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Novel amide- and sulfonamide-based aromatic ethanolamines: Effects of various substituents on the inhibition of acid and neutral ceramidases

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ABSTRACT

In the present study we describe the design and synthesis of a series of amide- and sulfonamide-based compounds as inhibitor of recombinant acid and neutral ceramidases. Inhibition of ceramidases has been shown to induce apoptosis and to increase the efficacy of conventional chemotherapy in several cancer models. B-13, lead in vitro inhibitor of acid ceramidase has been recently shown to be virtually inactive towards lysosomal acid ceramidase in living cells at lower concentrations and for a shorter time of treatment, suggesting the development of more potent inhibitors. In this study, a detailed SAR investigation has been performed to understand the effect of different substituents on the phenyl ring of amide- and sulfonamide-based compounds that partially resemble the structure of well-known inhibitors such as B-13, D-*e*-MAPP as well as NOE. Our results suggest that the electronic effects of the substituents on phenyl ring in B-13 and D-*e*-MAPP analogues have negligible effects either in enhancing the inhibition potencies or for selectivity towards aCDase over nCDase. However, the hydrophobicity and the steric effects of longer alkyl chains (*n*-Pr, *n*-Bu or *t*-Bu groups) at the phenyl ring were found to be important for an enhanced selectivity towards aCDase over nCDase.

1. Introduction

Sphingolipids (SLs) are major bioactive constituents of eukaryotic plasma membranes and play important roles in many cellular processes such as cell recognition, cell growth, cell differentiation, cell death and signaling.[1] A proper balance in concentrations of different SLs is crucial for cell homeostasis and normal cell development. The central molecule in the SL metabolism is ceramide (Cer), which is produced either by de novo biosynthesis, degradation of glycosphingolipids (GSLs) or by degradation of sphingomyelin, catalyzed by sphingomyelinases (SMases).[2] The produced Cer undergoes further degradation to sphingosine (Sph) and subsequently to sphingosine-1-phosphate (S1P) by various ceramidases (CDases) and sphingosine kinases (SKs), respectively. While Cer induces apoptosis and growth arrest,[3] the products Sph and S1P are known as anti-apoptotic agents, promoting cell growth and angiogenesis.[4,5] Therefore, it has been suggested that the equilibrium between ceramide and S1P is decisive for a final cellular response.[6] The cellular 'sphingolipid rheostat' is primarily regulated by three major classes of enzymes such as SMases, CDases and SKs. Ceramide is often accumulated upon the activation of SMases by external stimulation of cells with stress factors like inflammatory cytokines, UV radiation, X-rays, heat shock and oxidative stress followed by the degradation of sphingomyelin. However, the control over this process is rather more complex as the exact mechanism by which the respective SMases are activated, is still not completely understood. SMase-mediated degradation of sphingomyelin however only partially contributes to ceramide levels. On the other hand, the cleavage of Cer mediated by different ceramidases (CDases) is also important for the final cellular ceramide concentration. There are mainly three different CDases known such as acid ceramidase (aCDase),[7] neutral ceramidase (nCDase)[8] and alkaline ceramidase (alkCDase)[9–11] that can be characterized by their different pH-optima and subcellular localizations. The aCDase that localizes in the endo-lysosomal compartment is undoubtedly indispensable for the cellular ceramide degradation and a deficiency of aCDase leads to a massive accumulation of ceramide in lysosomes resulting in Farber disease.[12,13] Whereas, nCDase and alkCDase are known to localize in plasma membrane and endoplasmic reticulum (ER), respectively and both of them are also involved in degrading ceramide to sphingosine.

It has been shown that aCDase is up-regulated in prostate cancer[14] and melanomas,[15] leading to the hypothesis that aCDase could be a tumor marker.[13,15,14] Moreover, aCDase in tumor cells obviously confers resistance to chemo- and radiotherapy thus making the inhibition of this enzyme a potential target for cancer therapy.[16] Elevation of ceramide level by the modulation of various enzymes using multiple drug-like agents has been proposed previously for a successful treatment of cancer.[17,18] Considering these

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facts, the inhibition of ceramidases has been thought to be significantly important for the development of chemotherapeutic agents in anti-cancer therapy.

At present, three different lead structures such as B-13, D-*e*-MAPP and NOE are known as inhibitors of ceramidases (Fig. 1). It has been shown that B-13 selectively inhibits aCDase activity with an IC_{50} of about 10 μM when cell lysate was used as enzyme source.[19] Whereas, D-*e*-MAPP, a structurally modified B-13 analogue has been reported to selectively inhibit alkaline ceramidase activity derived from HL-60 cell extracts with an IC_{50} of about 1–5 μM.[20] In another report however, D-*e*-MAPP has been shown as a relatively poor inhibitor of aCDase in concentration dependent manner in human melanoma and HaCaT cells and it did not show any noticeable inhibition towards alkaline ceramidase.[21] The third compound *N*-oleoyl-ethanolamine (NOE) is a weak inhibitor of aCDase though with the ability to increase cellular Cer levels.[22] In further studies, two NOE analogues have been reported that significantly inhibited aCDase in cell culture (IC_{50} ~15 μM) and also at the same time exhibited cytotoxicity to A549 cells (IC_{50} ~40 μM).[23,24]

In very recent studies Bielawska and co-workers have developed different series of B-13 and D-*e*-MAPP analogues with the modification of various functionalities for an improvement of their in vitro and cellular activities.[25–27] In an attempt to develop inhibitors with preferential selectivity for localization in particular cellular compartments, they have incorporated several different functional groups like permanently cationic moieties for a mitochondrial accumulation.[25,26] For the inhibition of aCDase, they introduced an ω-*N,N*-dimethylamino group at the fatty acid part of B-13 skeleton to yield compound LCL-464, which was reported to accumulate in lysosomes.[27] Indeed, LCL-464 exhibited significantly higher inhibition than B-13 towards lysosomal aCDase in MCF7 cellular experiments, but on the other hand it showed only weak in vitro inhibition of aCDase, even in comparison to B-13. The same group has also studied the effect of *p*-NO_2 group in the amine-based B-13 analogues (LCL-204 and LCL-385) on their cell growth efficacy in MCF7 cells.[25,26] The effect of *p*-NO_2 group was found to be almost negligible as both of them exhibited similar IC_{50} values for cell growth. In contrast, Foroozesh and co-workers have developed a series of substituted benzylideneamino and benzylamino-based ceramide analogues with various electron donating and withdrawing groups in the phenyl ring to study their effect on MCF-7TN-R cancer cell viability.[28] Interestingly, detailed SAR studies revealed the electronic influence of different aromatic substituents on cell viability. Very recently, Gatt and Dagan have reported novel synthetic sphingolipid analogues that could elevate the cellular ceramide concentration leading to apoptosis.[29] Furthermore, some of the compounds were useful for the sphingolipid storage diseases such as Niemann–Pick and Gaucher diseases.

