Bioorganic & Medicinal Chemistry 20 (2012) 3173–3179

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

3-Deoxy-3,4-dehydro analogs of XM462. Preparation and activity on sphingolipid metabolism and cell fate

Luz Camacho [a,†], Fabio Simbari [a,†], Maria Garrido [a], José Luis Abad [a], Josefina Casas [a], Antonio Delgado [b], Gemma Fabriàs [a,*]

[a] Research Unit on Bioactive Molecules (RUBAM), Department of Biomedicinal Chemistry, Institute for Advanced Chemistry of Catalonia, Spanish Council for Scientific Research (IQAC-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain
[b] Unitat de Química Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona (UB), Avda. Joan XXIII s/n, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 9 February 2012
Revised 26 March 2012
Accepted 30 March 2012
Available online 6 April 2012

Keywords:
Sphingolipids
Ceramides
Ceramidase
Dihydroceramide desaturase
Cancer

ABSTRACT

Three analogs of the dihydroceramide desaturase inhibitor XM462 are reported. The compounds inhibit both dihydroceramide desaturase and acid ceramidase, but with different potencies depending on the *N*-acyl moiety. Other enzymes of sphingolipid metabolism, such as neutral ceramidase, acid sphingomyelinase, acid glucosylceramide hydrolase, sphingomyelin synthase and glucosylceramide synthase, are not affected. The effect on the sphingolipidome of the two best inhibitors, namely (*R*,*E*)-*N*-(1-hydroxy-4-(tridecylthio)but-3-en-2-yl)octanamide (RBM2-1B) and (*R*,*E*)-*N*-(1-hydroxy-4-(tridecylthio)but-3-en-2-yl)pivalamide (RBM2-1D), is in accordance with the results obtained in the enzyme assays. These two compounds reduce cell viability in A549 and HCT116 cell lines with similar potencies and both induced apoptotic cell death to similar levels than C8-Cer in HCT116 cells. The possible therapeutic implications of the activities of these compounds are discussed.

1. Introduction

Alterations in the cellular levels of the sphingolipid ceramide, a potent mediator of apoptosis and an inhibitor of cell proliferation, contribute to cancer outcome, progression, and resistance to both chemo- and radiotherapy. The cytotoxic and/or antiproliferative effect of many antitumor strategies is at least partly due to a boost of ceramide in a variety of tumor cell lines. Growing evidence points to important roles of acid ceramidase (aCDase) in cancer development and response of tumors to therapy.[1,2] Likewise, increasing efforts are being dedicated to the development of aCDase inhibitors as anticancer drugs, either alone or in combination with radio- or chemotherapy as sensitizers to overcome resistance.[1] Although the aCDase inhibitor *N*-oleoylethanolamine (NOE) has been extensively used as a pharmacological tool in tumor biology studies, its weak potency precludes its therapeutic use. Furthermore, NOE is not specific for aCDase, as it also inhibits the skin alkaline ceramidases[3] and the glucosylation of ceramides.[4] Much more interesting aCDase inhibitory properties have been exhibited by the compound B13 and its analogs with modified cell targeting properties, which have shown effective and selective suppression of aCDase activity with increased ceramide levels and apoptotic cell death in a wide range of cancer cell lines.[1]

The NOE backbone inspired the design and synthesis of NOE-analogs, which exhibited interesting aCDase inhibitory properties.[5,6] Among them, the corresponding aminoethanol pivaloylamides and octanoylamides with a *E*-1-hexadecenyl moiety at C2[5] were among the most potent ones (compounds E, Fig. 1). These compounds elicited cytotoxicity in A549 cells with higher potencies than NOE. Cells died by apoptosis, which was accompanied by an increase in ceramide levels. The absence of the characteristic sphingolipid C3 stereogenic centre in these inhibitors meant a valuable improvement from a synthetic point of view. Along this line, in this paper we report on a new family of inhibitors arising from the formal replacement of the allylic methylene unit of the above inhibitors with a sulphur bridge (compounds RBM2-1, Fig. 1). Interestingly, the sulphur atom at C5 position of the sphingoid backbone, present in compounds RBM2-1, is reminiscent of our previously reported mechanism-based dihydroceramide desaturase 1 (Des1) inhibitor XM462 (Fig. 1).[7] In this context, the new analogues here reported can be regarded as hybrid compounds containing the most relevant structural features of the above aCDase and Des1 inhibitors. Recent evidence supports that Des1 inhibition is of therapeutical interest.[8] It has been shown that some of the effects of fenretinide rely on its capacity to inhibit dihydroceramide desaturation.[9–11] Thus, in the human neuroblastoma cells SMS-KCNR, inhibition of Des1 and accumulation of endogenous dihydroceramides

* Corresponding author. Tel.: +34 93 4006100; fax: +34 93 2045904.
E-mail address: gemma.fabrias@iqac.csic.es (G. Fabriàs).
† Both authors contributed equally to this work and are listed in alphabetical order.

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.03.073

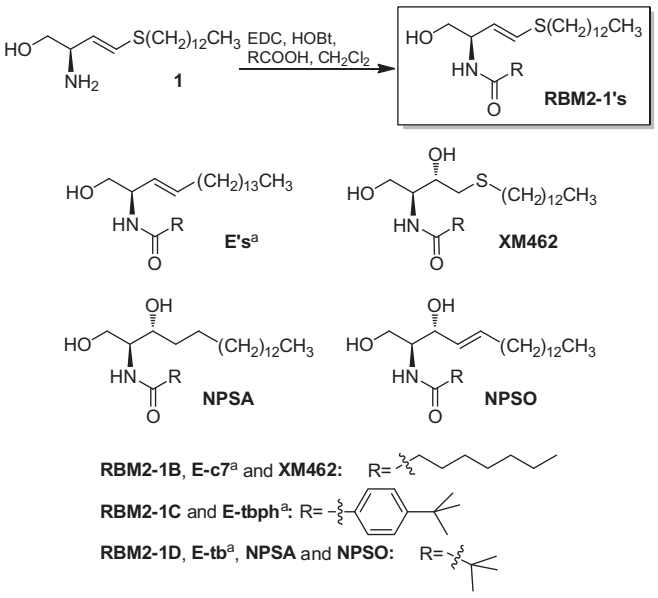

Figure 1. Compounds investigated in this study and their preparation. [a]Reported in *Chem. Phys. Lipids* **2008**, *156*, 33 and included here for comparison. XM462 was reported in *ChemMedChem* **2008**, *3*, 946. HOBt, hydroxybenzotriazole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NPSA, *N*-pivaloyldihydrosphingosine; NPSO, *N*-pivaloylsphingosine.

inhibited cell growth, with cell cycle arrest at G(0)/G(1) and a significant decrease in the amount of phosphorylated retinoblastoma protein.[11] In the A2780 human ovarian carcinoma cells, fenretinide incremented the production of dihydroceramide with a concomitant reduction of cell proliferation and induction of apoptosis.[12,13] In a recent paper, Mao et al.[14] have shown that in two different cell models, HeLa cervical tumor and human oral squamous cell carcinoma cells, the cytotoxicity elicited by fenretinide is not due to dihydroceramide, but to dihydrosphingosine resulting from dihydroceramide hydrolysis by the Golgi alkaline ceramidase (Asah3L or ACER2).[15] Des1 is also inhibited by resveratrol[16] and the cyclooxygenase 2 inhibitor celecoxib.[17] Inhibition of Des1 and increase of intracellular dihydroceramides has been linked to the induction of autophagy in different cancer cell lines.[9,16]

Both the synthesis of the new analogues RBM2-1B/D and their biological activity in two different human adenocarcinoma cell lines, namely lung A459 and colon HCT116, are reported in this article,[18] and their interest as new lead structures for the discovery of inhibitors of sphingolipid metabolizing enzymes with impact on cancer cell death is discussed.

