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Bioorganic & Medicinal Chemistry 16 (2008) 1032–1045
Bioorganic & Medicinal Chemistry

Novel analogs of D-*e*-MAPP and B13. Part 2: Signature effects on bioactive sphingolipids

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Received 8 June 2007; revised 16 August 2007; accepted 20 August 2007
Available online 24 August 2007

Abstract—Novel isosteric analogs of the ceramidase inhibitors (1*S*,2*R*)-*N*-myristoylamino-phenylpropanol-1 (D-*e*-MAPP) and (1*R*,2*R*)-*N*-myristoylamino-4′-nitro-phenylpropandiol-1,3 (B13) with modified targeting and physicochemical properties were developed and evaluated for their effects on endogenous bioactive sphingolipids: ceramide, sphingosine, and sphingosine 1-phosphate (Cer, Sph, and S1P) in MCF7 cells as determined by high-performance liquid chromatography–mass spectrometry (HPLC–MS/MS). Time– and dose–response studies on the effects of these compounds on Cer species and Sph levels, combined with structure–activity relationship (SAR) data, revealed 4 distinct classes of analogs which were predominantly defined by modifications of the *N*-acyl-hydrophobic interfaces: *N*-acyl-analogs (class A), urea-analogs (class B), *N*-alkyl-analogs (class C), and ω-cationic-*N*-acyl analogs (class D). Signature patterns recognized for two of the classes correspond to the cellular compartment of action of the new analogs, with class D acting as mitochondriotropic agents and class C compounds acting as lysosomotropic agents. The neutral agents, classes A and B, do not have this compartmental preference. Moreover, we observed a close correlation between the selective increase of C_{16}-, C_{14}-, and C_{18}-Cers and inhibitory effects on MCF7 cell growth. The results are discussed in the context of compartmentally targeted regulators of Sph, Cer species, and S1P in cancer cell death, emphasizing the role of C_{16}-Cer. These novel analogs should be useful in cell-based studies as specific regulators of Cer–Sph–S1P inter-metabolism, in vitro enzymatic studies, and for therapeutic development.

1. Introduction

The stimulus-controlled pathways of SPL metabolism provide a rich network of bioactive molecules with pivotal roles in the regulation of diverse cell functions. Of these bioactive SPLs, Cer is known to be a key modulator of cancer cell growth and apoptosis.[1] In contrast, the SPL metabolite S1P, which is generated from Cer via ceramidases (CDases) to yield Sph, and then subsequently phosphorylated by sphingosine kinases (SKs), promotes growth and opposes Cer-mediated apoptosis.[2,3] Accumulation of endogenous Cers, and perhaps their metabolites, occurs in response to a variety of external inducers, and can take place in different sub-cellular compartments depending on the inducer and what enzyme of SPL metabolism it regulates.[4,5] Thus, these compartmentally restricted SPLs may play distinct roles in cellular responses through their actions on varied SPL-specific intra- and extra-cellular targets.[6–11]

Because of the role of Cer in regulating cell growth and cell death, Cer metabolic and signaling pathways are considered potential targets for anticancer therapy. Many promising approaches have been used to evaluate this concept[12,13] including the application of cell-permeable, short-chain Cers,[14–16] liposomal formulations,[17,18] site-specific cationization,[19–24] and induction of endogenous Cer by modulation of SPL metabolizing enzymes.[20,25–38]

In our investigation of SPL chemistry and the search for new molecules that mimic the action of SPLs and/or reg-

Keywords: D-*e*-MAPP; B13; Ceramidases; Ceramidase inhibitors; Ceramide; Sphingosine; Sphingosine 1-phosphate; Lysosomes; Mitochondria; Cytotoxicity.
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doi:10.1016/j.bmc.2007.08.032

ulate their metabolism, we focused on analogs that affect Cer metabolism, attempting to target them to specific sub-cellular compartments.[24,26,27,34–37,39–44]

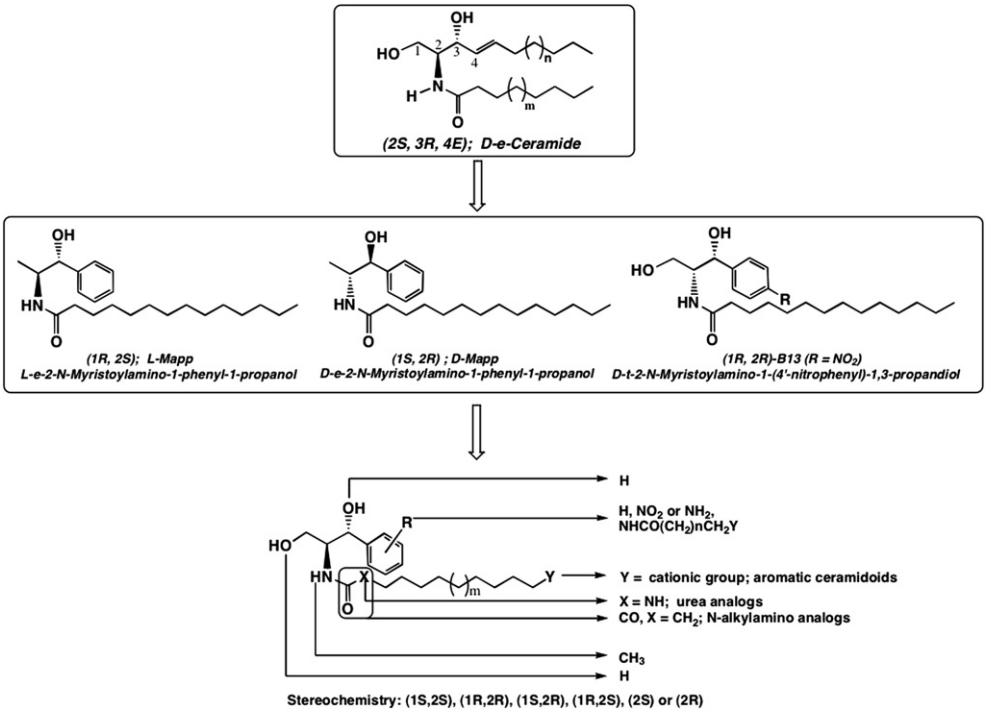
Previously, we synthesized a set of lipophilic phenyl-*N*-acyl-amino-alcohols (aromatic analogs of Cer) and confirmed that the active analogs increased endogenous Cer and inhibited CDases.[26,27,34,36] Experiments in HL60 cells and in in vitro studies showed that the most potent analogs from this group, D-*e*-MAPP, stereospecifically inhibited alkaline CDase, whereas its enantiomer, L-*e*-MAPP, served as a substrate for this enzyme.[26] Biological activity of D-*e*-MAPP was later confirmed by several investigators.[34,45–50] Another active analog, B13 (Scheme 1), which differs from D-*e*-MAPP in stereochemistry and functional groups, inhibited acid CDase, caused the release of cytochrome *C*, and induced apoptosis.[34,36] Biological activity of B13 was demonstrated in leukemia, malignant melanoma, colon, and prostate cancer cells, and in animal experiments of in vivo cancer growth.[34–36] In a previous study, we also showed that isosteric replacement of the amide group of Cer by urea or amine generated inhibitors of neutral CDase, thus illustrating the usefulness of this approach.[37] Moreover, in another recent study, we developed the concept of the fixed positive charge-dependent, cellular-targeting Cer, and demonstrated that fixed cationic Cer analogs target preferentially to the mitochondria.[19,21,23,24]

Extending these findings to the aromatic analogs of Cer, we have synthesized a new group of analogs of D-*e*-MAPP and B13 with specific structural features, improving and modifying their physicochemical and targeting properties to specific cell compartments (Scheme 1).[44] Based on known targeting behavior of alkylamines, we expected that some analogs will locate to lysosomes (e.g., *N*-alkylamino-analogs, class C).[51–54] In contrast, fixed cations are expected to be mitochondriotropic (aromatic ceramidoids, class D).[19,21,23,24] Finally, neutral analogs (parent amides, *N*-methyl-amides, class A, and urea-analogs, class B) may show no compartmental preferences as was shown for exogenous Cers.[55,56]

The results with MCF7 cells showed that all the new analogs were equally or more potent than the parent compounds.[44] Their activity was predominantly defined by the nature of the modification of the *N*-acyl-hydrophobic interfaces. The most potent compounds belonged to either class D, the aromatic ceramidoids, or to class C, the aromatic *N*-alkyl-amino-alcohols. Representative analogs were also evaluated by the National Cancer Institute for a full anticancer screening against a 60-human-tumor-cell assay (NCI's 60-cell line assay). Again, results showed a class-dependent activity, with classes C and D being the most effective.[44]

We expected that these new analogs, similar to the parent compounds, would inhibit CDases. Additionally, the action on CDases would have significant effects on the flux between the Cer species, Sph and S1P. Selected analogs are: D-*e*-MAPP, LCL16, LCL284, LCL120, and B13, LCL15, LCL204, LCL85 (Fig. 1).

