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Effective inhibition of acid and neutral ceramidases by novel B-13 and LCL-464 analogues

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ABSTRACT

Induction of apoptosis mediated by the inhibition of ceramidases has been shown to enhance the efficacy of conventional chemotherapy in several cancer models. Among the inhibitors of ceramidases reported in the literature, B-13 is considered as a lead compound having good in vitro potency towards acid ceramidase. Furthermore, owing to the poor activity of B-13 on lysosoamal acid ceramidase in living cells, LCL-464 a modified derivative of B-13 containing a basic ω-amino group at the fatty acid was reported to have higher potency towards lysosomal acid ceramidase in living cells. In a search for more potent inhibitors of ceramidases, we have designed a series of compounds with structural modifications of B-13 and LCL-464. In this study, we show that the efficacy of B-13 in vitro as well as in intact cells can be enhanced by suitable modification of functional groups. Furthermore, a detailed SAR investigation on LCL-464 analogues revealed novel promising inhibitors of aCDase and nCDase. In cell culture studies using the breast cancer cell line MDA-MB-231, some of the newly developed compounds elevated endogenous ceramide levels and in parallel, also induced apoptotic cell death. In summary, this study shows that structural modification of the known ceramidase inhibitors B-13 and LCL-464 generates more potent ceramidase inhibitors that are active in intact cells and not only elevates the cellular ceramide levels, but also enhances cell death.

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1. Introduction

Sphingolipids (SLs) that act as major signaling molecules are bioactive constituents of eukaryotic plasma membranes and play important roles in many cellular processes such as cell recognition, cell growth, cell differentiation, cell death.[1] Among different SLs, the central molecule is ceramide (Cer), which is produced either by de novo biosynthesis, degradation of glycosphingolipids (GSLs) or by degradation of sphingomyelin (SM), catalyzed by sphingomyelinases (SMases).[2,3] The produced Cer undergoes further degradation to sphingosine (Sph) by various ceramidases (CDases). Moreover, a further phosphorylation of Sph to sphingosine-1-phosphate (S1P) via sphingosine kinases (SKs) can occur. Proper equilibrium between Cer and S1P is crucial for a final cellular response, cell homeostasis and normal cell development as they induce contrasting cellular processes.[4–6] The concentration of cellular ceramide is highly dependent on the cleavage of produced Cer by different ceramidases (CDases). Based on the pH-optima and subcellular localizations, CDases can be classified into three major sub-types such as acid ceramidase (aCDase),[7] neutral ceramidase (nCDase)[8] and alkaline ceramidase (alkCDase).[9–11] A deficiency of aCDase leads to massive lysosomal accumulation of Cer, resulting in Farber disease.[12,13] On the other hand it has been shown that especially aCDase is up-regulated in prostate cancer[14] and melanomas,[15] leading to the hypothesis that aCDase could be a tumor marker.[14,15] Moreover, aCDase in tumor cells confers resistance to chemo- and radiotherapy therefore the inhibition of this enzyme has become a potential target for cancer therapy.[16] Based on these outcomes and indications, the inhibition of ceramidases has been thought to be significantly important for the development of chemotherapeutic agents in anti-cancer therapy.

Since last several years only few lead inhibitors of ceramidases could be developed such as B-13, D-*e*-MAPP and NOE (Fig. 1).[2] A further improvement on the potency of inhibitors becomes complicated as the exact molecular basis for the inhibition of ceramidases by the existing inhibitors is still unknown. Several research groups have attempted SAR studies for the development of new inhibitors mainly by various modifications on the existing inhibitors such as substituent, functional group, length of fatty acid, stereochemistry, etc. For example, two decades ago, Hannun and co-workers have reported that B-13 effectively inhibited the HL-60 cell growth in a dose-dependent manner with an IC_{50} of approximately 1.5 μM.[17] In that report, they have varied and optimized several

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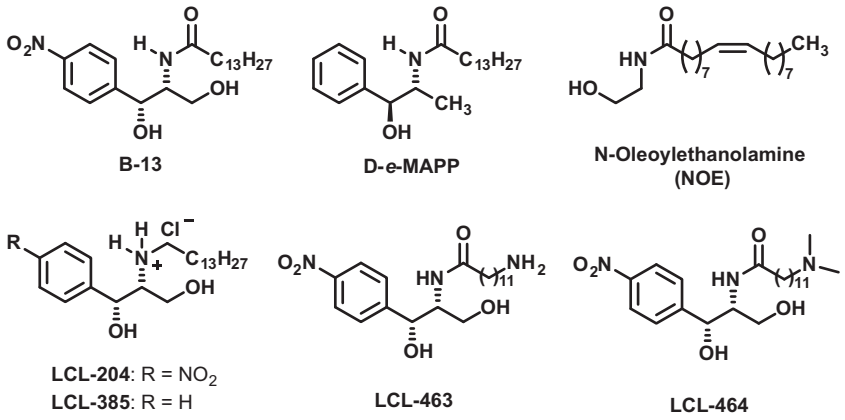


Figure 1. Chemical structures of some literature known inhibitors of ceramidases.

structural factors on *N*-acyl-phenylaminoalcohol analogues such as the length of fatty acid chain, importance of primary/secondary hydroxyl groups and stereochemistry of C1 and C2 atoms on the cell growth. The same group subsequently developed D-*e*-MAPP, a structurally modified B-13 analogue that selectively inhibits alkaline ceramidase activity derived from HL-60 cell extracts with an IC_{50} of about 1–5 μM.[18] It should be noted that D-*e*-MAPP differs from B-13 with respect to functional groups as well as the stereochemistry of chiral centers. The inhibitory activity of D-*e*-MAPP towards alkCDase was completely abolished upon the reversal of stereochemistry of chiral centers (L-*e*-MAPP). This compound rather acted as a competitive substrate analogue for alkCDase. However, in a contradictory report after several years, it has been shown that D-*e*-MAPP does not show any noticeable inhibition towards alkCDase, while it shows poor activity towards aCDase in a dose dependent manner in human melanoma and HaCaT cells.[19] This study also showed B-13 to be a potent and selective inhibitor of aCDase over alkCDase in HaCaT keratinocytes and human melanoma cells. The third compound *N*-oleoyl-ethanolamine (NOE) significantly increased the cellular Cer level although it acted only as a weak inhibitor of aCDase.[20] In further studies however, two NOE analogues have been reported that significantly inhibited aCDase in cellular studies (IC_{50} ~15 μM) and also at the same time exhibited cytotoxicity to A549 cells (IC_{50} ~40 μM).[21,22]