2. Results

2.1. Chemistry

Preparation of amides was carried out by *N*-acylation of the sphingoid backbone **1**[19] with the corresponding carboxylic acid in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride/hydroxybenzotriazole as coupling reagents (Fig. 1). *N*-Pivaloyldihydrosphingosine (NPSA) and *N*-pivaloylsphingosine (NPSO) were prepared following the same procedure.

2.2. Effect on enzymes of sphingolipid metabolism

The compounds were tested as aCDase inhibitors both in vitro and in intact cells, using a Farber cell line transduced to overexpress aCDase (Moh. pAS AcCer10X cell line). Since a modification of the reported assay was used,[20] the previously described compounds (E's, Fig. 1)[5] were also included in the screening as positive controls of inhibition and also for comparative purposes. In both series, pivaloylamides RBM2-1B and E-tb were the most active compounds, whose activity was higher in intact cells that in cell lysates (Fig. 2). Dose–response curves afforded in vitro IC_{50} values of 77, 315, and 51 μM for compounds RBM2-1B, RBM2-1C and RBM2-1D, respectively. Under the same conditions, but using RBM14C16 as substrate,[21] *N*-pivaloylsphingosine and *N*-pivaloyldihydrosphingosine, both at 16 μM, produced an about 50% inhibition of aCDase activity (Fig. 2).

The effect of compounds on neutral ceramidase (nCDase) was also determined in both intact and lysed cells transformed for overexpression of nCDase.[22] As previously found with structurally similar compounds,[5] none of the vinylthioethers modified nCDase activity as compared to controls (Fig. S1) either in vitro or in intact cells.

To assess the activity of the compounds on Des1, their effect on the formation of *N*-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine (CerC6NBD) from *N*-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-dihydrosphingosine (dhCerC6NBD) was tested following the standard assay.[7] The cell line HCT116 was used, as it is a very suitable model to determine Des1 inhibition (Muñoz-Olaya et al., unpublished). As shown in Figure 3A, amounts of CerC6NBD decreased significantly with the three compounds, as well as with XM462, which was used as positive control. Similar effects were found with the A549 cell line, previously used to investigate the activity of structurally related compounds[5] (data not shown). Compound RBM2-1B was the most potent one with both cell models. Its inhibitory activity was dose dependent with IC_{50} values around 18 μM with both HCT116 and A549 cell models (Fig. 3C). The other compounds produced a 50% inhibition at concentrations $\geqslant$ 100 μM (Fig. 3B).

The effect of the vinylthioethers RBM2-1B/D on other sphingolipid metabolism enzymes was also investigated following standard protocols (see Supplementary data). Neither compound affected the activities of either the acidic sphingomyelinase (Fig. S2A) or glucocerebrosidase (Fig. S2B). These activities decreased in the presence of desipramine and *N*-nonyl-1-deoxynojirimycin (NNDNJ), which were used as the respective positive controls of inhibition in parallel experiments. The effect of the compounds on the metabolization of CerC6NBD into both the glucosyl and the phosphocholine derivatives was also investigated. No marked effect was elicited by any of the compounds on the formation of either metabolite from the administered probe

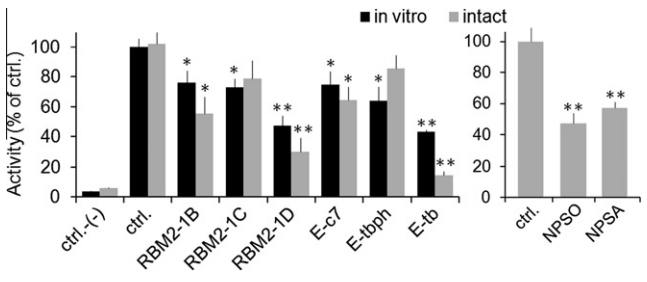

Figure 2. Activity of compounds as aCDase inhibitors. aCDase activities were determined in Moh. pAS AcCer10X cells with a fluorogenic substrate (16 μM). Compounds were tested at 16 μM (aCDase; $S/I = 1$) and the experiments were performed as detailed in Section 5. Data correspond to the mean% of control (±SD) of three experiments with triplicates. Means are statistically different from controls at: $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.005$ (unpaired two-tail *t*-test). Results are normalized to amount of protein (in vitro) or number of cells (intact), which was similar in all cases. Ctrl-(−) corresponds to Moh. pAS cells. NPSO, *N*-pivaloylsphingosine; NPSA, *N*-pivaloyldihydrosphingosine.

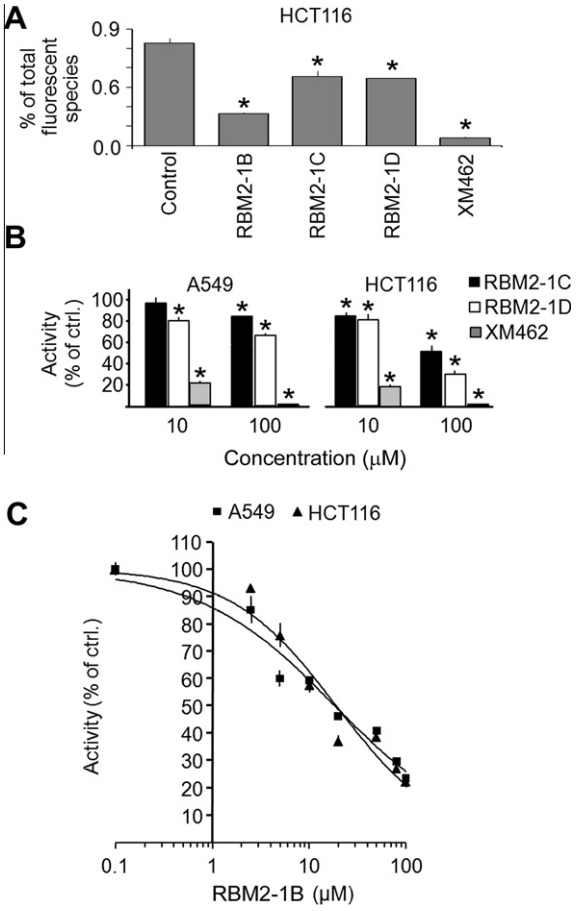

Figure 3. Effect of compounds on Des1 activity. (A) Percentage of CerC6NBD over total fluorescent species in HCT116 intact cells incubated for 4 h with both dhCerC6NBD and the test compounds at equimolar concentrations (10 μM). (B and C) Dose–response data obtained with cell lysates of the specified cell lines incubated for 4 h with dhCerC6NBD (10 μM) and different concentrations of RBM2-1C (B), RBM2-1D (B) and RBM2-1B (C). Samples were processed and analysed as detailed in Section 5. Data correspond to the mean ± SD of two (A) and three (B and D) experiments with triplicates. In C, regression analysis of data (sigmoidal dose–response with variable slope) affords IC_{50} values of 18.1 μM (A549) and 18.8 μM (HCT116). In all the experiments, XM462 (8 μM) was included as a positive control of inhibition.