Results from this study clearly distinguish class-dependent effects of these analogs on Cer species, Sph and S1P. However, distinct profiles were observed at low concentrations for D-*e*-MAPP and B13, previously identified inhibitors of the alkaline and acid CDases. The results are discussed in relation to a proposed compartment-specific action of these compounds.



Scheme 1. Ceramide, D-*e*-MAPP, and B13 structures and design for aromatic analogs.

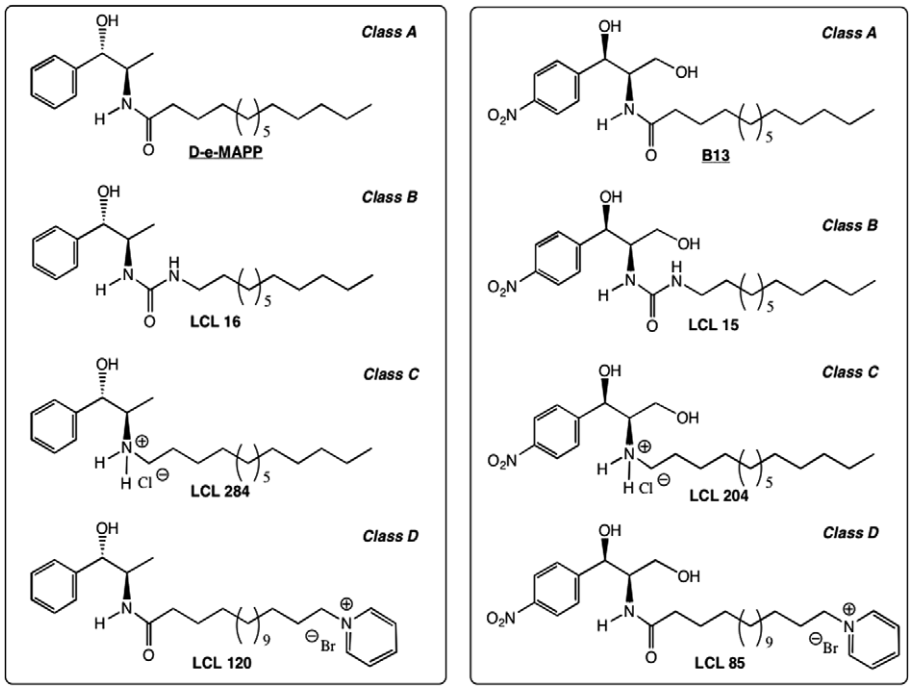


Figure 1. Chemical structures of LCL compounds used in this study.

2. Results and discussion

The compounds synthesized for this study represent the second generation of analogs that are based on the *N*-acyl part modifications of D-*e*-MAPP and B13 (Scheme 1).[44] The selected model compounds generally represent the C_{14}-analogs of the following groups of compounds: class A, D-*e*-MAPP and B13; class B, urea-analogs LCL16 and LCL15, in which the *N*-acyl group is replaced by a nonhydrolyzable urea-group; class C, LCL284 and LCL204, *N*-alkyl-amino-analogs in which the *N*-acyl-moiety was reduced to an *N*-alkyl-amino-group; and class D, LCL120 and LCL85, analogs containing an ω-pyridinium salt in the *N*-acyl-component (shown in Fig. 1).

2.1. Signature effects of D-*e*-MAPP and B13 analogs on endogenous Cer and Sph

To study the enhanced cytotoxicity of the novel D-*e*-MAPP and B13 analogs, we investigated their effects on endogenous SPLs. Concentration-dependent effects at 24 h are shown in Figures 2, 3, 5, and 6. Time-dependent effects for 10 μM treatments are shown in Figures 4 and 7.

The major (~94%) Cer components (C_n-Cers) of the MCF7 control cells are: $C_{24:1}$-, C_{24}-, and C_{16}-Cers and changes in these Cers are shown in Figures 3, 4, 6, and 7. The minor C_n-Cers are C_{14}-, C_{18}-, and C_{20}-Cers. After treatment with the selected analogs, these C_n-Cers followed the same pattern as that of C_{16}-Cer.

2.1.1. Effects of D-*e*-MAPP analogs

2.1.1.1. Concentration-dependent effects on Cer and Sph. D-*e*-MAPP analogs had significantly varied effects on endogenous Cer and Sph (Fig. 2a and b, respectively), as determined for 24 h treatment. The most effective analog was LCL284, which elevated Cer and decreased Sph in a concentration-dependent manner. The increase in Cer (180%) and decrease in Sph (80%) were observed even at 1 μM. D-*e*-MAPP showed a concentration-dependent biphasic effect on Cers: downregulation for the lower concentrations (1–10 μM D-*e*-MAPP) and an increase of Cers starting from 25 μM D-*e*-MAPP (Fig. 2a). A decrease in Sph was observed starting from 2.5 μM D-*e*-MAPP, reaching a plateau at about 10 μM D-*e*-MAPP (Fig. 2b). L-*e*-MAPP neither affected endogenous Cers nor Sph at concentrations ranging from 10 to 50 μM (not shown). LCL16 had no effect on Cer up to 10 μM; however, Cer increased 180% at 50 μM treatment (Fig. 2a). LCL16 had a biphasic effect on Sph, which increased up to 10 μM and decreased below control levels at higher concentrations of LCL16 (Fig. 2b). A similar effect was observed for LCL17, the enantiomer of LCL16 (data not shown). LCL120, the cationic analog from class D, increased Cer ~260% (10 μM LCL120) in a concentration-dependent manner (Fig. 2a). This compound also increased Sph, starting at 5 μM LCL20 and reached 230% for 10 μM LCL120 treatment (Fig. 2b). Its enantiomer, LCL420, followed the same pattern, though to a lesser extent (data not shown).

2.1.1.2. Effects on endogenous Cer species (C_n-Cers) versus Sph. The specific effects of the selected analogs on Cer species are shown in Figures 3a–d and 4a–d.

D-*e*-MAPP had a concentration-dependent effect on the major Cer components and Sph (24 h, Fig. 3a). Decreases in C_{16}-, C_{24}-Cers, and Sph accompanied by a small increase in $C_{24:1}$-Cer were observed up to 5 μM

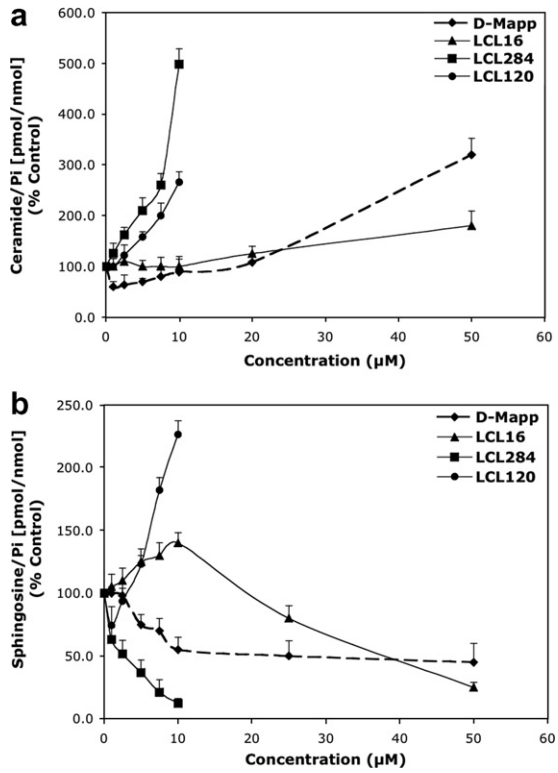


Figure 2. Concentration-dependent effects of the representative D-*e*-MAPP analogs on endogenous Cer and Sph in MCF7 cells for 24 h treatment. (a) Effects of D-*e*-MAPP, LCL16, LCL284, and LCL120 on total Cer. (b) Effects of D-*e*-MAPP, LCL16, LCL284, and LCL120 on Sph.

D-*e*-MAPP. At higher concentrations, all Cer species were variously elevated; however, no effect on C_{24}-Cer was observed. Sph was still decreased up to 50 μM D-*e*-MAPP.

