



The natural marine anhydrophytosphingosine, Jaspine B, induces apoptosis in melanoma cells by interfering with ceramide metabolism

Yahya Salma^{a,b,c}, Elodie Lafont^{a,b}, Nicole Therville^{a,b}, Stéphane Carpentier^{a,b}, Marie-José Bonnafé^d, Thierry Levade^{a,b,d}, Yves Génisson^c, Nathalie Andrieu-Abadie^{a,b,*}

^a Institut National de la Santé et de la Recherche Médicale, Inserm U858, Toulouse, France

^b Université Toulouse III Paul Sabatier, Institut de Médecine Moléculaire de Rangueil, IFR31, Toulouse, France

^c LSPCMB, UMR 5068, Université Toulouse III Paul Sabatier, Toulouse, France

^d Laboratoire de Biochimie Métabolique, CHU de Toulouse, France

ARTICLE INFO

Article history:

Received 24 March 2009

Accepted 4 May 2009

Keywords:

Jaspine B

Ceramide

Sphingomyelin

Melanoma

Apoptosis

ABSTRACT

Marine environment has frequently afforded a variety of biologically active compounds with strong anticancer and cytotoxic properties. In the present study, the mechanism of action of Jaspine B, an anhydrophytosphingosine derivative isolated from the marine sponge *Jaspis* sp., was investigated. Jaspine B was able to dose- and time-dependently decrease the viability of murine B16 and human SK-Mel28 melanoma cells. On these cells, Jaspine B treatment triggered cell death by typical apoptosis as illustrated by phosphatidylserine externalization, the release of cytochrome c and caspase processing. These effects were associated with increased intracellular ceramide levels owing to perturbed ceramide metabolism. Indeed, Jaspine B exposure strongly inhibited the activity of sphingomyelin synthase (SMS), an enzyme that converts *de novo* ceramide into the membrane lipid sphingomyelin. Moreover, whereas Jaspine B-induced cell death was enhanced in SMS1-depleted cells, it was strongly inhibited in cells that stably overexpress human SMS1. Finally, the cytotoxic effects of Jaspine B truncated analogs were also shown to be dependent on SMS activity.

Altogether, Jaspine B is able to kill melanoma cells by acting on SMS activity and consequently on ceramide formation, and may represent a new class of cytotoxic compounds with potential applications in anticancer melanoma therapy.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Cutaneous melanoma is one of the most aggressive forms of skin cancer with high metastatic potential and strong resistance to radiation, immunotherapy and chemotherapy [1]. The incidence of melanoma is rising at an alarming rate and has become a major public health concern in many countries. Despite extensive research the precise molecular determinants responsible for melanoma progression are yet to be delineated. Recent studies, however, have indicated that melanoma cells show dysfunction in

the apoptotic program [2] which provided exciting new targets for rationally designed anti-melanoma therapeutic strategies.

Manipulation of cellular sphingolipid (SL) metabolism has been proposed as a means to amplify or restore apoptosis [3]. Indeed, numerous studies have shown that increased intracellular levels of the SL metabolite ceramide inhibit cell proliferation and promote apoptosis of tumor cells (reviewed in [4]). Not only can genetic manipulation of ceramide-metabolizing enzymes lead to the elevation of intracellular concentration of ceramide and subsequent cell death but also addition of exogenous ceramide analogs with short- or long-acyl chain promotes apoptosis in various cancer cell lines and reduces in some cases, the mass of xenografted tumors in nude mice [5]. Three main metabolic pathways can modulate ceramide levels to produce non-antiproliferative sphingolipids: (i) biosynthesis of sphingomyelin (SM) through sphingomyelin synthase (SMS), (ii) biosynthesis of cerebroside through glucosyltransferases and (iii) production of sphingosine and then sphingosine-1-phosphate via ceramidase and sphingosine kinase [6]. In human melanoma cell lines, resistance to stress-induced apoptosis has been associated with low ceramide levels and, conversely, high sphingosine-1-phosphate levels and sphingosine kinase activity [7].

Abbreviations: C6-NBD ceramide, 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl) sphingosine; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-aminomethylcoumarin; FCS, fetal calf serum; GCS, glucosylceramide synthase; GlcCer, glucosylceramide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SL, sphingolipid; SM, sphingomyelin; SMS, sphingomyelin synthase; TLC, thin layer chromatography; zVAD-fmk, benzyloxycarbonyl valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone.

* Corresponding author at: U858 INSERM, Institut de Médecine Moléculaire de Rangueil BP84225, 31432 Toulouse Cedex 4, France. Tel.: +33 561 32 35 31; fax: +33 561 32 20 84.

E-mail address: nathalie.andrieu@inserm.fr (N. Andrieu-Abadie).

On the contrary, treatment with a ceramidase inhibitor was shown to increase ceramide content and elicit apoptosis [8]. As elevations in ceramide levels promote apoptosis in melanoma cells, the hypothesis that related analogs of ceramide could be used as chemotherapeutic agents has been proposed.

For instance, natural ceramide isolated from fungi or synthetic derivatives analogs have shown high toxicity against the human melanoma cell line SK-Mel1 [9,10]. Fungi contain phytosphingolipids that differ from mammalian SLs by the presence of a hydroxyl group at the carbon 4 instead of a *trans* double bond of the most abundant sphingoid long chain base, sphingosine. Natural or synthetic phytosphingolipids can kill human cancer cells with comparable or even higher potency than ceramide [11]. Indeed, *N*-acetylphytosphingosine has been reported to induce apoptosis in human T-cell lymphoma in a mitochondria-dependent manner [12]. Phytosphingosine, another phytosphingolipid, has been shown to enhance the apoptotic response to radiation [13], arsenic [14] or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant [15] cancer cells. These effects are mediated by the activation of several caspases and Bax translocation to the outer leaflet of mitochondria [16] but the impact on ceramide metabolism has never been studied.

Recent studies on the marine sponges *Pachastrissa* sp. [17] and *Jaspis* sp. [18] led to the isolation of a cyclic anhydrophytosphingosine, named pachastrissamine or Jaspine B, which was reported to exhibit strong cytotoxicity against different human solid tumor cell lines [17,18]. However, its mechanism of action remains to be clarified.

In this study, we investigated the molecular mechanisms of cell death induced by Jaspine B both in murine and human melanoma cells. We report here that Jaspine B induces apoptotic cell death in melanoma cells by a caspase-dependent pathway. Moreover, we show for the first time that this anhydrophytosphingosine disturbs intracellular SL metabolism by inhibiting SM synthesis and elevating ceramide content.

2. Materials and methods

2.1. Reagents

Natural Jaspine B was purified from crude extracts of the sponge *Jaspis* sp. kindly provided by Dr. C. Debitus (UMR 152 IRD, Toulouse, France). This raw material was chromatographed on silica gel eluted with ethyl acetate/methanol/30% ammonia (88:10:2, by vol.). The analytical thin layer chromatography (TLC) plates ($R_f = 0.4$ for Jaspine B eluting with the same solvent system) were visualised using two successive dipping solutions: (a) 0.5% NaIO₄ in H₂O and (b) 1.4% benzidine in H₂O. Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) and phytosphingosine were obtained from Cogem (Paris, France). zVAD-fmk was purchased from Bachem (Voisins-Le-Bretonneux, France). DMEM, trypsin–EDTA and fetal calf serum (FCS) were from Invitrogen (Cergy-Pontoise, France). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied from Euromedex (Mundolsheim, France). Other reagents were purchased from Sigma (Lisle d'Abeau, France). Original Jaspine B synthetic analogs were synthesized in the course of this work (LSPCMIB, UMR 5068, Toulouse, France).