Bielawska and co-workers have recently carried out several very detailed and interesting studies with aCDase inhibitors (LCL-analogues) containing basic amino functionalities that help the molecules to accumulate in lysosomes (Fig. 1).[23–25] A number of similar compounds were also developed previously by Gatt and co-workers as inhibitors of sphingolipid biosynthesis.[26,27] Lysosomal accumulation is also observed for a group of functional inhibitors, the tricyclic antidepressants such as imipramine and desipramine.[28] The major drawback is that the tricyclic compounds are highly non-selective in their nature of inhibition. In addition to inhibiting aCDase,[29] they inhibit acid sphingomyelinase[30] and lysosomal phospholipases.[31] These amine-based molecules generally are in equilibrium between the protonated and neutral forms depending on pH. Only the latter can permeate through lysosomal membranes and enter the lysosomes. However, in the acidic environment of the lysosome, compounds undergo full protonation leading to an increased local concentration of substances in lysosome. In lysosome these compounds interfere with the substrate (membrane) binding of lipid hydrolases, making them highly susceptible to proteolytic degradation.[32] Indeed, spatial preferences for the LCL-analogues such as LCL-204 and LCL-385 have been observed for the accumulation in acidic compartment of cells. Among different sets of LCL-analogues, LCL-204 has been further studied in details by several research groups. For example, it has been shown that the lysosomotropic aCDase inhibitor LCL-204 induces apoptosis in prostate cancer cells[33] and enhances the cytotoxicity of the viral protein apoptin in prostate cancer.[34] This compound was also found to be active in head and neck squamous cancer cells (HNSCC) in vitro and in vivo in a xenograft model sensitizing cells to Fas-ligand induced apoptosis.[35] A more detailed biochemical investigation however revealed LCL-204 and probably also LCL-385 are highly unspecific and induce unwanted lysosomal permeabilization and degradation of aCDase.[25]

Based on these observations, a second generation basic B-13 analogue (LCL-464) was tested by Bielawska and co-workers.[25] This compound has been shown to inhibit aCDase activity in cell extracts (50% at ~50 μM) with a lower potency than B-13 but much higher than that of LCL-204. Furthermore, LCL-464 caused an early inhibition of aCDase in cellular studies leading to decreased sphingosine levels and a specific increase in C14- and C16-ceramide contents. Unlike LCL-204, the new derivative LCL-464 neither induces lysosomal destabilization nor the degradation of aCDase.[25] These results show that the cellular inhibition of acid ceramidase depends both on their in vitro activity and on their ability to accumulate in cellular lysosomes. As both LCL-464[25] and B-13[17] have been shown previously to be moderate inhibitors of acid ceramidase, the present study was focused on the design of several B-13 and LCL-464 analogues with the intention to have more potent inhibitors of ceramidases. In the first category, some B-13 analogues have been developed with the modification of important functional groups. In a second series, a number of LCL-464 analogues have been designed with the basic structures of D-*e*-MAPP and some recently published B-13 analogues.[24] Inhibition potencies of new derivatives are studied towards both of acid and neutral ceramidases in order to better interpret results from cell culture experiments. Furthermore, apoptotic behavior as well as the effect of these compounds on the endogenous ceramide level has been studied to understand their pharmacokinetics of inhibition.

2. Results and discussion

Recently we have developed a series of new aromatic ethanolamine-based inhibitors with the partial modifications of B-13, D-*e*-MAPP as well as NOE structures and some of the new compounds exhibited significantly higher potency than the parent inhibitors towards acid and neutral ceramidases.[36] An enhanced selectivity towards aCDase over nCDase has been observed in those analogues upon the substitution of alkyl chains at the aromatic ring. In this

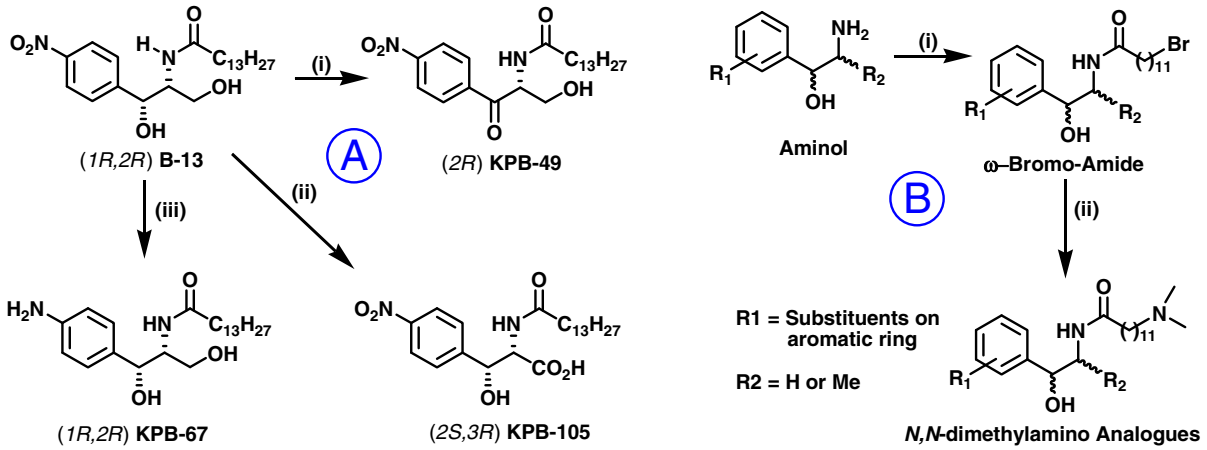
study, we also showed for the first time that the lead compound B-13 shows significant inhibition towards nCDase along with its good aCDase activity. Therefore, the elevation of endogenous ceramide level by B-13 might be the result of both, the inhibition of aCDase and nCDase. Furthermore, the potency of B-13 and some of the amide-based inhibitors could be enhanced significantly upon the conversion of amide group to the corresponding sulfonamide counterparts.

In the present study, we wanted to understand the effect of different functionalities in B-13 such as primary and secondary hydroxyl groups, *p*-nitro group etc. Accordingly, we have synthesized three different modified analogues with the alteration of one functional group at a time. To probe the importance of possible hydrogen bonding interactions in B-13 at the enzyme active site, the secondary hydroxyl group was converted to the corresponding ketone (KPB-49). As shown in Scheme 1A, KPB-49 could be synthesized from B-13 by a single step selective oxidation of the secondary hydroxyl group using fine powder of manganese dioxide. In contrast, the primary hydroxyl group could also be selectively oxidized to the corresponding carboxylic acid (KPB-105) using sodium hypochloride (NaOCl) in the presence of TEMPO as radical initiator. The electron withdrawing *p*-nitro group was converted to the electron donating amino group by the reduction mediated by zinc dust in concentrated acetic acid (Scheme 1A). All the new derivatives were screened along with B-13 for their inhibition potencies towards both of acid and neutral ceramidases. In another set of compounds, a series of *N,N*-dimethylamino substituted compounds has been synthesized following the method developed by Bielawska and co-workers with minor modifications.[25] These compounds could be synthesized in three steps starting from the corresponding aminols as shown in Scheme 1B. Some of the aminols could be prepared from the corresponding aldehydes using Henry reaction as reported previously.[37,38] The amino group of aminols with different substituents can be coupled with ω-bromo-acyl chloride to form ω-bromo-amides. Subsequently, the bromo group was reacted with *N,N*-dimethylamine leading to the formation of different *N,N*-dimethylamino substituted compounds as shown in Figure 2. All the new compounds were thoroughly characterized by NMR spectroscopic (^{1}H and ^{13}C) and ESI-MS spectrometric analyses. Optical rotation was measured for the stereochemically pure compounds.

The inhibitory potency of the newly synthesized inhibitors was evaluated using NBD-ceramide analogue as a substrate.[36] Very recently we have reported a detailed comparative kinetic study with possible combination of NBD and Nile Red (NR) substituted singly labeled ceramide analogues to understand their relative hydrolytic behavior towards ceramidases.[39] Among the four different combinations of NBD and NR-based substrates, Acyl-NBD-C_{12}-Cer was found to be superior candidate with respect to the ceramidase-mediated hydrolysis in vitro and therefore this substrate was chosen for the detailed inhibition studies in this report in the presence of recombinant human aCDase and nCDase as enzyme sources.

