leukemia cells CCRF-CEM (Table 1), proved to possess a good anti-proliferative activity, significantly higher than that of C2-ceramide, thus demonstrating that the polar portion of ceramide and C2-ceramide can be mimicked by a thiouracil or uracil ring. On the contrary, compounds **12** and **13** were practically inactive (Table 1),



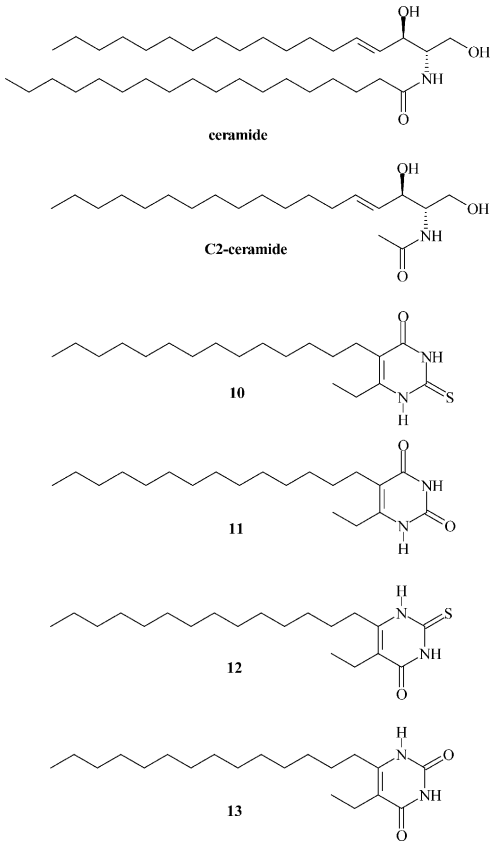
indicating that the position of the two side-chains linked to the heterocyclic nuclei seems to be important for the anti-proliferative effects.

Furthermore, uracil, thiouracil and 5,6-dimethyluracil were tested on CCRF-CEM cells at the same concentration and length of exposure as ceramide analogs **10** and **11**; none of the compounds affected the proliferation of CCRF-CEM cells, thus proving that the biologic activity of compounds **10** and **11** depends on the whole structure resembling ceramide and not only on the presence of the pyrimidine rings.

Compounds **10** and **11** also proved to be able to induce apoptosis on CCRF-CEM cells, while the amount of apoptotic DNA generated upon exposure to C2-ceramide was modest.

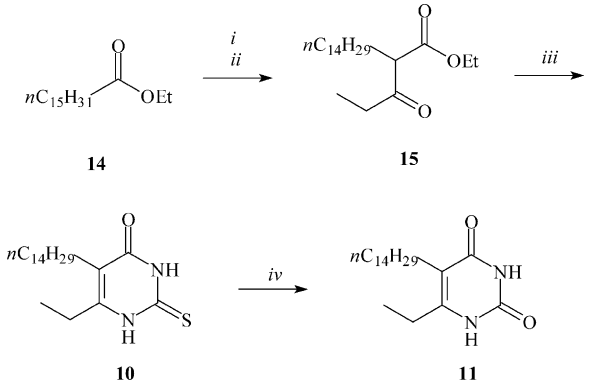
Compounds **10** and **11** as well as C2-ceramide, were unable to significantly increase the activity of caspase-3 over control values [20]. In order to explore in more detail the underlying mechanism of apoptosis induced by compounds **10** and **11**, the release of cytochrome *c* was investigated in CCRF-CEM cells [31]. Cells were exposed to compounds **10** and **11** and C2 ceramide at 50 μM, and analyzed by immunoblotting. Compounds **10**, **11** and C2-ceramide induced cytochrome *c* release, before the onset of nuclear apoptosis, in a caspase-3 independent manner. Compound **10** was the most active, while C2 ceramide displayed the lowest effect.

Compounds **10** and **11** thus shared important similarities with C2-ceramide, particularly the ability to induce cytochrome *c* release in conjunction with the onset of

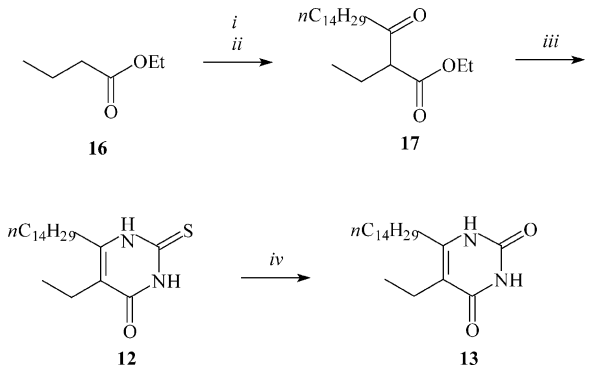


apoptosis, in a caspase-3 independent manner, as previously demonstrated for C2-ceramide in leukemic cells; [20,31] on the contrary, the profile of pharmacological activity of compounds **10** and **11** was markedly different from that of pyrimidines, including uracil, thiouracil and 5,6-dimethyluracil, which did not display a significant proliferation-suppressive ability in CCRF-CEM cells (Fig. 3).