Therefore, it was worthwhile to study the electronic effect of different substituents on well-known inhibitors such as B-13, D-*e*-MAPP and NOE for their inhibition towards ceramidases. In order to have a possible combination of differently substituted aromatics available with different functionalities, we decided to synthesize a set of compounds resembling either of B-13, D-*e*-MAPP or NOE lead structures. Such compounds could be conveniently synthesized by nitroaldol-reaction[30] using nitromethane and substituted benzaldehydes, followed by the subsequent reduction and acylation (Scheme 1). In a recent preliminary study, we have already shown that such compounds are candidate inhibitors of aCDase.[31]

2. Results and discussion

An improvement in the potencies of some amide-based B-13/D-*e*-MAPP analogues for the inhibition of aCDase activities as compared to B-13 in our preliminary study[31] prompted us to investigate further the molecular basis of ceramidase inhibition by these compounds with the variation of substituents on phenyl ring to have detailed structure–activity relationship (SAR) studies. Substituents on phenyl ring were chosen to understand the electron donating and electron withdrawing effects of different functional groups. As described in our previous study, the simplified B-13/D-*e*-MAPP analogues could be efficiently synthesized in three steps starting from the corresponding aldehydes.[31] These compounds could also be considered as aromatic analogues of ethanolamines, resembling the structure of NOE and analogues. Coupling of aldehydes with an excess amount of nitromethane in the presence of triethylamine (Henry reaction) led to the formation of corresponding racemic nitro-alcohol derivatives (Scheme 1). Due to the possible interference by the unreacted nitromethane for the subsequent hydrogenation reaction, the product was readily purified by a short column chromatography and subjected to hydrogenation reaction in the presence of hydrogen gas catalyzed by palladium on activated charcoal to form aminols. Treatment of the produced aminols with myristoyl chloride formed the corresponding amide-substituted DP- and KPB-analogues (Fig. 2). On the other hand, reaction of aminols with 1-dodecane-sulfonyl chloride produced new sulfonamide analogues as shown in Figure 2. In contrast to the sulfonamide-analogues of DP- and KPB-compounds, the sulfonamides KPB-70 and KPB-82, corresponding to B-13 and D-*e*-MAPP could be directly synthesized by a single step coupling of the commercially available amines (*1R,2R*)-2-amino-1-(4-nitrophenyl)-propane-1,3-diol and (*1S,2R*)-2-amino-1-(phenyl)-propan-1-ol with 1-dodecane-sulfonyl chloride. All the final amide- and sulfonamide-based inhibitors were thoroughly characterized by NMR (^1H and ^{13}C) spectroscopic and ESI-MS spectrometric analyses.

To evaluate the inhibition potency of newly synthesized amide- and sulfonamide-based compounds towards acid and neutral ceramidases, we have chosen fluorescently-labeled acyl-NBD-C_{12}-ceramide as a substrate and recombinant human aCDase and nCDase were used as enzyme sources. Ceramidase-mediated cleavage of amide bond in acyl-NBD-ceramide produces fluorescent NBD-fatty acid, which was quantified after TLC-separation. It should be noted that the absolute IC_{50} values in inhibition experiments are strongly dependent on the substrates used and the respective substrate concentrations, which prompted us to use literature-known inhibitors as reference.

As shown in Figure 3, most of the newly synthesized amides and sulfonamides exhibited significantly good inhibition towards recombinant aCDase. Inhibitory activities of different amide-based

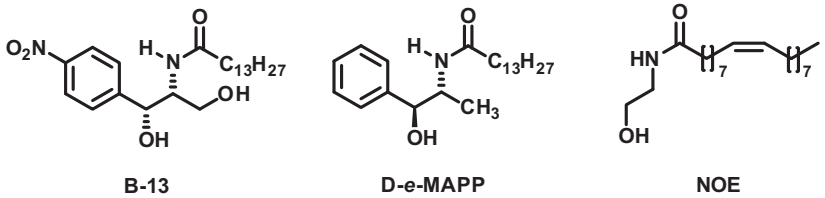
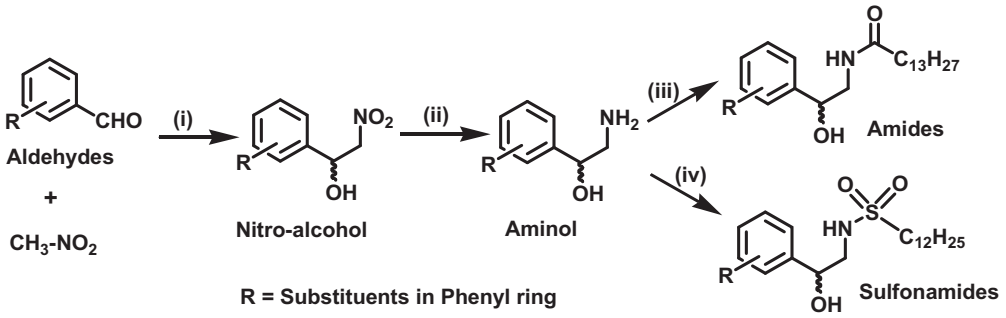


Figure 1. Current lead structures for the development of CDase inhibitors.



Scheme 1. General synthetic route to amide- and sulfonamide-based inhibitors. Reagents and conditions: (i) triethylamine, rt, 2–12 h; (ii) Pd-C/H_2, MeOH, rt, 12 h; (iii) Myristoyl chloride, aq NaOAc/THF (1:1), 0 °C to rt, 2–3 h; (iv) 1-dodecane-sulfonyl chloride, NEt_3, CH_2Cl_2, rt, 2–3 h.

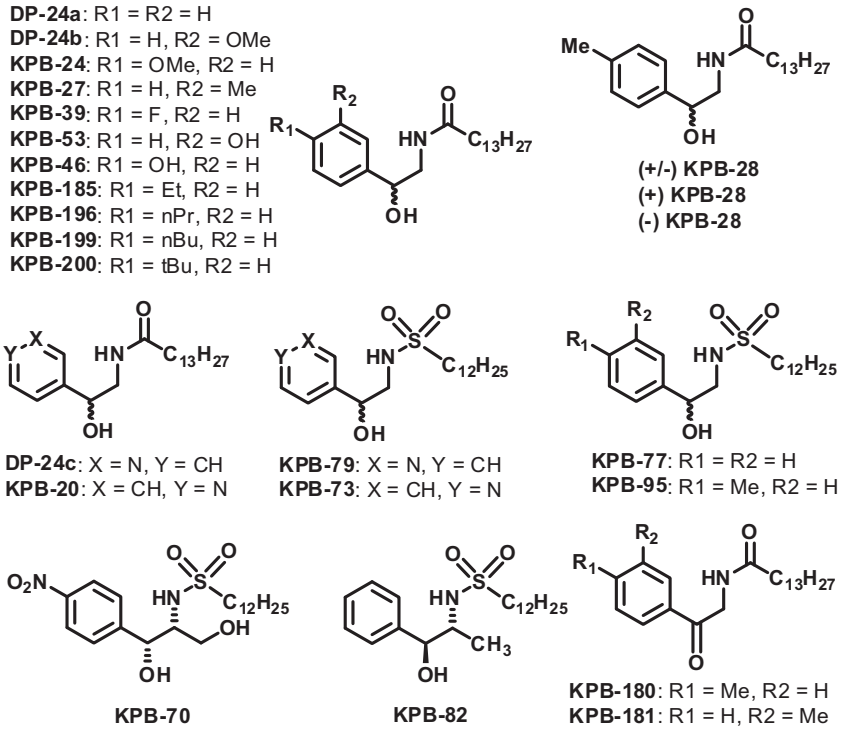


Figure 2. Chemical structures of amide- and sulfonamide-based compounds as potential inhibitors of ceramidases.

compounds are compared to that of B-13 having an electron withdrawing *p*-NO_2 group on phenyl ring. As observed previously and also in the present study that a more simplified analogue DP-24a lacking *p*-NO_2 and hydroxymethyl group of B-13, exhibited relatively higher inhibition than that of B-13 for aCDase (Fig. 3).[31] The observed enhancement in potency of DP-24a is probably due to a combined effect of nitro group and hydroxymethyl moiety. A similar enhancement in potency is also observed for D-*e*-MAPP that does not contain *p*-NO_2 and hydroxymethyl moieties (Fig. 3). Significant inhibition of aCDase activity by D-*e*-MAPP in the present study is in contrast with previous reports that show that D-*e*-MAPP exhibits relatively poor inhibition towards aCDase in HL-60 cell as well as in HaCat cells at pH 4.5.[20,21] However, a closer comparison of the activity of B-13 with DP-24a analogue having *p*-NO_2 group could not be performed, as the synthesis of *p*-NO_2 substituted DP analogue was unsuccessful owing to the complication with aromatic nitro group during the hydrogenation reaction. Therefore, the activities of newly synthesized compounds are compared to DP-24a that does not contain any substituent on the phenyl ring.