Ceramidase-mediated hydrolysis of the amide bond in Acyl-NBD-C_{12}-Cer produces fluorescent NBD-C_{12}-fatty acid, which was separated from the uncleaved substrate using thin layer chromatographic (TLC)-method and quantified by fluorescent imaging method. It should be mentioned that the absolute IC_{50}/EC_{50} values in an inhibition experiment are dependent on the substrate and enzyme used and also on their respective concentrations, which suggested us to consider literature-known inhibitors such as B-13, D-*e*-MAPP and LCL-464 as references during the inhibition experiments.

As shown in Figure 3 and also reported very recently from our group,[36] B-13 has been found to be a moderate inhibitor of recombinant human aCDase in vitro. The newly designed B-13 analogues such as KPB-49, KPB-67 and KPB-105 exhibited significantly higher inhibition than that of B-13 towards aCDase at a concentration of 80 μM (Fig. 3). For example, KPB-49 exhibited almost 1.5 times higher potency than B-13 towards inhibition of aCDase. Inhibition was found to be even more pronounced towards nCDase upon this modification as the inhibition potency of KPB-49 was almost double of B-13 (Fig. 4).These results indicate that the in vitro inhibitory activity of B-13 could be considerably improved upon the replacement of a hydrogen-bond donor atom (secondary hydroxyl group) with a hydrogen-bond acceptor (keto group). However, a similar modification of secondary hydroxyl group in B-13 analogues lacking primary hydroxyl group led to a weaker inhibition towards ceramidases as shown recently.[36] These observations suggest that the presence of at least one hydroxyl group as hydrogen-bond donor is possibly important for an effective inhibition towards ceramidases. To understand the role of primary hydroxyl group in B-13, compound KPB-105 was synthesized upon the selective oxidation of primary hydroxyl group to the corresponding carboxylate. A significant increase in inhibition (~1.75 times) was observed towards aCDase upon this modification. However, the scenario was opposite in case of nCDase as KPB-105 did not exhibit any noticeable inhibition towards nCDase. Unlike KPB-49, compound KPB-105 produced an enhanced selectivity towards aCDase over nCDase. The selective inhibition of KPB-105 towards aCDase may be highly influenced by the presence of pH-sensitive carboxylate group. For example, during the inhibition of aCDase (pH



Scheme 1. Synthetic route to different B-13 (A) and *N,N*-dimethylamino substituted compounds (B) as inhibitors of ceramidases. Reagents and conditions: (A) (i) MnO_2, CH_2Cl_2, 35 °C, 24 h; (ii) NaOCl, TEMPO, $NaHCO_3$, $(n\text{Bu})_4\text{NBr}$, CH_2Cl_2, H_2O, rt, 30 min; (iii) Zn dust, concd AcOH, rt to 70 °C, 30 min, (B) (i) Br-$C_{11}H_{23}$-COCl, 50% NaOAc (aq), THF, 2 h, 0 °C to rt; (ii) Me_2NH, 1.5 N NaOH, THF, reflux, 12 h.

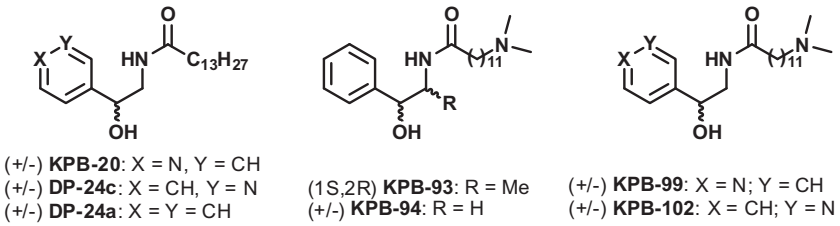


Figure 2. Chemical structures of already reported amide-based compounds (KPB-20, DP-24c and DP-24a) and the newly synthesized *N,N*-dimethylamino substituted compounds as inhibitors of ceramidases.

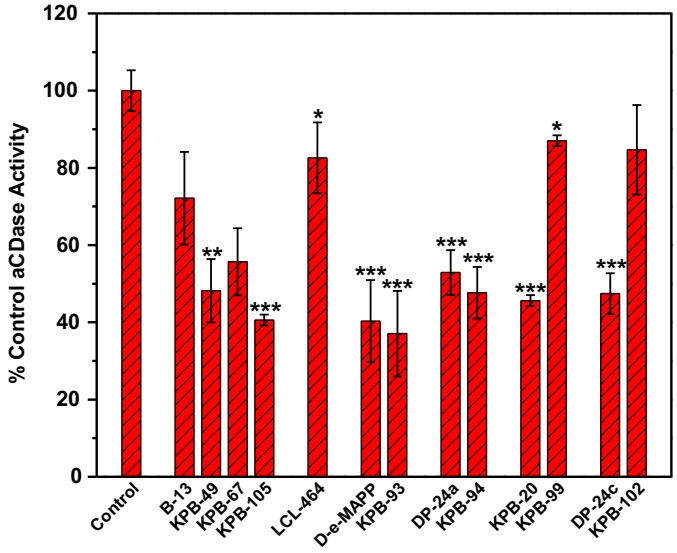


Figure 3. Plot for the relative potencies of various compounds for the inhibition of aCDase activity. The final concentrations of fluorescent substrate and inhibitors were 15 and 80 μM, respectively. Inhibitory data are shown as mean ± SD from three independent experiments under standard assay condition. Statistical significances of the results have been compared to the control reaction; *, $0.01 < p \leqslant 0.05$; **, $0.001 < p \leqslant 0.01$; ***, $p \leqslant 0.001$. The control value represents the percentage hydrolysis of the substrate by aCDase in the absence of any inhibitor.

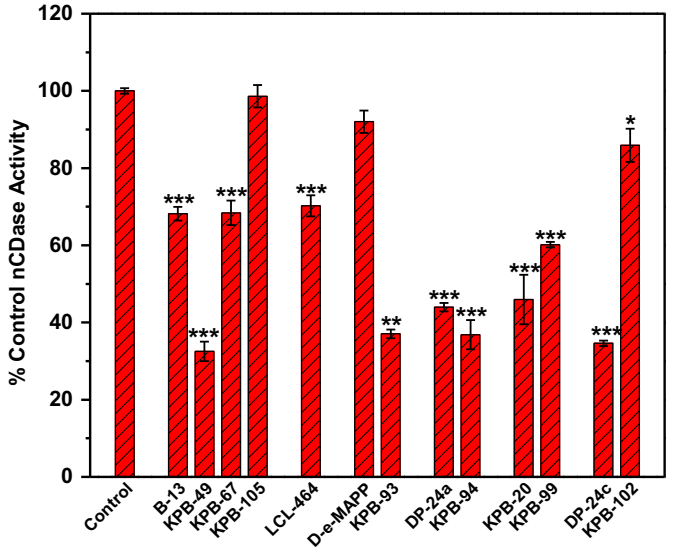


Figure 4. Plot for the relative potencies of various compounds for the inhibition of nCDase activity. The final concentrations of fluorescent substrate and inhibitors were 15 and 80 μM, respectively. Inhibitory data are shown as mean ± SD from three independent experiments under standard assay condition. Statistical significances of the results have been compared to the control reaction; *, $0.01 < p \leqslant 0.05$; **, $0.001 < p \leqslant 0.01$; ***, $p \leqslant 0.001$. The control value represents the percentage hydrolysis of the substrate by nCDase in the absence of any inhibitor.