2.2. Cell lines

Murine B16F10 and human SK-Mel28 melanoma cell lines were purchased from American Type Culture Collection (LGC, Molsheim, France). B16 mouse melanoma cells deficient in glucosylceramide synthase (also named GM95) were obtained from the RIKEN cell bank (Hiroshima, Japan). Human cervical carcinoma HeLa cells

engineered to overexpress a tagged-V5 SMS1 were kindly provided by Dr. J.C.M. Holthuis (Utrecht, The Netherlands) [19]. Cells were grown in a humidified 5% CO₂ atmosphere at 37 °C in DMEM medium containing Glutamax (2 mM), and heat-inactivated FCS (10%). Natural Jaspine B was added to the cells as ethanolic solution. Control cells were treated with the same concentration of solvent (which did not exceed 0.5%) as that for Jaspine B-treated cells.

2.3. Cell viability

After treatment with natural Jaspine B or its analogs, cell viability was evaluated by using the MTT assay based on the cleavage of the tetrazolium salt MTT to formazan crystals by metabolically active cells [20]. The formazan crystals formed were solubilized by adding dimethylsulfoxide for 1 h at 37 °C and quantified spectrophotometrically using an ELISA reader ($\lambda = 560$ nm).

2.4. Flow cytometry analyses

Cells were incubated for 1 h with or without 50 μ M zVAD-fmk and further incubated for 6 or 24 h in the presence or absence of 5 μ M Jaspine B. Phosphatidylserine externalization was evaluated by labeling cells with Annexin V-FITC (250 ng/ml) and propidium iodide (12.5 μ g/ml) (Immunotech, Marseille, France) for 10 min at 4 °C. Analyses were performed on a FACScan cytometer (Becton Dickinson, Le Pont de Claix, France).

2.5. Fluorogenic DEVD cleavage enzyme assay

After incubation with Jaspine B, cells were sedimented and washed with PBS. Cell pellets were homogenized in 10 mM HEPES (pH 7.4), 42 mM KCl, 5 mM MgCl₂, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM PMSF, and 2 μ g/ml leupeptin. Reaction mixtures contained 100 μ l of cell lysates and 100 μ l of 40 μ M Ac-DEVD-AMC. After 30 min incubation at room temperature, the amount of the released fluorescent product aminomethylcoumarin was determined at 351 and 430 nm for the excitation and emission wavelengths, respectively.

2.6. Western blot analyses

Equal amounts of proteins were separated in a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (PerkinElmer, Courtaboeuf, France). Proteins were detected using an ECL detection system (Pierce). Monoclonal anti-caspase-3, polyclonal anti-caspase-9, polyclonal anti-poly(ADP-ribose) polymerase (PARP) and polyclonal anti- β -actin (used as a control for protein loading) were purchased from Cell Signaling Technology and used at 1/1000 dilution. Monoclonal anti-V5 (0.2 μ g/ml) was from Invitrogen.

2.7. Immunofluorescence microscopy

Murine B16 (treated or not with 5 μ M Jaspine B for 6 h) or human HeLa cells were grown on glass coverslips for 24 h in DMEM supplemented with 10% FCS. Then, cells were fixed in PBS containing 4% (w/v) paraformaldehyde for 15 min, permeabilized with PBS containing 0.1% (w/v) Triton X-100 for 15 min, washed in PBS and stained for 1 h with a monoclonal anti-cytochrome c antibody (BD Pharmingen; Le Pont-de-Claix, France) for B16 cells or a monoclonal anti-V5 for HeLa cells. After rinsing with PBS, an Alexa Fluor 488 anti-mouse antibody (Molecular Probes/Invitrogen) was added for 1 h. Cells were also counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 μ g/mL) to visualize the nuclei. Coverslips were mounted using Prolong Gold anti-fade reagent

(Molecular Probes/Invitrogen) and examined under an Olympus fluorescence-equipped microscope.

2.8. Determination of *in situ* sphingomyelin synthase and glucosylceramide synthase activities

Melanoma cells were incubated with natural Jaspine B or its analogs in the absence of FCS for the indicated times and concentrations. Then, 5 μ M C₆-NBD-ceramide (Interchim, Montluçon, France) was added to the medium as an ethanolic solution. After incubation for 2 h at 37 °C, cellular lipids were extracted with chloroform/methanol (2:1, v/v) [21]. After centrifugation (1000 \times g, 10 min), the lower phases were concentrated under nitrogen and resolved by TLC developed in chloroform/methanol/30% ammonia/water (70:30:3:2, by vol.). C₆-NBD-ceramide (C₆-NBD-Cer), C₆-NBD-glucosylceramide (C₆-NBD-GlcCer) and C₆-NBD-sphingomyelin (C₆-NBD-SM) were eluted from the silica and quantified spectrofluorometrically (λ_{ex} = 470 nm and λ_{em} = 530 nm).

2.9. Ceramide quantification

Lipids were extracted by the Folch procedure with chloroform/methanol from cell lysates. Ceramide content was determined as previously reported [22] using *E. coli* membranes as a source of diacylglycerol kinase (the *E. coli* strain was a gift from Drs. D. Perry and Y.A. Hannun, Charleston, SC) and [³²P]γ-ATP (6000 Ci/mmol, PerkinElmer). Radioactive ceramide-1-phosphate was purified by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by vol.) and then scraped before counting radioactivity by liquid scintillation.

2.10. Gas chromatography analyses

Lipids were extracted from cell lysates and separated by TLC using chloroform/methanol/water (100:42:6, by vol.) and then chloroform/methanol/acetic acid (94:1:5, by vol.). The band corresponding to ceramide was scraped and the lipid eluted from the silica using chloroform/methanol (2:1, by vol.). Then, the fatty acid composition of ceramide was determined by gas chromatography as previously reported [23]. Briefly, after evaporation, the dried residue was dissolved in 0.2 ml of chloroform/methanol (2:1, by vol.) and transferred to glass screw-capped tubes. Two micrograms of nonadecanoic acid (Sigma) were added and mixed with 1 ml of dichloromethane/methanol (1:3, by vol.) and 0.2 ml of acetyl chloride. After incubation for 1 h at 75 °C, 4 ml of K₂CO₃ and 2 ml of hexane were added. After vigorous vortexing and centrifugation, the upper (hexane rich) phase was collected and washed with 2 ml of acetonitrile. The upper phase was evaporated under a nitrogen stream and dissolved in ethyl acetate. For analysis of long chain fatty acids, the ethyl acetate solution was injected into an Agilent 6890N gas chromatograph equipped with a CPSil5-CB column (Varian) and a flame ionization detector. Temperature was raised from 180 to 280 °C (3 °C/min) and nitrogen was used as a gas vector (at 0.3 ml/min). For analysis of very long chain fatty acids, the ethyl acetate solution was injected into a Carlo Erba 8000 gas chromatograph equipped with a CPSil88 column (Varian). Temperature increased from 150 to 255 °C. The gas vector and detector were as above.

2.11. Sphingolipid turnover

Sphingolipids were labeled by incubating cells for 6 h with 0.33 μ Ci/mL [3-³H]-sphingosine (PerkinElmer, France) in the presence or absence of Jaspine B. Then, cells were washed twice with PBS, harvested and centrifuged. Lipids were extracted and separated by TLC using chloroform/methanol/water (100:42:6, by

vol.). Radiolabeled sphingolipids were detected using a Berthold radiochromatoscanner, identified using lipid standards and then scraped before counting radioactivity by liquid scintillation.

2.12. Transfection with siRNA and treatment

To suppress the expression of SMS1, small interfering RNA (siRNA) targeting this enzyme (Dharmacon ON-TARGETplus SMARTpool with the following sequences: 5'-CCAAGGAAUUGUACCUCGA-3', 5'-CAACAUGUGUGGCGACUAU-3', 5'-CUGUAUUCUCUUAGCGCAU-3', 5'-AGACUAAACCCAACGGAAU-3') was transfected into B16 melanoma cells grown to 70–90% confluence using Hyperfect (Qiagen) reagent. For each transfection, 5 nM of siRNA were used and incubated in DMEM medium without FCS for 24 h. After transfection, C₆-NBD-ceramide (5 μ M) or Jaspine B (at the indicated concentrations) was added to the medium and incubated for 2 or 24 h to determine SMS activity or cell death, respectively.