As discussed previously, compound LCL-464 with the introduction of a polar and basic *N,N*-dimethylamino group at the end of hydrophobic fatty acid chain of B-13 exhibited significantly less inhibition towards in vitro aCDase activities.[27] The remarkable drop in in vitro aCDase inhibition by LCL-464 is probably due to a partial disruption of the major hydrophobic interaction between aCDase and the positively charged fatty acid chain in LCL-464 in an acidic assay condition. Therefore, for a further modification, we decided to introduce a basic group at the phenyl ring by the replacement of phenyl ring in DP-24a with weakly basic pyridyl group (DP-24c and KPB-20). Unlike LCL-464, both the pyridyl substituted compounds exhibited significantly higher inhibitions than B-13 and even slightly higher potency than DP-24a (Fig. 3). However, the potential of these compounds in cellular medium and subsequently in animal studies is important to understand their pharmacological impacts.

To understand the electronic effect of different functionalities at the phenyl ring on their inhibitory activities, we have considered a series of compounds such as (i) compounds with electron

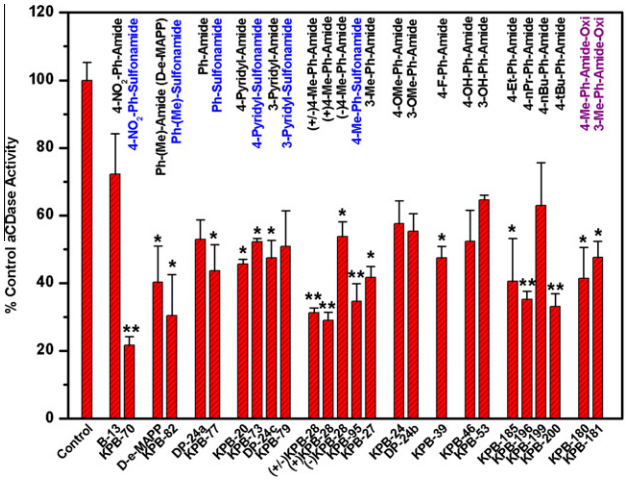


Figure 3. Plot for the relative potencies of B-13, DP- and KPB-analogues for the inhibition of acid ceramidase activity. The final concentrations of fluorescent substrate and inhibitors were 15 μM and 80 μM, respectively. Inhibitory data shown in the plot represent average of three independent experiments under standard assay condition and the results are shown as mean ± SD values. Statistical significances of the results have been compared to the potency of B-13. *, $0.01 \leqslant p \leqslant 0.05$; **, $0.001 \leqslant p \leqslant 0.01$. The control value represents the percentage hydrolysis of the substrate by aCDase in the absence of any inhibitor.

withdrawing group (–I and/or –M effect; e.g., $-NO_2$ and –F) or (ii) compounds with electron donating group (+I and/or +M effect; e.g., –Me, –Et, –*n*Pr, –*n*Bu, –OMe, –OH groups). An almost comparable inhibition of compound KPB-39 with an electron withdrawing fluoride group (–I effect) on the aromatic ring with that of DP-24a indicates negligible effect of electron withdrawing groups on the inhibitory potencies. Unfortunately, a more detailed comparison with other halogen substituted KPB-analogues was not possible as the coupling of fatty acid part with the corresponding aminol was unsuccessful, probably due to extreme instability of those aminols. Then we studied the effect of different electron donating groups (+M/+I effects) such as methoxy, hydroxyl and alkyl groups. In contrast to almost negligible effects of both *para*- and *meta*-substituted methoxy derivatives (KPB-24 and DP-24b), the corresponding hydroxyl-substituted (KPB-46 and KPB-53) and methyl-substituted compounds (KPB-28 and KPB-27) exhibited significantly improved aCDase inhibition (Fig. 3). Particularly, a notable difference in the potency was observed for the 4-Me analogue KPB-28 as compared to the corresponding 3-Me analogue (KPB-27). Eventually, KPB-28 (+I effect) was found to be the most active amide-based inhibitor of aCDase in the present study. With a possible hint for the effect of hydrophobic methyl group at phenyl ring on its inhibitory activity, we planned to synthesize and screen the higher homologues with ethyl, *n*-propyl, *n*-butyl and *tert*-butyl groups (KPB-185, KPB-196, KPB-199 and KPB-200). All the higher homologues of alkyl-substituted compounds were more active than DP-24a and almost equally active as KPB-28 except the *n*-butyl-substituted compound KPB-199. Significantly higher activity of *t*-Bu substituted compound KPB-200 and a relatively much weaker activity of the corresponding *n*-Bu analogue KPB-199 indicate the major influence of hydrophobic long hydrocarbon chain and a minor importance of steric constraints for an effective inhibition of aCDase activities. In order to investigate the effect of secondary hydroxyl group as a potential hydrogen bond donor, we have oxidized the secondary hydroxyl groups in the most active amide-derivative KPB-28 and its 3-Me analogue (KPB-27), to the corresponding ketones (KPB-180 and KPB-181). A slight (~10%) decrease in the inhibition potency was observed upon this modification, indicating a lower importance of secondary hydroxyl group for an effective inhibition of aCDase activity.

It should be mentioned that B-13 and D-*e*-MAPP structures differ by functional groups such as nitro group and primary hydroxyl group along with the stereochemical differences, suggesting these features to be decisive for selectivity between aCDase and alkCDase. Since in the newly synthesized compounds, the methyl or hydroxymethyl groups are replaced by simple hydrogen, we found it worthwhile to examine the selectivity-pattern of the newly synthesized amide-based inhibitors and we thus tested them for an inhibition of nCDase, as well. To our surprise, B-13 showed a similar—if not better—inhibition of rec. human nCDase than that of aCDase. This fact was surprising, since B-13 is known for many years as lead compound inhibiting aCDase. Indeed, Bai et al. observed a significant increase in cellular ceramide after treatment with B-13 although under the conditions applied, the lysosomal aCDase activity was not affected.[27] Our result obviously supports their hypothesis that an amidase or ceramidase other than aCDase is also inhibited by B-13 in living cells.

As shown for aCDase, most of the amide-based inhibitors exhibited higher potency than B-13 for nCDase. A comparison with the inhibitory actions of these inhibitors towards aCDase and nCDase generally revealed only little selectivity for one of the enzymes. Only the alkyl-substituted compounds KPB-196 and KPB-200 showed considerably low inhibition towards nCDase, while being among the most active inhibitors of aCDase. The potent inhibition of nCDase by methoxy-substituted (+M) compounds KPB-24 and DP-24b suggest that, unlike in the study of Foroozesh, this effect is rather due to steric or hydrophobic effects of the substituents on the phenyl ring, rather than electronic effects. Moreover, we oxidized the Me-substituted derivatives KPB-28 and KPB-27 at their secondary hydroxyl groups (KPB-180 and KPB-181), converting the hydroxyl groups into keto groups and thus turning them into hydrogen bond acceptors instead of hydrogen bond donors. Again, a substantial decrease in their nCDase activity (~30%) was observed, whereas the same modification resulted only in a little drop of aCDase inhibition (~10%). These observations suggest that, as compared to aCDase, nCDase has relatively narrow window or less flexibility for an effective binding of an inhibitor to the enzyme, which could be one of the reasons why there are not many effective inhibitors known for nCDase in the literature.

