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Targeting (cellular) lysosomal acid ceramidase by B13: Design, synthesis and evaluation of novel DMG-B13 ester prodrugs

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Abstract

Acid ceramidase (ACDase) is being recognized as a therapeutic target for cancer. B13 represents a moderate inhibitor of ACDase. The present study concentrates on the lysosomal targeting of B13 via its N, N-dimethylglycine (DMG) esters (DMG-B13 prodrugs). Novel analogs, the isomeric mono-DMG-B13, LCL522 (3-O-DMG-B13•HCl) and LCL596 (1-O-DMG-B13•HCl) and di-DMG-B13, LCL521 (1,3-O, O-DMG-B13•2HCl) conjugates, were designed and synthesized through N, N-dimethyl glycine (DMG) esterification of the hydroxyl groups of B13. In MCF7 cells, DMG-B13 prodrugs were efficiently metabolized to B13. The early inhibitory effect of DMG-B13 prodrugs on cellular ceramidases was ACDase specific by their lysosomal targeting. The corresponding dramatic decrease of cellular Sph (80-97% Control/1h) by DMG-B13 prodrugs was mainly from the inhibition of the lysosomal ACDase.

Keywords

B13; DMG-B13 prodrugs; inhibitors; acid ceramidase; lysosomes; cancer

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1. INTRODUCTION

Bioactive sphingolipid (SPL) metabolites, specifically ceramide species (C_n -Cer), sphingosine (Sph), and sphingosine 1-phosphate (S1P), are increasingly recognized for their important roles as signaling molecules involved in regulation of survival, proliferation, and cell death.^{1,2} Acid ceramidase (ACDase, ASAH1) is a 55kDa polypeptide that is processed, under the normal growth conditions, into the α - and β -subunits (α -ACDase and β -ACDase) within the lysosomes.^{3,4} The role of this enzyme is to hydrolyze lysosomal Cers, thus providing Sph, which in turn can serve as a substrate of Sph kinases to synthesize S1P or as a substrate for ceramide synthases for the regeneration of Cers. Nonetheless, a cell type specific nuclear expression of ASAH1 has been reported recently identifying its key role for nuclear lipid metabolism in regulating gene transcription.⁵ Thus, ACDase represents a new target for cancer therapy due to its importance in regulating Cer-Sph-S1P inter-metabolism and nuclear gene transcription.⁵⁻¹³ Moreover, elevation of ACDase has been observed in many cancer cell lines and tumor tissues, particularly in the late stage of tumors, as compared to normal conditions.^{8-10,12} Inhibition of cellular ACDase sensitizes tumors to therapies and advances tumor cell killing. It may therefore represent an efficient and promising strategy for cancer treatment.

Over the last decade, our group has been focusing on development of ACDase inhibitors based on the structural and stereochemical modifications of ceramide. We have synthesized a set of lipophilic phenyl-N-acyl-amino-alcohols (aromatic analogs of Cer) that acts as anticancer agents, and we have demonstrated that the active analogs increased endogenous Cer and decreased endogenous Sph via inhibition of CDases.¹⁴⁻¹⁶ Among them, B13, (1R, 2R)-2-(tetradecanoylamino)-1-(4'-nitrophenyl)-1, 3-propanediol, which induces apoptosis in many cancer cell lines, functions as an ACDase inhibitor.¹⁶ This discovery made B13 a potential key compound to develop ACDase-related cancer research.^{11,16-19}

However, B13, as a neutral lipophilic molecule, may not be able to specifically and efficiently reach and accumulate in the lysosomes where mainly ACDase resides.^{3,4} Our previously published results displayed a dual action of B13 on cellular Cer and Sph, with an increase of Cer and decrease of Sph at high doses, and with no effect on Cer and no effect or a small increase of Sph especially during early treatments with low doses.¹⁹ These results suggest the possible effects of B13 on unidentified proteins that may transport B13 to the lysosomes; action of B13 on ACDase in compartments other than lysosomes, perhaps in the nucleus where the presence of this enzyme and its inhibition by B13 has been reported⁵; or possible B13 effects on other enzymes, perhaps on other CDases, ceramide synthases (CerSs) or acyl-transferases (ATases), that regulate Cer and Sph levels independently of ACDase, as previously suggested.¹⁹

Several structurally varied analogs of B13 have been developed so far.^{11-13,19-26} Our first choice for lysosomal analogs of B13 was based on its amide group modifications that resulted in (1R, 2R)-2-(N-tetradecylamino)-1-(4'-nitrophenyl)-1, 3-propanediol hydrochloride (LCL204), and (1R, 2R)-2-N-(12'-N,N-dimethylaminododecanoylamino)-1-(4'-nitrophenyl)-1,3-propanediol (LCL464), Scheme 1.^{11,13,19} These analogs increased cytotoxicity of B13, decreased cellular Sph and specifically increased C_{14} -, C_{16} -, and C_{18} -

Cer. Unfortunately, LCL204 also caused induction of lysosomal destabilization, resulting in a proteolytic degradation of ACDase very early in the process.^{12,19} Additional structural modifications toward B13 have been carried out by others.^{25,26} Some analogs showed improved effects on ACDase inhibition in an *in vitro* assay as compared to B13. Interestingly, *in vitro* results of B13 action on ACDase and neutral CDase (NCDase) showed a similar and low inhibition on both isoforms of CDase.²⁶ Thus, the current work also reevaluates B13 specificity to ACDase inhibition.

The goal of the present study is the “lysosomal delivery” of B13 via its DMG-modified prodrugs, Scheme 2, which are expected to cause B13 accumulation in lysosomes, given the ionizable amines, and inhibition of lysosomal ACDase. This approach represents a novel concept for targeted drug release into a specific cellular compartment.

In this manuscript we discuss design and present synthesis of DMG-B13 prodrugs. Cellular metabolism of DMG-B13 prodrugs, specificity to cellular ACDase, and early effects on Sph-Cer balance and lysosomal targeting are studied in MCF7 cells where ASAHI has a predominant cytoplasmic localization in contrary to the nuclear compartment where ASAHI was detected at low levels only.⁵ Results are compared to the action of B13.

2. RESULTS AND DISCUSSION

2.1. Evaluation of B13 as ACDase inhibitor

Our *in vitro* results showed a dose-dependent effect of B13 on ACDase activity when an acidic MCF7 cell lysate was used as an enzymatic source ($Y=2.267-0.02047X$, $R^2=0.9627$), IC_{50} 27.7 μ M, Fig.1A). 50 μ M B13 almost completely inhibited activity of this enzyme (95%).

NCDase and AlkCDase activity results showed no inhibitory effects of B13 on those enzymes at concentrations ranging from 1–50 μ M (Fig.1B). In addition, our results indicate that B13, up to 250 μ M, did not inhibit those enzymes (not shown), thus indicating the specificity of B13 to ACDase and corroborating our previously published results.^{16,27} B13 action on NCDase seems to contradict recently published results²⁶ that show almost equal inhibitory effects of B13 on the acid and the neutral CDases (~30%/80 μ M).

In MCF7 cells, there was little change in the *cellular* activity of ACDase except at high concentrations (~ 50% C inhibition /50 μ M/5h and 20–30% inhibition/50 μ M, 24h), as shown in Fig. 1C. These results were paralleled by decreased level of α -ACDase protein (~40% C/ 50 μ M/5h, western blot, not shown) suggesting that B13 may affect ACDase stability non-specifically.