2.13. Statistical analyses

Data are presented as means \pm S.E.M. Student's *t*-test was used for statistical comparisons among groups and differences were considered statistically significant when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3. Results

3.1. Structure of Jaspine B and phytosphingosine

Fig. 1 shows the structures of the natural Jaspine B and the D-ribo-phytosphingosine. Jaspine B is a C₁₈ anhydrophytosphingosine derivative in which the C-1 and the C-4 position are connected through an ether linkage, as the result of a formal dehydration. Central to its structure is thus a rigid tetrahydrofuran ring bearing three different residues in an all-*cis* stereochemical arrangement: an amino group, a hydroxyl group and an aliphatic chain. This cyclic framework corresponds to the C-2 to C-4 portion of D-ribo-phytosphingosine (the configuration at C-4 being however inverted) whereas the C-1 hydroxyl group is importantly missing.

3.2. Jaspine B triggers apoptotic melanoma cell death

The first set of experiments aimed at evaluating the cytotoxic effect of natural Jaspine B on melanoma cell lines. As shown in Fig. 2, Jaspine B exhibited a dose-dependent (Fig. 2A) and time-dependent (Fig. 2B) cytotoxic action on murine B16 and human SK-Mel28 (Fig. 2C) melanoma cells. Jaspine B displayed a strong cytotoxicity with an IC₅₀ of 0.5 μ M after 24 h incubation whereas phytosphingosine was approximately 100 times less toxic (Fig. 2A). Addition of FCS (10%) to the incubation medium resulted in moderately increased IC₅₀ values (~2.5 μ M), however, Jaspine B was still highly cytotoxic under these conditions (Fig. 2A). To ascertain that the effect of Jaspine B was not a detergent effect leading to unspecific lysis of melanoma cells, we examined its impact on B16 cell phosphatidylserine externalization, one of the earliest hallmarks of apoptosis. As shown in Fig. 2D, 5 μ M Jaspine B led to an increased proportion of annexin-V-single-positive cells plus annexin-V/propidium iodide-double-positive cells after 24 h treatment whereas there was no significant change after 6 h exposure. These effects were accompanied by morphological alterations such as nuclear condensation without cell membrane permeability indicating apoptotic cell death (data not shown). Preincubation of the cells with the broad-spectrum caspase inhibitor, zVAD-fmk, resulted in a significant reduction (~50%) of the annexin V-positive cell population (Fig. 2D) suggesting that

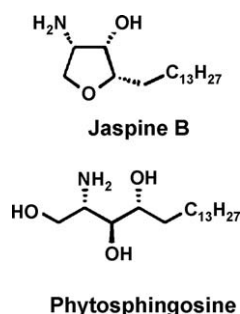


Fig. 1. Structures of Jaspine B and phytosphingosine.

Jaspine B cytotoxic effect was partially dependent on caspase activity.

To substantiate the involvement of caspases in Jaspine B-induced B16 melanoma cell death, we then investigated the effect of the natural anhydrophytosphingosine on caspase activation. As illustrated in Fig. 3A, Jaspine B actively promoted the cleavage of caspase-9 and -3 as well as the caspase substrate PARP. Consistent with these results, caspase-3-like activity, as measured by the cleavage of the fluorogenic tetrapeptide substrate Ac-DEVD-AMC, which contains the cleavage site found in several caspase-3 and caspase-7 targets, increased in B16 cells within 4 h, being sub-optimal at 6 h post-treatment (Fig. 3B). To further dissect the apoptotic cascade activated by Jaspine B in melanoma cells, we examined mitochondrial-related events. As shown in Fig. 3C, translocation of cytochrome c from mitochondria to the cytosol was observed in Jaspine B-treated cells by immunofluorescence microscopy.

Taken together, these results demonstrate for the first time that Jaspine B induces apoptotic cell death in melanoma cells, with a site of action likely lying upstream of mitochondria.

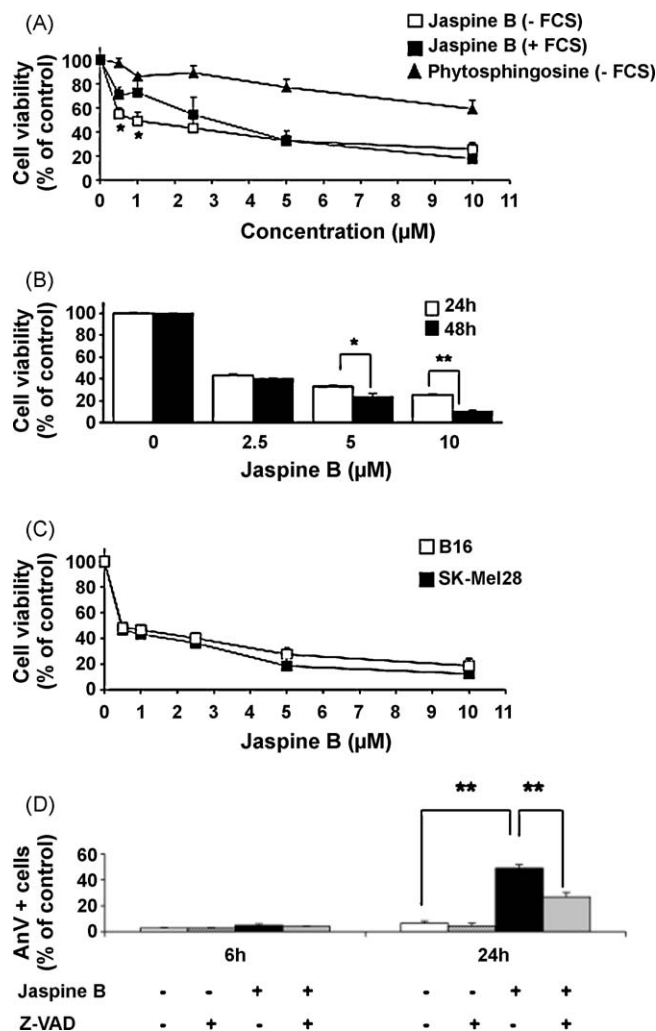


Fig. 2. Effect of natural Jaspine B on melanoma cell viability. Cells were incubated for 24 (A–C) or 48 h (B) with the indicated concentrations of Jaspine B or phytosphingosine in the absence (–FCS) or presence (+FCS) of FCS in the medium. Then, cell viability was assessed by MTT. (A) Comparative cytotoxic effects of Jaspine B and phytosphingosine on B16 cell viability. (B) Time- and dose-dependent effect of Jaspine B on B16 cell viability. (C) Comparative cytotoxic effects of Jaspine B on murine B16 and human SK-Mel28 melanoma cells. Data are expressed as percentage of the values measured in the absence of the molecules and are the mean \pm S.E.M. of at least three different experiments performed in triplicate. (D) B16 mouse melanoma cells were pre-incubated for 1 h with 50 μM zVAD-fmk and further incubated for the indicated times with or without 5 μM Jaspine B. Then cells were analyzed by flow cytometry after annexin-V-FITC and propidium iodide labeling. Percentages of annexin-V-single-positive cells plus annexin-V/propidium iodide-double-positive cells are indicated. Values are means \pm S.E.M. of three different experiments.