The effect of stereochemistry at C1 and C3 positions in B-13 and D-*e*-MAPP has been studied previously by Bielawska et al.[20,19] Studies on the stereoiosmers of B-13 and related compounds revealed that the stereochemistry at the carbon center bearing the secondary hydroxyl group is important for ceramidase activity.[19] On the other hand, the inhibition potency of D-*e*-MAPP towards alkaline ceramidase could be completely abolished by the reversal of stereochemistry of both the chiral centers (L-*e*-MAPP). L-*e*-MAPP was rather found to be a competitive substrate analogue for alkaline ceramidase.[20] Therefore, to understand the importance of stereochemistry of the carbon attached to the secondary hydroxyl group on their inhibitory activities in the newly synthesized aromatic ethanolamines, we have separated the enantiomers of one of the most active amide KPB-28 via preparative chiral HPLC. The optically pure enantiomers such as (+) KPB-28 and (−) KPB-28 along with the racemic (±) KPB-28 were screened for their potencies towards both acid and neutral ceramidases. However, the comparative inhibition studies with enantiomers and racemic KPB-28 revealed almost similar potencies, indicating no significant stereochemical effect of the chiral carbon atoms (Figs. 3 and 4). Furthermore, this experiment indicates that the inhibitory potencies of the racemic inhibitors in the present study are the average inhibition potency of both the enantiomers. To further prove whether one of the enantiomer act as a competitive substrate for ceramidases, we have carried out the experiment by treating both optically pure and the racemic inhibitors with ceramidase and products were analyzed to detect the hydrolysis of inhibitors.

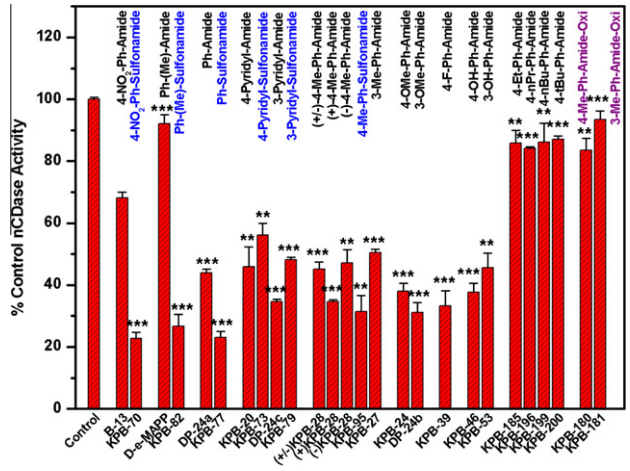


Figure 4. Plot for the relative potencies of B-13, DP- and KPB analogues for the inhibition of neutral ceramidase activity. The final concentrations of fluorescent substrate and inhibitors were 15 μM and 80 μM, respectively. Inhibitory data shown in the plot represent average of three independent experiments under standard assay condition and the results are shown as mean ± SD values. Statistical significances of the results have been compared to the potency of B-13. **, $0.001 \leqslant p \leqslant 0.01$; ***, $p \leqslant 0.001$.The control value represents the percentage hydrolysis of the substrate by nCDase in the absence of any inhibitor.

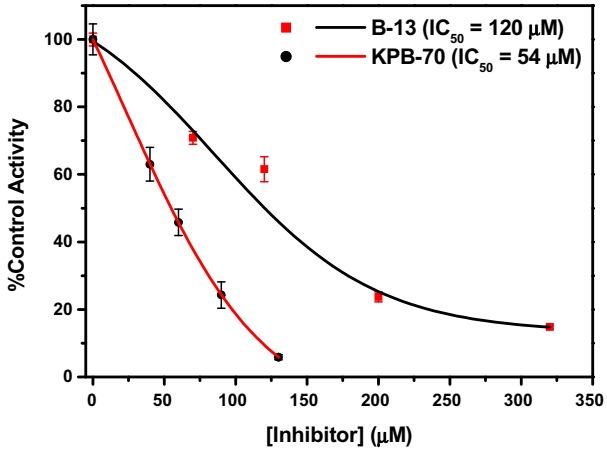


Figure 5. Plot for the effect of varying concentrations of B-13 and KPB-70 for the inhibition of nCDase activities. IC_{50} values represent the concentration of inhibitors required for 50% inhibition of control activities. Inhibitory data shown in the plot represent average of three independent experiments under standard assay condition and the results are shown as mean ± SD values.

However, both the amide and sulfonamide-based inhibitors were found to be stable towards ceramidase-mediated amide hydrolysis (see Supplementary data Fig. S43). This is in contrast to the results obtained for the regulation of cell growth and differentiation by different stereoisomers of B-13 and D-*e*-MAPP, having two chiral centers.[19]

In a final set of compounds, we replaced the amide groups of the most interesting inhibitors by sulfonamide groups. Sulfonamide groups are biologically more stable than amides and may also mimic the tetrahedral transition state of amide cleavage,[32] which makes this modification particularly interesting for substrate-analogue ceramidase inhibitors. The sulfonamide analogue of B-13 (KPB-70), D-*e*-MAPP (KPB-82) and of some selective DP- and KPB-analogues were synthesized and studied for their inhibition potencies. In contrast to the most of the newly synthesized racemic amides and sulfonamides, KPB-70 and KPB-82 were optically pure sulfonamides identical to their amide counterparts such as B-13 and D-*e*-MAPP. Interestingly, both the sulfonamides exhibited significantly higher inhibitory potency than their parent amides (B-13 and D-*e*-MAPP) for the inhibition of both aCDase and nCDase, making them most potent inhibitors in the series of amide- and sulfonamide-based compounds (Figs. 3 and 4). Furthermore, replacement of amide moiety in DP-24a with sulfonamide (KPB-77) led to a significant improvement in the potency for both aCDase and nCDase. Particularly, the enhancement was more pronounced for nCDase, making KPB-77 almost equally active as KPB-70. In contrast, the inhibitory activities of pyridyl-based sulfonamides (KPB-73 and KPB-79) were slightly less than the corresponding amides KPB-20 and KPB-24c, respectively. The activity of KPB-95, the sulfonamide analogue of KPB-28, was found to be at least similar or better than its parent compound for the inhibition of ceramidases. The higher potency of sulfonamides as compared to amides could be due to the presence of an additional hydrogen bond acceptor in sulfonamides.

As KPB-70 was found to be best inhibitor in the series of amide- and sulfonamide-based compounds employed in the present study, we studied the dose-dependency for the inhibition of neutral ceramidase with a wide range of concentrations and compared to that of B-13. As shown in Figure 5, KPB-70 was found to be significantly more potent than B-13 over the entire range of concentrations and the IC_{50} value was less than half of that for B-13 for the inhibition of nCDase under the identical assay conditions.

3. Conclusion

In summary, we provide a detailed SAR study for an inhibition of both recombinant human aCDase and nCDase using a series of amide- and sulfonamide-based inhibitors. Surprisingly, most of the inhibitors including B-13 showed little selectivity for one of the two ceramidases. In our in vitro study the electronic contribution of different substituents on phenyl ring in B-13 and D-*e*-MAPP analogues neither was important for the inhibition potencies towards both of aCDase and nCDase nor did it influence the enzyme selectivity. In contrast, several alkylated and oxidized compounds showed potent inhibition of aCDase while nCDase activity was hardly affected. Furthermore, the activity of B-13 and D-*e*-MAPP could be significantly enhanced by the replacement of amide group with a sulfonamide moiety. Overall, this study reveals that aCDase has much higher tolerance than nCDase for accepting wide range of inhibitors with altered geometries highlighting the difference in both enzymes' binding pocket geometries. However, the pharmacological significance of the lead compounds of the present study could be better justified only after investigating their efficacy in living cells, which will be performed in due course.