Next, we performed a time-course treatment with 10 μM D-*e*-MAPP (Fig. 4a) observing an immediate decrease of all Cer species and Sph (30% of control). C_{24}- and $C_{24:1}$-Cer slowly recovered over time; however, C_{16}-Cer and Sph were below control levels up to 24 h. C_{14}- and C_{18}-Cers followed the pattern of C_{16}-Cer.

LCL16 had concentration-dependent effects on Cer species and Sph (24 h, Fig. 3b). Downregulation of C_{16}-Cer and upregulation of $C_{24:1}$-Cer were observed up to 10 μM of LCL16. An increase in C_{16}-Cer was observed only for the highest concentration tested (50 μM). LCL16 had almost no effect on C_{24}-Cer up to 50 μM. Sph was slightly increased for the lower concentration (up to 140% for 10 μM LCL16) and decreased to 25% in response to 50 μM of LCL16. A similar but weaker pattern was observed for LCL17 (data not shown). Treatment with 10 μM LCL16 over the time course (Fig. 4b) had a biphasic effect on endogenous SPLs. At early time points (1–2.5 h), C_{24}-, $C_{24:1}$-Cers, and Sph rapidly increased and no effect was observed on C_{16}-Cer. Later, all Cers and Sph were decreased below the control levels subsequent to their slow recovery at 24 h. However, C_{16}-Cer was permanently decreased below the control level up to 24 h. C_{18}- and C_{14}-Cers were also decreased, but less than that observed with C_{16}-Cer. After the time course, up to 48 h, C_{16}-Cer recovered to control levels, but Sph decreased to 25% (data not shown).

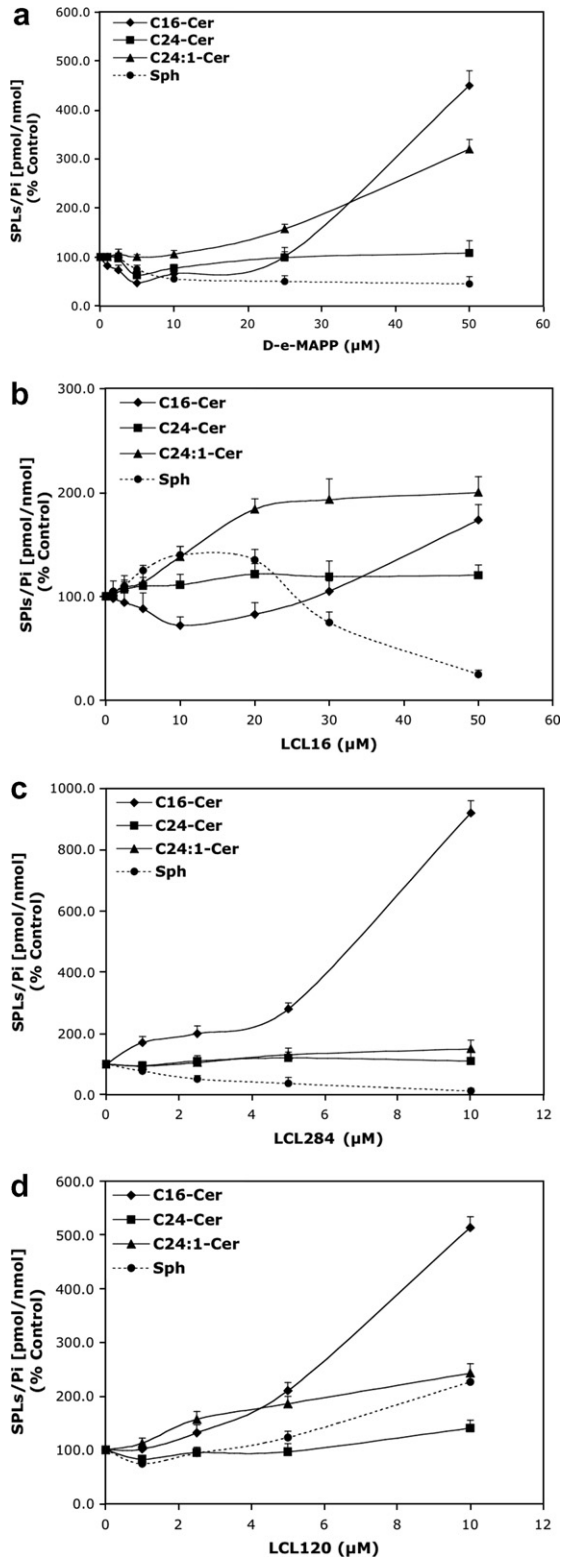


Figure 3. Concentration-dependent regulatory effects of representative D-*e*-MAPP analogs on balance of endogenous Cer species and Sph for 24 h treatment. (a) D-Mapp; (b) LCL16; (c) LCL284; and (d) LCL120.

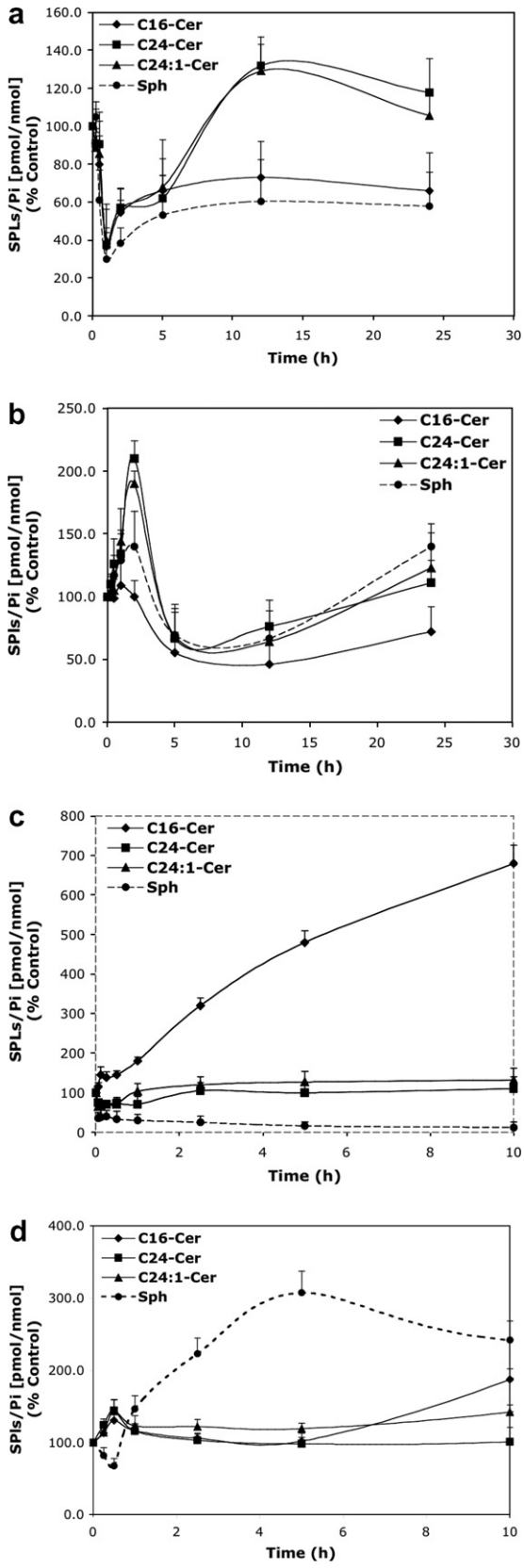


Figure 4. Time-dependent effects of representative D-*e*-MAPP analogs (10 μM) on balance of endogenous Cer species and Sph. (a) D-Mapp; (b) LCL16; (c) LCL284; (d) LCL120.