Functionally, there was a moderate effect of B13 on cellular Sph (Fig. 1D). A decrease in Sph levels was observed only for the high concentration (30 μ M or higher). If the bulk of Sph derives from the action of ACDase, these results indicate relatively poor effectiveness of B13 on ACDase in MCF7 cells. Furthermore, the strongest effects on Sph by B13 at 5h were partially recovered after a prolonged incubation with this compound (70% inhibition/5h to 40% inhibition/24h as observed for 50 μ M B13). B13 applied at a low concentration had no

effects on cellular Cer whereas 50 μ M B13 increased cellular Cer to 140% after 1h of treatment (data not shown) and to ~ 270% after 24 h.¹⁹ However, this high dose treatment was beyond the lethal dose for 24 h treatments.¹¹

In summary, B13 inhibited ACDase activity both *in vitro* and in cells but only at the high concentrations, affecting both Sph and Cer. Moreover, the effects of 50 μ M B13 on cellular Cer were not specific as to the Cer species, contrary to the effects of LCL204 and LCL464, our previously reported lysosomotropic inhibitors of ACDase.^{12,19}

Thus, facts of a high expression of the lysosomal ACDase versus relatively low expression of the nuclear ACDase⁵ and high levels of B13 in the nuclear fraction of MCF7 cells (data not shown) may suggest that B13 may rather cause a low efficiency effect on the nuclear form of this enzyme. Therefore, all these results prompted us to improve the cellular action of B13, i.e., increase its delivery and compartmental targeting to the lysosomes that may intensify B13's efficacy.

2.2. Principles of design and synthesis of the lysosomal B13-prodrugs through DMG modification

The prodrug approach is commonly used to improve the physicochemical and targeting properties of the parent drug. In general, a "prodrug" should represent a pharmacologically inactive chemical that can be converted into an active drug through metabolizing enzymes *in vivo*. Addition of a metabolically labile group should ultimately result in improvement of pharmacokinetics, safety, and therapeutic efficacy of the active compound.^{28–32}

Knowing that the whole B13 structure is essential for its inhibitory function, we decided to apply dimethyl glycine (DMG) as a lysosomotropic modifier (Scheme 2). The DMG-approach, previously reported for the lysosomal modification of other classes of bioactive alcohols, should release B13 from its hydrolysable DMG esters, thus improve B13 lysosomal targeting.^{33,34} DMG is a non-protein amino acid naturally found in animal and plant cell, which is produced as an intermediate in the metabolism of choline to glycine. DMG is formed from trimethylglycine (betaine) and is absorbed by the small intestine and transported by portal circulation to the liver. DMG is not toxic except at very high doses; the LD₅₀ of its hydrochloride salt is 3.9g/kg in rats. DMG has demonstrated the ability to enhance immune system function and to advance neurological function, as well as antiviral and antibacterial defenses.^{35,36}

In addition, DMG can form a hydrochloride salt, thus improving B13's solubility and its prodrug's stability. Scheme 2 depicts the synthetic approach and chemical structures of DMG-B13 prodrugs.

The B13 molecule contains two hydroxyl groups, the primary (3-OH) and the secondary (1-OH), providing possibilities for the formation of three distinct esters. Three different reaction sequences were then developed, and the following hydrochloride salts of B13 prodrugs were efficiently synthesized: 1,3-O, O-DMG-B13 (**LCL521**), 3-O-DMG-B13 (**LCL522**) and 1-O-DMG B13 (**LCL596**). We produced pure **LCL521** in 60% yields by coupling of B13 with excess of DMG in the presence of DCI and TEA or DIPEA in

anhydrous DCM, after the treatment of the formed free base of 1,3-O, O-DMG-B13 with HCl. **LCL522** was prepared in a similar fashion in 52% yield, but the proportions of key reagents were modified to drive esterification only on the primary hydroxyl group. In order to synthesize **LCL596**, a three-step procedure was developed. First, the 3-OH group of B13 was protected by the trityl group; then the DMG pro moiety was introduced into the 1-OH group. The protected 3-O-trityl mono ester of B13 was subjected to BF₃ ether solution in anhydrous methanol to give a free base of **LCL596**. Final treatment of the formed 1-O-DMG-B13 base with HCl produced **LCL596** in 36% overall yield.

As expected, the introduction of the DMG moiety into B13 markedly improved B13's water solubility. For instance, the solubility of LCL521 was ~430 mg/mL at 25°C versus only 8µg/mL for B13 under the same conditions.

2.3. Metabolism and uptake of DMG-B13 prodrugs in MCF7 cells

2.3.1. Cellular metabolism of the mono- and di-DMG-B13 prodrugs—Next, the ability of LCL521, 522 and 596 to be metabolized to B13 was evaluated (Figs. 2A and 2B). A time-dependent increase of B13 from LCL522 and 596 was observed up to 2–5 h when the released B13 had reached a plateau (Fig. 2A). Reciprocally, the mono-DMG B13 prodrugs were decreased in a time-dependent manner starting from 0.5 h and were almost completely eliminated after 5–10h of treatment.

Metabolism of di-DMG-B13, LCL521 was different from that of the mono-DMG analogs, as shown in Fig.2B. LCL521 (10 µM) was partially and slowly metabolized to B13 and unexpectedly to a mono-DMG intermediate, a metabolite dubbed CX (30%/15 min, of the total mixture), whose presence was noticed even for the longer treatments (Fig. 2B1). The level of cellular LCL521 was almost stable up to 2h, followed by its slow decrease; its presence was noticed for 24h of treatment (27 % of total mixture). B13 was increased slowly in a time-dependent manner, reaching 32% of total mixture/10h. CX represented a stable mono-DMG-B13 metabolite that was at plateau over the treatment time (50–70 units/0.25-10h)). LCL521 was a major component of the mixture up to 5 h of treatment. For 10h of treatment, all components of the mixture (LCL521, CX and B13) were at similar levels (~70–90 unit). CX represented a major metabolite of 1 µM LCL521 over the treatment time up to 10h (~80% of total mixture, Fig. 2B2).

2.3.2. Total cellular uptake of LCL521 as compared to the mono-DMG-B13 prodrugs

—Total cellular uptake of 10 µM LCL522, 596 and 521, representing the sum of all cellular components, is shown in Fig.2C. All prodrugs were very quickly taken up by the cells, with similar levels for the mono-DMG prodrugs but much higher levels for LCL521, the di-DMG-B13. Cellular uptake of LCL522 and LCL596 was at a plateau over the experimental time (15 min-24h); LCL521 showed a time-dependent small increase in its cellular uptake up to 2h, remained at plateau up to 10 h, and then decreased at 24h of treatment. Those results may indicate stronger cellular permeability and stability for LCL521, as well as greater stability of its major metabolite CX.

As shown in Fig.2D, the cellular level of B13 released from all 10 µM DMG- B13 prodrugs was at a comparable level of 10 µM B13 treatment. During early treatments (1–2h), cellular

levels of B13 from direct deliveries were higher than levels generated from prodrugs (e.g. B13/B13 (LCL521)/B13 (LCL522/B13 (LCL596); 50:25:25:25/0.25h). During later treatments (5 and 10h), cellular levels of B13 from direct delivery and B13 released from LCL521 were at comparable levels.

The results indicate that B13 modification by introduction of DMG ester moieties did not improve the overall cellular delivery of B13; however the following findings suggest that cellular (lysosomal) effectiveness of B13 was markedly improved.

2.3.3. Cellular transformation of LCL521 to metabolite CX—Comparative LC-MS/MS results of metabolite CX and the mono-DMG-B13 compounds, LCL522 and LCL596, showed identical fragmentation patterns recorded under the same applied conditions (mass transition: 508.1 – 405.1; loss of the DMG moiety, as well as chromatographic retention time: 2.53 min), which suggests that the cellular CX may represent a mixture of the mono-DMG-B13 regioisomers.

Although, as shown in Fig.2A, both LCL522 and LCL596 were quickly and almost equally metabolized to B13 (~ 90–95 % for 5h of treatment), the formation of a three-component mixture (LCL521, CX and B13) was always observed for LCL521 treatments with metabolite CX being present even for 24h time point (Fig.2B1 and 2B2).

Identification of the major and stable CX metabolite of LCL521 will require further investigation.