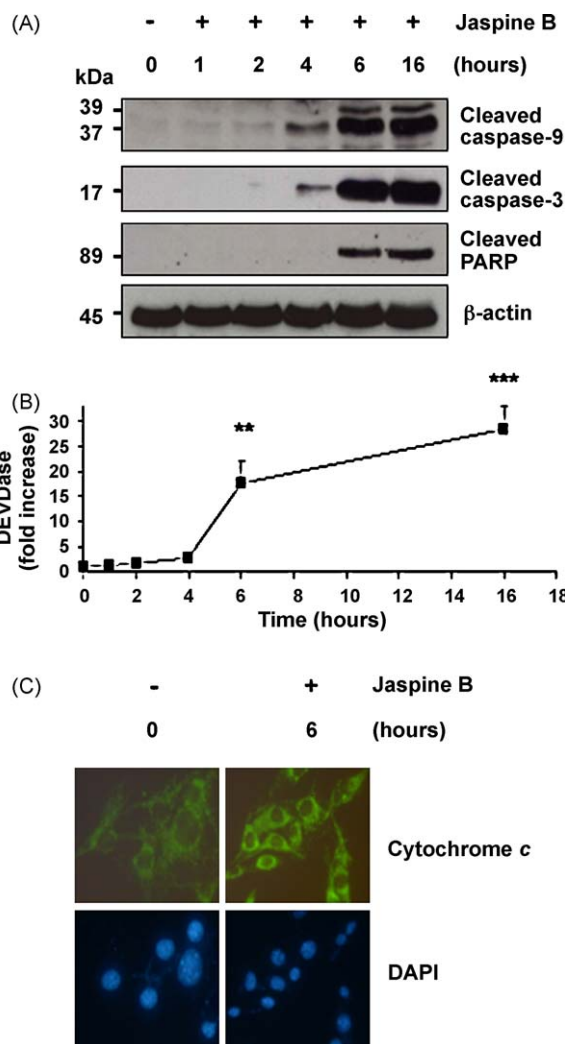


Fig. 3. Jaspine B induces apoptosis in melanoma cells. B16 mouse melanoma cells were incubated for the indicated times in the absence or presence of 5 μM Jaspine B. (A) Proteins were extracted and analyzed by Western blotting for caspase-9, caspase-3 and PARP cleavage. Anti-β-actin antibody was used as a control. (B) Caspase-3-like (or DEVDase) activity was assessed as described in Section 2. Data are means \pm S.E.M. of three independent experiments. (C) Cells were labeled with DAPI or anti-cytochrome c as indicated and examined by fluorescence microscopy. Data are representative of a least three independent experiments.

3.3. Jaspine B induces ceramide accumulation and inhibits sphingomyelin synthase activity in melanoma cells

As ceramide has been identified as an important mediator of cancer cell death [4,24], the effects of the anhydrophytosphingosine Jaspine B on ceramide metabolism were investigated. As illustrated in Fig. 4A, Jaspine B treatment led to a time-dependent accumulation of ceramide, which was detectable as early as 4 h after addition of Jaspine B. Moreover, gas chromatography analysis of ceramide molecular species confirmed that the levels of several fatty acids contained in ceramide, including both long chain and very long chain fatty acids, were elevated upon Jaspine B treatment (Fig. 4B).

As inhibition of SM and glucosylceramide (GlcCer) synthesis can account for ceramide elevation, we evaluated the role of SMS and GlcCer synthase (GCS) in Jaspine B-triggered apoptosis of B16 melanoma cells. To this end, we measured the *in situ* SMS and GCS activities by incubating intact living cells with a fluorescent analog of ceramide that is converted into fluorescent SM and GlcCer (Fig. 5A). Under these conditions, Jaspine B elicited a dose-dependent (Fig. 5B) and time-dependent (Fig. 5C) decrease in SMS activity. In contrast, GCS activity was not decreased but rather tended to be slightly enhanced (without reaching statistical significance) upon Jaspine B treatment.

To confirm the inhibitory effect of Jaspine B on SMS activity, we next evaluated the changes in the SL pattern by incubating B16 cells with [3-³H]sphingosine in the absence or presence of 5 μ M Jaspine B. After 6 h, the SL pattern was profoundly perturbed in Jaspine B-treated cells as compared to untreated control cells. Notably, Jaspine B caused a strong increase in [3-³H]sphingosine-labeled ceramide content ($248 \pm 110\%$) whereas the level of radiolabeled SM decreased ($44 \pm 6\%$). In sharp contrast, no variation was observed for the cellular content of GlcCer ($90 \pm 18\%$) (data not shown).

3.4. Manipulation of sphingomyelin synthase but not glucosylceramide synthase activity affects Jaspine B toxicity

To further investigate the relationship between Jaspine B-induced SMS inhibition and cell death, we knocked-down the Golgi membrane SMS1 isoform by a siRNA approach. As shown in Fig. 6A, transfection of SMS1 siRNA in B16 melanoma cells decreased by 70% the activity of SMS and significantly potentiated the inhibitory effect of the natural anhydrophytosphingosine Jaspine B. Under these conditions, SMS1 siRNA-transfected cells were more sensitive to Jaspine B treatment than their control counterparts (Fig. 6B).

Reciprocally, to evaluate the impact of SMS1 overexpression on Jaspine B-induced cell death, we used HeLa cells stably overexpressing human V5-tagged SMS1 and displaying higher SMS activity (Fig. 6C). Of particular interest was the finding that the cytotoxic effects of Jaspine-B were partially antagonized in HeLa cells overexpressing SMS1 as compared to mock-transfected cells (Fig. 6D). Collectively, our data show that a decrease in SMS1 expression can sensitize cancer cells to Jaspine B treatment, whereas its increase can confer resistance to this natural molecule.

Of note, GCS deficiency did not affect melanoma cell viability upon Jaspine B exposure. Indeed, when the sensitivity to Jaspine B of variant B16 melanoma cells which are completely deficient in GlcCer (Fig. 7A), GCS activity (Fig. 7B) and all higher glycosphingolipids [21,25] was compared with that of the parental B16 melanoma cell line, no difference was observed (Fig. 7C).

3.5. Jaspine B analogs kill melanoma cells through a SMS-dependent pathway

In order to gain insights into the structure–activity relationship of Jaspine B, we synthesized three analogs of the natural

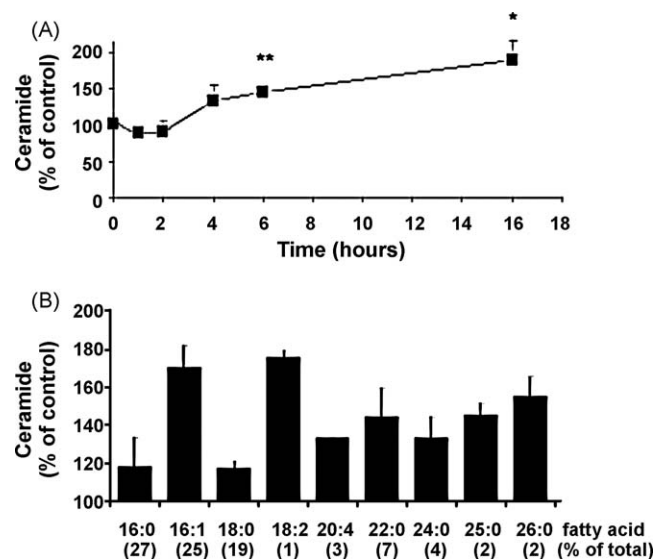


Fig. 4. Jaspine B triggers ceramide accumulation in B16 melanoma cells. Cells were incubated for the indicated times (A) or 6 h (B) in the absence or presence of 5 μ M Jaspine B. (A) Total intracellular ceramide content was determined by the diacylglycerol kinase assay as described in Section 2. Data are expressed as percentage of the values measured in the absence of Jaspine B and are the mean \pm S.E.M. of three independent experiments. (B) The fatty acids contained in ceramide were separated and quantified by gas chromatography. Data are expressed as percentage of the values measured in the absence of Jaspine B and are the mean \pm S.E.M. of two experiments.