LCL284 exerted a very strong effect on Cer species and Sph. Concentration-dependent increases of C_{14}-, C_{16}-,

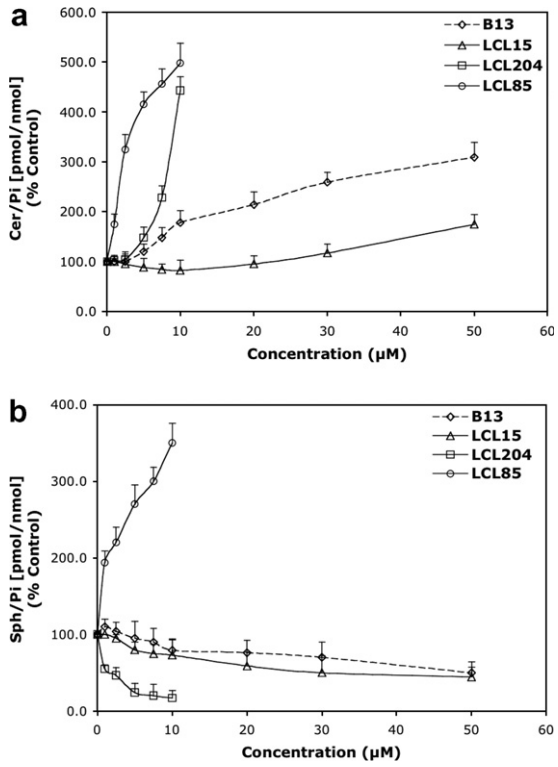


Figure 5. Concentration-dependent effects of the representative B13 analogs on endogenous Cer and Sph in MCF7 cells for 24 h treatment. (a) Effect of B13, LCL15, LCL204, and LCL85 on total Cer. (b) Effect of B13, LCL15, LCL204, and LCL85 on Sph.

and C_{18}-Cers were observed, and C_{24}- and $C_{24:1}$-Cers were virtually unaffected at 24 h of treatment, even for the lowest concentration of LCL284 used (1 μM, Fig. 3c). This effect on Cer species was observed almost immediately (5 min) starting with the upregulation of C_{14}-, C_{16}-, and C_{18}-Cers and downregulation of C_{24}- and $C_{24:1}$-Cers, as shown for 10 μM LCL284 (Fig. 4c). Over this time course, the last two Cers slowly recovered to control levels after 2.5 h, reaching a plateau at 24 h. The increase in C_{14}-Cer, the minor component of endogenous Cer, was the most significant. Levels of C_{14}-, C_{16}-, and C_{18}-Cer after treatment with 10 μM LCL284 for 2.5 h were elevated 430%, 330%, and 260%, respectively. LCL289 (enantiomer of LCL284) followed the pattern of LCL284, but was less effective in elevating C_{14}-, C_{16}-, and C_{18}-Cers (data not shown). Both enantiomers exerted inhibitory effects on Sph. As shown in Figure 4c, LCL284 caused an immediate (within 5 min of treatment) and permanent decrease in Sph (to 25% of control). A permanent decrease in Sph was also observed for the lower concentration as shown in Figure 3c.

LCL120 caused a concentration-dependent increase of C_{14}-, C_{16}-, and $C_{24:1}$-Cers and Sph without affecting C_{24}-Cer as shown for 24 h treatment (Fig. 3d). LCL420, the enantiomer of LCL120, caused similar changes in Cers, but to a lesser extent and without affecting Sph (data not shown). Over the time course (Fig. 4d), 10 μM LCL120 caused a biphasic effect on endogenous Cer species and Sph. The early increase of all Cer species (140–190%, the highest for C_{14}-

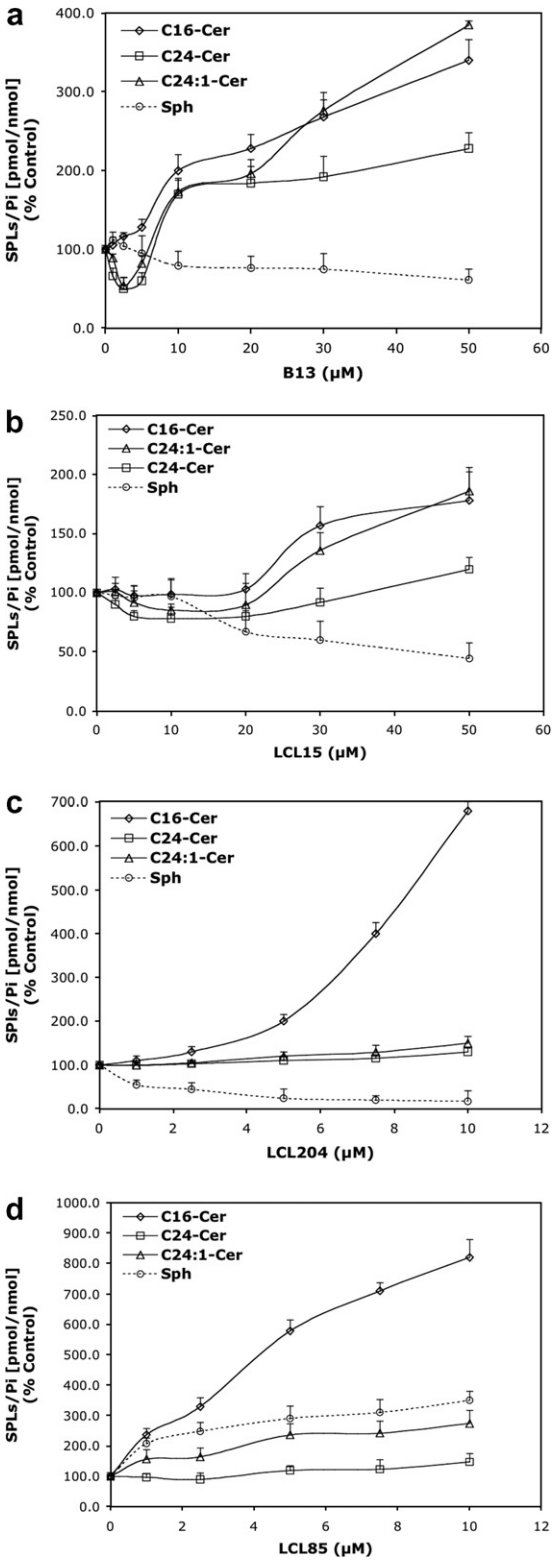


Figure 6. Concentration-dependent regulatory effects of representative B13 analogs on the balance of endogenous Cer species and Sph for 24 h treatment. (a) B13; (b) LCL15; (c) LCL204; and (d) LCL85.

Cer) and the decrease in Sph (60%) was observed within 30 min. This was followed by an increase in Sph (300% at 5 h) and a time-dependent formation of C_{14}-, C_{16}-, and $C_{24:1}$-Cers (310%, 157%, and 142%, respectively, for 10 h treatment). C_{24}-Cer recovered to the control level at 1 h and was not further elevated.

2.1.2. Effects of B13 analogs

2.1.2.1. Concentration-dependent effects on endogenous Cer and Sph. The observed differences in the induced levels of Cer and Sph (Fig. 5a and b, respectively) by B13 analogs followed the pattern of changes recognized previously for D-*e*-MAPP analogs (Fig. 2a and b) and were similarly correlated with their structural modifications.

Cer was efficiently elevated by class C and D analogs (LCL204 and LCL85, Fig. 5a); however, these analogs had different effects on Sph (Fig. 5b). LCL85, the mitochondriotropic B13 analog, increased Sph in a concentration-dependent manner. In contrast, LCL204, the lysosomotropic B13 analog, decreased Sph in a concentration-dependent fashion. The other lysosomotropic analogs tested, LCL385, LCL343, and LCL18, also decreased Sph and elevated endogenous Cer (data not shown).

B13 elevated Cer starting at 5 μM treatment, and Cer increased ~300% with 50 μM treatment (Fig. 5a). A small decrease in Sph was noticed at 10 μM (75% of control) and Sph was further decreased to ~50% of control at 50 μM (Fig. 5b).

LCL15, urea-B13, was the least potent in elevating Cer, which only increased 170% when treated with 50 μM LCL15 (Fig. 5a). At low concentrations of LCL15 (2.5–10 μM), a small decrease in Cer was observed (~90% control for 10 μM treatment). LCL15 decreased Sph more efficiently than B13, starting at 5 μM treatment (80% of control) and decreasing to 45% of control with 50 μM (Fig. 5b).

2.1.2.2. Effects on endogenous Cer species and Sph. Next, the specific effects of the selected compounds on Cer molecular species (C_n-Cer) were determined (Figs. 6a–d and 7a–d).

B13 had a concentration-dependent effect on Cer species (24 h, Fig. 6a). A decrease in C_{24}- and $C_{24:1}$-Cer, and a small increase in C_{16}-Cer were observed at low concentrations, whereas the higher concentrations of B13 increased all Cer species. B13 (50 μM) increased $C_{24:1}$-, C_{16}-, and C_{24}-Cers to 380%, 340%, and 220%, respectively. The minor components of endogenous Cer, C_{14}- and C_{18}-Cers, were also elevated. B13 (10 μM) induced early changes in endogenous Cer species and Sph (Fig. 7a). Small increases in C_{16}- and C_{14}-Cers (up to 120%) and a small decrease in C_{24}- and $C_{24:1}$-Cers (to 80%), followed by an increase of all Cer species (C_{16}-Cer was the most greatly effected) were observed. Interestingly, the early upregulation of C_{16}- and C_{14}-Cer and the downregulation of C_{24}- and $C_{24:1}$-Cer were accompanied by an increase in Sph. Over time, Sph was decreased, returning to control levels (12 h treatment), followed by a further small decrease (~80% of control) at 24 h (Fig. 7a).