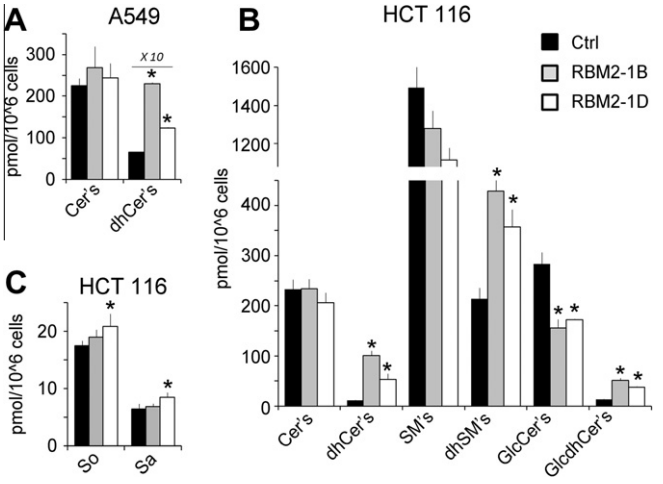

Figure 4. Effect of compounds RBM2-1B and RBM2-1D on the sphingolipidome. Cells were incubated with the compounds (10 μM) for 24 h (A549) or 14 h (HCT116) and then lipids were extracted and analyzed as detailed in Section 5. Data correspond to the mean ± SD of one representative experiment with triplicates. Asterisk indicates statistically significant difference from the mean ($p \leqslant 0.05$; unpaired two-tail *t*-test). (So: sphingosine; Sa: dihydrosphingosine).

(Fig. S3). However, amounts of the sphingomyelin analogue were reduced significantly to about 80% of control by incubations with RBM2-1C and RBM2-1D. While the decreased production of the phosphocholine derivative was accompanied with a rise of CerC6NBD in incubations with RBM2-1D, CerC6NBD did not accumulate in the presence of RBM2-1C, but was converted into the glucosylceramide analogue, which increased over the control by about a 30%.

2.3. Effect on the sphingolipidome

In the A549 cell line, neither RBM2-1B nor RBM2-1D produced a significant increase in total ceramides (Figs. 4A and S4), although a slight but significant rise occurred on C18-ceramide (mean pmol/10^6 cells: ctrl., 4.8 ± 0.5; RBM2-1B, 11.5 ± 2.1, $p \leqslant 0.005$; RBM2-1D, 7.0 ± 1.1, $p \leqslant 0.028$). In contrast, both compounds induced high increments of all dihydroceramide species (Figs. 4A and S4) (mean total pmol/10^6 cells ± SD: ctrl., 6.5 ± 0.42; RBM2-1B, 22.9 ± 4.10, $p \leqslant 0.002$; RBM2-1D, 12.4 ± 2.08, $p \leqslant 0.01$; 3.5 and 1.9-fold increase with RBM2-1B and RBM2-1D, respectively), while RBM2-1C did not modify the total amounts of either ceramides or dihydroceramides (data not shown).

In the HCT116 cell line, RBM2-1B and RBM2-1D produced 8.5 and 4.5-fold increases in total dihydroceramides, respectively (Fig. 4B) and these rises were statistically significant as compared to controls for all the several *N*-acyl species (Fig. S5) (mean total pmol/10^6 cells: ctrl., 11.8 ± 1.13; RBM2-1B, 100.8 ± 9.69, $p \leqslant 0.00009$; RBM2-1D, 53.5 ± 10.89, $p \leqslant 0.00273$). These increments were accompanied with significant increases in both dihydrosphingomyelins (dhSM's) and glucosyldihydroceramide(s) GlcdhCer's (Fig. 4B), a significant reduction in total glucosylceramide(s) (GlcCer's) (Fig. 4B) and a marginally significant decrease of total sphingomyelins (SM's). Although total ceramides (Cer's) were not modified by any compound, both elevated significantly the production of C18-ceramide (mean pmol/10^6 cells ± SD: ctrl., 3.9 ± 0.3; RBM2-1B, 16.0 ± 1.6, $p \leqslant 0.00025$; RBM2-1D, 10.8 ± 1.7, $p \leqslant 0.0025$) and reduced that of C24-Cer and C16-SM (Fig. S5). Analysis of long chain bases showed that RBM2-1B did not modify either sphingosine or dihydrosphingosine levels. In contrast, the amounts of both bases exhibited a slight but significant increase with RBM2-1D. No significant changes in the amounts of sphingosine-1-phosphate occurred with any treatment (mean pmol/10^6 cells ± SD of three experiments with triplicates: Ctrl., 1.49 ± 0.70; RBM2-1B, 1.25 ± 0.22, $p \geqslant 0.43$; RBM2-1D, 2.17 ± 0.56, $p \geqslant 0.16$), while amounts of dihydrosphingosine-1-phosphate were barely above background in all experimental groups.

2.4. Metabolization of RBM2-1B and 1D

Ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF) analysis of the same extracts used to characterize sphingolipidomes revealed that both compounds, RBM2-1B and RBM2-1D, are mainly metabolized to their C1-*O*-phosphocholine derivatives (Table 1), which shows their suitability as substrates of sphingomyelin synthase 1 and/or 2. Similar metabolization was found to occur in both cells lines examined.

2.5. Effect on cell fate

Similar effects on cell viability were elicited by the vinylthioethers RBM2-1B and RBM2-1D in the two different cell lines, A549 and HCT116 (Table 2). Flow cytometry analysis with propidium iodide and Annexin V showed that a 14 h treatment with both

Table 1
MS-Based assignments of RBM2-1B and RBM2-1C metabolites present in extracts of A549 and HCT116 cells

Compound[a]	Theoretical *m*/*z*	Measured *m*/*z* (error, ppm)		Amounts[b] (%conversion[c])	
		A549	HCT116	A549	HCT116
RBM2-1B	428.3562	427.3577 (+3.5)	428.3580 (+4.2)	5645 ± 1031	3645 ± 398
PC-RBM2-1B	593.4117	593.4100 (−2.9)	593.4092 (−4.3)	1589 ± 360 (22)	965 ± 104 (21)
RBM2-1D	386.3105	386.3102 (+3.2)	386.3102 (+2.4)	2563 ± 425	1481 ± 204
PC-RBM2-1D	551.3633	551.3633 (−2.8)	551.3630 (−3.3)	1318 ± 62 (34)	781 ± 53 (35)

[a] RBM1-1B-PC and RBM2-1D-PC refer to the phosphocholine derivatives of RBM2-1B and RBM2-1D, respectively.
[b] The amounts (mean pmols ± SD, $n = 3$) of RBM2-1B and RBM2-1D relative to *N*-dodecylsphingosine and of RBM1-1B-PC and RBM2-1D-PC relative to *N*-dodecylsphingosylphos-phorylcholine.
[c] [PC-RBM2-1/(PC-RBM2-1 + RBM2-1)] × 100.

compounds triggered apoptotic cell death to similar levels than C8-Cer, which was used as a positive control of apoptosis stimulation. Moreover, the three compounds promoted also necrotic cell death with the molecule exhibiting the best apoptosis/necrosis ratio being RBM2-1B (Fig. 5).