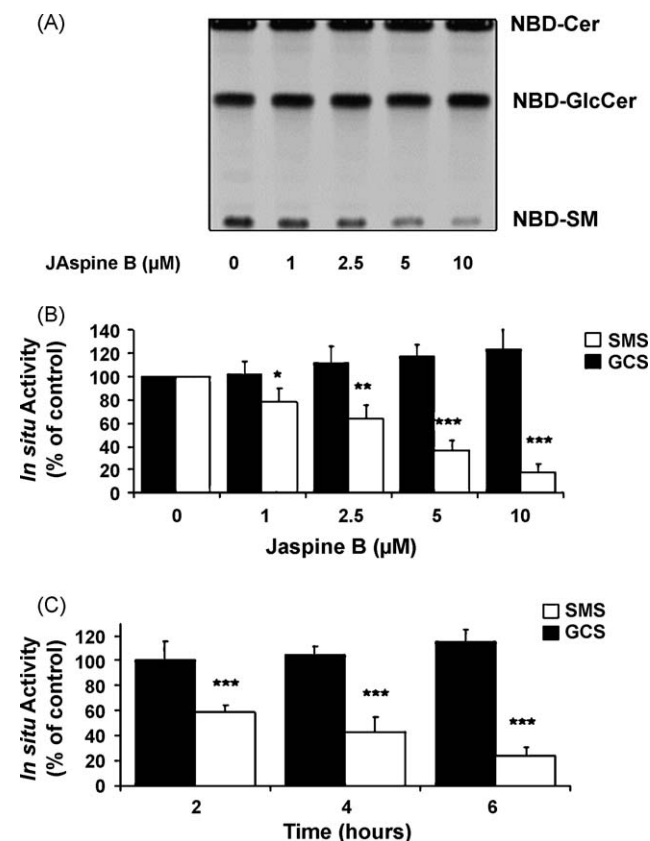


Fig. 5. Jaspine B inhibits sphingomyelin synthase activity in B16 melanoma cells. Cells were incubated for 6 h in the absence or presence of the indicated concentrations of Jaspine B and further incubated up to 2 h in the presence of C6-NBD-ceramide (5 μ M). After washing the cells, cellular lipids were extracted, separated by TLC, and photographed under UV light (A). The specific activities of GCS and SMS were determined by quantifying the fluorescence of SM and GlcCer (B). Time-dependent effect of 5 μ M Jaspine B on SMS activity (C). Results are expressed as the percentage of the activities measured in the absence of Jaspine B. Data are means \pm S.E.M. of at least three independent experiments.

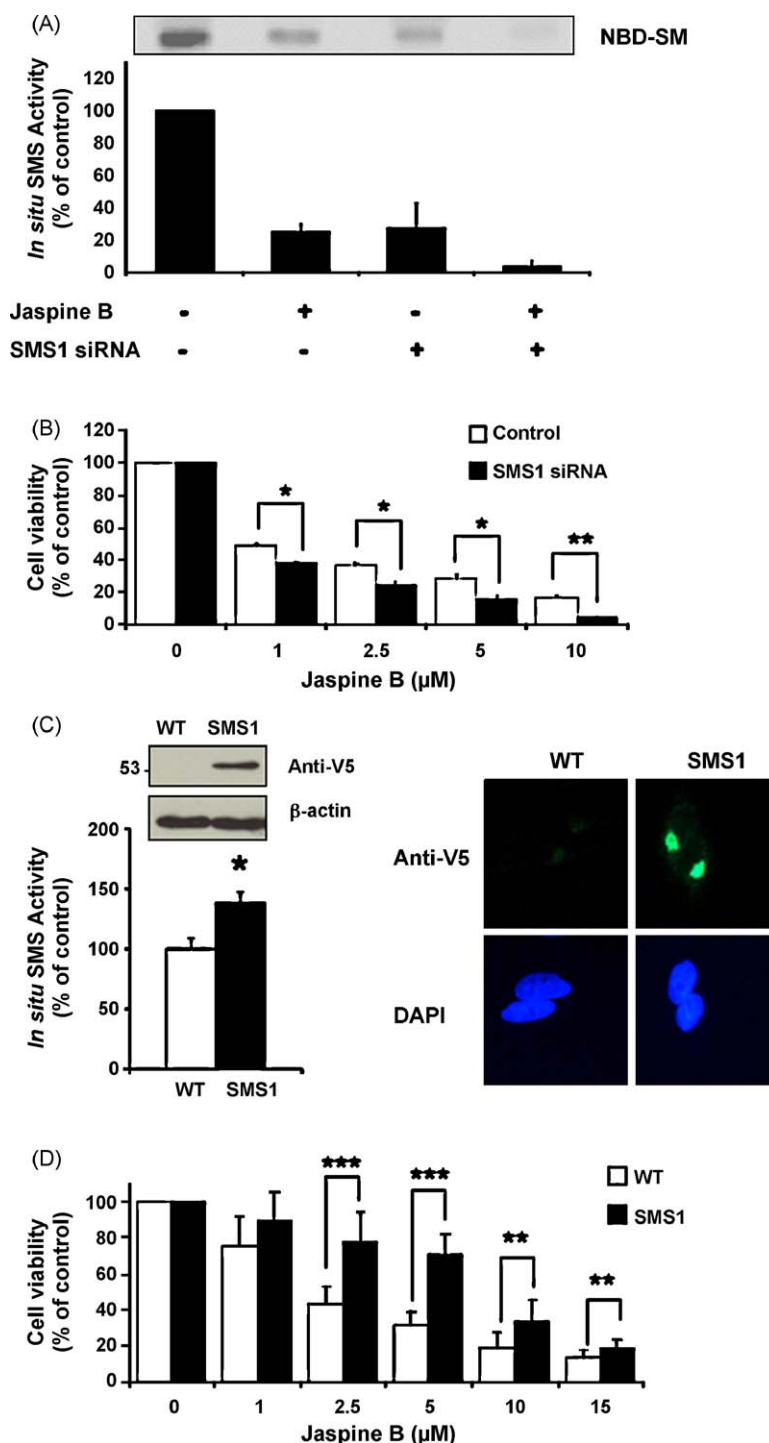


Fig. 6. Modulation of SMS1 activity affects Jaspine B-induced cell death of cancer cells. (A) Effect of SMS1 siRNA on SMS activity. B16 melanoma cells were incubated for 24 h in the absence or presence of 5 nM SMS1 siRNA and further incubated up to 2 h with C6-NBD-ceramide (5 μ M). Cells were collected and the fluorescence of NBD-C6-SM was quantified. (B) Effect of SMS1 siRNA on B16 melanoma cell viability. Control and SMS1 siRNA-transfected cells were incubated for 24 h with the indicated concentrations of Jaspine B. Then, cell viability was assessed by MTT. Results are representative of three independent experiments. (C) *In situ* SMS activity (left panel) and SMS1 expression (left panel, inset) and localization (right panel) were evaluated in mock-transfected (WT) and SMS1-V5 overexpressing (SMS1) HeLa cells by using C6-NBD-ceramide and anti-V5 antibody, respectively. Anti- β -actin antibody was used as a control for Western blotting. (D) Mock-transfected and SMS1-V5 overexpressing HeLa cells were incubated for 24 h in the presence or absence of 5 μ M Jaspine B and cell viability was evaluated by MTT assay. Data are the mean \pm S.E.M. of at least three different experiments performed in triplicate.

anhydrophytosphingosine by means of an original asymmetric synthetic route. Compound **1** bears a truncated C8 aliphatic chain in place of the C14 present in the natural product whereas compound **3** incorporates a *p*-fluorophenylethyl appendage. In addition, we also prepared from **1** the *N*-hexyl secondary amine **2**, a double-chained isomer of Jaspine B comprising the same total

number (18) of carbon atoms with different motifs at the C2 and C4 positions (Fig. 8A).