Furthermore, compound **10** was evaluated in vivo for its anti-tumor activity and toxicity. The administration of compound **10** at 2, 10, 50 and 200 mg/kg to seven animals per dose level xenografted with the WiDr



Scheme 1. Reagents and conditions: (i) LDA, 0 °C, THF, 30 min; (ii) propionyl chloride, r.t., 12 h; (iii) thiourea, EtONa–EtOH, 90 °C, 1 h; (iv) chloroacetic acid (10%), reflux, 12 h.



Scheme 2. Reagents and conditions: (i) LDA, 0 °C, THF, 30 min; (ii) nC$_{14}$H$_{29}$COCl, r.t., 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 50% effect level) of CCRF-CEM human T-leukemia cells by compounds **10**–**13** and C2-ceramide

Comp.	IC_{50} (μM, mean ± SD)
10	1.7 ± 0.2
11	7.9 ± 0.9
12	not reached
13	not reached
C2-ceramide	31.6 ± 4.6

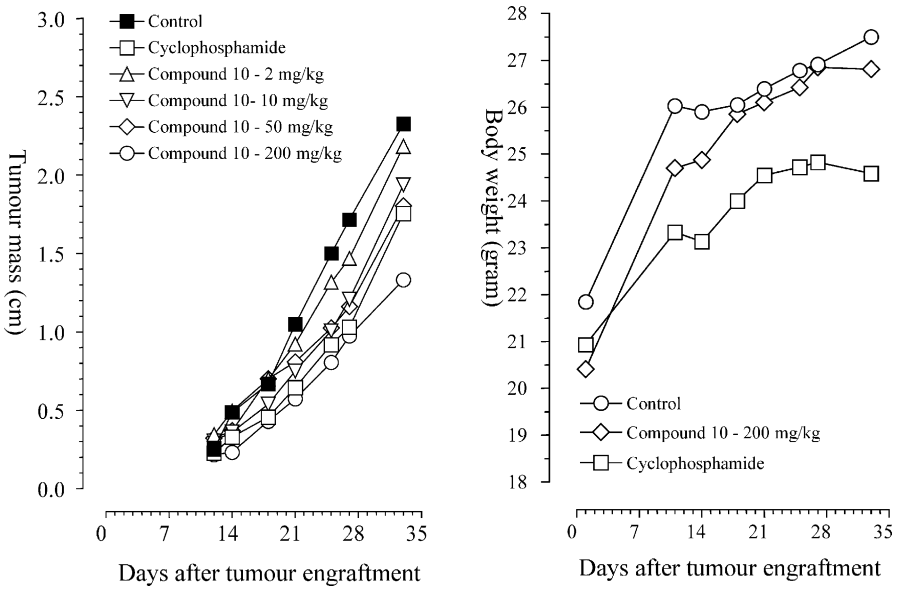


Fig. 3. Increase in tumor mass (left) and changes in body weight (right) as a function of time in athymic mice xenografted with WiDr human colon cancer cells and treated with compound **10** (2, 10, 50 and 200 mg/kg), cyclophosphamide (20 mg/kg) and sterile water as a control vehicle. The symbols represent the mean values of seven animals per treatment.

human colon cancer cells resulted in a dose-dependent reduction of tumor growth. At the end of the fifth week of study, a significant decrease in tumor growth was detected in animals given compound **10** at 50 and 200 mg/kg; the latter dose level was the most effective, with a 42.7% reduction of tumor volume compared with controls. Animals given cyclophosphamide 20 mg/kg showed a lower tumor mass reduction (24.6%). Compound **10** was also well tolerated; no toxic deaths or signs of toxicity were observed; the body weight of animals given 200 mg/kg was similar to that of controls at the end of the fifth week, while that of mice given cyclophosphamide was smaller.

Compounds **10** may thus represent the lead of a new class of anti-tumor agents with enhanced activity on apoptosis-controlling pathways, as compared with the well characterized C2-ceramide.

Future research will be directed at developing modifications of the chemical structure of compounds **10** and **11**, in order to try to elucidate the structure–activity relationships in this class of conformationally restrained analogs of ceramide, not only in terms of the interaction with the biological target, but also taking into account cell penetration.

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