No changes in cell cycle were observed in HCT116 cells after incubation for 14 h with either RBM2-1B or RBM2-1D (20 μM) (data not shown).

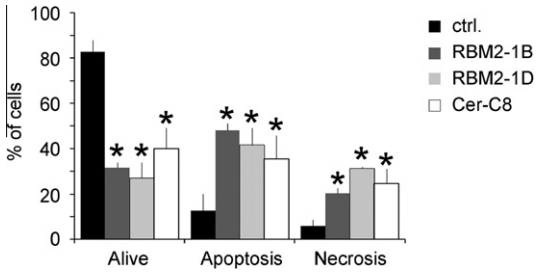

Figure 5. Analysis of apoptosis. HCT116 cells were incubated without (ctrl) or with RBM2-1B (15 μM), RBM2-1D (15 μM) or *N*-octanoylsphingosine (Cer-C8, 30 μM) for 14 h. Apoptosis was determined by flow cytometry using Annexin V and propidium iodide as dyes. Data correspond to the mean ± SD of 3 experiments with triplicates. Apoptosis includes both propidium iodide (−)/Annexin V (+) and propidium iodide (+)/Annexin V (+) cells. Asterisks denote statistical significance at $p \leqslant 0.05$.

3. Discussion

The importance of sphingolipid metabolic pathways as targets for drug discovery has promoted the search for specific enzyme inhibitors. The acid ceramidase has attracted special attention because of its role in cancer development and resistance to therapy.[1] In this context, a number of aCDase inhibitors are currently known, including several C2-substituted aminoethanol amides reported by our group[5] (see Fig. 1). In contrast, the interest of Des1 as a therapeutic target in cancer has emerged more recently after the discovery that some drugs inhibit Des1 and elevate dihydroceramides (dhCer's) levels as part of their mode of action.[8] These include fenretinide,[11] celecoxib,[17] resveratrol,[16] and specific forms of vitamin E.[23,24] Besides these drugs, two rationally designed Des1 inhibitors, namely GT11[25,26] and XM462,[7] have been reported. The latter is a dihydroceramide analog with a sulfur atom replacing the natural C5-methylene unit. Therefore, the vinylthioethers reported here can be regarded as hybrid analogs that combine the most characteristic structural features of the above aCDase and Des1 inhibitors. Biological studies have shown that the three compounds inhibit both enzymes, aCDase and Des1, although their selectivity is modulated by the *N*-acyl substituent. Thus, while the *p*-*tert*-butylphenylamide is a very poor inhibitor of both aCDase and Des1, the pivaloylamide is a better aCDase inhibitor and the linear octanoyl group confers preferential activity over Des1.

The pivaloylamide RBM2-1D was the most potent aCDase inhibitor of the three analogs. Its activity is similar in vitro, but lower in intact cells, than that found for E-tb. The higher metabolization of RBM2-1D (34% conversion into the C1-*O*-phosphocholine metabolite) (Table 1) as compared to E-tb (16% conversion into the C1-*O*-phosphocholine metabolite[5]) may account in part for this difference. The aCDase inhibition data reinforce the importance of the *tert*-butyl group in the amide function for inhibitory activity. In this regard, compound DM102, the *Z*-isomer of E-tb, inhibited aCDase in different prostate and breast cancer cell lines and synergized with weakly cytotoxic agents at inducing apoptotic cell death.[27,28] Furthermore, Antoon et al.[29] reported that *N*-pivaloylsphingosine inhibits the viability and clonogenic survival in several breast cancer cell lines. Similar activities were reported by the same authors for a ceramide analog with the pivaloylamide unit.[30] Although the effect of these compounds on aCDase activity was not determined, we found that both *N*-pivaloylsphingosine and *N*-pivaloyldihydrosphingosine produce a 50% and 40% inhibition of aCDase activity, respectively, as measured in intact Moh. pAS AcCer10X cells with the fluorogenic substrate in the usual conditions followed in our laboratories (Fig. 2A). Therefore, it is possible that inhibition of this enzyme and the subsequent increase in ceramides is responsible for the effects observed by Antoon et al.[29,30]

Table 2
Cytotoxicity of compounds[a]

Compound	A549	HCT116
RBM2-1B	19 ± 0.2	28 ± 0.1
RBM2-1C	76 ± 0.9	81 ± 1.4
RBM2-1D	24 ± 0.4	30 ± 1.1

[a] Number of viable cells was determined with the MTT test after 24 h of treatment. Data correspond to the LD_{50} values (μM) obtained by regression analysis of the dose–response curves constructed with data from two experiments with triplicates.

Compound RBM2-1B was the most active Des1 inhibitor, showing similar IC_{50} values in the two cell lines used as source of enzyme. However, its activity is lower than that of XM462.[7,16] This difference can be explained assuming a higher affinity of the latter for the enzyme by virtue of its C4-OH group, which is not present in RBM2-1B. On the other hand, since XM462 could arise in cells from metabolism of RBM2-1B, this possibility was investigated and ruled out in the sphingolipidome analyses, as no traces of XM462 or its metabolites were detected in extracts from cells treated with RBM2-1B. Likewise, no evidence of dehydration of XM462 to RBM2-1B was obtained in the UPLC-TOF analyses of lipids extracted from cells incubated with XM462.

The observed effects of the compounds on the sphingolipidome were in accordance with the overall data. Thus in the two cell lines examined, both RBM2-1B and RBM2-1D increased dihydroceramide levels, although RBM2-1B produced a significantly higher increment than RBM2-1D (A549, $p \leqslant 0.017$, HCT116, $p \leqslant 0.005$; see Fig. 4B), in agreement with its higher potency as Des1 inhibitor.

It is worth noting that 24 h treatments with compounds E-c7 and E-tb induced an accumulation of ceramides, but not dihydroceramides, in A549 cells.[5] Therefore, unlike RBM2-1B, compounds E-c7 and E-tb do not appear to inhibit Des1 in a similar concentration range, underscoring the importance of the sulfur atom at C5 for Des1 inhibition.