We investigated the potential cytotoxic effect of these novel short chain synthetic Jaspine B analogs on murine B16 melanoma cells. As illustrated in Fig. 8B, compounds **1** and **2**, that share the same aliphatic C12 skeleton, exhibited a dose-dependent cytotoxic

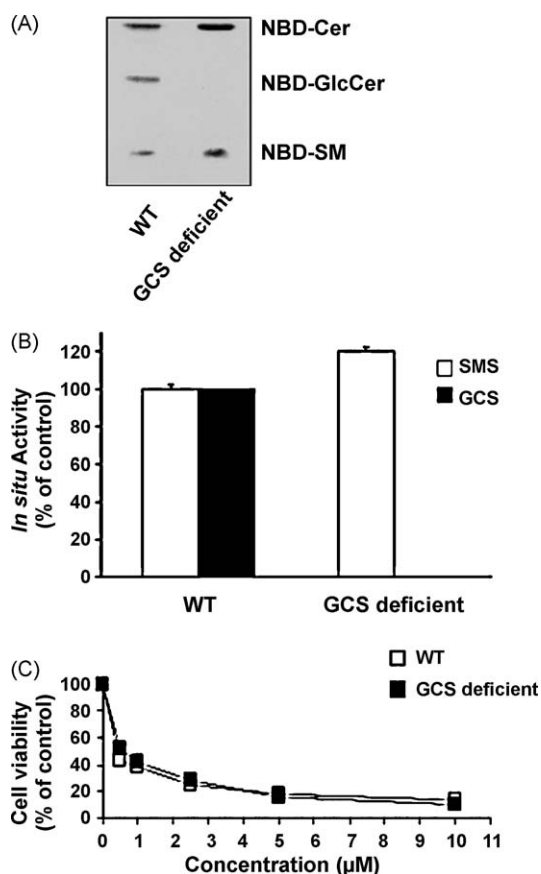


Fig. 7. Effect of Jaspine B on GCS-deficient B16 melanoma cells. *In situ* SMS and GCS activities were measured in wild-type (WT) and GCS-deficient (GCS-deficient) B16 melanoma cells. Cells were incubated up to 2 h in the presence of C6-NBD-ceramide (5 μM). After washing the cells, cellular lipids were extracted, separated by TLC, and photographed under UV light (A). The specific activities of GCS and SMS were determined by quantifying the fluorescence of SM and GlcCer (B). (C) Cells were incubated for 24 h in the presence of the indicated concentrations of Jaspine B, and cell viability was assessed by MTT. Data are expressed as percentage of the values measured in the absence of Jaspine B and are the mean ± S.E.M. of five different experiments performed in triplicate.

action whereas compound **3** had no effect at the concentrations used. The single-chained compound **1** displayed a higher cytotoxicity than its double-chained analog **2** and its IC₅₀ (2.5 μM) was comparable to that of the effect observed with the natural product. The activity of derivative **1** thus suggests a relatively moderate influence of the aliphatic chain length on cytotoxicity, while the decrease in potency for derivative **2** indicates the importance of the primary amine function. Of interest was the finding that only compounds **1** and **2** inhibited SM synthesis, reinforcing the notion of a strong relationship between Jaspine B-induced SMS inhibition and cell death.

4. Discussion

Apoptosis plays an important role in cancer development and progression. Dysfunction in the signaling pathways of programmed cell death may be associated with proliferation of malignant cells while correction of such defects may selectively induce apoptosis in cancer cells [26]. One of the focus of cancer treatment today attempts to amplify or restore apoptosis in tumor cells by acting on pro- or antiapoptotic molecules.

Increasing evidence suggests that the marine environment contains different classes of biologically active compounds with strong anticancer and cytotoxic properties [27]. In particular,

marine sponges are organisms from which several potent cytotoxic bioactive compounds, including alkaloids, steroids, terpenes, peptides, macrolides and polyketides have been isolated. Among them, are some cytotoxic sphingosine-related compounds like sphingosine-4-sulfates [28], penaresidin B [29] and α-galactosylceramide [30]. This latter molecule was shown to exert a strong antitumor effect against various malignant tumors by activating natural killer T (NKT) cells indirectly through stimulation of secretion of cytokines [31].

In this study, we have demonstrated that the natural marine compound Jaspine B, an anhydrophytosphingosine, inhibits the growth of human and murine melanoma cells (and also of human cervix epitheloid carcinoma). On melanoma cells, Jaspine B treatment induces cell death by typical apoptosis as illustrated by phosphatidylserine externalization, the release of mitochondrial cytochrome c into the cytosol, the cleavage of initiator caspase-9 and effector caspase-3. However, inhibition of caspase activity by the synthetic peptide inhibitor zVAD did not completely block phosphatidylserine externalization suggesting that Jaspine B could also trigger a non-apoptotic form of cell death. Indeed, it has been shown that synthetic diastereoisomeric Jaspine B derivatives could induce autophagy in human alveolar epithelial A549 cells [32]. In B16 melanoma cells, the cytotoxic effect of Jaspine B was not affected by the presence of 3-methyladenine (10 mM), a widely used inhibitor of autophagy, suggesting that autophagy is not an important mode of cell death induced by Jaspine B in melanoma cells (data not shown). However, the apoptotic effect induced by Jaspine B is consistent with the observations that phytosphingolipid-treated cancer cells displayed several features of apoptosis [16,33]. Phytosphingolipids are abundant in yeasts and plants. Also, some mammalian cells, specifically those in skin, intestines, and kidney, have been shown to contain a considerable amount of phytosphingosine-based SLs. In skin, phytosphingosine is a lipid occurring naturally in the stratum corneum, both in its free form and as part of the major fraction of ceramides [34]. However, the effect of phytosphingosine on melanoma development has never been examined. Furthermore, a specific mode of action might be expected from Jaspine B due to its unique structural features.

We have also shown that Jaspine B treatment leads to an increase in cellular ceramide level before the onset of apoptosis and the activation of caspases. This proapoptotic SL is produced by cancer cells in response to exposure to ionizing radiation and most chemotherapeutic agents, however, some chemo- and radio-resistant tumor cells exhibit defects in ceramide generation supporting the emerging role of ceramide as a tumor suppressor lipid [3,4]. Strategies that target the enzymes of ceramide clearance (SMS, GCS or ceramidases), resulting in enhanced ceramide levels in cancer cells, have been developed as novel approaches for anticancer chemotherapy. For instance, the acid ceramidase inhibitor B13 has been shown to sensitize xenografted prostate tumors to radiation and to reduce tumor volume [35]. Moreover, downregulation of GCS by siRNA results in a significant inhibition in the expression of P-glycoprotein (P-gp), an ATP-binding cassette transporter implicated in drug resistance, and sensitizes breast cancer cells to chemotherapeutic agents [36].

Jaspine B-mediated increases in tumor cell ceramide can result from distinct mechanisms: (i) stimulation of the *de novo* synthetic pathway, (ii) increase in sphingomyelinase-dependent catabolism of SM, or (iii) inhibition of ceramide metabolism by SMS, GCS or ceramidases. In this study, we found for the first time that Jaspine B-induced inhibition of sphingomyelin synthesis likely contributes to the modification of SL pattern, including SM decrease and ceramide increase, and cell death induction. The biosynthesis of SM is catalyzed by SMS, an enzyme that transfers the phosphocholine moiety from phosphatidylcholine onto the primary hydroxyl group of ceramide generating SM and diacylglycerol [37]. There are