4.5), the likely active form is carboxylic acid (–COOH) whereas, the corresponding carboxylate (COO^-) might be the most active species at neutral pH during the inhibition of nCDase (pH 7.0). This might be an interesting aspect for the design of selective inhibitors modulated by pH of the cellular medium/environment. A third modification with the conversion of electron withdrawing nitro group to the basic amino group (KPB-67) also led to a slightly increased inhibition towards aCDase while the effect was negligible towards nCDase. This modification however made KPB-67 to be more or at least equally potent as B-13 towards ceramidases. With the presence of primary amino group in the aromatic ring, KPB-67 could be considered as an analogue of lysosomotropic inhibitor LCL-464. While LCL-464 exhibited almost similar inhibition as B-13, compound KPB-67 was found to be almost 1.5 times more potent than LCL-464 towards aCDase.

As the lysosomotropic inhibitor LCL-464 exhibited moderate inhibition towards both aCDase and nCDase in the in vitro assays, we decided to screen the newly developed analogues with the variation of substituents as shown in Figure 2. In addition to comparing their inhibitory potencies to LCL-464, we also included the corresponding amide counterparts, reported recently from our group, such as KPB-20, DP-24c and DP-24a to understand the effect of basic *N,N*-dimethylamino group towards their activities. While D-*e*-MAPP has been reported to be a potent inhibitor of alkaline ceramidase in HL-60 cells[18] subsequent reports revealed this compound to be relatively poor inhibitor of both aCDase and nCDase in HaCaT keratinocytes.[19] With these complications in its activity in different experimental conditions and with different cellular lysates as enzyme sources, we decided to screen the potency of D-*e*-MAPP using recombinant human aCDase and nCDase as sources of enzymes. Interestingly, in our present study, difference in inhibition potency of D-*e*-MAPP towards recombinant human aCDase and nCDase has been observed. For example, while D-*e*-MAPP exhibited significant inhibition towards aCDase (60% inhibition at 80 μM concentration, Fig. 3), it showed very weak inhibitory activity for nCDase (8%). These results indicate the selectivity of D-*e*-MAPP towards aCDase over nCDase. However, the weak activity of D-*e*-MAPP towards nCDase could be dramatically enhanced upon the incorporation of *N,N*-dimethylamino group. For example, while D-*e*-MAPP exhibited very weak inhibition (8%) towards nCDase, the corresponding *N,N*-dimethylamino derivative (KPB-93) showed almost 2.5 times higher potency (63% inhibition), which was even much higher than the already reported amino-substituted compound LCL-464 (30%). These observations indicate that *N,N*-dimethylamino derivatives of D-*e*-MAPP is more effective inhibitors of ceramidases than the parent compound at least in the in vitro inhibition assays.

As shown in our previous reports, more simplified amide-substituted analogues of B-13 such as DP-24a, KPB-20 and DP-24c also exhibited relatively higher inhibition potency towards

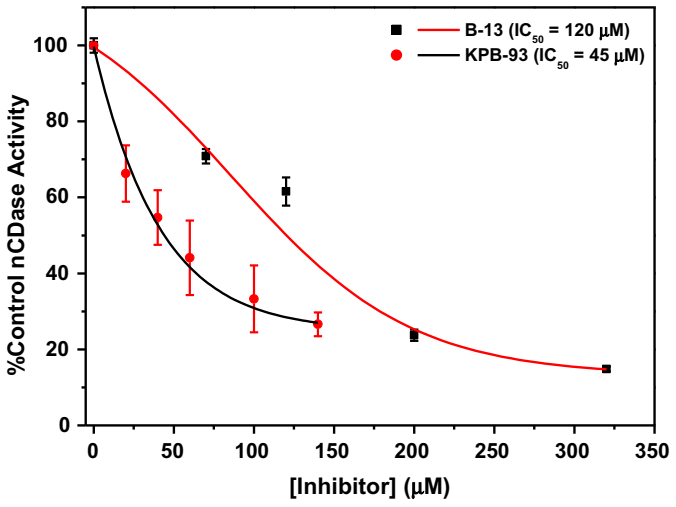


Figure 5. Plot for the effect of concentrations of B-13 and KPB-93 for the inhibition of nCDase activities. IC_{50} values represent the concentration of inhibitors required for 50% inhibition of control activities. Inhibitory data are shown as mean ± SD from three independent experiments under standard assay condition.

both of aCDase and nCDase.[36,38] To see the effect of *N,N*-dimethylamino group on their potencies, we compared the activities of KPB-94, KPB-99 and KPB-102 to the corresponding parent compounds. As shown in Figures 3 and 4, while the activities of KPB-94 and DP-24a were comparable, a much weaker inhibition was observed for the *N,N*-dimethylamino derivatives (KPB-99 and KPB-102) of the pyridyl-substituted compounds. This observation supports the assumption that the incorporation of a weakly basic group apart from the hydrophobic fatty acid part in B-13 and D-*e*-MAPP analogues is a better choice for the development of more potent ceramidase inhibitors. However, this could be verified only after screening the compounds in cellular as well as in animal model experiments.

As KPB-93 was found to be one of the most active inhibitors among the new *N,N*-dimethylamino-substituted compounds in the present study, we further studied the dose-dependency of this compound and compared to that with B-13 towards the inhibition of nCDase. As shown in Figure 5, KPB-93 exhibited much higher potency than B-13 over the entire range of concentrations studied and the IC_{50} value of KPB-93 was found to be almost 2.5 times lower than B-13 under the identical experimental condition.

To assess the pro-apoptotic potential of our compounds, we have carried out apoptosis studies. In the present study, apoptotic cell death in MDA-MB-231 breast cancer cells was investigated, detecting the occurrence of DNA fragmentation by using an ELISA. Cells were incubated with various inhibitors at two different concentrations (25 and 50 μM) to see their apoptotic behavior. As shown in Figure 6, most of the newly synthesized inhibitors showed significantly higher fragmentation (~150–400%) than the control, indicating the occurrence of apoptosis. At 50 μM concentration of inhibitors, at least double or even more fragmentation was observed for most compounds. Induction of apoptosis by compounds in the present study is probably the result of significant inhibition of ceramidases and consequently an increase of cellular ceramide levels. Among the modified B-13 analogues and newly synthesized basic *N,N*-dimethylamino substituted compounds, KPB-67 having a primary amino group in the phenyl ring of B-13 was found to be most potent, showing a three- to fourfold enhancement of DNA fragmentation, while a more detailed investigation revealed an EC_{50} of 59.02 μM (Fig. 6). Obviously, the presence of a polar primary amino group in the phenyl ring of B-13 may have high significance for the development of a potent chemotherapeutic agent.