4. Experimental section

4.1. Materials and method

Chemicals were purchased from Sigma–Aldrich or Acros. Acyl-C_{12}-NBD ceramide 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 F_{254} on aluminum sheets (Merck KGaA). ^1H (500 MHz and 300 MHz) and ^{13}C (125 MHz and 75 MHz) NMR spectra were obtained on a Bruker Avance III 500 MHz and 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 pure enantiomers

of KPB-28 was determined using Perkin-Elmer 241 Polarimeter. The inhibition data have been plotted using Origin 6.1 software version and the statistical significance was assessed using students *T*-test with IBM SPSS software. Compounds DP-24a, DP-24b and DP-24c were synthesized following the literature procedure.[31]

4.2. General procedure for the synthesis of nitro-alcohols

To a stirred solution of aldehyde (1.50 mmol) in nitromethane (10.0 mL) was added triethylamine (1.0 mL) and the resulting solution was stirred at room temperature until consumption of the aldehyde was indicated by TLC. The solution was diluted with ethyl acetate (200 mL), washed with sodium hydrogen sulfite, saturated sodium hydrogen carbonate, water and brine. The combined organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluents. As most of the nitro-alcohols were quite unstable, they were immediately used for the next step without detailed characterization.

4.3. General procedure for the reduction of nitro-alcohols to aminols

The nitro-alcohol was dissolved in dry methanol and charged with a catalytic amount of palladium on activated charcoal (10 mol %). The reaction vessel was evacuated and aerated with hydrogen gas. The reaction mixture was stirred at room temperature until the consumption of the nitro-alcohol was indicated by TLC with the formation of a very polar spot, active with ninhydrin staining. The solution was filtered through a pad of Celite and the solvent was evaporated to dryness. The crude product was directly used for the next step without any purification.

4.4. General procedure for the preparation of amide-based ceramidase inhibitors

To an ice-cooled stirred solution of the aminol (1.23 mmol) in 3 mL of THF and 4 mL of 50% aqueous solution of sodium acetate was dropwise added myristoyl chloride (1.23 mmol) in THF (1 mL). The reaction mixture was allowed to attain room temperature over 1 h and stirred at room temperature for another 2–3 h. The completion of the reaction was monitored using TLC technique. The reaction mixture was diluted with ethyl acetate and extracted with water and brine solution. The combined organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford the crude product as white or yellowish solid. The crude product was purified by silica gel column chromatography using cyclohexane, ethyl acetate and methanol as eluent.

4.5. Compound KPB-20

Aminol used (0.17 g, 1.23 mmol); Yield: 0.20 g (47%) as white amorphous solid. Mp: 88–90 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.88 (t, *J* = 6.5 Hz, 3H), 1.25 (br, 20H), 1.58 (m, 2H), 2.17 (m, 2H), 3.31 (m, 1H), 3.71 (m, 1H), 4.85 (d, *J* = 7.0 Hz, 1H), 6.48 (d, *J* = 4.3 Hz, 1H), 7.32 (d, *J* = 3.7 Hz, 2H), 8.47 (br, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 25.7, 29.3, 29.5, 29.6, 31.9, 36.4, 47.1, 72.2, 121.2, 149.2, 151.8, 175.3. EI-MS: *m/z* calcd for $C_{21}H_{36}N_2O_2$: 348.2777 $[M]^+$; observed: 348.2777.

4.6. Compound KPB-24

Aminol used (0.27 g, 1.61 mmol); Yield: 0.28 g (46%) as white solid. Mp: 98–100 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.89 (t, *J* = 7.0 Hz, 3H), 1.26 (br, 20H), 1.59–1.65 (m, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 3.31–3.37 (m, 1H), 3.64–3.69 (m, 1H), 3.81 (s, 3H), 4.80 (dd, *J*1 = 3.3 Hz, *J*2 = 7.8 Hz, 1H), 5.88 (s, 1H), 6.89 (d, *J* = 8.8 Hz, 2H), 7.29 (d, *J* = 8.6 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 25.7, 29.3, 29.4, 29.5, 29.7, 31.9, 36.7, 47.5, 55.3, 73.5, 113.9, 127.1, 133.9, 159.3, 174.7. EI-MS: *m/z* calcd for $C_{23}H_{39}NO_3$: 377.2930 $[M]^+$; observed: 377.2930.

4.7. Compound KPB-27

Aminol used (0.25 g, 1.65 mmol); Yield: 0.27 g (44%) as white crystalline solid. Mp: 68–70 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.88 (t, *J* = 7.0 Hz, 3H), 1.25 (br, 20H), 1.57 (br, 2H), 2.14 (dd, *J*1 = 7.7 Hz, *J*2 = 12.3 Hz, 2H), 2.33 (d, *J* = 2.6 Hz, 3H), 3.26–3.31 (m, 1H), 3.62–3.69 (m, 1H), 4.75 (m, 1H), 6.30 (br, 1H), 7.07 (d, *J* = 7.4 Hz, 1H), 7.12 (d, *J* = 7.7 Hz, 1H), 7.15 (s, 1H), 7.19–7.23 (m, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 21.4, 22.7, 25.7, 29.3, 29.4, 29.5, 29.7, 31.9, 36.6, 47.5, 73.5, 122.9, 126.5, 128.3, 128.5, 138.1, 141.9, 174.8. EI-MS: *m/z* calcd for $C_{23}H_{39}NO_2$: 361.2981 $[M]^+$; observed: 361.2981.

4.8. Compound (±) KPB-28

Aminol used (0.23 g, 1.52 mmol); Yield: 0.24 g (43%) as white crystalline solid. Mp: 86–88 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.88 (t, *J* = 7.0 Hz, 3H), 1.18–1.30 (m, 23H), 1.40–1.46 (m, 2H), 2.03–2.13 (m, 2H), 3.81 (t, *J* = 4.3 Hz, 2H), 4.13–4.17 (m, 1H), 5.20 (d, *J* = 2.8 Hz, 1H), 6.25 (d, *J* = 8.4 Hz, 1H), 7.54 (d, *J* = 8.7 Hz, 2H), 8.17 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 21.1, 22.7, 25.7, 29.3, 29.5, 29.6, 29.7, 31.9, 36.6, 47.4, 73.7, 125.8, 129.2, 137.5, 138.8, 174.8. EI-MS: *m/z* calcd for $C_{23}H_{39}NO_2$: 361.2981 $[M]^+$; observed: 361.2981.

4.9. Compound (+) KPB-28

^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, *J* = 6.7 Hz, 3H), 1.26 (s, 23H), 1.57–1.67 (m, 2H), 2.19 (t, *J* = 7.8 Hz, 2H), 2.35 (s, 3H), 3.34 (dddd, *J*1 = 5.0 Hz, *J*2 = 7.6 Hz, *J*3 = 14.1 Hz, 1H), 3.70 (dddd, *J*1 = 3.3 Hz, *J*2 = 6.8 Hz, *J*3 = 14.1 Hz, 1H), 4.82 (dd, *J*1 = 3.3 Hz, *J*2 = 7.5 Hz, 1H), 5.83 (br, 1H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.25 (d, *J* = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 21.1, 22.7, 25.7, 29.2, 29.5, 29.5, 29.6, 31.9, 36.6, 47.4, 73.8, 125.7, 129.2, 137.6, 138.8, 174.7. EI-MS: *m/z* calcd for $C_{23}H_{39}NO_2$: 361.2981 $[M]^+$; observed: 361.2981. $[\alpha]_D^{25}$ = +1.24 (*c* 7.22 mg/mL in MeOH).

4.10. Compound (−) KPB-28

^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, *J* = 6.7 Hz, 3H), 1.26 (s, 23H), 1.61 (m, 2H), 2.19 (t, *J* = 7.8 Hz, 2H), 2.35 (s, 3H), 3.34 (dddd, *J*1 = 5.0 Hz, *J*2 = 7.6 Hz, *J*3 = 14.1 Hz, 1H), 3.70 (dddd, *J*1 = 3.4 Hz, *J*2 = 6.8 Hz, *J*3 = 14.1 Hz, 1H), 4.82 (dd, *J*1 = 3.3 Hz, *J*2 = 7.6 Hz, 1H), 5.83 (br, 1H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.25 (d, *J* = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 21.1, 22.7, 25.7, 29.2, 29.4, 29.5, 29.6, 31.9, 36.6, 47.4, 73.8, 125.7, 129.2, 137.6, 138.8, 174.7. EI-MS: *m/z* calcd for $C_{23}H_{39}NO_2$: 361.2981 $[M]^+$; observed: 361.2981. $[\alpha]_D^{25}$ = −1.11 (c = 7.20 mg/mL in MeOH).