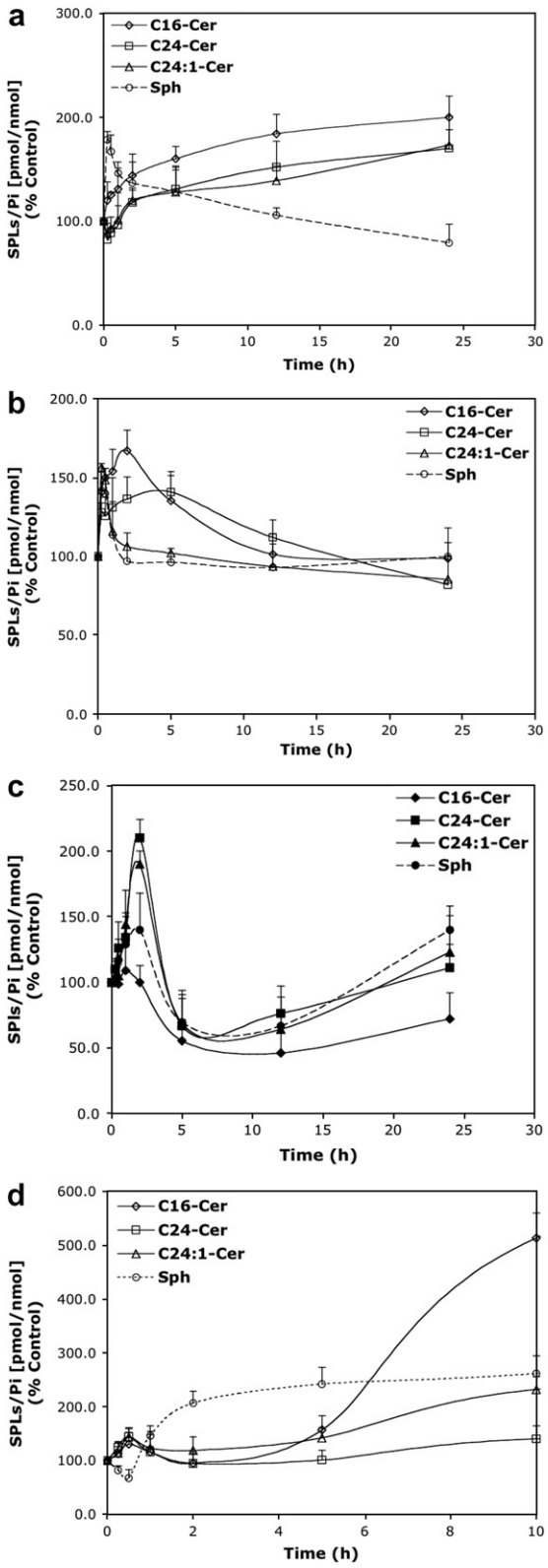


Figure 7. Time-dependent effects of representative B13 analogs (10 μM) on the balance of endogenous Cer species and Sph. (a) B13; (b) LCL15; (c) LCL204; and (d) LCL85.

LCL15 stimulated Cer species in a concentration-dependent manner, and inhibited Sph (Fig. 6b). C_{24}- and $C_{24:1}$-Cers were slightly decreased (to 80%) by LCL15 concentrations up to 20 μM and $C_{24:1}$-Cer increased with LCL15 concentrations beyond 30 μM. C_{16}-Cer was elevated at concentrations of LCL15 starting at 25 μM. Sph decreased when treated with 20 μM LCL15. However, LCL15 (10 μM) increased (up to 30 min) all C_n-Cers and Sph (Fig. 7b). Over time, C_{16}- and C_{24}-Cers were progressively increased (up to 2.5 h); however, Sph and $C_{24:1}$-Cer decreased rapidly, reaching control levels at 2.5 h. C_{16}- and C_{24}-Cer were slowly decreased to control level (~18 h of treatment).

LCL204 caused a concentration-dependent increase of C_{16}-Cer, no changes for C_{24}- and $C_{24:1}$-Cers, and a decrease in Sph (Fig. 6c). C_{14}- and C_{18}-Cers were also effectively elevated, with C_{14}-Cer being increased the most (850% for 10 μM, data not shown). Sph was effectively decreased, even for the lowest concentration of LCL204 tested (1 μM).

In a time course study, treatment with 10 μM LCL204 immediately decreased Sph and C_{24}- and $C_{24:1}$-Cers and increased C_{16}- and C_{14}-Cers (Fig. 7c). Long-chain Cers recovered to control levels at 1 h and stayed relatively unchanged up to 24 h. C_{16}-Cer (and C_{14}-, C_{18}-Cers) gradually increased over the time course. Sph was permanently decreased up to 24 h.

LCL85 induced a concentration-dependent increase of Sph (up to 350%), C_{16}- and C_{14}-Cers (up to ~800%), $C_{24:1}$-Cer (270%), and C_{24}-Cer (140%) (24 h, Fig. 6d). Next, we investigated the time dependence of these effects on endogenous Sph and Cer species. As shown in Figure 7d, all Cer species were increased and Sph decreased within the first 0.5 h of treatment. With the time progression, Sph recovered quickly and increased over the time course. All Cer species were lowered to control levels after 1 h treatment and were unchanged for the next 4 h. Thereafter, we observed a steady increase in Cer species up to 24 h, with the highest effects seen with C_{14}-Cer (760%, data not shown). However, at the end of the experiment, the level of C_{16}-Cer (820%, Fig. 6d) surpassed that of C_{14}-Cer. In comparison, $C_{24:1}$-Cer increased ~260%, and C_{24}-Cer increased ~130% (24 h, Fig. 6d).

2.2. Elucidation of specific patterns in the Cer species-Sph levels and balance induced by the action of D-*e*-MAPP and B13 analogs

Analysis of the previously described results revealed several distinctive class-dependent patterns on the cellular levels C_{16}-Cer, $C_{24:1}$-Cer, and Sph. These patterns are elucidated and discussed below and illustrated in Table 1.

Pattern I was characterized by the upregulation of C_{16}-, $C_{24:1}$-Cers and downregulation of Sph, and this was identified for the neutral compounds: D-*e*-MAPP, B13, LCL16, and LCL15, when applied at a high concentration (Figs. 3a and b and 6a and b), and for class D for early treatments (Figs. 4d and 7d).

Pattern II was distinguished by the upregulation of C_{16}-Cer and downregulation of $C_{24:1}$-Cer and Sph. This was

Table 1. Identification of the specific patterns in the Cer species-Sph level and balance induced by the action of D-*e*-MAPP and B13 analogs

Pattern	C_{16}-Cer	$C_{24:1}$-Cer	C_{24}-Cer	Sph	Class	Compound	LC[a]	ET[b]
I	↑[c]	↑	↑	↓[d]	A and B	D-*e*-MAPP, B13, LCL15, and LCL16		
I	↑	↑	↑	↓	D	LCL85 and LCL120		x
II	↑	↓	↓	↓	C	LCL284 and LCL204		x
III	↑	—[e]	—	↓	C	LCL284 and LCL204		
IV	↑	↑	—	↑	D	LCL120 and LCL85		
V	↓	↑	—	↑	B	LCL15 and LCL16	x	
VI	↑	↑	↑	↑	B	LCL15 and LCL16	x	x
VII	↓	—	↓	↓	A	D-*e*-MAPP	x	
VIII	↓	↓	↓	↓	A	D-*e*-MAPP	x	x
IX	—	↓	↓	—	A	B13	x	
X	↑	↓	↓	↑	A	B13	x	x

[a] LC, low concentration.
[b] ET, early treatment.
[c] ↓, downregulation.
[d] ↑, upregulation.
[e] —, no effect.

a signature pattern for the early effects of treatment with the lysosomotropic class C agents (Figs. 4c and 7c). Extended treatments with these analogs followed *Pattern III*, consistent with the upregulation of C_{16}-Cer, downregulation of Sph, and the absence of an effect on $C_{24:1}$- and C_{24}-Cers (Figs. 3c, 4c, 6c, and 7c).

Pattern IV was characterized by the upregulation of C_{16}- and $C_{24:1}$-Cers and Sph with no detectable effect on C_{24}-Cer. This pattern was a characteristic of the mitochondriotropic analogs, class D (Figs. 3d, 4d, 6d, and 7d).

It is noteworthy that the neutral analogs applied at low concentrations did not follow the SPL profile described by *Pattern I*.