2.4. Evaluation of DMG-B13 prodrugs as ACDase inhibitors

2.4.1. Effects of DMG-B13 prodrugs on ACDases activities—The *in vitro* assay was performed with the free bases of 20 μ M and 50 μ M LCL596, LCL522, and LCL521 (Fig. 3A). As expected, prodrugs showed significantly lower inhibitory effects as compared to B13, especially for 50 μ M concentration demonstrating an importance of the free hydroxyl groups of B13 for enzymatic inhibition (Fig. 3A and Fig. 1A). In summary, the highest inhibition was observed for B13 (IC₅₀ ~30 μ M, Fig. 1A), whereas the other compounds had moderate effects only.

2.4.2. Effects of DMG-B13 prodrugs on cellular ACDase activity—We evaluated effects of these compounds on the *cellular* activity of ACDase (Fig. 3B) and ACDase protein (Fig. 3C) in MCF7 cells.

All 10 μ M DMG-B13 prodrugs caused early (1h) inhibitory effects on the *cellular* ACDase activity as shown in Fig. 3B. LCL521 acted as the most potent inhibitor, followed by LCL522 and LCL596, whereas B13 had only a small inhibitory effect. Interestingly, LCL521 applied at a lower concentration, 1 μ M, showed an effect similar to 10 μ M treatment, suggesting a high efficiency on this enzyme inhibition.

The early effects of 10 μ M DMG-B13 prodrugs on ACDase (as detected by the western blot via its cleaved α -subunit, α -ACDase) were investigated next (Fig. 3C). Results were compared to the effects of 10 μ M B13 and 10 μ M LCL204, our previous lysosomotropic

inhibitor (Scheme 1). LCL521 caused a time-dependent decrease of the α -ACDase form, whereas LCL522 and B13 had no effects, or rather slightly increased α -ACDase levels. LCL204 caused also a time-dependent decrease of the α - form, however, this effect was much lower and delayed comparing to the action of LCL521. Interestingly, 1 μ M LCL521 did not affect cellular ACDase protein (not shown). These results suggest differences among the cellular action of B13, the mono- and the di-DMG-B13 prodrugs and an accelerated response of ACDase to LCL521 in comparison to LCL204. Importantly, 1 μ M LCL521 acted as a potent inhibitor of cellular ACDase activity, whereas 10 μ M LCL521 had an additional, decreased affect on the α -form of this enzyme.

2.4.3. Effects of DMG-B13 prodrugs on the cellular Sph-Cer balance—The early effects (1h) on cellular Sph-Cer balance are shown in Fig. 4A and 4B. Unlike B13, a very strong decrease of Sph (~97-60%C) was observed after treatments with all DMG-B13 prodrugs. LCL521 was the most effective compound on decreasing cellular Sph (97% C/ 10 μ M and 80%C/1 μ M), Fig. 4A. However, cellular Sph was almost completely recovered (5h) after treatments with the mono-DMG-B13 prodrugs, whereas LCL521 maintained suppression (95%C/10 μ M and 50%/1 μ M), results not shown.

The mono-DMG-B13 prodrugs (10 μ M) showed almost no increase (100%C-105%C) in cellular Cer (Fig. 4B), nor effects on specific Cn-Cer species (not shown). On the other hand, 10 μ M LCL521 caused an increase in cellular Cer (130%/1h) with the preference to C₁₆-Cer (not shown). Furthermore, a correlation between decrease of Sph and increase of Cer can be found if results are expressed in absolute values (pmols). Control samples (1x10⁶ MCF7 cells) usually contain ~ 10 times more Cer (140 pmols) than Sph (~ 10 pmols). Summarizing results, a decrease of Sph by LCL522 and 596 (~4–6 pmols) was accompanied by an expected small increase of Cer, (~10 pmol). Whereas a fast and almost complete loss of cellular Sph by 10 μ M LCL521 (9 pmol) accompanied by increase of Cer (40 pmol) suggested a more profound action on cellular ACDase, it can also suggest a possible additional effect on other Cer related enzymes. Our results on the regulation of cellular key SPLs, Sph and Cer, showed a higher effect of the di-DMG-B13 prodrug over the mono-DMG-B13 prodrugs, which may require a higher concentration to reach efficiency of LCL521. Most importantly, these prodrugs showed an appropriate correlation on the cellular Sph-Cer balance.

In summary, corresponding results on the inhibition of cellular ACDase activity and decrease of Sph proved inhibitory effects of DMG-B13 prodrugs on cellular ACDase. LCL521 applied at a higher concentration, similarly to LCL204, affected also ACDase protein (decrease of the cleaved α - form of this enzyme).

2.4.4. Specificity of DMG-B13 prodrugs to the cellular ACDase—Results presented in the previous section showed the DMG-B13 prodrugs' action on the cellular ACDase but did not prove their specificity to this enzyme. For this reason, effects of a DMG-B13 prodrug, especially LCL521, and B13 on the cellular neutral and alkaline CDases, in addition to ACDase, were investigated as well. The increase of the cellular levels of Sph in response to the overexpression of NCDase (ASAH2) or AlkCDase (ACER2, one of ACERs) was used as the readout of cellular activity of these two enzymes. To over

express ASAH2 or ACER2, we used an inducible expression system (TET-ON) in which overexpression is ideally induced by tetracyclin (Tet), but not by its vehicle ethanol (Et) as described.⁴² We used HeLa cell lines that were stably transfected with the ASAH2- or ACER2-expressing plasmid or empty vector. Effects of the overexpression of those enzymes in HeLa cell lines: HeLa-ASAH2 and HeLa-ACER2 in comparison to HeLa-Vector cells, and effects of the treatments with B13, LCL521 and vehicle (Et) on the cellular Sph are shown in Fig.5.

Results showed that HeLa-ACER2 cells had a much higher levels of Sph (110 pmoles or 0.05/0.44 pmole/nmole Lipid Phosphate), Fig. 5A, and lower level of total Cer, (200 pmoles, not shown) than HeLa-Vector cells. These results suggest that overexpression of ACER2 increased the generation of Sph by hydrolyzing Cer in cells. Therefore, Vector and ACER2 cell lines were appropriate for testing if compounds inhibit ACER2 cellular activity.

In contrast, HeLa-ASAH2 cells had slightly lower levels of Sph than vector cells (Fig. 5A) whereas total Cer was increased (~ 1.7 fold, not shown), suggesting that NCDase may not contribute to the generation of Sph in HeLa cells. Therefore, HeLa-ASAH2 cells were not appropriate for testing our compounds. To test effects of DMG-B13 prodrugs and B13 on the cellular NCDase, we applied an approach similar to the cellular ACDase activity assay, e.g. using MCF7 cell neutral lysate for the enzymatic reaction (Fig. 6).

2.4.4.1. Effects on the cellular AlkCDase in HeLa-ACER2 cells: As shown in Fig. 5A, treatment with B13 did not affect the Sph increase by ACER2 overexpression, suggesting that this compound does not inhibit ACER2 cellular activity. This is consistent with the *in vitro* results (Fig. 1B). 1 μ M LCL521 slightly enhanced Sph increase, suggesting that at a low concentration, LCL521 does not inhibit ACER2 cellular activity. 10 μ M LCL521, however, slightly decreased the Sph level, suggesting that this compound at a high concentration may inhibit ACER2 activity. To further investigate this point, data are also expressed as a ratio of CDase/Vector (Fig. 5B) based on the fact that ACDase is present in both vector and HeLa-ACER2 cells and that strong and unique inhibitory effects of LCL521 on ACDase could be unexploited properly. And indeed, results showed the most increase of Sph from LCL521 treatment (2 folds/1 μ M and 5 folds/10 μ M) whereas B13 caused a slight decrease of Sph B13 (0.85 fold).

These results suggest that LCL521 did not inhibit cellular AlkCDase whereas B13 may have a very small inhibitory effect on this cellular enzyme.

2.4.4.2. Effects on the cellular NCDase in MCF7 cells: We used MCF7 cells and evaluated effects of 10 μ M DMG-B13 prodrugs on the *cellular* activity of NCDase after 1h of treatment (Fig. 6). LCL521 did not show inhibition of NCDase activity, whereas B13, LCL522 and LCL596 had a small, ~ 10–20% inhibitory effects on this enzyme.