In contrast to the marked rise in total Cer content produced by analogs of D-*e*-MAPP and B13,[31] both RBM2-1B and RBM2-1D did not induce Cer accumulation, which can be explained, at least in part, by their activity as Des1 inhibitors. Interestingly, however, both RBM2-1B and RBM2-1D provoked a significant rise in C18-ceramide in the two cell lines studied. Whether the rise of this specific ceramide is of importance in the observed cell fate is unknown, although it has been reported that ceramides with different chain lengths may have distinct functions in the regulation of tumor progression. Thus, in head and neck squamous cell carcinoma tumors, C18-ceramide inhibits, while C16-ceramide induces tumor growth.[32]

Analysis of long chain bases showed that RBM2-1D, but not RBM2-1B, produced a small but significant increase in the levels of both sphingosine and dihydrosphingosine (Fig. 4C). Since RBM2-1D is a more potent aCDase inhibitor than RBM2-1B, decreased aCDase activity may cause up-regulation of other ceramidases, such as the nCDase, the alkaline ceramidase Asah3 and/or the alkaline dihydroceramidase (Asah3L).[14] Thus, nCDase and/or Asah3 activity would augment the production of extralysosomal sphingosine to counterbalance the decreased intralysosomal sphingosine production, while elevated dihydrosphingosine levels would result from enhanced Asah3L activity.[14] Therefore, the lower dihydroceramide levels present in HCT116 cells treated with RBM2-1D as compared to RBM2-1B ($p \leqslant 0.017$; see Fig. 4B) might result from lower inhibition of Des1 together with augmented Asah3L activity. The latter would not occur with RBM2-1B because its effect on aCDase is not strong enough to up-regulate other ceramidase activities. A recent report showing that knockdown of alkaline ceramidase *ACER3* up-regulated the expression of *ASAH3L*[33] supports that this could also occur by sustained inhibition of aCDase.

The three compounds were similarly toxic to the two cell lines examined. Conversely, RBM2-1C, which exhibited the poorest activity on sphingolipid metabolism, was also the least toxic compound, in agreement with the similar trend found for the previously reported compounds E-c7, E-tbph and E-tb.[5] This result supports that cell death induced by RBM2-1B and RBM2-1D is mediated by altered sphingolipid metabolism. Sphingolipidomics data point to dihydrosphingolipids and/or C18-ceramide as the active mediators of RBM2-1B and RBM2-1D activity. However, given their structural similarity to ceramide, the possibility that the compounds are active by themselves cannot be ruled out with the available data. In this regard, in prostate cancer cell models, DM102 synergized with fenretinide at decreasing cell viability by induction of apoptosis. However, blocking ceramide generation failed to rescue cells from cytotoxicity, which casts some doubt on the involvement of ceramide in the cytotoxic response induced by the cotreatment.[28]

Since it has been reported that dihydroceramide accumulation induces autophagy in a number of cell lines,[9,16] the effect of RBM2-1B on autophagy in HCT116 cells was examined. The pair RBM2-1B/HCT116 was chosen for this study because they afforded the highest dihydrosphingolipid increases upon treatments. In agreement with the literature,[34] higher LC3-II levels were found in cells cultured in the absence of serum. The LC3-II increase was enhanced by co-incubation with protease inhibitors, indicating that the LC3-II build up results from an enhanced autophagic flux. Furthermore, HCT116 cells responded to XM462 with autophagy enhancement, as previously found in a gastric cancer cell line.[16] However, RBM2-1B failed to induce autophagy in the same experimental setup. The reasons for this difference are unknown, but two possibilities can be invoked. First, the HCT116 cell line may not be a good model of dihydroceramide-mediated autophagy, as it is less responsive to XM462 treatment in terms of dihydroceramide accumulation than the HGC27 cell line used in our previous study.[9,16] Second, autophagy may indeed have been induced upon RBM2-1B treatment, but at earlier time points than the one examined, in which cells were already showing signs of death.

Flow cytometry analysis of HCT116 cells showed that apoptosis was triggered by RBM2-1B (and RBM2-1D). Moreover, apoptosis was induced to a similar extent by C8-Cer. Although the role of ceramides as sphingolipid mediators of apoptotic cell death has been extensively documented, involvement of dhCer's in apoptosis induction is far from proven. Most data come from experiments using drugs that increase dhCer's, such as γ-tocopherol, γ-tocotrienol, fenretinide, resveratrol and celecoxib.[8] However, exogenously added dihydroceramides have been generally used as the inactive counterparts of pro-apoptotic ceramides.[8] Likely, the proapoptotic action of RBM2-1B is independent of its activity as a Des1 inhibitor. Indeed, RBM2-1D, which is a less potent Des1 inhibitor than RBM2-1B, induces apoptosis to a similar extent. Further studies are necessary to decipher the precise mechanisms involved in the propapoptotic activity of RBM2-1B and RBM2-1D. In the case of RBM2-1D, the possible involvement of dihydrosphingosine deserves further attention. Merrill and co-workers showed that silencing the *ASAH1* gene in breast cancer MCF7 cells reduced the autophagy induced by fenretinide. In the light of these results, the authors suggested that dihydrosphingosine accumulation contributes to fenretinide cytotoxicity.[35] In a recent work, Mao et al.[14] proved that the Golgi alkaline ceramidase (Asah3L) is involved in the release of dihydrosphingosine upon treatment with fenretinide. The authors show that the drug increases ASAH3L expression with enhanced hydrolysis of dhCer's, the preferred Asah3L substrate, and provokes dihydrosphingosine production and cell death. As mentioned above, dihydrosphingosine rose upon treatment of cells with RBM2-1D, but not RBM2-1B, which is a weaker inhibitor of aCDase. This result suggests upregulation of Asah3L and augmented hydrolysis of dhCer's with increased formation of dihydrosphingosine upon aCDase inhibition. RBM2-1B is not potent enough at inhibiting aCDase to provoke upregulation of Asah3L as a response.

Finally, metabolism of RBM2-1B and RBM2-1D occurs mainly through the formation of the C1-*O*-phosphocholine derivatives in both A549 and HCT116 cell lines, with RBM2-1D exhibiting a higher incorporation of phosphocholine (PC) (PC-RBM2-1D/RBM2-1D = 35/65; PC-RBM2-1B/RBM2-1B = 22/78). Competition with the substrate may thus be the reason why RBM2-1D reduces the formation of the sphingomyelin derivative of CerC6NBD (Fig. S3). Therefore, improving the metabolic stability of RBM2-1B and RBM2-1D at C1OH would likely increase their potency in vivo. Further research along this line is ongoing in our laboratories.

4. Conclusions

In summary, the vinylthioether structure reported in this article emerges as an interesting scaffold in the discovery of inhibitors of sphingolipid metabolism. As exemplified with RBM2-1B and RBM2-1D, selectivity towards specific enzymes can be achieved by changing the *N*-acyl group. Thus, while the linear octanoyl moiety in RBM2-1B confers selectivity against dihydroceramide desaturase, the pivaloyl group of RBM2-1D renders the compound selective for acid ceramidase. The pro-apoptotic activity of both RBM2-1B and RBM2-1D in HCT116 cells warrants the interest of further studies with both compounds in other cancer cells lines.

5. Experimental

5.1. General protocol for the acylation of sphingoid bases

To a solution of the corresponding acid (0.22 mmol) and hydroxybenzotriazole (32 mg, 0.25 mmol) in anhydrous CH_2Cl_2 (4 mL) was added solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (62 mg, 0.3 mmol) at room temperature under argon atmosphere. After disappearance of the white precipitate, the resulting solution was stirred for 5 min and then transferred dropwise via syringe to a vigorously stirred solution containing **1**[19] (60 mg, 0.20 mmol) and Et_3N (56 μL, 0.40 mmol) in anhydrous CH_2Cl_2 (4 mL). After stirring for 1 h at room temperature under argon, the mixture was diluted with CH_2Cl_2 (5 mL) and washed with saturated $NaHCO_3$ solution (5 mL) and brine (2 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and solvent was evaporated to afford a crude residue, which was purified by flash chromatography (two gradients: hexane/MTBE 0–20% and then CH_2Cl_2/MeOH 0–3%) to afford the pure acylated products.