Thus, LCL16 induced upregulation of $C_{24:1}$-Cer and Sph and downregulation of C_{16}-Cer (Fig. 3b, *Pattern V*). However, for the short treatment (up to 2.5 h), we observed an increase of long-chain Cers and Sph (Fig. 4b, *Pattern VI*) followed by a decrease of all Cer species and Sph below control levels (at 5 h) and then a slow recovery of all but C_{16}-Cer at 24 h (Fig. 4b).

D-*e*-MAPP downregulated C_{16}-Cer, C_{24}-Cer, and Sph and did not change $C_{24:1}$-Cer (Fig. 3a, *Pattern VII*). However, for the short treatment, all SPLs decreased significantly below the control level (30–40% of control, Fig. 4a, *Pattern VIII*).

B13 decreased $C_{24:1}$- and C_{24}-Cers, minimally increased C_{16}-Cer, and did not detectably affect Sph (Fig. 6a, *Pattern IX*), but during the short treatment, an increase of Sph was observed (Fig. 7a, *Pattern X*).

Pattern I may indicate the inhibitory action on a 'non-Cer-species (C_n-Cer)-specific' CDase, accounting for non-specific elevation of Cers and downregulation of Sph.

Pattern II, identified above for the lysosomotropic agents, may result from their inhibitory effect on acid CDase, with the enzyme selectively involved in the hydrolysis of C_{16}-Cer. Alternatively, acid CDase may hydrolyze all Cers, but this could be accompanied by the selective resynthesis of $C_{24:1}$-Cer from the liberated Sph, giving the appearance of a selectivity for C_{16}-Cer.

However, *Pattern III*, observed after extended treatment with these agents, may indicate a more complete inhibition of acid CDase, that is, no increase of Sph or $C_{24:1}$-Cer. Importantly, the rapid and dramatic decrease in Sph was not recovered by the action of other SPL-metabolizing enzymes, indicating that the main source of cellular Sph is via the hydrolytic action of acid CDase. Fast proteolysis of acid CDase occurred in response to LCL204,[40,43] a result that support our earlier observations.

The dual pattern of $C_{24:1}$-Cer changes observed under *Patterns II* and *III* could also be consistent with inhibitory effects of these agents on acid SMase at the higher concentrations.[40]

Interestingly, we also observed that LCL204 and LCL284 decreased endogenous S1P. Thus, 10 μM LCL204 caused a rapid (at 0.5 h) and permanent (up to 24 h) decrease of S1P to 15% of control (data not shown).

Pattern IV was observed for the aromatic ceramidoids LCL120 and LCL85, class D. Starting at 1 h treatment, an increase of Sph was followed by increases of all Cers except for the C_{24}-Cer species. This pattern could not be explained by the action on CDases alone. Considering aromatic ceramidoids as mitochondriotropic agents,[24] their possible enzymatic targets should be located in or around the mitochondria. These results raise the intriguing possibility of the existence of a mitochondrial C_n-Cer–Sph balance, and this balance may be regulated by either CDase/Cer synthase enzymes, or perhaps via some specific transferases: *O*-acyl-Cers transferases,[57,58] or *O*-acyl-Sph transferases (1- and 3-*O*-acyl-Sph formed by the theoretical transfer of the *N*-acyl group of Cer to its primary or secondary hydroxyl groups).[59]

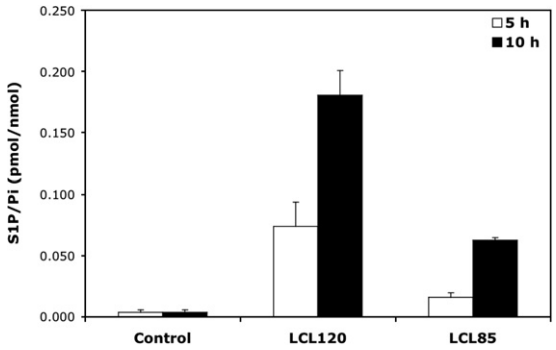


Figure 8. Effect of 10 μM aromatic ceramidoids on S1P formation.

Because the increase in S1P was observed only after treatment with the aromatic ceramidoids (Fig. 8, time treatment between 5 and 10 h), we suggest that this S1P was generated from the mitochondrial pool of Sph.

Contrary to this, *Pattern I*—typical for the inhibition of CDases—was identified for the short treatment with these compounds, suggesting that cationic analogs affected some CDases, possibly on their way to mitochondria.

Patterns V and *VI* were identified in response to low concentrations of LCL16. Longer treatment with 10 μM LCL16 suggests an increased activity of CDase using C_{16}-Cer as a substrate to form long-chain Cers, especially $C_{24:1}$-Cer, from the liberated Sph (*Pattern V*). Supporting this theory, an increase in acid CDase expression after LCL16 was observed with Western blot experiments (Fig. 9). *Pattern VI* identified for the early treatments suggested activation of Cer- and Sph-related transferases. However, LCL16 acted as a typical CDase inhibitor (*Pattern I*) if applied at a high concentration.

Patterns VII and *VIII* were observed for the low concentration of D-*e*-MAPP. The decrease in C_{16}-Cer suggested increased activity of CDase using C_{16}-Cer as a substrate (*Pattern VII*). Increased acid CDase expression after D-*e*-MAPP was observed with Western blot experiments (Fig. 9), suggesting indirect action on this enzyme. However, *Pattern VIII* observed for its early effects suggested action on targets other than CDase/Cer synthase enzymes (e.g., specific acyl transferases).

Pattern IX, which was observed for the low concentrations of B13, suggested an effect on enzymes utilizing the long-chain Cers, however *Pattern X*, which was observed during early treatment, suggests an effect on CDase using long-chain Cers as substrates to generate Sph and transfer it to C_{16}-Cer.

In summary, all analogs showed regulatory effects on Cer species and Sph, emphasizing their effects on C_{16}-Cer changes. The identified patterns depended on the group classification, concentration used, and time treatments. Changes in Cer species and Sph were related to the effects of these compounds on CDase or Cer-associated transferase enzymes.

2.3. Lysosomotropic properties of *N*-alkylamino-analogs of D-*e*-MAPP and B13 analogs

Recently published studies with LCL204 in DU145 prostate cancer cells showed that this compound localized to lysosomes resulting in rapid destabilization followed by the specific degradation of the key ceramide

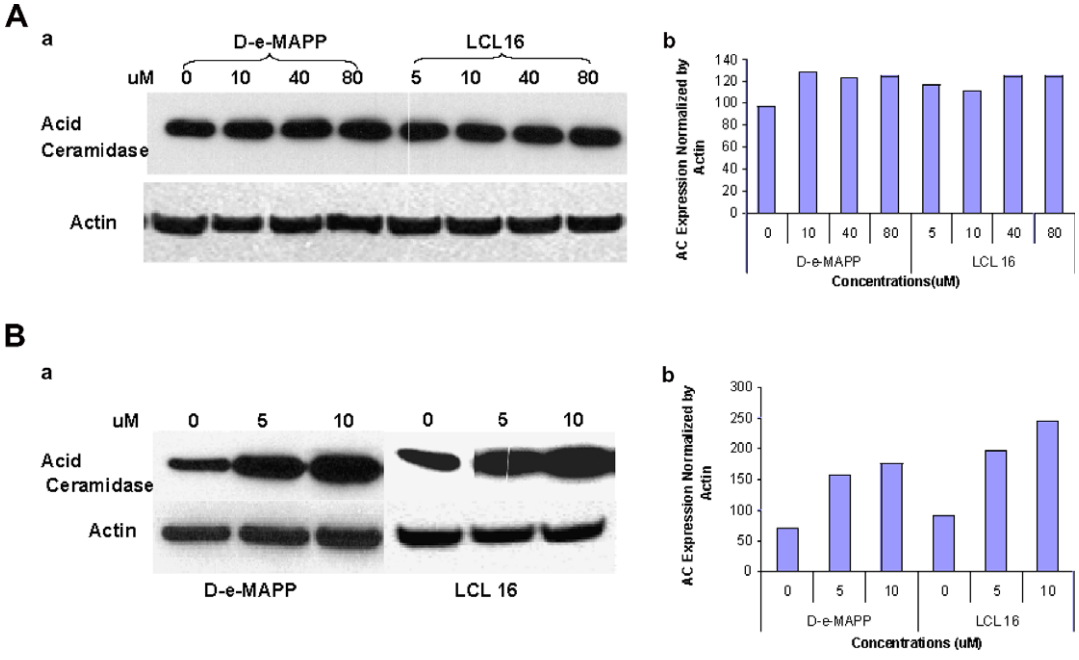


Figure 9. Effect of D-*e*-MAPP and LCL16 on ACDase. (A) 5 h treatment; (A-a) Western blotting; (A-b) densitometry analysis. (B) 24 h treatment; (B-a) Western blotting; (B-b) densitometry analysis.