4.11. Compound KPB-39

Aminol used (0.15 g, 0.97 mmol); Yield: 0.15 g (42%) as white amorphous solid. Mp: 78–80 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, *J* = 7.0 Hz, 3H), 1.28 (br, 20H), 1.60–1.65 (m, 2H), 2.22 (t, *J* = 7.6 Hz, 2H), 3.33–3.38 (m, 1H), 3.69 (d, *J* = 14.2 Hz, 1H), 4.87 (d, *J* = 7.5 Hz, 1H), 5.98 (br, 1H), 7.06 (t, *J* = 8.6 Hz, 2H), 7.36 (dd, *J*1 = 5.5 Hz, *J*2 = 8.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 25.7, 29.2, 29.3, 29.5, 29.7, 31.9, 36.6, 47.7, 73.4,

115.3, 115.4, 127.4, 127.5, 137.6, 161.4, 163.3, 175.1. EI-MS: *m/z* calcd for $C_{22}H_{36}FNO_2$: 365.2730 $[M]^+$; observed: 365.2726.

4.12. Compound KPB-46

Aminol used (0.12 g, 0.78 mmol); Yield: 0.16 g (57%) as white solid. Mp: 104–106 °C. ^{1}H NMR (MeOH-d_4, 500 MHz, ppm): δ = 0.92 (t, *J* = 7.0 Hz, 3H), 1.31 (br, 20H), 1.55–1.61 (m, 2H), 2.18 (t, *J* = 7.5 Hz, 2H), 3.35–3.37 (m, 1H), 3.42 (dd, *J*1 = 5.3 Hz, *J*2 = 13.5 Hz, 1H), 4.67 (dd, *J*1 = 5.3 Hz, *J*2 = 7.5 Hz, 1H), 6.77 (d, *J* = 8.6 Hz, 2H), 7.21 (d, *J* = 8.5 Hz, 2H). ^{13}C NMR (MeOH-d_4, 125 MHz, ppm): 14.5, 23.8, 27.0, 30.3, 30.5, 30.6, 30.7, 30.8, 33.1, 37.1, 47.9, 73.3, 116.1, 128.5, 134.6, 158.1, 176.6. ESI-MS: *m/z* calcd for $C_{22}H_{35}NO_3$: 362.2701 $[M+H]^+$; observed: 362.2701.

4.13. Compound KPB-53

Aminol used (0.2 g, 1.31 mmol); Yield: 0.23 g (48%) as yellowish crystalline solid. Mp: 68–70 °C. ^{1}H NMR (MeOH-d_4, 500 MHz, ppm): δ = 0.92 (t, *J* = 7.0 Hz, 3H), 1.31 (br, 20H), 1.57–1.62 (m, 2H), 2.19 (t, *J* = 7.5 Hz, 2H), 3.33 (dd, *J*1 = 8.4 Hz, *J*2 = 12.9 Hz, 2H), 3.45 (dd, *J*1 = 4.8 Hz, *J*2 = 13.6 Hz, 1H), 4.70 (dd, *J*1 = 5.0 Hz, *J*2 = 7.6 Hz, 1H), 6.71 (dd, *J*1 = 1.6 Hz, *J*2 = 7.6 Hz, 1H), 6.85 (d, *J* = 6.6 Hz, 2H), 7.16 (t, *J* = 8.0 Hz, 1H). ^{13}C NMR (MeOH-d_4, 125 MHz, ppm): 14.5, 23.8, 27.1, 30.3, 30.5, 30.7, 30.8, 33.1, 37.1, 48.1, 73.6, 114.1, 115.5, 118.4, 130.4, 145.5, 158.6, 176.7. EI-MS: *m/z* calcd for $C_{22}H_{37}NO_3$: 363.2773 $[M]^+$; observed: 363.2773.

4.14. Compound KPB-185

Aminol used (0.30 g, 1.81 mmol); Yield: 0.41 g (62%) as yellowish amorphous solid. Mp: 86–88 °C. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.89 (t, *J* = 6.6 Hz, 3H), 1.19–1.26 (br, 25H), 1.53 (br, 2H), 2.09 (t, *J* = 7.5 Hz, 2H), 2.61 (q, *J* = 7.5 Hz, 2H), 3.21–3.27 (m, 1H), 3.55–3.58 (m, 1H), 4.71 (d, *J* = 5.4 Hz, 1H), 6.58 (br, 1H), 7.12 (d, *J* = 7.6 Hz, 2H), 7.22 (d, *J* = 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 14.6, 22.7, 25.8, 29.3, 29.4, 29.5, 29.7, 31.9, 36.7, 51.0, 76.6, 126.3, 127.4, 128.1, 140.8, 174.2. ESI-MS: *m/z* calcd for $C_{24}H_{39}NO_2$: 362.3054 $[M+H]^+$; observed: 362.3054.

4.15. Compound KPB-196

Aminol used (0.20 g, 1.12 mmol); Yield: 0.38 g (70%) as white amorphous solid. Mp: 100–102 °C. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.86–0.95 (m, 6H), 1.27 (br, 18H), 1.48–1.64 (m, 4H), 2.06 (t, *J* = 8.1 Hz, 2H), 2.54 (t, *J* = 7.8 Hz, 2H), 3.21 (m, 1H), 3.53 (m, 1H), 4.69 (d, *J* = 7.7 Hz, 1H), 4.90 (s, 1H), 6.84 (t, *J* = 4.36 Hz, 1H), 7.07 (d, *J* = 8.1 Hz, 2H), 7.19 (d, *J* = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 13.8, 14.1, 22.7, 24.5, 25.8, 29.3, 29.4, 29.5, 29.6, 29.8, 31.9, 36.5, 47.5, 73.0, 125.7, 128.4, 139.3, 141.9, 174.9. ESI-MS: *m/z* calcd for $C_{25}H_{43}NO_2$: 412.3186 $[M+Na]^+$; observed: 412.3186.

4.16. Compound KPB-199

Aminol used (0.29 g, 1.50 mmol); Yield: 0.31 g (52%) as light yellow solid. Mp: 78–80 °C. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.86–0.95 (m, 6H), 1.26 (br, 18H), 1.31–1.38 (m, 2H), 1.52–1.62 (m, 2H), 2.11 (t, *J* = 7.6 Hz, 2H), 2.58 (t, *J* = 8.1 Hz, 2H), 3.23–3.30 (m, 1H), 3.57–3.63 (m, 1H), 4.74 (d, *J* = 5.2 Hz, 1H), 6.41 (br, 1H), 7.12 (d, *J* = 7.8 Hz, 2H), 7.22 (d, *J* = 7.9 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.0, 14.1, 22.4, 22.7, 25.7, 29.3, 29.4, 29.6, 29.7, 31.9, 33.6, 35.4, 36.6, 47.4, 73.3, 125.7, 128.4, 139.1, 142.4, 174.8. ESI-MS: *m/z* calcd for $C_{26}H_{45}NO_2$: 426.3343 $[M+Na]^+$; observed: 426.3343.