In summary, LCL521 acted as a specific inhibitor of the cellular ACDase, whereas the mono DMG-B13 prodrugs, similarly as B13, affected also the cellular neutral and alkaline ceramidases, but with a low efficiency. These results may also suggest that metabolism of LCL522 and LCL596 to B13 may partially occur before they reach the lysosomes.

2.5. Lysosomal action of DMG-B13 prodrugs

DMG-B13 prodrugs, representing lipophilic amines, can accumulate in the lysosomes, a process known as lysosomal trapping.³⁷ They may affect ASMase whose lysosomal location is generally accepted.³⁸ To prove lysosomal action of DMG-B13 prodrugs, their cellular effects on the lysosomal pH changes and the lysosomal ASMase activity were investigated as well.

2.5.1. Effect on the lysosomal stability—The effects of 10 μ M DMG-B13-prodrugs on lysosomal stability were examined by LysoTracker Red (LTR) uptake, and results were also compared to the action of 10 μ M B13 and 10 μ M LCL204, a previously published lysosomal inhibitor of ACDase (FIG. 7A). The early, 1h treatments with LCL521 and LCL204 caused decrease of LTR uptakes (~ 80% C - 70% C, respectfully), whereas B13 and LCL522 had no decreasing effects. Extended, 5h treatments caused a complete recovery in LTR uptake by LCL521, small increasing tendency for LCL204 but below the control level, increase for LCL522 (to ~180% C), and no change for B13. These results suggest a lysosomal action of LCL521 and LCL522. Importantly, a lysosomal destabilization effect of LCL521 was not permanent in contrast to LCL204.

2.5.2. Effect on the lysosomal ASMase activity—The early effects, 1h treatments, with DMG-B13 prodrugs on ASMase activity in MCF7 cells are shown in FIG. 7B. Results showed inhibition only by LCL521 (85% C/10 μ M and 30% C/1 μ M, respectfully). These effects are comparable to the action of LCL204 (95% C/10 μ M). 10 μ M treatments with the mono-DMG-B13 analogs: LCL522, 596 as well as B13 did not inhibit activity of this enzyme.

These results suggest a lysosomal delivery of LCL521 whereas LCL522 and LCL596 may require applications at higher concentrations or a longer treatment time.

Moreover, LCL521 acted as inhibitor of the lysosomal ACDase and ASMase whereas LCL522 and LCL596 may be specific to the lysosomal ACDase.

3. SUMMARY AND CONCLUSIONS

Here, we report development of a compartmentally-targeted B13 prodrugs that lack efficacy on ACDase inhibition *in vitro* but are converted to the active metabolite in cells with significantly enhanced effectiveness on this enzyme.

Our results indicate that B13 is a specific inhibitor of ACDase showing almost complete inhibition of this enzyme at 50 μ M in an *in vitro* assay. We want to further emphasize the needs for the enzymatic assays standardization (enzymatic substrates and sources, and assay conditions) in order to provide reliable and comparable results.²⁶ The inhibitory effect of B13 on ACDase in MCF7 cells was noticed only at the high concentrations, which suggest that B13 is recognizable and can reach cellular ACDase. Future investigation of the compartmental effects of B13 on lysosomal versus nuclear ACDase is needed.

Application of the DMG prodrugs resulted in significant liberation of B13. There was a fast and complete release of B13 from the mono DMG prodrugs and a significantly slower release of B13 from di-DMG prodrug with persistent accumulation. Importantly, the generation of B13, which we assume occurs in the lysosome due to the ionizable amine on the prodrugs, was accompanied by a markedly improved cellular action of B13 that was observed from all DMG-B13 prodrugs, especially LCL521. Considering the cellular CDases, LCL521 showed specificity to the ACDase whereas the mono DMG-B13 prodrugs, in addition to the inhibition of the ACDase, had also a small effect on the NCDase, similar to B13, suggesting that their partial metabolism may occur before they reached the lysosomes. All DMG-B13 prodrugs caused an early decrease of cellular Sph, as opposed to the action of B13 itself. Our previous results specified that inhibition of ACDase by LCL204 and desipramine also resulted in very early and profound drop in the cellular Sph,^{19,39} thus, early effects on Sph may be partially taken as a measure of cellular inhibition of ACDase, presumably the lysosomal ACDase, based on fact that LCL521 inhibited also the lysosomal ASMase. We also predict that cellular B13, generated from DMG-B13 prodrugs, could be trapped in the lysosomes, nonetheless more effectively for LCL521. Alternatively, coupled with the unique detection of a metabolite CX, a stable mono DMG-B13, LCL521 may be involved in the formation of strong complexes with certain lysosomal proteins to play an important role in the early and persistent inhibition of ACDase. Therefore, appropriate validation of LCL521, a “lysosomal mixture” (B13+ CX +LCL521), is necessary to tune and further optimize the described drug targeting approach. Cell fractionation studies toward separation of lysosomes or labeling experiments with the appropriate fluorescent LCL521 and B13 analogs should be considered for the further exploration of the cellular action of LCL521. Importantly, our recently published collaborative results in a xenograft mice model of prostate cancer showed that LCL521 completely prevented tumor relapse after combination with ionization radiation.⁴⁰

Taken together, DMG-B13 prodrug modification to the DMG-B13 prodrugs, especially to LCL521, defines a novel approach to target enzymes of sphingolipid metabolism in a compartment-specific manner.

4. EXPERIMENTAL

Material and Methods

4.1. Chemistry

4.1.1. Chemicals: All solvents and general reagents were purchased from Sigma-Aldrich and Fluka. [9,10-³H] Palmitic acid was purchased from American Radiolabeled Chemical, Inc. (St. Louis, MO). (2S, 3R, 4E) [³H] C₁₆-Cer (specific activity: 4x10⁶ cpm/nmol) and (2S, 3R, 4E) C₁₆-Cer were synthesized in the Lipidomic Facility, Medical University of South Carolina. B13 and LCL204 were synthesized by Lipidomics Facility Synthetic Unit, MUSC, as described before.^{11,14} DMG-B13 prodrugs, LCL521, 522 and 596, used in the study were synthesized by the Lipidomics Facility, Synthetic Unit, MUSC. Synthetic outline for the preparation of B13 prodrugs is shown in Scheme 2.

TLC, ^1H NMR, EA and MS confirmed purity and conformation of the synthesized compounds. Reaction progress was monitored by analytical normal phase thin layer chromatography (NP TLC) using aluminum sheets coated with 0.25 mm Silica Gel 60-F₂₅₄ (Merck). Detection was performed by fluorescence quenching at 254 nm with the PMA reagent (ammonium heptamolybdate tetrahydrate to cerium sulfate, 5:2, w/w in 125 ml of 10% H_2SO_4). Flash chromatography was performed using EM Silica Gel 60 (230–400 mesh) in combination with the indicated solvent systems. NMR spectra were recorded on Bruker AVANCE 400 or 600 MHz spectrometer equipped with Oxford Narrow Bore Magnet. Chemical shifts were reported in ppm on the δ scale from the internal standard of residual chloroform (7.27 ppm) and methanol (4.87 ppm). Mass spectral data were recorded in a positive ion electrospray ionization (ESI) multiple reaction monitoring (MRM) mode on Thermo Fisher TSQ Quantum/Accela LC/MS/MS liquid chromatography/ triple quadrupole mass spectrometry system. Samples in a methanol solution were injected through Accel autosampler. ESI voltage of 4.5 kV and capillary temperature of 200°C were applied throughout the analyses. Specific structural features were established by fragmentation pattern upon electrospray (ESI/MS/MS) conditions, specific for each of the compound studied.