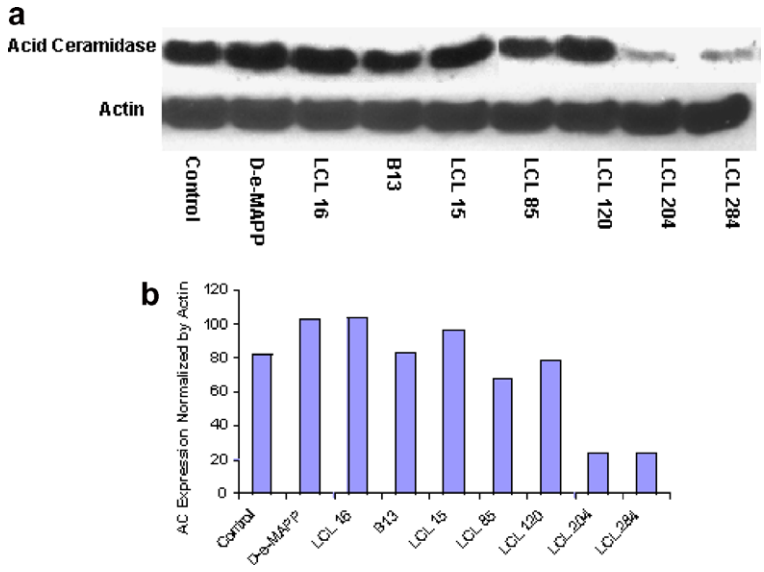


Figure 10. Effects of 10 μM selected inhibitors on ACDase after 5 h of treatment. (a) Western blotting; (b) densitometry analysis.

metabolizing proteins, acid CDase and acid SMase, by a lysosomal protease.[40,42,43]

Similar experiments performed in MCF7 cells with selected analogs evaluated in this study showed that only analogs from class C—LCL284 and LCL204—but not analogs from the other classes, caused lysosomal destabilization and degradation of acid CDase as shown for the 10 μM concentration after 5 h treatment (Fig. 10).

2.4. Endogenous C_{16}-Cer and cell cytotoxicity

SAR studies on cell-growth inhibition (shown for 48 h of treatment)[44] correlated well with the increase of C_{16}-Cer caused by the lysosomo- and mitochondriotropic analogs and B13, but not by D-*e*-MAPP or urea analogs, class B (Fig. 11). A linear relationship was found for the B13 family: LCL85, LCL204, B13, and LCL15 with LCL85 increasing C_{16}-Cer to the greatest degree over the IC_{50} value of this analog (Fig. 12b). In contrast,

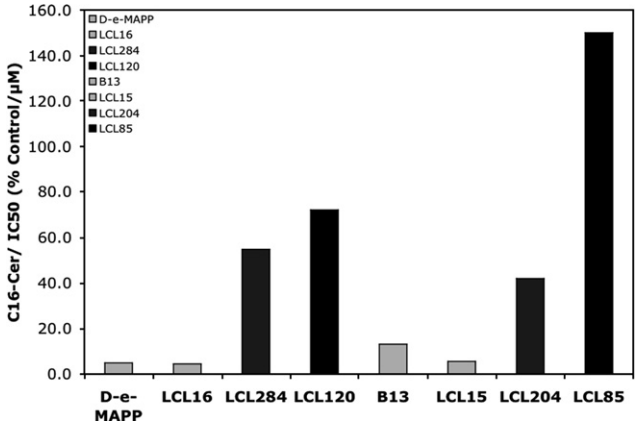


Figure 11. Correlation between the increased endogenous $C1_{16}$-Cer and MCF7 cell-growth inhibition (IC_{50} values) caused by D-*e*-MAPP and B13 and their representative analogs.

members of the D-*e*-MAPP family did not show a similar linear relationship for these parameters (Fig. 12a). The class B compounds, LCL15 and LCL16, did not increase C_{16}-Cer at the corresponding IC_{50} values of the analogs. These results suggest that: (i) cell-growth inhibition caused by B13 and analogs from classes C and D follows increases of endogenous C_{16}-Cer, (ii) differences between D-*e*-MAPP and B13 most likely reflect their distinctive effects on SPL metabolism, and (iii) cell-growth inhibition by LCL16 and LCL15 is related to a different mechanism of action than for class C and D analogs because no increase of C_{16}-Cer was observed.

Analogs C and D were similarly correlated regarding the increase of C_{14}- and C_{18}-Cers (data not shown). However, the long-chain Cers, C_{24}-Cer, and $C_{24:1}$-Cer, were not correlated in this manner. In general, C_{24}-Cer was not elevated by these treatments, and $C_{24:1}$-Cer was only slightly increased by the mitochondriotropic agents. This is also consistent with studies in MCF-7 cells which demonstrated a role for C_{24}- and $C_{24:1}$-Cers, generated by neutral SMase, in regulating cell cycle growth but not apoptosis[60].

3. Summary and conclusions

The compounds developed and examined in this study were grouped into 4 classes (A–D) based on their chemical structures, activity on MCF7 cell growth, and effects on endogenous SPLs: Cer species and Sph. The 4 groups were as follows: class A, *N*-acyl-analogs (neutral); class B, urea-analogs (neutral); class C, *N*-alkyl-analogs (lysosomotropic analogs); and class D, ω-cationic analogs (mitochondriotropic analogs). Specific class-dependent effects of the representative analogs (Fig. 1) on endogenous Cer and Sph were defined. The neutral analogs were not effective in elevating endogenous Cer; however, they had different effects on endogenous Sph. Mitoc-

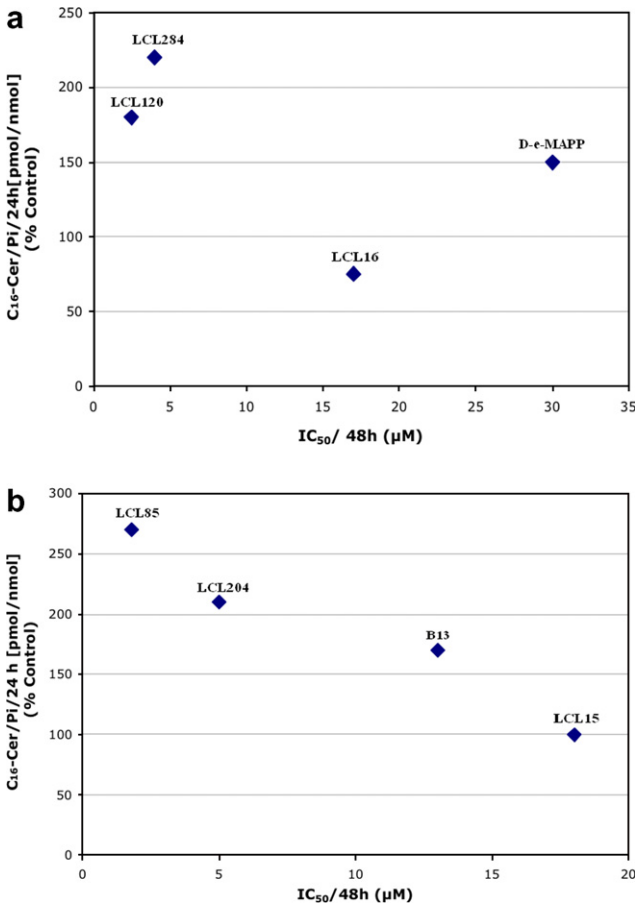


Figure 12. Correlation between increase of endogenous C16-Cer at 24 h and inhibition of cell growth at 48 h (IC_{50}). (a) D-*e*-MAPP family; (b) B13 family.

hondrio- and lysosomotropic analogs were very effective in elevating endogenous Cer, and they exhibited different profiles on endogenous Sph.

Class A. D-*e*-MAPP and B13, when applied at a high concentration, upregulated C_{16}-, $C_{24:1}$-Cers, and downregulated Sph, indicating an inhibitory action on a 'non-Cer-species (C_n-Cer)-specific' CDase. However, at a low concentration D-*e*-MAPP upregulated $C_{24:1}$-Cer and downregulated C_{16}-Cer and Sph, suggesting an increased activity of CDase using C_{16}-Cer as a substrate that could also lead to production of $C_{24:1}$-Cer through selective resynthesis from the liberated Sph. This possibility requires further study.