Interestingly, and in contrast to the apoptosis data, a cell viability assay using the AlamarBlue® blue reagent showed only minor and non-significant responses for most compounds (data not shown). Although this result was unexpected, it may indicate that our ceramidase inhibitors are specifically pro-apoptotic, rather than being cytotoxic. To understand whether the addition of inhibitors indeed enhances endogenous ceramide levels in the cells, we have determined the total ceramide levels in MDA-MB-231 cancer cells upon treatment of cells with various inhibitors of interest. Cells were incubated at 37 °C for 24 h with two different concentrations of inhibitors (25 and 50 μM) and the ceramide levels were quantified using mass spectrometry. As shown in Figure 7, at 50 μM concentration, all the compounds with the exception of KPB-99 increased total ceramide content in cells to at least 150% or more upon their incubation. B-13 showed a significant elevation of total ceramide content (350%), which is in agreement with earlier reports by Bielawska and co-workers.[25] It should be noted that

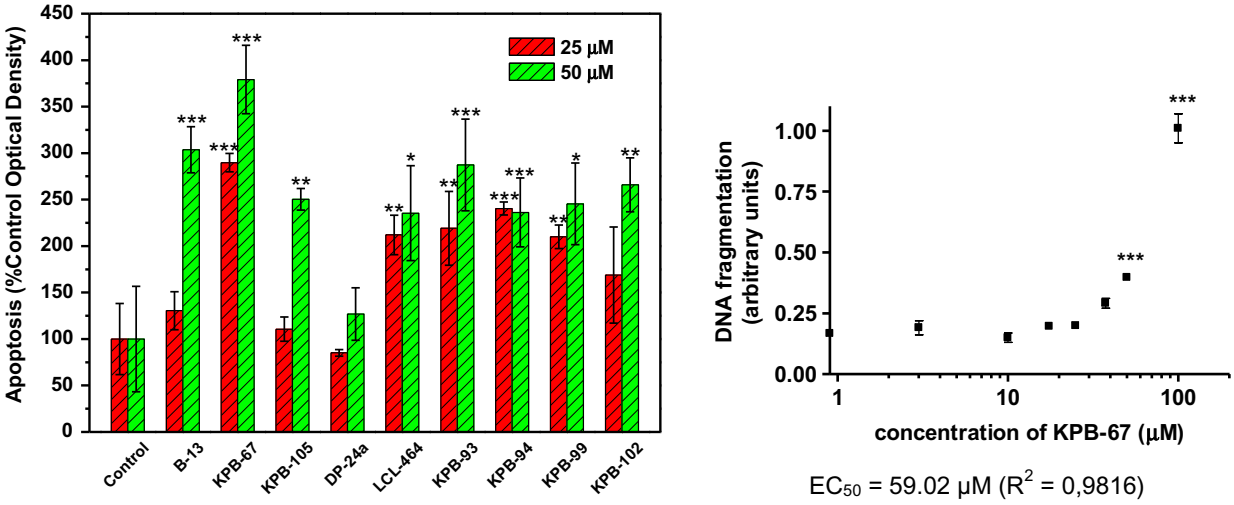


Figure 6. Left: Induction of apoptosis by different ceramidase inhibitors Data are expressed as % of control and are means ± S.D ($n = 3$). Right: Dose–response curve for KPB-67. Data are expressed as optical density. MDA-MB-231 cells were treated for 24 h with either vehicle (DMSO) or 25 μM (red) and 50 μM (green) of the different compounds. Thereafter, DNA fragmentation as a measure of apoptosis was determined by a cell death detection plus ELISA as described in the methods part. Statistical significances of the results have been compared to the control reaction; *, $0.01 < p \leqslant 0.05$; **, $0.001 < p \leqslant 0.01$; ***, $p \leqslant 0.001$.

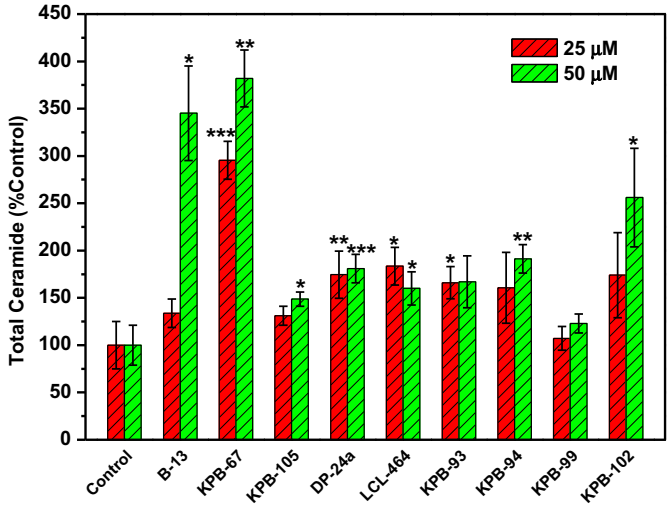


Figure 7. Effect of ceramidase inhibitors on the cellular ceramide content. A final concentration of 25 μM (red) and 50 μM (green) of various inhibitors were incubated with MDA-MB-231 breast cancer cells for 24 h at 37 °C for their effect on cellular ceramide level. Data are shown as mean ± SD from three independent experiments under standard assay condition. Statistical significances of the results have been compared to the control reaction; *, $0.01 < p \leqslant 0.05$; **, $0.001 < p \leqslant 0.01$; ***, $p \leqslant 0.001$.

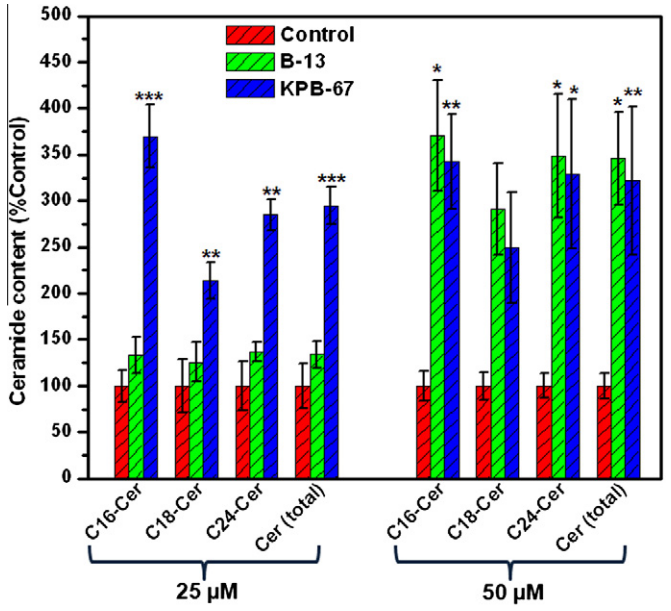


Figure 8. Effect of B-13 and KPB-67 on the elevation different ceramide levels in MDA-MB-231 cells at two different concentrations. Data shown in the plot represent average of three independent experiments under standard assay condition and the results are shown as mean ± SD values. Statistical significances of the results have been compared to the control reaction; *, $0.01 < p \leqslant 0.05$; **, $0.001 < p \leqslant 0.01$; ***, $p \leqslant 0.001$.

the elevation was much higher at 50 μM concentration (350%) of B-13 than at 25 μM (150%). For highly polar B-13 analogues such as KPB-67 and KPB-105, it has been observed that, as compared to KPB-105, the primary amino substituted compound KPB-67 showed a significant increase in total ceramide level upon the incubation at both the concentrations. For example, while KPB-105 increased the ceramide level by ~150%, the enhancement was around 300–380% for KPB-67. KPB-67 was the compound that exhibited highest increase in ceramide level among all the inhibitors including LCL-464 and other newly developed *N,N*-dimethylamino derivatives tested in the present study. The elevation of total ceramide level by B-13 and KPB-67 is in agreement with the results on their apoptotic behavior and this clearly indicates that the apoptosis of MDA-MB-231 cells upon the administration of inhibitors such as B-13 or KPB-67 is most probably due to the inhibition of ceramidases leading to the accumulation of cellular ceramide. Among the *N,N*-dimethylamino-substituted compounds, KPB-102 showed reasonable elevation of ceramide in comparison to other derivatives.