Synthetic compounds: B13, LCL521, 522 and 596 used in the study were synthesized by the Lipidomics Facility, MUSC (Scheme 2)

4.1.2. Synthesis and characterization of DMG-B13 ester prodrugs

4.1.2.1. (1R, 2R)-2-N-(tetradecanoylamino)-1-(4'-nitrophenyl)-propyl-1, 3 -O, O-di-(N,N-dimethylamino) acetate dihydrochloride (1,3-di-DMG-B13, LCL521): The synthesis was carried out according to established methods with small modification.⁴¹ Briefly, N, N-dimethyl glycine (103mg, 1mmol) and 1,1'-carbonyldiimidazole (162mg, 1mmol) were mixed in anhydrous CH_2Cl_2 (10mL) and stirred for 2 hour before adding B13 (42.3mg, 0.1mmol) followed by a small amount of either triethyl amine (TEA) or diisopropylethylamine (DIPEA). The reaction mixture was stirred at room temperature until there was no trace of starting material. The product was evaporated to dryness, and the residue was purified through column chromatography using chloroform-methanol-ammonium hydroxide conc. (15: 1: 0.025; v/v/v) to isolate wax solid freebase. Then, at 4°C, 2M hydrochloride ether solution was added drop-wise, with mixing, into the obtained freebase ethyl acetate solution. The reaction mixture was maintained at 4°C until TLC showed no trace of starting material and kept at the same condition for another 30min before evaporation to dryness. The residue was further dried down under high vacuum. Target hydrochloride salt was recrystallized from ethyl acetate to give 40mg (60%) of LCL521 as a light yellow microcrystalline powder. TLC (CHCl_3 - CH_3OH - $\text{NH}_3\cdot\text{H}_2\text{O}$ conc., 10:1:0.1, v/v/v), R_f = 0.63; $[\alpha]_D^{23.6}$ -5.12 (c 0.5, CH_3OH), ^1H NMR (400 MHz, CD_3OD); 8.29 (d, J =8.8 Hz, 2H, ArH), 7.70 (d, J =8.8 Hz, 2H, ArH), 6.27 (d, J =4.0 Hz, 1H, CONH), 4.80 (m, 1H, CH), 4.59 (dd, J =5.2, 7.2 Hz, 1H, CH), 4.44 (s, 2H), 4.22 (dd, J =17.0, 8.1Hz, 4H, COCH_2N), 3.01 (s, 12H, $2\text{N}(\text{CH}_3)_2$), 2.21 (m, 2H, CH_2), 1.48 (m, 2H, CH_2), 1.00–1.40 (m, 20H), 0.93 (t, J =6.6 Hz, CH_3); ^{13}C -NMR (150.9 MHz, CD_3OD) δ 175.15, 165.61, 164.94, 148.00, 143.38, 127.60, 123.31, 75.45, 64.63, 56.94, 56.79, 51.26, 43.41, 35.47, 31.68, 29.39, 29.36, 29.25, 29.14, 29.07, 28.88, 25.49, 22.34, 13.05; ESI-MS (CH_3OH , relative

intensity, %): m/z calcd. for $C_{31}H_{53}N_4O_7$, 593.34. Found: 593.40 ($[MH^+-2HCl]$, 60). Anal. Calcd for $C_{31}H_{54}Cl_2N_4O_7 \cdot 2H_2O$ (701.71) C, 53.06; H, 8.33; N, 7.98; Cl, 10.10. Found: C, 52.52; H, 8.35; N, 7.76; Cl, 10.95.

4.1.2.2. (1R, 2R)-2-N-(tetradecanoylamino)-1-hydroxyl-1-(4'-nitrophenyl)propyl-3-O-(N,N-dimethylamino) acetate hydrochloride (3-DMG-B13, LCL522): This compound was prepared using the same method as LCL521, except the ratio of N, N-dimethyl glycine to B13 was 1.0 : 1.1. Pure LCL522 was obtained from its corresponding free base in overall 52% yield after crystallization from *n*-hexane–ethyl acetate (1:4; v/v) as a light yellow waxy solid. TLC (chloroform-methanol-ammonium hydroxide conc., 10 : 1 : 0.1; v/v/v), R_f = 0.39; $[\alpha]_D^{22.9}$ -10.7 (c 0.5, CH_3OH) 1H NMR (400 MHz, CD_3OD) δ 8.22 (d, J =8.8 Hz, 2H, ArH), 7.68 (d, J =8.8 Hz, 2H, ArH), 5.11 (d, J =2.5 Hz, 1H, CH), 4.67 (dd, J =5.2, 5.2 Hz, 1H, CH), 4.54 (m, 1H, CH), 4.33 (dd, J =8.3, 8.3 Hz, 1H, CH), 4.14 (Q, J =17 Hz, 2H, $COCH_2N$), 2.99 (s, 6H, $N(CH_3)_2$), 2.95 (t, J =7.5 Hz, CH_2), 2.10 (m, 2H, CH_2), 1.10–1.40 (m, 20H), 0.93 (t, J =7.0 Hz, CH_3); ^{13}C -NMR (150.9 MHz, CD_3OD) δ 176.71, 167.37, 151.27, 148.80, 128.43, 124.26, 71.94, 67.55, 58.16, 54.36, 44.66, 36.89, 33.18, 30.88, 30.82, 30.68, 30.60, 30.57, 30.14, 27.05, 23.84, 14.53; ESI-MS (CH_3OH , relative intensity, %): m/z calcd for $C_{27}H_{46}N_3O_6$, 508.34. Found: 508.40 ($[MH^+-HCl]$, 70). Anal. Calcd for $C_{27}H_{46}ClN_3O_6H_2O$ (562.1) C, 57.7; H, 8.6; N, 7.48; Cl, 6.31. Found: C, 56.7; H, 8.14; N, 7.00; Cl, 7.70.

4.1.2.3. (1R, 2R)-2-N-(tetradecanoylamino)-1-(4'-nitrophenyl)-3-hydroxylpropyl-1-O-(N,N-dimethylamino) acetate hydrochloride (1-DMG-B13, LCL596)

(A) (1R, 2R)-2-N-(tetradecanoylamino)-1-(4'-nitrophenyl)-1-hydroxylpropyl-3-O-trityl (3-Tr-B13): To the stirred solution of B13 (422.5mg, 1mmol) in anhydrous CH_2Cl_2 (100mL), we added trityl chloride (250.9mg, 0.9mmol) and a catalytic amount of DMAP. The reaction was stopped when trace amount of trityl chloride was observed. After evaporation, the residue was purified by flush column chromatography (ethyl acetate-hexane, 1:2; v/v) to obtain 600.1mg (90.3%) of pure 3-Tr-B13 as light yellow oil. R_f =0.35; $[\alpha]_D^{20.9}$ -24.7 (c0.5, CH_3OH). 1H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, J =8.8 Hz, 2H, ArH), 7.44 (d, J =8.4 Hz, 2H, ArH), 7.25–7.40 (m, 15H, ArH), 5.97 (d, J =8.4 Hz, 1H, CONH), 5.06 (t, J =3.6, 4.0 Hz, 1H, CH), 4.20 (m, 1H, CH), 3.96 (d, J =3.6 Hz, 1H, OH), 3.43 (dd, J =4.8, 4.8 Hz, 1H, CH), 3.33 (dd, J =3.6, 3.6 Hz, 1H, CH), 2.06 (td, J =7.6, 2.0 Hz, 2H, CH_2), 1.13–1.48 (m, 20H), 0.86 (t, J =7.2, 6.4 Hz, 3H, CH_3).