Class B. LCL16 and LCL15, when applied at a high concentration, upregulated C_{16}-, $C_{24:1}$-Cers, and downregulated Sph, indicating inhibitory action on a 'non-Cer-species (C_n-Cer)-specific' CDase. However, at a low concentration, LCL16 upregulated $C_{24:1}$-Cer and Sph and downregulated C_{16}-Cer, suggesting an increased activity of CDase using C_{16}-Cer as a substrate.

Class C. These analogs caused an immediate and permanent decrease of Sph and a time-dependent effect on Cer species. Short treatment with LCL284 and LCL204 upregulated C_{16}-Cers and downregulated C_{24} and $C_{24:1}$-Cers and Sph, indicating an inhibitory effect on 'C_{16}-Cer-specific acid CDase' and suggesting an early participation of acid CDase in a turn-over process, involving selective hydrolysis of C_{16}-Cer and resynthesis $C_{24:1}$-Cer from the liberated Sph. Extended treatment with these analogs increased C_{16}-Cers and decreased Sph with almost no effect on C_{24}- and $C_{24:1}$-Cers, suggesting a complete inhibition of this enzyme.

Class D. These analogs caused biphasic effects on Cer species and Sph. For the short treatment, LCL120 and LCL85 upregulated C_{16}-, $C_{24:1}$-Cers, and downregulated Sph, indicating the inhibition of 'non-Cer-species-specific CDase' and suggesting possible effects on some CDases on their way to the target compartments. For the extended treatment (starting from 1 h), these analogs upregulated Sph followed by increases of all Cer species but C_{24}-Cer. This pattern could not be explained by the action on CDases alone, suggesting possible effects on some acyl transferases.

The neutral analogs D-*e*-MAPP and B13, which have already been established as inhibitors of alkaline and acid CDases, and results with LCL16 suggested that they act as inhibitors of non-Cer-species-specific CDases (decrease Sph and increase all Cer species, except C_{24}-Cer in response to D-*e*-MAPP) when used at a high concentration. However, distinctive differences were noticed when these inhibitors were used at a lower concentration, suggesting their effects on C_{16}-Cer-specific CDase (or related enzymes). A parallel increase of acid CDase suggests an indirect effect on the synthesis of this protein.

Examining the actions of the lysosomotropic inhibitors, which have been shown to rapidly degrade acid CDase and acid SMase,[40] we suggest that a biological role of acid CDase is to hydrolyze C_{16}-Cers, and possibly provide Sph for the selective resynthesis of S1P or $C_{24:1}$-Cer. This analysis suggests that acid CDase may act as a regulator of pro-apoptotic and anti-apoptotic activities in the cancer cells. Inhibition of the hydrolytic activities of this enzyme permanently decreased Sph, eliminating resynthesis of $C_{24:1}$-Cer and S1P. The sharp drop in Sph was not compensated by the action of other SPL-metabolizing enzymes that could generate Sph. Competition between Cer synthases and SK activities on Sph released from C_{16}-Cer may be the key issue for the formation of S1P or $C_{24:1}$-Cer. This raises a question about the connections between S1P and $C_{24:1}$-Cer. An additional question is also raised about the source of C_{16}-Cer, since these analogs also caused degradation of acid SMase.

Parallel experiments performed by our group on DU145 cells overexpressing acid CDase additionally supported the hypothesis proposed here regarding the biological roles of acid CDase.[61] Results from these experiments showed a significant decrease of C_{16}-Cer and an increase of C_{24}- and $C_{24:1}$-Cers.

The independently synthesized AD2646, sharing the structure of LCL204 shown here (the free base of

LCL204, Fig. 1), acted as a bioactive molecule in HL60 cells, elevating endogenous Cer formed from exogenously added pyrenedodecanoic acid, inhibiting biosynthesis of cellular SM and glycosphingolipids from exogenous fluorescent Cers, and inhibiting human acid CDase in vitro.[28,30,32] These results indicate multimodal actions of AD2646 on various SPL enzymes. Considering that AD2646 acts as lysosomotropic agents presented here, lysosomal dysfunction in HL60 cells is also predicted in response to this agent.

Regarding analogs from class D, the mitochondrial agents, we suggest the existence of a mitochondrial (or mitochondrial-associated) metabolic pathway that regulates interconversion of Cer and Sph, and consequently affects the levels and balance of these two bioactive SPLs. This metabolic pathway may involve a CDase/Cer synthase set of enzymes, and/or could perhaps involve the action of specific *O*-acyl-transferases as suggested in the Part 1[44] based on the activity profile of LCL120 and LCL420. These *O*-acyl transferases, some of which have been described,[57,58] may release C_{16}-Cer and Sph from their O-acylated forms—*O*-acyl-C_{16}-Cer and *O*-acyl-Sph. Because increased S1P was observed only after treatment with the aromatic ceramidoids, we suggest that this S1P may be generated from a mitochondrial pool of Sph. The observed increase of S1P also suggested the action of a mitochondrial or mitochondrially associated SK.

SAR on cell-growth inhibition and effects on endogenous Cer species showed a significant and intimate correlation between the cytotoxic effects observed at 48 h of treatment with the active analogs and the increase of C_{16}-Cer observed up to 24 h of treatment. Aromatic ceramidoids represented the most potent analogs which increased C_{16}-Cer and Sph. The combined action of these two lipids could account for the increased apoptotic effects of the ceramidoids.

4. Experimental

4.1. D-*e*-MAPP and B13 analogs

Compounds used for this study were prepared as presented in the first part of this series, Novel Analogs of D-*e*-MAPP and B13. Part 1: Synthesis and evaluation as potential anticancer agents[44] where detailed synthetic procedures and compound characterization are listed under Chemistry (Experimental section).

4.2. Cell culture

MCF7 cells (breast adenocarcinoma, pleural effusion) were purchased from American type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS, Summit Biotechnology, CO, USA) and maintained under standard incubator conditions (humidified atmosphere 95% air, 5% CO_2, 37 °C).

4.3. Cell experiments

Cells were seeded at a density of ~50%, corresponding to 1×10^6 cells, in 10 mL of 10% fetal calf serum (FCS) and after 24 h incubation, cells were treated with LCL compounds dissolved in ethanol, keeping ethanol level at 0.1%. Control cells were prepared under identical condition and treated with the same amount of ethanol.

4.4. HPLC–MS/MS analysis of endogenous Cers and Sph

Advanced analyses of Cer species and Sph were performed by the Lipidomics Core at MUSC on a Thermo Finnigan TSQ 7000, triple-stage quadrupole mass spectrometer operating in a Multiple Reaction Monitoring (MRM) positive ionization mode as described.[62] Briefly, cells were isolated from the culture media, washed twice with a cold PBS and scraped into PBS. Cell pellets were fortified with the internal standards IS (17C base D-*erythro*-sphingosine: 17Sph, 17C D-*erythro*-sphingosine-1-phosphate: 17S1P, D-*erythro*-*N*-palmitoyl-13C-D-*erythro*-sphingosine: $13C_{16}$-Cer, and *N*-heptadecanoyl-D-*erythro*-sphingosine: $18C_{17}$-Cer) and extracted into a one-phase solvent system with ethyl acetate/iso-propanol/water (60:30:10%, v/v), ~4 mL from which a 1-mL portion was separated and used for determination of phospholipids levels (Pi was used for data normalization) after reextraction by the method of Bligh and Dyer. The remaining extract was used for analysis of S1P, Sph, and Cer species after evaporation under nitrogen and reconstitution in 100 μl of acidified (0.2% formic acid) methanol. Samples were injected on the HP1100/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3 μm particle size column, with 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and IS were collected and processed using the Xcalibur software. Quantitative analysis of endogenous SPLs and cellular level of compounds used for cell treatments were based on calibration curves generated by spiking an artificial matrix with known amounts of the target analyte synthetic standards and an equal amount of the IS. The target analyte peak area ratios from the samples were similarly normalized to their respective IS and compared to the calibration curves using a linear regression model. Final results were expressed as the level of the particular SPLs/phospholipids (Pi) and expressed as SPLs/Pi (pmol/nmol). Changes in SPLs were expressed in comparison to the control cells representing treatment with the equal amount of ethanol only.

4.5. Acid CDAase by Western blot

Western blots of ACDase expression levels were performed as previously described.[40]

Acknowledgments

Financial support was provided by the NIH NCI—IPO1-CA097132. Special acknowledgment is for NCRR—

CO6RR018823 providing laboratory space for Lipidomics Core in CRI building. We thank Dr. Jennifer Schnellmann for editorial assistance.

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