To understand the influence of inhibitors on different ceramides such as C16-Cer, C18-Cer and C24-Cer, we have chosen the highly active inhibitors (B-13 and KPB-67) for further detailed studies. As shown in Figure 8, the effect of these compounds on different ceramides was analyzed at 25 and 50 μM concentrations. In general, the elevation of C16-Cer was highest, while the elevation was found to be least for C18-Cer. The increase of all ceramides was much lower for B-13 at lower concentration (25 μM) but it was found to be significantly higher at 50 μM concentration.

3. Conclusion

In summary, we report the design and synthesis of two series of novel analogues of B-13 and LCL-464 having improved inhibition potencies towards aCDase and nCDase in vitro and in intact cells. This study suggests that the in vitro potency of B-13 could be enhanced further with a suitable modification of functional groups. While B-13 did not show much selectivity, replacement of the primary hydroxyl group with a carboxylic acid led to an enhanced selectivity towards aCDase over nCDase. Furthermore, the inhibitory activity of D-*e*-MAPP and some of the simplified B-13 analogues could be increased upon the incorporation of polar basic *N,N*-dimethylamino group at the fatty acid chain. While the replacement of *p*-nitro group in B-13 with a primary amino group (KPB-67) had marginal effect on the in vitro inhibition of aCDase and nCDase, this modification made the molecule to be highly active at the cellular level leading to apoptosis with significant elevation of endogenous ceramide content, suggesting a more detailed investigation of KPB-67 in future experimental therapies.

4. Experimental section

4.1. Materials and method

Chemicals were purchased from Sigma–Aldrich or Acros. Acyl-C_{12}-NBD-Cer was purchased from Avanti-Polar Lipids, Inc. Recombinant neutral ceramidase (nCDase) was obtained from R&D systems, Inc. Solvents were freshly distilled whenever required for the reaction. All the moisture sensitive reactions were carried out under dry argon atmosphere. Thin layer chromatographic (TLC) studies are performed on pre-coated silica gel 60 F254 on aluminum sheets (Merck KGaA). ^{1}H (500 or 300 MHz) and ^{13}C (125 or 75 MHz) NMR spectra were obtained on a Bruker Avance III 500 MHz or Bruker Avance DPX 300 MHz NMR spectrometers. Chemical shifts are cited with respect to Me_4Si as internal standard. Mass spectral studies were carried out on a Hewlett-Packard GCMS 5995-A mass spectrometer with ESI-MS or EI-MS modes. Optical rotation of the stereochemically pure compounds was determined using Perkin-Elmer 241 Polarimeter. The inhibition data were plotted using Origin 6.1 or Graph Pad Prism 4 software and the statistical significance was assessed using students *T*-test with IBM SPSS software. Previously published compounds from our group such as DP-24a/DP-24c[38] and KPB-20[36] were synthesized following the literature procedure.

4.1.1. Synthesis of *N*-((*R*)-3-hydroxy-1-(4-nitrophenyl)-1-oxopropan-2-yl)tetradecanamide (KPB-49)

To a stirred solution of B-13 (50.0 mg, 0.11 mmol) in dichloromethane was added fine powder of manganese dioxide (0.1 g, 1.14 mmol) and the reaction mixture was stirred at 35 °C for 24 h. The mixture was filtered through a pad of celite to remove the unreacted MnO_2. The solvent was evaporated to obtain the crude product as white solid. The compound was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluent. The solvent was evaporated to afford the pure product as yellowish amorphous solid. Yield: 15 mg (33%), mp: 78–80 °C. R_f = 0.30 (100% Ethyl acetate). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, J = 6.5 Hz, 3H), 1.27–1.32 (m, 20H), 1.64–1.70 (m, 2H), 2.29–2.35 (m, 2H), 3.94–4.02 (m, 2H), 5.61–5.64 (m, 1H), 6.75 (d, J = 6.5 Hz, 1H), 8.22 (d, J = 9.0 Hz, 2H), 8.37 (d, J = 8.5 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 25.6, 29.2, 29.3, 29.5, 29.6, 31.9, 36.5, 57.2, 64.1, 124.1, 129.9, 138.9, 150.8, 174.0, 196.1. $[\alpha]_D^{25}$ +7.1 (*c* 0.01 in MeOH). ESI-MS: *m/z* calcd for $C_{23}H_{36}N_2O_5$: 419.2551 $[M-H]^-$; observed: 419.2553.

4.1.2. Synthesis of *N*-((1*R*,2*R*)-1-(4-aminophenyl)-1,3-dihydroxypropan-2-yl)tetradecanamide (KPB-67)

The compound was synthesized following the literature procedure with minor modifications.[24] To a stirred solution of B-13 (0.10 g, 0.23 mmol) in acetic acid (80%, 2.0 mL) was added zinc dust (0.15 g, 2.29 mmol). After 10 min, the reaction mixture was heated to 70 °C for 30 min. The unreacted zinc dust was removed by filtration. The filtrate was diluted with ethyl acetate and extracted with saturated sodium bi-carbonate solution. The combined organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated and the compound was purified by silica gel column chromatography using cyclohexane/ethyl acetate and finally with ethyl acetate/methanol mixtures as eluent. The solvent was evaporated to afford the pure product as light reddish solid. Yield: 30 mg (34%), mp: 112–114 °C. R_f = 0.35 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, J = 7.0 Hz, 3H), 1.27 (br, 20H), 1.62 (m, 2H), 2.15 (m, 2H), 2.76 (m, 2H), 3.61 (dd, $J1$ = 5.5 Hz, $J2$ = 11.0 Hz, 2H), 3.70 (dd, $J1$ = 3.1 Hz, $J2$ = 11.0 Hz, 1H), 4.10 (m, 1H), 5.65 (d, J = 4.3 Hz, 1H), 6.66 (d, J = 8.3 Hz, 2H), 7.00 (d, J = 8.3 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 25.7, 29.2, 29.3, 29.5, 29.7, 31.9, 36.2, 36.8, 53.2, 64.9, 115.4, 127.2, 130.0, 145.1, 174.1. $[\alpha]_D^{25}$ +5.7 (*c* 0.01 in MeOH). ESI-MS: *m/z* calcd for $C_{23}H_{40}N_2O_3$: 393.3115 $[M+H]^+$; observed: 393.3112.

4.2. Synthesis of LCL-464 analogues

These compounds were synthesized following the literature procedure with minor modifications.[25]

4.2.1. Preparation of 12-bromododecanoyl chloride

12-Bromododecanoic acid (0.30 g, 1.10 mmol) was dissolved in dry cyclohexane (4.0 mL) by stirring at 45 °C for 5 min. To this well-stirred solution, one drop of dry pyridine followed by oxalyl chloride (0.14 mL, 1.65 mmol) were added over 1 min. The reaction mixture was heated to 50 °C for 30 min. After stirring for an additional 30 min at room temperature, the reaction mixture was evaporated to dryness by purging dry argon gas into the reaction flask and dried under the high vacuum. The freshly prepared 12-bromododecanoyl chloride was dissolved in 3 mL anhydrous THF and used directly to the next step without purification.