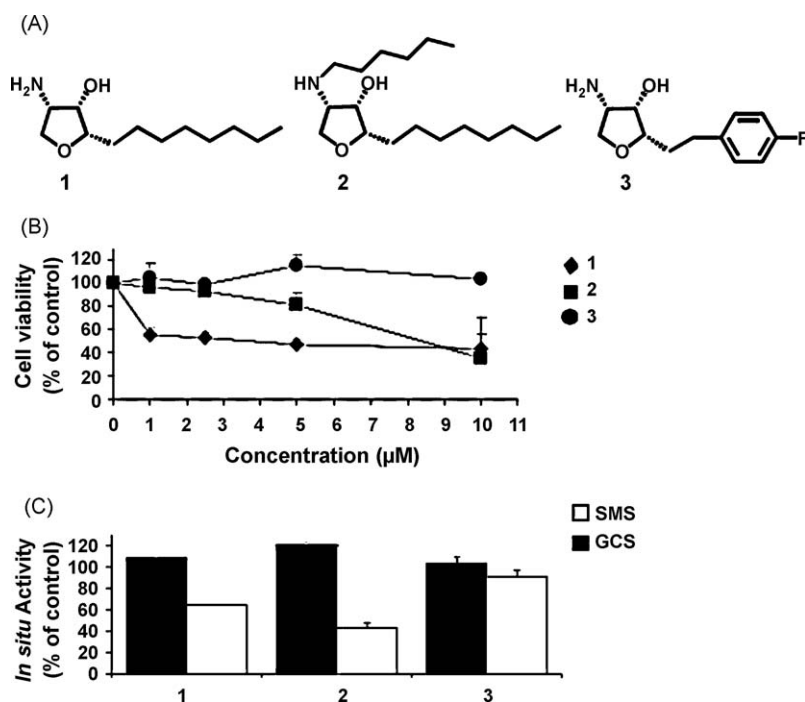


Fig. 8. Effect of synthetic truncated analogs of Jaspine B on cell viability and SMS activity in B16 melanoma cells. (A) Structure of Jaspine B analogs **1**, **2** and **3**. (B) Cells were incubated for 24 h in the presence or absence of the indicated concentrations of compound **1**, **2** or **3** and cell viability was assessed by MTT. Data are the mean \pm S.E.M. of three different experiments performed in triplicate. (C) Cells were incubated for 6 h in the absence or presence of 5 μ M of compound **1**, **2** or **3** and further incubated up to 2 h with C6-NBD-ceramide (5 μ M). Cells were collected and the fluorescence of NBD-C6-SM and NBD-C6-GlcCer were quantified. Results are expressed as the percentage of the activities measured in the absence of Jaspine B. Data are means \pm S.E.M. of at least three independent experiments.

two isoforms of mammalian SMS genes [19,38]. The corresponding proteins, SMS1 and SMS2, are located in the *cis*-, medial-Golgi and at the plasma membrane, respectively. Moreover, a SMS activity has been described in the nucleus, associated to nuclear membranes and chromatin [39].

Regarding the role of SMS in cell death, some studies reported that downregulation of SMS1 confers resistance to stress-induced apoptosis [40,41]. Recently, this notion has been challenged with the observation that suppression of SMS1 by siRNA is associated with enhanced ceramide production and apoptosis after photo-damage [42]. Moreover, SMS1 overexpression was partially protective against staurosporine-induced apoptosis in oligodendrogloma cells [43]. Pharmacological inhibition of SMS by the antiviral xanthate compound D609 has also been reported to induce death in human monocytic leukemia cells [44]. Both SMS1 and SMS2 can be inhibited by D609, however, most of the biological activities of this compound have been largely attributed to its inhibitory effect on phosphatidylcholine-specific phospholipase C [45,46]. The natural marine anhydrophytosphingosine Jaspine B has emerged from this study as a particularly active compound that can inhibit SM synthesis and lead to accumulation of cellular ceramide. Although our data show that manipulation of SMS1 by genetic inhibition or overexpression of the enzyme affects Jaspine B-induced cell death, future studies are under progress to determine whether Jaspine B acts as a direct inhibitor of SMS1, SMS2 or both enzymes.

Altogether, these observations support the fact that Jaspine B-like anhydrophytosphingosines represent attractive molecules in order to design future drug candidates with improved properties against melanoma progression. Using various sources of chirality, several enantiospecific [47–49], and asymmetric [50] syntheses of Jaspine B have been already reported. Herein, we have focused on new truncated analogs of the natural product. Indeed, in view of the amphiphilic nature of Jaspine B one might expect it to behave

as a tensioactive substance.¹ It thus appeared important in this regard to assess the impact of the nature, the length and the number of the lipophilic chains. Moreover, truncated sphingolipids such as ceramides are known to exhibit an enhanced cell penetration [51,52]. Our results showed that single- and double-chained aliphatic truncated analogs **1** and **2** were metabolically active, thus pointing out opportunities for structural modulations in this series. Moreover, the absence of activity for the aromatic derivative **3** allowed us to confirm the relationship between Jaspine B-induced SMS inhibition and cell death. Our findings support the potential of original synthetic Jaspine B analogs in the design of cytotoxic SMS inhibitors that could synergize with classical antitumoral molecules as it was shown for phyto-sphingosine [13,14].

Acknowledgments

We thank Drs. D. Perry and Y.A. Hannun (Charleston, SC) for providing bacteria to obtain DAG kinase and Dr. J. Holthuis (Utrecht, The Netherlands) for the kind gift of HeLa cells overexpressing SMS1-V5. We are indebted to Dr. C. Debitus for kindly providing a crude extract of *Jaspis* sp. This study was supported by INSERM, CNRS and Paul Sabatier University. Y.S. is a recipient of an MESR fellowship.

References

- [1] Thompson JF, Scolyer RA, Kefford RF. Cutaneous melanoma. *Lancet* 2005;365:687–701.

¹ The potential tensioactive behavior of Jaspine B chlorhydrate was analyzed by the technique of the hanging drop. Plotting the surface tension against Log[C] clearly indicated a tensioactive behavior with a critical micelle concentration (CMC) of 500 μ M. This value is typical of a cationic surfactant.