5.1.1. Compound RBM2-1B (72% yield)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.27 (d, 1H, J = 15.3 Hz, CH=C*H*–S), 5.74 (br s, 1H, N*H*CO), 5.46 (dd, 1H, J_1 = 15.3, J_2 = 6.8 Hz, C*H*=CH–S), 4.61–4.54 (m, 1H, C*H*NH), 3.74–3.62 (m, 2H, CH_2OH), 2.67 (t, 2H, J = 7.4 Hz, S–CH_2), 2.48 (br s, 1H, CH$_2$O*H*), 2.21 (t, 2H, J = 7.6 Hz, COCH_2), 1.68–1.57 (m, 4H, S–CH$_2$CH_2, COCH$_2$CH_2), 1.42–1.34 (m, 2H, S–CH$_2$CH$_2$CH_2), 1.25 (br s, 26H, 13CH$_2$), 0.88 (t, 6H, J = 6.8 Hz, 2CH$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.7 (CO), 128.7 (CH=CH–S), 123.0 (*C*H=CH–S), 65.7 (*C*H$_2$OH), 53.8 (*C*HNH$_2$), 37.0 (*C*OCH$_2$), 32.4 (*S*CH$_2$), 32.0, 31.8, 29.8, 29.8, 29.8, 29.6, 29.5, 29.4, 29.3, 29.3, 29.3, 29.1, 29.0, 25.9 (14*C*H$_2$), 22.8, 22.7 (2*C*H$_2$CH$_3$), 14.3, 14.2 (2*C*H$_3$). $[\alpha]_D$ = −3.1 (*c* 0.90, $CHCl_3$). HRMS: calculated for $C_{25}H_{50}NO_2S$ $(M+1)^+$: 428.3562; Found: 428.3560. Anal. Calcd for $C_{25}H_{49}NO_2S$: C, 70.20; H, 11.55; N, 3.27. Found: C, 70.23; H, 11.58; N, 3.31.

5.1.2. Compound RBM2-1C (73% yield)

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (d, 2H, J = 8.4 Hz, Ar-H), 7.43 (d, 2H, J = 8.4 Hz, Ar-H), 6.59 (d, 1H, J = 7.6 Hz, N*H*CO), 6.32 (d, 1H, J = 15.3 Hz, CH=C*H*–S), 5.56 (dd, 1H, J_1 = 15.3, J_2 = 6.6 Hz, C*H*=CH–S), 4.80–4.72 (m, 1H, C*H*NH), 3.76 (qd, 2H, J = 11.1, 4.5 Hz, CH_2OH), 2.66 (t, 2H, J = 7.4 Hz, S–CH_2), 1.60 (quint, 2H, J = 7.0 Hz, S–CH$_2$CH_2), 1.41–1.34 (m, 2H, S–CH$_2$CH$_2$CH_2), 1.32 (s, 9H, 3CH$_3$), 1.25 (br s, 18H, 9CH$_2$), 0.87 (t, 3H, J = 6.8 Hz, CH$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.6 (CO), 155.4, 131.3, 128.9 (CH=*C*H–S), 127.0, 125.6, 122.9 (*C*H=CH–S), 65.6 (*C*H$_2$OH), 54.2 (*C*HNH$_2$), 35.1 (*C*(CH$_3$)$_3$), 32.4 (*S*CH$_2$), 32.0 (CH$_2$), 31.3 (C(*C*H$_3$)$_3$), 29.8, 29.8, 29.7, 29.6, 29.5, 29.3, 29.3, 29.0, (9*C*H$_2$), 22.8 (*C*H$_2$CH$_3$), 14.3 (*C*H$_3$). $[\alpha]_D$ = +2.1 (*c* 1.25, $CHCl_3$). HRMS: calculated for $C_{28}H_{48}NO_2S$ $(M+1)^+$: 462.3406; Found: 462.3400. Anal. Calcd for $C_{28}H_{47}NO_2S$: C, 72.83; H, 10.26; N, 3.03. Found: C, 72.84; H, 10.28; N, 3.01.

5.1.3. Compound RBM2-1D (84% yield)

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.24 (d, 1H, J = 16.0 Hz, CH=C*H*–S), 5.95 (d, 1H, J = 6.8 Hz, N*H*CO), 5.47 (dd, 1H, J_1 = 15.3, J_2 = 6.7 Hz, C*H*=CH–S), 4.57–4.51 (m, 1H, C*H*NH), 3.75–3.72 (m, 1H, CH_2OH), 3.70–3.62 (m, 1H, CH_2OH), 2.75 (br s, 1H, CH$_2$O*H*), 2.66 (t, 2H, J = 7.4 Hz, S–CH_2), 1.61 (quint, 2H, J = 7.4 Hz, S–CH$_2$CH_2), 1.40–1.33 (m, 2H, S–CH$_2$CH$_2$CH_2), 1.25 (br s, 18H, 9CH$_2$), 1.21 (s, 9H, C(CH_3)$_3$), 0.87 (t, 3H, J = 6.8 Hz, CH$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 179.0 (CO), 128.7 (CH=*C*H–S), 123.0 (*C*H=CH–S), 65.9 (*C*H$_2$OH), 53.8 (*C*HNH$_2$), 38.9 (*C*(CH$_3$)$_3$), 32.4, 32.0, 29.8, 29.8, 29.7, 29.6, 29.5, 29.3, 29.3, 29.0 (10*C*H$_2$), 27.7 (C(*C*H$_3$)$_3$), 22.8 (2*C*H$_2$CH$_3$), 14.3 (*C*H$_3$). $[\alpha]_D$ = −2.3 (*c* 1.73, $CHCl_3$). HRMS: calculated for $C_{22}H_{43}NO_2S$ $(M+1)^+$: 386.3093; Found: 386.3090. Anal. Calcd for $C_{22}H_{43}NO_2S$: C, 68.52; H, 11.24; N, 3.63. Found: C, 68.55; H, 11.21; N, 3.65.

5.2. Cells

A549,[36] Moh. pAS and Moh. pAS AcCer10X[37] cells were cultured as reported. HCT116 cells (clone 40–16) were maintained at 37 °C and 5% CO2 in low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin. All the experiments were performed at about 80% confluence.