4.2.2. Coupling of acyl chloride with amines

To a well-stirred ice-cooled solution of the corresponding amine (1.00 mmol) in THF (5.0 mL) and 50% aqueous solution of sodium acetate (5.0 mL), the freshly prepared 12-bromododecanoyl chloride (1.10 mmol) was added dropwise over 1 min. The reaction mixture was allowed to attain room temperature and stirred at room temperature for another 2 h and the completion of the reaction was monitored by TLC method. The mixture was diluted with ethyl acetate and extracted with water followed by brine solution. The combined organic extract was dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure to afford crude product. This crude material was purified by silica gel column chromatography using cyclohexane and ethyl acetate mixture or ethyl acetate and methanol mixture as eluent.

4.2.3. Preparation of LCL-464 analogues

To a solution of the product (0.76 mmol) in a mixture of THF (8.0 mL) and 1.5 N NaOH solution (4.0 mL) (2:1), *N,N*-dimethylamine (1.52 mmol) was added at room temperature and the reaction mixture was refluxed overnight. The solution was diluted with CH_2Cl_2 and extracted with water and brine solution. The combined organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to afford the crude product as yellowish oil. The product was purified by silica gel column chromatography using cyclohexane, ethyl acetate and methanol mixture in the presence of 1% NEt_3.

4.2.3.1. Synthesis of 12-(dimethylamino)-*N*-((1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl)dodecanamide (KPB-93). Yield: (47%) as light yellow amorphous solid. Mp: 52–54 °C. R_f = 0.2 (25% MeOH in ethyl acetate with 2% NEt_3). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.95 (d, J = 7.0 Hz, 3H), 1.24 (br, 14H), 1.42 (t, J = 6.5 Hz, 2H), 1.58 (t, J = 6.0 Hz, 2H), 2.16 (s, 6H), 2.23 (t, J = 7.5 Hz, 2H), 4.23 (t, J = 6.5 Hz, 1H), 4.78 (s, 1H), 5.15 (br, 1H), 6.11 (br, 1H), 7.22 (t, J = 7.0 Hz, 1H), 7.28–7.33 (m, 4H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.0, 25.7, 27.2, 27.4, 29.1, 29.2, 29.3, 29.4, 36.8, 45.0, 50.8, 59.6, 75.9, 126.2, 127.2, 128.0, 141.6, 173.5. $[\alpha]_D^{25}$ +12.9 (*c* 0.01 in MeOH). ESI-MS: *m/z* calcd for $C_{23}H_{40}N_2O_2$: 377.3163 $[M+H]^+$; observed: 377.3163.

4.2.3.2. Synthesis of 12-(dimethylamino)-*N*-(2-hydroxy-2-phenylethyl)dodecanamide (KPB-94). Yield: (41%) as light yellow amorphous solid. Mp: 60–62 °C. R_f = 0.25 (25% MeOH in ethyl acetate with 2% NEt_3). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 1.24 (br, 14H), 1.39 (br, 2H), 1.55 (br, 2H), 2.11 (s, 6H), 2.17 (t, J = 7.5 Hz, 2H), 3.20–3.24 (m, 1H), 3.59–3.62 (m, 1H), 4.72 (d, J = 8.0 Hz, 1H), 5.44 (br, 1H), 6.41 (br, 1H), 7.21–7.33 (m, 5H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 25.7, 27.3, 27.4, 29.2, 29.3, 29.4, 29.5, 36.6, 45.1, 47.3, 59.7, 73.0, 125.8, 127.5, 128.3, 142.5, 174.3. ESI-MS: *m/z* calcd for $C_{22}H_{38}N_2O_2$: 363.3006 $[M+H]^+$; observed: 363.3002.

4.2.3.3. Synthesis of 12-(dimethylamino)-*N*-(2-hydroxy-2-(pyridin-4-yl)ethyl)dodecanamide (KPB-99). Yield: (39%) as off-white amorphous solid. Mp: 61–63 °C. R_f = 0.10 (25% MeOH in ethyl acetate with 2% NEt_3). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 1.22 (br, 14H), 1.38 (br, 2H), 1.55 (br, 2H), 2.11–2.21 (m, 10H), 3.19–3.26 (m, 1H), 3.62–3.67 (m, 1H), 4.76 (t, J = 7.5 Hz, 1H), 6.64 (br, 1H), 7.27 (d, J = 6.0 Hz, 2H), 8.42 (dd, $J1$ = 4.0 Hz, $J2$ = 2.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 25.6, 27.4, 29.1, 29.2, 29.3, 29.4, 29.5, 36.4, 45.2, 46.9, 59.7, 71.6, 121.1, 149.4, 152.0, 174.6. ESI-MS: *m/z* calcd for $C_{21}H_{37}N_3O_2$: 364.2959 $[M+H]^+$; observed: 364.2961.

4.2.3.4. Synthesis of 12-(dimethylamino)-*N*-(2-hydroxy-2-(pyridin-3-yl)ethyl)dodecanamide (KPB-102). Yield: (37%) as off-white amorphous solid. Mp: 54–56 °C. R_f = 0.10 (25% MeOH in ethyl acetate with 2% NEt_3). ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 1.27 (br, 14H), 1.44 (br, 2H), 1.61 (t, J = 6.5 Hz, 2H), 2.17–2.27 (m, 10H), 3.31–3.36 (m, 1H), 3.67–3.71 (m, 1H), 4.86 (t, J = 4.5 Hz, 1H), 6.42 (br, 1H), 7.28 (t, J = 8.0 Hz, 1H), 7.74 (d,

J = 8.0 Hz, 1H), 8.48 (s, 1H), 8.55 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 25.6, 27.3, 29.1, 29.2, 29.3, 29.4, 30.3, 36.5, 45.1, 47.4, 59.7, 71.2, 123.4, 133.8, 138.0, 147.7, 148.8, 174.8. ESI-MS: *m/z* calcd for $C_{21}H_{37}N_3O_2$: 364.2959 $[M+H]^+$; observed: 364.2962.

4.2.3.5. Synthesis of (2*S*,3*R*)-3-hydroxy-3-(4-nitrophenyl)-2-(tetradecanamido)propanoic acid (KPB-105). To a solution of B-13 (0.20 g, 0.47 mmol) in CH_2Cl_2 (1.0 mL) and 2,2,6,6-tetramethyl piperidine-*N*-oxide (TEMPO, 1.0 mol %, 0.7 mg in CH_2Cl_2), 0.9 mL of saturated solution of sodium bicarbonate with 5.3 mg of KBr and 10.0 mg of tetrabutylammonium bromide hydrate were added. The mixture was cooled to 0 °C with external ice-bath and 1.5 mL of NaOCl (5% solution), 0.8 mL of saturated sodium bicarbonate solution and 1.5 mL of brine solution was added. The reaction mixture was stirred at 0 °C for 30 min. The solution was diluted with CH_2Cl_2, extracted with aqueous solution and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography using cyclohexane/ethyl acetate and ethyl acetate/methanol mixtures, respectively. The solvent was evaporated to afford the pure product as yellowish semi-solid. Yield: 76 mg (37%). R_f = 0.1 (25% MeOH in ethyl acetate). ^{1}H NMR (CD_3OD, 500 MHz, ppm): δ = 0.92 (t, J = 7.0 Hz, 3H), 1.05 (br, 2H), 1.31 (br, 20H), 2.14 (m, 2H), 4.74 (br, 1H), 5.49 (br, 1H), 7.68 (d, J = 8.6 Hz, 2H), 8.17 (d, J = 7.8 Hz, 2H). ^{13}C NMR (CD_3OD, 125 MHz, ppm): 14.5, 23.8, 26.9, 30.2, 30.5, 30.6, 30.8, 33.1, 37.2, 60.6, 74.2, 124.0, 128.5, 148.5, 151.5, 175.7. $[\alpha]_D^{25}$ +9.1 (*c* 0.01 in MeOH). ESI-MS: *m/z* calcd for $C_{23}H_{36}N_2O_6$: 435.2501 $[M-H]^-$; observed: 435.2493.