4.17. Compound KPB-200

Aminol used (0.30 g, 1.50 mmol); Yield: 0.28 g (45%) as light yellow amorphous solid. Mp: 108–110 °C. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, *J* = 6.7 Hz, 3H), 1.26 (s, 18H), 1.30 (s, 10H), 1.57 (m, 2H), 2.14 (t, *J* = 7.5 Hz, 2H), 3.27–3.31 (m, 1H), 3.62–3.65 (m, 1H), 4.76 (d, *J* = 5.3 Hz,1H), 6.31 (br, 1H), 7.26 (d, *J* = 8.1 Hz, 2H), 7.35 (d, *J* = 7.9 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 22.7, 25.7, 29.3, 29.4, 29.6, 29.7, 31.3, 31.9, 34.5, 36.6, 47.3, 73.3, 125.3, 125.6, 138.8, 150.7, 174.8. ESI-MS: *m/z* calcd for $C_{26}H_{45}NO_2$: 426.3343 $[M+Na]^+$; observed: 426.3342.

4.18. Synthesis of sulfonamide analogues

To a solution of amine (0.73 mmol) in the presence of triethylamine (1.2 mL, 0.87 mmol) in CH_2Cl_2 (3.0 mL) was added dropwise a solution of 1-dodecanesulfonyl chloride (0.19 mg, 0.73 mmol) in CH_2Cl_2 (1.0 mL). The reaction mixture was stirred for 2–3 h at room temperature and the completion of the reaction was monitored by TLC. The mixture was diluted with CH_2Cl_2 and extracted with water followed by brine solution. The combined organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to obtain the crude product as semi-solid compound. The product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluent.

4.19. Compound KPB-70

Aminol used (50 mg, 0.23 mmol); Yield: 51 mg (49%) as white amorphous solid. Mp: 94–96 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, *J* = 6.7 Hz, 3H), 1.28 (br, 16H), 1.37–1.44 (m, 2H), 1.84–1.94 (m, 2H), 2.49 (d, *J* = 4.5 Hz, 1H), 2.61 (d, *J* = 3.6 Hz, 1H), 2.70 (d, *J* = 3.6 Hz, 1H), 3.05–3.13 (m, 2H), 4.73 (t, *J* = 4.6 Hz, 1H), 7.64 (d, *J* = 8.4 Hz, 2H), 8.28 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 22.9, 28.3, 29.0, 29.3, 29.4, 29.5, 29.6, 31.9, 42.4, 52.4, 71.8, 124.0, 127.0, 147.0, 147.9. ESI-MS: *m/z* calcd for $C_{21}H_{36}N_2O_6S$: 443.2221 $[M-H]^-$; observed: 443.2221.

4.20. Compound KPB-73

Aminol used (0.09 g, 0.64 mmol); Yield: 0.14 g (58%) as light yellow semi-solid. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, *J* = 7.0 Hz, 3H), 1.26 (br, 16H), 1.36–1.41 (m, 2H), 1.74–1.81 (m, 2H), 3.00–3.03 (m, 2H), 3.20 (dd, *J*1 = 7.9 Hz, *J*2 = 13.6 Hz, 1H), 3.44 (d, *J* = 13.0 Hz, 1H), 4.90 (dd, *J*1 = 3.2 Hz, *J*2 = 7.8 Hz, 1H), 5.50 (br, 1H), 7.33 (d, *J* = 4.2 Hz, 2H), 8.49 (s, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 23.6, 28.3, 29.1, 29.3, 29.5, 29.6, 31.9, 49.9, 53.0, 71.8, 121.2, 149.5, 150.8. EI-MS: *m/z* calcd for $C_{19}H_{34}N_2O_3S$: 370.2290 $[M]^+$; observed: 370.2290.

4.21. Compound KPB-77

Aminol used (0.1 g, 0.73 mmol); Yield: 0.14 g (52%) white amorphous solid. Mp: 76–78 °C. ^{1}H NMR1H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, *J* = 11.0 Hz, 3H), 1.27 (br, 18H), 1.71–1.82 (m, 2H), 2.95–3.00 (m, 2H), 3.21–3.28 (m, 1H), 3.39–3.44 (m, 1H), 4.88–4.91 (m, 1H), 7.30–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 23.6, 26.9, 28.3, 29.1, 29.3, 29.5, 29.6, 31.9, 50.3, 53.0, 73.3, 125.9, 128.3, 128.7, 140.9. ESI-MS: *m/z* calcd for $C_{20}H_{35}NO_3S$: 368.9760 $[M-H]^-$; observed: 368.9674.

4.22. Compound KPB-79

Aminol used (65 mg, 0.47 mmol); Yield: 75 mg (43%) as white amorphous solid. Mp: 74–76 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.87 (t, *J* = 11.0 Hz, 3H), 1.25 (br, 18H), 1.70–1.80 (m, 2H), 2.96–

3.01 (m, 2H), 3.20–3.27 (m, 1H), 3.37–3.41 (m, 1H), 4.80–4.81 (br, 1H), 4.90–4.93 (m, 1H), 5.73 (br, 1H), 7.28 (t, J = 6.0 Hz, 1H), 7.75 (d, J = 13.5 Hz, 1H), 8.41 (d, J = 6.0 Hz, 1H), 8.53 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 22.7, 23.6, 28.3, 29.1, 29.3, 29.5, 29.6, 31.9, 50.2, 53.0, 71.0, 123.7, 134.4, 147.5, 148.7. EI-MS: *m/z* calcd for $C_{19}H_{34}N_2O_3S$: 370.2290 $[M]^+$; observed: 370.2290.

4.23. Compound KPB-82

Aminol used (70 mg, 0.46 mmol). Yield: 73 mg (41%) as white amorphous solid. Mp: 66–68 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.90 (t, J = 6.5 Hz, 3H), 1.09 (d, J = 7.0 Hz, 3H), 1.28 (br, 14H), 1.35–1.39 (m, 2H), 1.73–1.78 (m, 2H), 2.95 (t, J = 6.0 Hz, 2H), 3.72–3.75 (m, 1H), 4.80 (br, 1H), 4.89 (d, J = 3.5 Hz, 1H), 7.29–7.37 (m, 5H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 15.9, 22.7, 23.6, 28.3, 29.1, 29.3, 29.5, 29.6, 31.9, 53.9, 55.2, 126.3, 127.8, 128.4, 140.5. ESI-MS: *m/z* calcd for $C_{21}H_{37}NO_3S$: 382.2421 $[M-H]^-$; observed: 382.2422.

4.24. Compound KPB-95

Aminol used (0.2 g, 1.32 mmol); Yield: 0.24 g (47%) as light yellow amorphous solid. Mp: 50–52 °C. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ = 0.89 (t, J = 6.6 Hz, 3H), 1.26 (s, 20H), 1.72–1.90 (m, 2H), 2.34 (s, 3H), 2.93–3.02 (m, 1H), 3.17–3.27 (m, 2H), 3.60 (dd, $J1$ = 10.2 Hz, $J2$ = 13.0 Hz, 1H), 5.03 (dd, $J1$ = 1.9 Hz, $J2$ = 10.0 Hz, 1H), 7.15 (d, J = 8.0 Hz, 2H), 7.26 (dd, $J1$ = 3.5 Hz, $J2$ = 8.8 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): 14.1, 21.2, 22.1, 22.7, 28.6, 29.0, 29.3, 29.4, 29.5, 29.6, 29.6, 31.9, 45.1, 50.3, 52.9, 59.7, 70.6, 73.3, 125.9, 126.1, 129.3, 137.3, 137.8, 137.9. ESI-MS: *m/z* calcd for $C_{21}H_{37}NO_3S$: 382.2421 $[M-H]^-$; observed: 382.2421.