5.3. Enzyme inhibition

aCDase activity was measured both in vitro and in intact cells as reported[20,21] using Moh. pAS AcCer10X cells[37] and the fluorogenic substrate RBM14C12.nCDase activity was determined as described[22,38] using *N*-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]lauroyl]-D-*erythro*-sphingosine (CerC12NBD) as the substrate and Moh. pAS transfected with nCDase. Twenty-four hours before transfection, cells were plated at a density of 2.5 × 105 cells/mL in 6 well-plates (35 mm, 9.46 cm^2, 1 mL/well). Then cells were transfected with pcDNA5/TO (empty vector) or pcDNA5/TO-*ASAH2* in lipofectamine 2000 (10 μL, 0.4 μg DNA/μL). Twenty-four hours after transfection cells were collected by trypsinization and washed twice with phosphate buffered-saline (PBS). The cell pellets were suspended in 1 mM Tris–HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (100 μL, 5–6 mg protein/mL), lysed by sonication (10 min, ultrasonic ice-cold bath), and incubated at 37 °C for 1 h with 5 μM CerC12NBD in reaction buffer (25 mM Tris–HCl, pH 7.5, 1% sodium cholate) with or without the test compounds (50 μM final concentration). The reaction was terminated by the addition of methanol (1 mL) and 50 μL were injected into a high performance liquid chromatography with a fluorescence detector (HPLC-FD).

To determine nCDase inhibition in intact cells, 24 h prior to transfection, 2.5x105 cells per well were plated in 6 well-plates. Then cells were transfected with pcDNA5/TO (empty vector) or pcDNA5/TO-*ASAH2* with lipofectamine 2000 (10 μL, 0.4 μg DNA/μL). Twenty-four hours after transfection cells were treated with the test compounds (50 μM) or vehicle for 4 h. Cells were collected, lysed and nCDase activity was determined as described above.

Des1 activity was determined in both A549 and HCT116 cells, which were seeded at a density of 2x10^5 cells/well in 6 well-plates. Twenty-four hours later, the medium was removed and fresh medium containing 16 μM each of both substrate and test compound was added. After incubation for 4 h, both media and cells were collected separatedly. One milliliter of MeOH was added to the medium and 25 μL were injected into the HPLC-FD. Cells were trypsinized (trypsin–EDTA, 0.4 mL), media (0.6 mL) and methanol (1 mL) were sequentially added and 100 μL were injected into the HPLC-FD system. Equipment and analysis conditions were as detailed previously.[7] Compound XM462[7] was used as positive control of Des1 inhibition.

5.4. Lipidomics

Sphingolipid analysis by UPLC-TOF was carried out following the standard protocol used in our laboratories.[39] Long chain bases and long chain base phosphates were analyzed with a system consisting of a Waters Alliance 2690 liquid chromatography pump equipped with an autosampler and connected to a Quattro LC triple-quadrupole mass spectrometer from Micromass (Manchester, UK). Separation was achieved on a Purospher STAR-RP-18 column (125 × 2 mm, 5 μm) (Merck, Darmstadt) using a gradient of two mobile phases: A, methanol/water/formic acid (74/25/1 v/v/v); B, methanol/formic acid (99/1 v/v), both also contained 5 mM ammo-

nium formate. A linear gradient was programmed as follows: 0.0 min, 50% B; 2 min, 50% B; 7 min, 100% B; 17 min, 100% B; 19 min, 50% B; 26 min, 50% B. The flow rate was 0.3 mL/min. MS/MS detection was performed with an electrospray interface operating in the positive ion mode acquiring the following selected reaction monitoring transitions: C17-dihydrosphingosine, 288-252, collision energy 18 eV; sphingosine, 300-264, collision energy 16 eV; dihydrosphingosine, 302-266, collision energy 18 eV; C17-dihydrosphingosine-1-phosphate, 368-252, collision energy 18 eV; sphingosine-1-phosphate, 380-264, collision energy 16 eV and dihydrosphingosine-1-phosphate, 382-266, collision energy 18 eV.

5.5. Cell viability

Cells were seeded in 96-well plates at a density of 10^5 cells/mL. After 24 h, media were removed and replaced with fresh media with and without the test compounds at the specified concentrations. The number of viable cells was determined after the specified times with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

5.6. Flow cytometry

Cells are seeded in a 96 well-plate (0.1 mL, 2.5×10^5 cells/mL) in low glucose DMEM supplemented with FBS (10%) and penicillin–streptomycin (1%). Twenty-four hours after seeding, medium was replaced with fresh medium (0.1 mL) containing the test compounds at the specified concentrations (Fig. 5). The same volume of vehicle (ethanol) was added to controls. After 15 h, cells were washed twice with PBS (0.2 mL), collected by trypsinization and stained with the Dead Cell Apoptosis Kit from Invitrogen following the manufacturer's instructions. Cells were analyzed with a Guava easyCyte 8HT (Millipore).

Acknowledgments

Financial support from the Ministerio de Ciencia e Innovación (Projects SAF2008-00706 and SAF2011-22444) and Generalitat de Catalunya (SGR 2009 1072), and predoctoral fellowships from Generalitat de Catalunya (SGR 2005 01063 to L.C.), Ministerio de Educación y Ciencia (F.S.) and the Spanish Council for Scientific Research (M.G.) are acknowledged. We thank Dr. Meritxell Egido-Gabás, Pedro Rayo and Eva Dalmau for their excellent technical assistance and Dr. Carmen Bedia for kindly providing the pcDNA5/TO-*ASAH2* construct.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.073.