4.2.3.6. Acid ceramidase (aCDase) assay. The acid ceramidase assay was performed in sodium acetate buffer (200 mM, pH 4.5) with 200 mM sodium chloride and 0.1% Triton-X100. The stock solutions of the fluorescent substrate and all the inhibitors were prepared in DMSO. Human recombinant acid ceramidase (aCDase) was used as the enzyme source for the cleavage of the fluorescent substrate. The total volume of the assay mixture was 100 μL. The final concentration of the substrate and inhibitors were 15 and 80 μM, respectively and the total protein concentration in the assay mixture was 60 μg/mL. In all the assay mixtures the volume of DMSO was maintained less than 5% (v/v). The assay mixture was incubated for 8 h at 37 °C. The enzymatic reaction was quenched by adding 200 μL of chloroform/methanol (2:1) mixture. The assay mixture was vortexed well and centrifuged to separate the organic and aqueous layers. Fifty microliters of the organic layer was transferred into new sets of vials and the total amount was spotted on non-fluorescent silica gel TLC plates pre-coated on aluminum sheets (mobile phase: cyclohexane/ethyl acetate/acetic acid = 40:60:2). The fluorescent spots of the cleaved and uncleaved substrate on the TLC plates were detected by Fluorescent Imaging System (Kodac Image Station 4000 MM PRO) using 430 and 550 nm as excitation and emission wavelengths for the NBD-linked substrate. The percentage ceramidase activity and the inhibition were determined by the quantification of fluorescent intensity of the cleaved and uncleaved substrates.

4.2.3.7. Neutral ceramidase (nCDase) assay. Human recombinant neutral ceramidase (nCDase) (R&D Systems GmbH) was used as the enzyme source for the cleavage of the fluorescent substrates. The neutral ceramidase assay was performed in sodium acetate buffer (200 mM, pH 7.0) with 200 mM sodium chloride and 0.1% Triton-X100. The stock solutions of the fluorescent substrates were prepared in DMSO. The total volume of the assay mixture was 100 μL. The final concentration of the substrates and inhibitors were 15 and 80 μM, respectively. The final concentration of recombinant enzyme was 25 ng/mL. In all the assay mixtures the volume of DMSO was maintained less than 5% (v/v). The mixture was incubated for 2 h at 37 °C and the enzymatic reaction was quenched by adding 200 μL of chloroform/methanol (2:1) mixture. The mixture was vortexed thoroughly and centrifuged at 10,000 rpm for 2 min. to separate the organic layer. Fifty microliters of the organic layer was transferred into new sets of vials and the percentage hydrolysis was determined as mentioned for the aCDase activities.

4.2.3.8. Apoptosis assay (DNA fragmentation). Apoptosis was detected by measuring the appearance of cytoplasmic histone-associated DNA fragments by photometric enzyme-linked immunoassay (Cell Death Detection ELISA Plus, Roche Diagnostics). MDA-MB-231 cells were seeded in 96 well plates for 24 h and then treated with the indicated concentrations of the compounds in Dulbecco's modified Eagle medium (DMEM) for 24 h. The preparation of cell lysates and the assay procedures were performed according to the manufacturer's protocol.

4.2.3.9. Cell viability assay. Cell vitality was detected by measuring the fluorescence of the AlamarBlue® reagent (AlamarBlue reagent, Invitrogen). MDA-MB-231 cells were seeded in 96 well plates for 24 h and then treated with different concentrations of the compounds in Dulbecco's modified Eagle medium (DMEM) for 48 or 72 h. AlamarBlue reagent was added and fluorescence was monitored after 2 and 4 h, according to the manufacturer's protocol.

4.2.3.10. Determination of cellular ceramide content. Cells (MDA-MB-231) upon the administration of different ceramidase inhibitors were collected, transferred into siliconized glass tubes and dissolved in 1 mL of methanol. Different ceramides in the samples were then extracted by a modified Bligh & Dyer extraction method.[40] As an internal standard, 20 pmol of C17-ceramide in methanol was added, followed by the addition of 1 mL each of chloroform, aqua dest and methanol. After 2 min of intensive stirring, 1 mL chloroform was added. Subsequently, the sample was stirred again for 2 min and 1 mL of aqua dest was added. The mixture was intensely vortexed and centrifuged for 5 min (800×g). The lower organic phase was transferred into a siliconized glas tube and the aqueous phase was further extracted twice with 1 mL of chloroform. Solvent in the combined organic phase was evaporated using a Speed Vac SC201 ARC vacuum system (Thermo Fischer Scientific, Dreieich, Germany) and the dried lipids were re-dissolved in 200 μL of methanol by rigorous vortexing and sonicating on ice. Sample analysis was performed using rapid resolution liquid chromatography/mass spectrometry (LC–MS). The LC equipment consisted of an Agilent 1200 Series binary pump, degasser and autosampler (Agilent Technologies, Waldbronn, Germany). A quadrupole/time-of flight (QTOF) 6530 mass spectrometer equipped with Jet-Stream Technology operating in the positive Electrospray Ionization (ESI) mode was used for the detection (Agilent Technologies, Waldbronn, Germany). Highly pure nitrogen gas for the mass spectrometer was produced by nitrogen generator (Parker Balston, Maidstone, UK). Chromatographic separations were obtained using a ZORBAX Eclipse XDB-C18 (C18, 4.6 × 50 mm, 1.8 μm particle size, 80 Å pore size, Agilent Technologies, Waldbronn, Germany). The injection volume per sample was 10 μL. An isocratic solvent system consisting of acetonitrile/2-propanol 3:2 with 1% formic acid with a flow rate of 1 mL/min over 15 min was used. For mass spectrometric measurements the following ion source conditions and gas settings for positive LC–MS/MS were adjusted: sheat gas temperature = 400 °C, sheat gas flow = 9 L/min, nebulizer pressure = 30 psig, drying gas temperature = 350 °C, drying gas flow = 8 L/min, capillary voltage = 2000 V, fragmentor voltage = 355 V, nozzle voltage = 2000 V. All ceramides gave the same

fragment ion of *m/z* 264.27 at different retention times, depending on their chain length. Quantification was performed using Mass Hunter Software. Calibration curves of reference ceramides were performed from 1 to 100 pmol and were constructed by linear fitting, using the least squares linear regression calculation. The resulting slope of the calibration curve was used to calculate the concentration of the respective analytes in the unknown samples.

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Supplementary data

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