- [2] Hersey P. Apoptosis and melanoma: how new insights are effecting the development of new therapies for melanoma. *Curr Opin Oncol* 2006;18: 189–96.
- [3] Modrak DE, Gold DV, Goldenberg DM. Sphingolipid targets in cancer therapy. *Mol Cancer Ther* 2006;5:200–8.
- [4] Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat Rev Cancer* 2004;4:604–16.
- [5] Padron JM. Sphingolipids in anticancer therapy. *Curr Med Chem* 2006;13: 755–70.
- [6] Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* 2008;9:139–50.
- [7] Bektas M, Jolly PS, Muller C, Eberle J, Spiegel S, Geilen CC. Sphingosine kinase activity counteracts ceramide-mediated cell death in human melanoma cells: role of Bcl-2 expression. *Oncogene* 2005;24:178–87.
- [8] Raisova M, Goltz G, Bektas M, Bielawska A, Riebeling C, Hossini AM, et al. Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes. *FEBS Lett* 2002;516:47–52.
- [9] Leon F, Brouard I, Rivera A, Torres F, Rubio S, Quintana J, et al. Isolation, structure elucidation, total synthesis, and evaluation of new natural and synthetic ceramides on human SK-MEL-1 melanoma cells. *J Med Chem* 2006;49:5830–9.
- [10] Leon F, Brouard I, Torres F, Quintana J, Rivera A, Estevez F, et al. A new ceramide from *Suillus luteus* and its cytotoxic activity against human melanoma cells. *Chem Biodivers* 2008;5:120–5.
- [11] Hwang O, Kim G, Jang YJ, Kim SW, Choi G, Choi HJ, et al. Synthetic phytoceramide induce apoptosis with higher potency than ceramides. *Mol Pharmacol* 2001;59:1249–55.
- [12] Han Y, Kim Y, Kang H, Hong SH, Kim YH, Lim DS, et al. N-Acetylphyto-sphingosine-induced apoptosis of Jurkat cells is mediated by the conformational change in Bak. *Apoptosis* 2006;11:581–8.
- [13] Park MT, Kim MJ, Kang YH, Choi SY, Lee JH, Choi JA, et al. Phytosphingosine in combination with ionizing radiation enhances apoptotic cell death in radiation-resistant cancer cells through ROS-dependent and -independent AIF release. *Blood* 2005;105:1724–33.
- [14] Park MT, Kang YH, Park IC, Kim CH, Lee YS, Chung HY, et al. Combination treatment with arsenic trioxide and phytosphingosine enhances apoptotic cell death in arsenic trioxide-resistant cancer cells. *Mol Cancer Ther* 2007;6: 82–92.
- [15] Choi SY, Kim MJ, Chung HY, Lee SJ, Jang YJ. Phytosphingosine in combination with TRAIL sensitizes cancer cells to TRAIL through synergistic up-regulation of DR4 and DR5. *Oncol Rep* 2007;17:175–84.
- [16] Park MT, Kang JA, Choi JA, Kang CM, Kim TH, Bae S, et al. Phytosphingosine induces apoptotic cell death via caspase 8 activation and Bax translocation in human cancer cells. *Clin Cancer Res* 2003;9:878–85.
- [17] Kuroda I, Musman M, Ohtani II, Ichiba T, Tanaka J, Gravalos DG, et al. Pachastrissamine, a cytotoxic anhydrophytosphingosine from a marine sponge, *Pachastrissa* sp.. *J Nat Prod* 2002;65:1505–6.
- [18] Ledroit V, Debitus C, Lavaud C, Massiot G, Jaspines A. B: two new cytotoxic sphingosine derivatives from the marine sponge *Jaspis* sp.. *Tetrahedron Lett* 2003;44:225–8.
- [19] Huitema K, van den Dikkenberg J, Brouwers JF, Holthuis JC. Identification of a family of animal sphingomyelin synthases. *EMBO J* 2004;23:33–44.
- [20] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986;89:271–7.
- [21] Veldman RJ, Mita A, Cuvillier O, Garcia V, Klappe K, Medin JA, et al. The absence of functional glucosylceramide synthase does not sensitize melanoma cells for anticancer drugs. *FASEB J* 2003;17:1144–6.
- [22] Bielawska A, Perry DK, Hannun YA. Determination of ceramides and diglycerides by the diglyceride kinase assay. *Anal Biochem* 2001;298:141–50.
- [23] Moser HW, Moser AB. Measurement of saturated very long chain fatty acids in plasma. In: Hommes F, editor. *Techniques in Diagnostic Human Biochemical Genetics*. New York: Wiley-Liss, Inc.; 1991. p. 177–91.
- [24] Segui B, Andrieu-Abadie N, Jaffrezou JP, Benoist H, Levade T. Sphingolipids as modulators of cancer cell death: potential therapeutic targets. *Biochim Biophys Acta* 2006;1758:2104–20.
- [25] Ichikawa S, Nakajo N, Sakiyama H, Hirabayashi Y. A mouse B16 melanoma mutant deficient in glycolipids. *Proc Natl Acad Sci USA* 1994;91:2703–7.
- [26] Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001;411:342–8.
- [27] Mayer AM, Gustafson KR. Marine pharmacology in 2003–2004: anti-tumour and cytotoxic compounds. *Eur J Cancer* 2006;42:2241–70.
- [28] Alam N, Wang W, Hong J, Lee CO, Im KS, Jung JH. Cytotoxic sphingosine 4-sulfates from the sponge *Spirastrella abata*. *J Nat Prod* 2002;65:944–5.
- [29] Ohshita K, Ishiyama H, Takahashi Y, Ito J, Mikami Y, Kobayashi J. Synthesis of penaresidin derivatives and its biological activity. *Bioorg Med Chem* 2007;15:4910–6.
- [30] Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* 1998;188: 1521–8.
- [31] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci USA* 1998;95:5690–3.
- [32] Canals D, Mormeneo D, Fabrias G, Llebaria A, Casas J, Delgado A. Synthesis and biological properties of Pachastrissamine (jaspine B) and diastereoisomeric jaspines. *Bioorg Med Chem* 2009;17:235–41.
- [33] Nagahara Y, Shinomiya T, Kuroda S, Kaneko N, Nishio R, Ikekita M. Phytosphingosine induced mitochondria-involved apoptosis. *Cancer Sci* 2005;96:83–92.
- [34] Schurer NY, Plewig G, Elias PM. Stratum corneum lipid function. *Dermatologica* 1991;183:77–94.
- [35] Samsel L, Zaidel G, Drumgoole HM, Jelovac D, Drachenberg C, Rhee JG, et al. The ceramide analog, B13, induces apoptosis in prostate cancer cell lines and inhibits tumor growth in prostate cancer xenografts. *Prostate* 2004;58: 382–93.
- [36] Gouaze V, Liu YY, Prickett CS, Yu JY, Giuliano AE, Cabot MC. Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multi-drug-resistant breast cancer cells to anticancer drugs. *Cancer Res* 2005;65: 3861–7.
- [37] Merrill Jr AH, Jones DD. An update of the enzymology and regulation of sphingomyelin metabolism. *Biochim Biophys Acta* 1990;1044:1–12.
- [38] Tafesse FG, Ternes P, Holthuis JC. The multigenic sphingomyelin synthase family. *J Biol Chem* 2006;281:29421–5.
- [39] Albi E, Magni MV. Sphingomyelin synthase in rat liver nuclear membrane and chromatin. *FEBS Lett* 1999;460:369–72.
- [40] Van der Luit AH, Budde M, Zerp S, Caan W, Klarenbeek JB, Verheij M, et al. Resistance to alkyl-lysophospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin and cholesterol-deficiency in lipid rafts. *Biochem J* 2007;401:541–9.
- [41] Ding T, Li Z, Hailemariam T, Mukherjee S, Maxfield FR, Wu MP, et al. SMS overexpression and knockdown: impact on cellular sphingomyelin and diacylglycerol metabolism, and cell apoptosis. *J Lipid Res* 2008;49:376–85.
- [42] Separovic D, Semaan L, Tarca AL, Awad Maitah MY, Hanada K, Bielawski J, et al. Suppression of sphingomyelin synthase 1 by small interference RNA is associated with enhanced ceramide production and apoptosis after photodamage. *Exp Cell Res* 2008;314:1860–8.
- [43] Kilkus JP, Goswami R, Dawson SA, Testai FD, Berdyshev EV, Han X, et al. Differential regulation of sphingomyelin synthesis and catabolism in oligodendrocytes and neurons. *J Neurochem* 2008;106:1745–57.
- [44] Meng A, Luberto C, Meier P, Bai A, Yang X, Hannun YA, et al. Sphingomyelin synthase as a potential target for D609-induced apoptosis in U937 human monocytic leukemia cells. *Exp Cell Res* 2004;292:385–92.
- [45] Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced “acidic” sphingomyelin breakdown. *Cell* 1992;71:765–76.
- [46] Yamamoto H, Hanada K, Nishijima M. Involvement of diacylglycerol production in activation of nuclear factor kappaB by a CD14-mediated lipopolysaccharide stimulus. *Biochem J* 1997;325(Pt 1):223–8.
- [47] Du Y, Liu J, Linhardt RJ. Stereoselective synthesis of cytotoxic anhydrophytosphingosine pachastrissamine (jaspine B) from D-xylitol. *J Org Chem* 2006;71:1251–3.
- [48] Prasad KR, Chandrakumar A. Stereoselective synthesis of cytotoxic anhydrophytosphingosine pachastrissamine [jaspine B]. *J Org Chem* 2007;72:6312–5.
- [49] Passiniemi M, Koskinen A. Stereoselective total synthesis of pachastrissamine (jaspine B). *Tetrahedron Lett* 2008;49:980–3.
- [50] Abraham E, Brock EA, Candela-Lena JI, Davies SG, Georgiou M, Nicholson RL, et al. Asymmetric synthesis of N,O,O,O-tetra-acetyl D-lyxo-phytosphingosine, jaspine B (pachastrissamine), 2-epi-jaspine B, and deoxoprosophylline via lithium amide conjugate addition. *Org Biomol Chem* 2008;6:1665–73.
- [51] Jeckel D, Wieland F, Dieter J. Truncated ceramide analogs as probes for sphingolipid biosynthesis and transport. *Adv Lipid Res* 1993;26:143–60.
- [52] Luberto C, Hannun YA. Use of short-chain ceramides. *Methods Enzymol* 2000;312:407–20.