(B) (1R, 2R)-2-N-(tetradecanoylamino)-1-(4'-nitrophenyl)-3-O-trityl-propyl-1-O-(N,N-dimethylamino)acetate (1-DMG-3-Tr-B13): N, N-dimethyl glycine (148.5mg, 1.44mmol) and 1,1'-carbonyldiimidazole (233.5 mg, 1.44mmol) were mixed in anhydrous CH_2Cl_2 (30 mL) and stirred for 2 hours under the exclusion of moisture before adding 3-Tr-B13 (320mg, 0.48 mmol), followed by a small amount of either TEA or DIPEA. The reaction was stopped when a trace amount of starting material was observed (min. 24h). The solvent was evaporated to dryness, residue was purified by the column chromatography (ethyl acetate-hexane-TEA, 1: 1: 0.04; v/v/v) to get pure waxy solid of 1-DMG-3-Tr-B13 221.7mg (61.4%). 1H NMR (400 MHz, CD_3OD) δ 8.05 (d, J =8.8 Hz, 2H, ArH), 7.38 (d, J =8.8 Hz, 2H, ArH), 7.23–7.36 (m, 15H, ArH), 6.28 (d, J =7.2 Hz, 1H, CONH), 6.06 (d, J =9.4 Hz, 1H,

CH), 4.53 (m, 1H, CH), 3.25 (dd, $J=4.8, 4.8$ Hz, 1H, CH), 3.16 (d, $J=1.3$ Hz, 2H, CH₂), 2.88 (dd, $J=3.2, 3.2$ Hz, 1H, CH), 2.30 (s, 6H, N(CH₃)₂), 2.14 (dt, $J=7.4, 2.1$ Hz, 2H, CH₂), 1.56 (m, 2H, CH₂), 1.25 (m, 22H), 0.88 (t, $J=6.6$ Hz, 3H, CH₃)

(C) (1R, 2R)-2-N-(tetradecanoylamino)-1-(4'-nitrophenyl)-3-hydroxypropyl-1-O-(N,N-dimethylamino)acetate hydrochloride (1-DMG-B13, LCL596): To a stirred and cooled up to +4°C solution of 1-DMG-3-Tr-B13 (51 mg, 0.07 mmol) in dry CH₂Cl₂ (10 mL), Et₂O·BF₃ (15 µL) was added. The formed reaction mixture was stirred for 1 hr at r.t. The solvent was evaporated to dryness, and the obtained residue was purified by column chromatography (chloroform-methanol-ammonium hydroxide conc., 10 : 1 : 0.1, v/v/v) to give 31 mg (91%) of pure 1-DMG-B13 free base as a pale yellow waxy solid. TLC (ethyl acetate-methanol, 11 : 1, v/v); $R_f = 0.22$; $[\alpha]_D^{25} -23.9$ (c 0.5, CHCl₃). This material was dissolved in anhydrous ethyl acetate (4 mL), and 2M HCl solution in a dry diethyl ether (0.3 mL) was added dropwise at +4°C. The reaction mixture was stirred at +4°C for an additional 30 min. The mixture was evaporated under reduced pressure to dryness. Residue was dried down under high vacuum. The crude hydrochloride was recrystallized from dry ethyl acetate-hexane (3:1, v/v) to give 24 mg (72%) of LCL-596 as a pale yellow microcrystalline powder. TLC (chloroform-methanol-ammonium hydroxide conc., 10 : 1 : 0.1, v/v/v), $R_f = 0.33$; $[\alpha]_D^{22.3} +1.26$ (c 0.55, CH₃OH) ¹H NMR (400 MHz, CD₃OD) δ 8.22 (d, $J=8.8$ Hz, 2H, ArH), 7.69 (d, $J=8.8$ Hz, 2H, ArH), 5.13 (d, $J=2.6$ Hz, 1H, CH), 4.64 (dd, $J=5.4, 5.4$ Hz, 1H, CH), 4.54 (m, 1H, CH), 4.33 (dd, $J=2.8, 7.9$ Hz, 1H, CH), 4.20 (dd, $J=17.0, 13.4$ Hz, 2H, NCH₂), 3.02 (s, 6H, N(CH₃)₂), 2.11 (m, 2H, CH₂), 1.00–1.40 (m, 20H), 0.93 (t, $J=7.0$ Hz, 3H, CH₃); ¹³C-NMR (150.9 MHz, CD₃OD) δ 175.17, 165.7, 149.82, 147.25, 126.99, 122.75, 70.38, 65.85, 56.61, 52.87, 43.19, 43.17, 35.41, 31.69, 29.40, 29.37, 29.35, 29.33, 29.19, 29.11, 29.08, 28.66, 25.56, 22.35, 13.07; ESI-MS (CH₃OH, relative intensity, %): m/z calcd for C₂₇H₄₆N₃O₆, 508.34. Found: 508.35 ([MH⁺-HCl], 70); Anal. Calcd for C₂₇H₄₆ClN₃O₆ (544.1) C, 59.6; H, 8.52; N, 7.72; Cl, 6.52. Found: C, 58.2; H, 8.31; N, 7.24; Cl, 6.98.

4.2. Biology

4.2.1. Cell Culture: MCF7 human breast adenocarcinoma cell line was obtained from ATCC (Manassas, VA), cultured in complete medium (RPMI 1640 medium supplemented with 10% FBS and 100 µg/ml Normocin), and maintained under standard incubator condition (humidified atmosphere with 5% CO₂ at 37°C). Cells in the exponential growth phase were harvested from the culture and used in all experiments. HeLa T-Rex cells stably expressing human alkCDase 2 (ACER2), or NCDase (ASAH2) or empty vector, which previously generated in the lab, were maintained in MEM medium as described.⁴²

4.2.2. In vitro Acid Ceramidase (ACDase) Activity Assay: The ACDase activity assay was processed under acidic conditions (MCF7 cells lysed, pH 4.5) as previously described.¹³ Briefly, protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL), equal amounts of [9,10-³H] D-e-C₁₆-Cer and test compound were mixed with 0.2% TritonX-100 and 0.4% cholate and dried down under nitrogen followed by additions of equal amount of the protein and assay buffer (0.2 M acetate buffer, 0.5% Triton X-100, pH 4.5). Assay was implemented at 37°C for an hour and terminated by addition of Dole's alkaline solution. [9,10-³H] palmitic acid, a hydrolytic

product of ACDase was extracted and counted to calculate the enzyme activity. Radioactivity was quantified using LS 6500 multipurpose scintillation counter (Beckman Coulter).

4.2.3. *In vitro* Neutral Ceramidase (NCDase) Activity Assay: The NCDase activity assay was processed as previously described with a minor modification.^{27,43} Briefly, enzyme activity was measured using D-e-C₁₂-NBD-Cer at a final concentration of 50μM in a total volume of 100μl (25mM Tris buffer, 0.3% Triton-X100, pH 7.5). The enzymatic reaction was started by addition of 10μg protein of HEK293 cells that previously overexpressed NCDase. Reaction mixtures were incubated at 37 °C for 1 h before terminated by the addition of 300μl chloroform-methanol (1:1, v/v). Organic phase was spotted on TLC plate and then developed with chloroform-methanol-25% ammonia (90:20:0.5, v/v/v) to separate NBD-C₁₂-Cer from NBD-dodecanoic acid. Rate of conversion of the fluorescent-labeled substrate and product was analyzed and quantified with a PhosphorImager Storm 860) system. Results were quantified by densitometric analysis using Image Quant software.

4.2.4. *In vitro* Alkaline Ceramidase (AlkCDase) Activity Assay: The AlkCDase activity assay was processed as described previously.⁴² Briefly, microsomes of HeLa cells overexpressing ACER2 were incubated with D-e-C₁₈:1- Cer substrate (150μM) and the various amount of test compound in the assay buffer (25mM Tris-HCl, 5mM CaCl₂ and MgCl₂, 0.15% Nonidet P-40, pH 8.5) in a total volume of 40μl. The reaction mixtures were incubated at 37°C for 30min and stopped by extraction with chloroform-methanol (2:1, v/v). To determine the enzyme activity, the level of Sph released from Cer by the AlkCDase action was measured in lipid extract by HPLC after OPA derivatization using 17C-D-e-Sph as an internal standard.

4.2.5. Cellular activity of Acid Ceramidase (ACDase) Assay: The cellular ACDase activity was measured as previously described.^{13 12} Briefly, MCF7 cells were seeded in 100mm dishes overnight before being treated accordingly. Cells were then washed with cold PBS and lifted by gently scraping followed by the in vitro assay protocol described under 4.2.2.