References and notes

1. Gangoiti, P.; Camacho, L.; Arana, L.; Ouro, A.; Granado, M. H.; Brizuela, L.; Casas, J.; Fabrias, G.; Abad, J. L.; Delgado, A.; Gomez-Munoz, A. *Prog. Lipid Res.* **2010**, *49*, 316.
2. Liu, X.; Elojeimy, S.; Turner, L. S.; Mahdy, A. E.; Zeidan, Y. H.; Bielawska, A.; Bielawski, J.; Dong, J. Y.; El-Zawahry, A. M.; Guo, G. W.; Hannun, Y. A.; Holman, D. H.; Rubinchik, S. *Front. Biosci.* **2008**, *13*, 2293.
3. Houben, E.; Uchida, Y.; Nieuwenhuizen, W. F.; De Paepe, K.; Vanhaecke, T.; Holleran, W. M.; Rogiers, V. *Skin Pharmacol. Physiol.* **2007**, *20*, 187.
4. Spinedi, A.; Di Bartolomeo, S.; Piacentini, M. *Biochem. Biophys. Res. Commun.* **1999**, *255*, 456.
5. Bedia, C.; Canals, D.; Matabosch, X.; Harrak, Y.; Casas, J.; Llebaria, A.; Delgado, A.; Fabrias, G. *Chem. Phys. Lipids* **2008**, *156*, 33.
6. Grijalvo, S.; Bedia, C.; Triola, G.; Casas, J.; Llebaria, A.; Teixido, J.; Rabal, O.; Levade, T.; Delgado, A.; Fabrias, G. *Chem. Phys. Lipids* **2006**, *144*, 69.
7. Munoz-Olaya, J. M.; Matabosch, X.; Bedia, C.; Egido-Gabas, M.; Casas, J.; Llebaria, A.; Delgado, A.; Fabrias, G. *ChemMedChem* **2008**, *3*, 946.
8. Fabrias, G.; Munoz-Olaya, J.; Cingolani, F.; Signorelli, P.; Casas, J.; Gagliostro, V.; Ghidoni, R. *Prog. Lipid Res.* **2011**, *51*, 82.
9. Zheng, W.; Kollmeyer, J.; Symolon, H.; Momin, A.; Munter, E.; Wang, E.; Kelly, S.; Allegood, J. C.; Liu, Y.; Peng, Q.; Ramaraju, H.; Sullards, M. C.; Cabot, M.; Merrill, A. H., Jr. *Biochim. Biophys. Acta* **1864**, *2006*, 1758.
10. Schulz, A.; Mousallem, T.; Venkataramani, M.; Persaud-Sawin, D. A.; Zucker, A.; Luberto, C.; Bielawska, A.; Bielawski, J.; Holthuis, J. C.; Jazwinski, S. M.; Kozhaya, L.; Dbaibo, G. S.; Boustany, R. M. *J. Biol. Chem.* **2006**, *281*, 2784.
11. Kraveka, J. M.; Li, L.; Szulc, Z. M.; Bielawski, J.; Ogretmen, B.; Hannun, Y. A.; Obeid, L. M.; Bielawska, A. *J. Biol. Chem.* **2007**, *282*, 16718.
12. Valsecchi, M.; Aureli, M.; Mauri, L.; Illuzzi, G.; Chigorno, V.; Prinetti, A.; Sonnino, S. *J. Lipid Res.* **1832**, *2010*, 51.
13. Illuzzi, G.; Bernacchioni, C.; Aureli, M.; Prioni, S.; Frera, G.; Donati, C.; Valsecchi, M.; Chigorno, V.; Bruni, P.; Sonnino, S.; Prinetti, A. *J. Biol. Chem.* **2010**, *285*, 18594.
14. Mao, Z.; Sun, W.; Xu, R.; Novgorodov, S.; Szulc, Z. M.; Bielawski, J.; Obeid, L. M.; Mao, C. *J. Biol. Chem.* **2010**, *285*, 29078.
15. Xu, R.; Jin, J.; Hu, W.; Sun, W.; Bielawski, J.; Szulc, Z.; Taha, T.; Obeid, L. M.; Mao, C. *FASEB J.* **1813**, *2006*, 20.
16. Signorelli, P.; Munoz-Olaya, J. M.; Gagliostro, V.; Casas, J.; Ghidoni, R.; Fabrias, G. *Cancer Lett.* **2009**, *282*, 238.
17. Schiffmann, S.; Sandner, J.; Schmidt, R.; Birod, K.; Wobst, I.; Schmidt, H.; Angioni, C.; Geisslinger, G.; Grosch, S. *J. Lipid Res.* **2009**, *50*, 32.
18. Abad, J. L.; Fabrias, G.; Casas, J.; Garrido, M.; Camacho, L.; Simbari, F.; Delgado, A. Spain Patent P201031642, 2010.
19. Nieves, I.; Garrido, M.; Abad, J. L.; Delgado, A. *Synlett* **2010**, 2950.
20. Bedia, C.; Camacho, L.; Abad, J. L.; Fabrias, G.; Levade, T. *J. Lipid Res.* **2010**, *51*, 3542.
21. Bedia, C.; Casas, J.; Garcia, V.; Levade, T.; Fabrias, G. *ChemBioChem* **2007**, *8*, 642.
22. Kono, M.; Dreier, J. L.; Ellis, J. M.; Allende, M. L.; Kalkofen, D. N.; Sanders, K. M.; Bielawski, J.; Bielawska, A.; Hannun, Y. A.; Proia, R. L. *J. Biol. Chem.* **2006**, *281*, 7324.
23. Jiang, Q.; Rao, X.; Kim, C. Y.; Freiser, H.; Zhang, Q.; Jiang, Z.; Li, G. *Int. J. Cancer* **2012**, *130*, 685.
24. Jiang, Q.; Wong, J.; Fyrst, H.; Saba, J. D.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17825.
25. Triola, G.; Fabrias, G.; Casas, J.; Llebaria, A. *J. Org. Chem.* **2003**, *68*, 9924.
26. Triola, G.; Fabrias, G.; Llebaria, A. *Angew. Chem., Int. Ed. Engl.* **1960**, *2001*, 40.
27. Flowers, M.; Fabrias, G.; Delgado, A.; Casas, J.; Abad, J. L.; Cabot, M. C. *Breast Cancer Res. Treat.* **2011**. http://dx.doi.org/10.1007/s10549-011-1768-8.
28. Gouazé-Andersson, V.; Flowers, M.; Karimia, R.; Fabriás, G.; Delgado, A.; Casas, J.; Cabot, M. C. *Prostate* **2011**, *71*, 1064.
29. Antoon, J. W.; Liu, J.; Ponnapakkam, A. P.; Gestaut, M. M.; Foroozesh, M.; Beckman, B. S. *Cancer Chemother. Pharmacol.* **2010**, *65*, 1191.
30. Antoon, J. W.; Liu, J.; Gestaut, M. M.; Burow, M. E.; Beckman, B. S.; Foroozesh, M. *J. Med. Chem.* **2009**, *52*, 5748.
31. Bielawska, A.; Bielawski, J.; Szulc, Z. M.; Mayroo, N.; Liu, X.; Bai, A.; Elojeimy, S.; Rembiesa, B.; Pierce, J.; Norris, J. S.; Hannun, Y. A. *Bioorg. Med. Chem.* **2008**, *16*, 1032.
32. Ponnusamy, S.; Meyers-Needham, M.; Senkal, C. E.; Saddoughi, S. A.; Sentelle, D.; Selvam, S. P.; Salas, A.; Ogretmen, B. *Future Oncol.* **2010**, *6*, 1603.
33. Hu, W.; Xu, R.; Sun, W.; Szulc, Z. M.; Bielawski, J.; Obeid, L. M.; Mao, C. *J. Biol. Chem.* **2010**, *285*, 7964.
34. Morselli, E.; Tasdemir, E.; Maiuri, M. C.; Galluzzi, L.; Kepp, O.; Criollo, A.; Vicencio, J. M.; Soussi, T.; Kroemer, G. *Cell Cycle* **2008**, *7*, 3056.
35. Sims, K.; Fluke, K.; Symolon, H.; Zheng, W.; Munter, E.; Momin, A.; Pack, C.; Haynes, C.; Kelly, S.; Allegood, J.; Wang, E.; Merrill, A. H. *FASEB J.* **2007**, *21*, 779.6. (Abstract A976).
36. Villorbina, G.; Canals, D.; Carde, L.; Grijalvo, S.; Pascual, R.; Rabal, O.; Teixido, J.; Fabrias, G.; Llebaria, A.; Casas, J.; Delgado, A. *Bioorg. Med. Chem.* **2006**, *15*, 50.
37. Medin, J. A.; Takenaka, T.; Carpentier, S.; Garcia, V.; Basile, J. P.; Segui, B.; Andrieu-Abadie, N.; Auge, N.; Salvayre, R.; Levade, T. *Hum. Gene Ther.* **1999**, *10*, 1321.
38. Tani, M.; Okino, N.; Mitsutake, S.; Ito, M. *J. Biochem.* **1999**, *125*, 746.
39. Canals, D.; Mormeneo, D.; Fabrias, G.; Llebaria, A.; Casas, J.; Delgado, A. *Bioorg. Med. Chem.* **2009**, *17*, 235.