4.2.6. Cellular activity of Neutral Ceramidase (NCDase) Assay: Briefly, MCF7 cells were seeded in 100mm dishes overnight before being treated accordingly. Cells were then washed with cold PBS and lifted by gently scraping followed by cell pellet lysed under the neutral condition, and protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL). The NCDase activity assay was processed using equal amounts of [9,10-³H] D-e-C₁₆-Cer mixed with 0.3% TritonX-100, and dried down under nitrogen. Lipid film was then homogenized with water, followed by additions of equal amount of the protein and assay buffer (50mM Tris-HCl, 0.3% Triton X100, 1mM CaCl₂, pH 7.1). Reaction was incubated at 37°C for an hour and terminated by Dole-s alkaline solution. [9,10-³H] palmitic acid, a hydrolytic product of NCDase, was extracted and counted to calculate the enzyme activity. Radioactivity was quantified using LC6500 multipurpose scintillation counter (Beckman Coulter).⁴³

4.2.7. Cellular activity of Alkaline Ceramidase (ACER2) or Neutral Ceramidase

(ASAH2) Assays: HeLa cells overexpressing ASAH2, ACER2, or empty vector were grown to a 60% confluence before they were treated with tetracycline (5 ng/ml) overnight. Next day, the cells were then treated with B13 (10 μ M), LCL521 (1 or 10 μ M), or ethanol (the vehicle control for both B13 and LCL521) for 1 h before they were harvested and processed as described in the previous studies⁴². Sphingolipids were extracted from these cell samples and were analyzed by LC-MS/MS.

4.2.8. Cellular activity of Acid Sphingomyelinase (ASMase) Assay: The assay was processed as described previously using MCF7 cell lysates.⁴⁴ Briefly, following treatment, cells were washed twice in cold PBS and scraped in ice-cold lysis buffer (0.2% Triton X-100, 50 mM Tris-HCl, pH 7.4, 1:200 phosphatase inhibitor mixtures 1 and 2, 1:200 protease inhibitor mixture, and 1.0 mM EDTA). Lysates were sonicated briefly (2 x 10 s pulses), and cellular debris and unbroken cells were pelleted by centrifugation at 1,000 x g for 1 min at 4 °C. Equal amounts of clarified lysate (in a total volume of 100 μ l lysis buffer) were added to 100 μ l of reaction mixture containing 100 μ M porcine brain SM and 1 x 10⁵ cpm of choline-[methyl-¹⁴C] SM (specific radioactivity = 1.5 x 10⁵ cpm/ μ l) presented in micelles containing 0.2% Triton X-100 in sodium acetate buffer (250 mM, pH 5.0) supplemented with 1.0 mM EDTA. Assays were run for 30 min at 37 °C, and the reaction was stopped by adding 1.5 ml of chloroform-methanol (2:1, v/v) followed by addition of 0.4 ml water (Folch extraction). Phases were separated by centrifugation at 2000g for 5 min at room temperature. 800 μ l of upper (aqueous) phase was counted by scintillation. The assay was linear with respect to time and protein/volume, and substrate hydrolysis was less than 10%.

4.2.9. Acid Ceramidase Protein Expression by Western Blot: ACDase protein expression was performed as previously described.¹³ Briefly, MCF7 cells were seeded in 100mm dishes overnight and treated accordingly. Then cells were washed with cold PBS and lifted by gently scraping the plates. Cells were then lysed with regular lysate buffer containing protease inhibitors. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Cell lysates corresponding to 35 μ g of proteins per sample were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked for one hour at room temperature in TBS containing 0.1% Tween-20 and 5% non-fat dry milk, and incubated overnight at 4°C with the primary antibody mouse monoclonal anti-ACDase (Pharmingen, San Diego, CA). After washing 3 times with TBS-T, membranes were incubated 1 hour with the secondary antibody goat anti-mouse IgG-HRP conjugate (Sigma, St. Louis, MO). Membranes were then washed 3 times with TBS-T, and protein bands were visualized with the super signal HRP substrate (Pierce, Rockford, IL). For each sample, the density of ACDase protein bands were normalized by the actin band, and results were expressed as % Control.

4.2.10. Lysosomal Stability Assay: Lysosomal stability was measured using the LysoTracker Red (LTR) dye DND-99 (Molecular probes, Oregon).⁸ Briefly, MCF7 cells were seeded 5x10⁵/well in 6 well-plate overnight. Media were then replaced with 2% FBS

which contained the same amount of either ethanol or the test compounds in ethanol solution (ethanol, 0.1%) and incubated at 37°C for 1 and 5 hours before adding 200nM LTR for 30 mins at 37°C. Then, cells were lifted with trypsin, washed twice with PBS, and re-suspended in 0.5ml PBS. LTR fluorescence was measured using FACS analysis (564–606nm). Decrease in the fluorescence intensity corresponded to the increase in the lysosomal pH value, and a minimum of 10,000 events were scored for each sample. Each dose was done in triplicate. Results were expressed as % Control.

4.3. HPLC-MS/MS Analysis—Advanced analyses of endogenous Sph and Cers and intracellular B13 and DMG-B13 prodrugs were performed on ThermoFisher TSQ Quantum liquid chromatography/ triple-stage quadrupole mass spectrometer (LC/MS/MS) system, operating in a multiple reaction monitoring (MRM) positive ionization mode, as previously described.⁴⁵ Briefly, after the treatment, culture media was removed and cells were washed two times with cold PBS before gently scraping the plate. Cell pellets were fortified with the internal standards ISs: 17C base *D-erythro*-sphingosine: 17Sph, 17S1P, 17C₁₆-Cer, 17C_{24:1}-Cer, 13C base *D-erythro*-sphingosine: 13C₁₆-Cer and 18C base *D-erythro*-sphingosine: 18C₁₇-Cer, then extracted into a one-phase solvent system with ethyl acetate/iso-propanol/water (60/30/10% v/v). Lipid extract was divided into two portions and used for MS analysis and for determination of the level of lipid phosphate after further re-extraction by Bligh and Dyer method (used for MS data normalization).⁴⁶ Original lipid extracts, after evaporation and reconstitution in 100 µl of acidified (0.2% formic acid) methanol, were injected on the Accela/TSQ Quantum LC/MS/MS system and gradient-eluted from the BDS Hypersil C8, 150 x 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 system.

Quantitative analyses were based on the calibration curves generated by spiking an artificial matrix with known amounts of the target synthetic standards and an equal amount of the internal standard. The target analyte peak area ratio 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 levels of the particular compound (pmols) normalized to cellular Lipid Phosphate, Pi (nmols). Changes were expressed in comparison to the control samples representing treatments with the equal amount of ethanol only.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

B13	(1R, 2R)-2-(tetradecanoylamino)- 1-(4'-nitrophenyl)-1, 3-propandiol
DMG	N, N-dimethyl glycine
LCL521	1,3-O, O-DMG-B13 dihydrochloride
LCL522	3-O-DMG-B13 hydrochloride
LCL596	1-O-DMG-B13 hydrochloride
CX	stable mono-DMG-B13
ACDase	acid ceramidase
NCDase	neutral ceramidase
AlkCDase	alkaline ceramidase
ASM	acid sphingomyelinase
Sph	sphingosine
Cer	ceramide
C_n-Cer	N-acyl-ceramide species
S1P	sphingosine 1-phosphate
dhSph	dihydrosphingosine
dhCer	dihydroceramide
DCI	1,1'-carbonyldiimidazole
DCM	dichloromethane
TEA	triethyl amine
DIPEA	diisopropylethylamine

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Fig. 1. Effects of B13 on CDases' activities in an *in vitro* assays and at the cellular levels in MCF7 cells

All results are presented as mean $\pm$ st dev. of duplicates. * p value < 0.05 (vs Control).

A. ACDase activity in an *in vitro* assay. Results are expressed as pmol of released [3 H] palmitic acid /mg protein/h, and are presented as % Control. **B. NCDase and AlkCDase activities, in an *in vitro* assays.** NCDase results are expressed as a rate of the conversion of the fluorescence released from NBD- C_{12} -Cer to NBD-dodecanoic acid, and are presented as %Control. AlkCDase results are expressed as pmol of cellular Sph released from D-e- $C_{18:1}$ -Cer using microsomes of HeLa cells overexpressing ACER2, and are presented as % Control. **C. Cellular ACDase activity assay.** After treatment with B13 or vehicle control, as was described in the text, cells were isolated and ACDase activity was measured by *in vitro* assay as described under A. Results are expressed as pmol of released [3 H] palmitic acid/mg protein/h, and are presented as % Control. **D. Release of cellular Sph by B13.** Results are expressed as Sph (pmol) /nmol Lipid Phosphate, and are presented as % Control.

Fig. 2. Metabolism and uptake of DMG-B13 prodrugs in MCF7 cells, time course
MCF7 cells were treated with either vehicle or 10 μ M inhibitors for 15min, 1, 2, 5, 10, 15 and 24h. Cellular levels of each compound were quantified by LC-MS/MS analysis of lipid extracts from cell pellets. Results are presented as mean $\pm$ st dev. of duplicates.

A. Metabolism of 10 μ M mono-DMG B13-prodrugs, LCL522 and LCL596. Results are expressed as pmol of cellular LCL522, LCL596 and B13/ nmol of Lipid Phosphate. **B.**

Metabolism of di-DMG B13-prodrug, LCL521. B1. 10 μ M LCL521; B2. 1 μ M LCL521.

Results are expressed as pmol of LCL521, CX and B13/nmol Lipid Phosphate. **C. Total cellular uptake of 10 μ M DMG-B13 prodrugs.** Results represent the sum of all cellular components of DMG-B13 prodrugs or B13 itself and are expressed as levels of compounds (pmol)/nmol Lipid Phosphate. **D. Comparison between the cellular levels of B13 released from 10 μ M DMG-B13 prodrugs and 10 μ M B13 applied directly.** Results are presented as level of B13 (pmol)/nmol Lipid Phosphate.

C1.

Fig. 3. Effects of DMG-B13 prodrugs on ACDase activity, in an *in vitro* assay, and in MCF7 cells
A. ACDase activity in an *in vitro* assay. Results are expressed as pmol of released [^3H] palmitic acid/mg protein/h, and presented as % Control. Results are presented as mean $\pm$ st dev. of duplicates; * p value < 0.05 (vs control). **B. Cellular ACDase activity assay.** After treatment with inhibitors or vehicle control for 1h, as was described in the text, cells were isolated and ACDase activity was measured through *in vitro* assay as described under A. Results are expressed as pmol of released [^3H] palmitic acid/mg protein/h, and presented as % Control. Results are presented as mean $\pm$ st dev. of quadruplicates; * p value < 0.05 (vs control), ** p value < 0.05 (vs B13). **C. ACDase protein level.** MCF7 cells were treated with either 10 μM DMG-B13 prodrugs, B13 or LCL204 for 1h and 5h. ACDase protein expression was visualized by western blot as described. The density of the α -ACDase protein bands was normalized to the actin, and results were expressed as % Control. **C1.** Western blots **C2.** Fluorescence densities normalized to the actin * p value < 0.05 (vs Control).

Fig. 4. Effects of DMG-B13 prodrugs on the cellular Sph-Cer balance

MCF7 cells were treated with B13, B13 prodrugs or vehicle control for 1 h as was described in the text. Cell pellets were isolated, lipids were extracted and cellular levels of Sph and Cer were measured by LC-MS/MS approach. **A. Effect on cellular Sph.** Results are expressed as pmol Sph /nmol Lipid Phosphate, and are presented as % Control. All results are presented as mean± st dev. of duplicates, * p value < 0.001 (vs Control); ** p value < 0.001 (vs B13). **B. Effect on cellular Cer.** Results are expressed as pmol total Cer/nmol Lipid Phosphate, and are presented as % Control. All results are presented as mean± st dev. of duplicates, * p value < 0.05 (vs Control)

Fig. 5. Specificity of DMG-B13 prodrugs to the cellular ACDase. Effects on the AlkCDase and the NCDase activities

The increase in the cellular levels of Sph in response to the overexpression of the NCDase or AlkCDase in HeLa cells: ASAH2-HeLa or ACER2-HeLa, respectively, was used as the readout of cellular activity of these two enzymes, as was described in the text. After treatments for 1h, cell pellets were isolated, lipids were extracted and cellular levels of Sph and Cer were measured by LC-MS/MS approach. **A. Effects on the cellular Sph.** Results are expressed as pmol Sph /nmol Lipid Phosphate. **B. Effects on the ratio of the cellular Sph in ACER2-HeLa or ASAH2-HeLa cells versus Vector-HeLa cells.** Results are expressed as a ratio of the levels of Sph (pmol) normalized to Lipid Phosphate (nmol).

Fig. 6. Effect of DMG-B13 prodrugs on the cellular activity of NCDase in MCF7 cells. Cellular NCDase activity assay

After MCF7 cell treatment with inhibitors or vehicle control for 1h, as was described in the text, cells were isolated and NCDase activity was measured by *in vitro* assay as described in the text. Results are expressed as pmol of released [^3H] palmitic acid/mg protein/h, and presented as % Control. Results are presented as mean $\pm$ st dev. of duplicates.

Fig. 7. Lysosomal action of DMG-B13 prodrugs

A. Effect on the lysosomal pH changes. MCF7 cells were treated with either inhibitors or vehicle control for 1 and 5h before incubation with Lysotracker Red (LTR, tracking of acidic organelles) for additional 30min. Fluorescence relative intensity was measured by FACS analysis (564-606nm). Decrease in the fluorescence intensity corresponded to the increase in the lysosomal pH, and a minimum of 10,000 events were scored for each sample. Each dose was triplicated. Results were expressed as % Control, * p value < 0.05 (vs control).

B. Effect on the lysosomal ASMase activity. MCF7 cells were treated with either inhibitors or vehicle control for 1 h, cell pellets were isolated, cell lysates were prepared and used to measure activity of the cellular ASMase by release of [methyl- ^{14}C] choline from choline-[methyl- ^{14}C] SM as was described in the text. Results are expressed as formed nmol Cer /mg protein/h, and presented as % Control. Results are presented as mean $\pm$ st dev. of triplicates, * p value < 0.05 (vs control)

Scheme 1.

Chemical structures of previously developed lysosomotropic B13 analogs

Scheme 2.

Synthetic outline for the preparation of DMG-B13 prodrugs

Reagents and conditions: (i) N, N-dimethyl glycine: 1,1'-carbonyldiimidazole: B13 = 1:1:1.1, CH₂Cl₂, r.t.; (ii) N,N-dimethyl glycine: 1,1'-carbonyldiimidazole: B13=10:10:1, CH₂Cl₂, r.t.; (iii) B13: Trityl Chloride = 1:0.9, DMAP (Cat.), CH₂Cl₂, r.t.; (iv) N,N-dimethyl glycine: 1,1'-carbonyldiimidazole: Trityl-B13 = 1:1:0.9, r.t.; (v) 80% acetic acid or BF₃ in MeOH solution, CH₂Cl₂, r.t.; (vi) 2M HCl in ethyl ether, 4°C.

(i) N, N-dimethyl glycine: 1,1'-carbonyldiimidazole: B13=10:10:1, CH₂Cl₂, r.t.; (ii) N,N-dimethyl glycine: 1,1'-carbonyldiimidazole: B13=1:1:1.1, CH₂Cl₂, r.t.; (iii) B13: Trityl Chloride =1:0.9, DMAP (Cat.), CH₂Cl₂, r.t.; (iv) N,N-dimethyl glycine: 1,1'-carbonyldiimidazole: Trityl-B13= 1:1:0.9, r.t.; (v) 80% acetic acid or BF₃ in MeOH solution, CH₂Cl₂, r.t.; (vi) 2M HCl in ethyl ether, ethyl acetate, 4°C.