4.25. Synthesis of KPB-180

To a stirred solution of KPB-28 (50 mg, 0.14 mmol) in CH_2Cl_2 (4 mL) was added pyridinium chlorochromate (PCC, 90 mg, 0.41 mmol) and the reaction was continued for 12 h at room temperature. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated and the crude product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluents. The solvent was evaporated to afford the pure product as white semi-solid. Yield: 37 mg (74%). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, J = 6.7 Hz, 3H), 1.26 (s, 22H), 1.68 (m, 2H), 2.45 (s, 3H), 2.80 (t, J = 7.4 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 8.17 (d, J = 8.2 Hz, 2H), 9.20 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 21.9, 22.7, 24.0, 29.0, 29.3, 29.4, 29.6, 29.7, 31.9, 37.4, 129.6, 129.8, 131.2, 146.5, 173.9, 185.4. ESI-MS: *m/z* calcd for $C_{23}H_{37}NO_2$: 360.2897 $[M+H]^+$; observed: 360.2897.

4.26. Synthesis of KPB-181

To a stirred solution of KPB-27 (50 mg, 0.14 mmol) in CH_2Cl_2 (4 mL) was added pyridinium chlorochromate (PCC, 90 mg, 0.41 mmol) and the reaction was continued for 12 h at room temperature. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated and the crude product was purified by silica gel column chromatography using cyclohexane and ethyl acetate as eluents. The solvent was evaporated to afford the pure product as white solid. Yield: 34 mg (68%). Mp: 68–70 °C. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ = 0.88 (t, J = 6.7 Hz, 3H), 1.25 (s, 22H), 1.68 (m, 2H), 2.30 (t, J = 7.8 Hz, 2H), 2.42 (s, 3H), 4.76 (d, J = 4.2 Hz, 2H), 6.57 (br, 1H), 7.40 (m, 2H), 7.78 (d, J = 8.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 14.1, 21.3, 22.7, 25.7, 29.3, 29.5, 29.6, 31.9, 36.6, 46.4, 125.1, 128.5, 128.8, 134.4, 134.9, 138.8, 173.4, 194.5. ESI-MS: *m/z* calcd for $C_{23}H_{37}NO_2$: 382.2717 $[M+Na]^+$; observed: 382.2716.

4.27. 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 substrates and all the inhibitors were prepared in DMSO. Partially purified human recombinant acid ceramidase (aCDase) expressed from SF9 cells[33] was used as the enzyme source for the cleavage of the fluorescent substrates. The total volume of the assay mixture was 100 μL. The final concentration of the substrates and inhibitors were 15 μM 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 substrates on the TLC plate were detected by Fluorescent Imaging System (Kodak Image Station 4000 MM PRO) using 430 nm and 550 nm as excitation and emission wavelengths for the NBD-linked substrate and product. The percentage ceramidase activity and the inhibition were determined by the quantification of fluorescent intensity of the cleaved product and uncleaved substrates.

4.28. 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 μM 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.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.031.

References and notes

1. Kolter, T.; Sandhoff, K. *Angew. Chem., Int. Ed.* **1999**, *38*, 1532.
2. Delgado, A.; Casas, J.; Llebaria, A.; Abad, J. L.; Fabrias, G. *Biochim. Biophys. Acta* **2006**, *1758*, 1957.
3. Pettus, B. J.; Chalfant, C. E.; Hannun, Y. A. *Biochim. Biophys. Acta* **2002**, *1585*, 114.

4. Maceyka, M.; Payne, S. G.; Milstien, S.; Spiegel, S. *Biochim. Biophys. Acta* **2002**, *1585*, 193.
5. Taha, T. A.; Mullen, T. D.; Obeid, L. M. *Biochim. Biophys. Acta* **2006**, *1758*, 2027.
6. Spiegel, S.; Milstien, S. *Nat. Rev. Mol. Cell. Biol.* **2003**, *4*, 397.
7. Li, C. M.; Park, J. H.; He, X.; Levy, B.; Chen, F.; Arai, K.; Adler, D. A.; Disteche, C. M.; Koch, J.; Sandhoff, K.; Schuchman, E. H. *Genomics* **1999**, *62*, 223.
8. Liu, B.; Hassler, D. F.; Smith, G. K.; Weaver, K.; Hannun, Y. A. *J. Biol. Chem.* **1998**, *273*, 34472.
9. El Bawab, S.; Bielawska, A.; Hannun, Y. A. *J. Biol. Chem.* **1999**, *274*, 27948.
10. El Bawab, S.; Roddy, P.; Qian, T.; Bielawska, A.; Lemasters, J. J.; Hannun, Y. A. *J. Biol. Chem.* **2000**, *275*, 21508.
11. Tani, M.; Okino, N.; Mitsutake, S.; Tanigawa, T.; Izu, H.; Ito, M. *J. Biol. Chem.* **2000**, *275*, 3462.
12. Koch, J.; Gartner, S.; Li, C. M.; Quintern, L. E.; Bernardo, K.; Levran, O.; Schnabel, D.; Desnick, R. J.; Schuchman, E. H.; Sandhoff, K. *J. Biol. Chem.* **1996**, *271*, 33110.
13. Ehlert, K.; Frosch, M.; Fehse, N.; Zander, A.; Roth, J.; Vormoor, J. *Pediatr. Rheumatol. Online J.* **2007**, *5*, 15.
14. Musumarra, G.; Barresi, V.; Condorelli, D. F.; Scire, S. *Biol. Chem.* **2003**, *384*, 321.
15. Liu, X.; Cheng, J. C.; Turner, L. S.; Elojeimy, S.; Beckham, T. H.; Bielawska, A.; Keane, T. E.; Hannun, Y. A.; Norris, J. S. *Expert Opin. Ther. Targets* **2009**, *13*, 1449.
16. Saad, A. F.; Meacham, W. D.; Bai, A.; Anelli, V.; Elojeimy, S.; Mahdy, A. E.; Turner, L. S.; Cheng, J.; Bielawska, A.; Bielawski, J.; Keane, T. E.; Obeid, L. M.; Hannun, Y. A.; Norris, J. S.; Liu, X. *Cancer Biol. Ther.* **2007**, *6*, 1455.
17. Radin, N. S. *Eur. J. Biochem./FEBS* **2001**, *268*, 193.
18. Radin, N. S. *Med. Hypotheses* **2001**, *57*, 96.
19. Bielawska, A.; Linardic, C. M.; Hannun, Y. A. *J. Biol. Chem.* **1992**, *267*, 18493.
20. Bielawska, A.; Greenberg, M. S.; Perry, D.; Jayadev, S.; Shayman, J. A.; McKay, C.; Hannun, Y. A. *J. Biol. Chem.* **1996**, *271*, 12646.
21. Raisova, M.; Goltz, G.; Bektas, M.; Bielawska, A.; Riebeling, C.; Hossini, A. M.; Eberle, J.; Hannun, Y. A.; Orfanos, C. E.; Geilen, C. C. *FEBS Lett.* **2002**, *516*, 47.
22. Ueda, N.; Yamanaka, K.; Yamamoto, S. *J. Biol. Chem.* **2001**, *276*, 35552.
23. Grijalvo, S.; Bedia, C.; Triola, G.; Casas, J.; Llebaria, A.; Teixido, J.; Rabal, O.; Levade, T.; Delgado, A.; Fabrias, G. *Chem. Phys. Lipids* **2006**, *144*, 69.
24. Bedia, C.; Canals, D.; Matabosch, X.; Harrak, Y.; Casas, J.; Llebaria, A.; Delgado, A.; Fabrias, G. *Chem. Phys. Lipids* **2008**, *156*, 33.
25. Bielawska, A.; Bielawski, J.; Szulc, Z. M.; Mayroo, N.; Liu, X.; Bai, A.; Elojeimy, S.; Rembiesa, B.; Pierce, J.; Norris, J. S.; Hannun, Y. A. *Bioorg. Med. Chem.* **2008**, *16*, 1032.
26. Szulc, Z. M.; Mayroo, N.; Bai, A.; Bielawski, J.; Liu, X.; Norris, J. S.; Hannun, Y. A.; Bielawska, A. *Bioorg. Med. Chem.* **2008**, *16*, 1015.
27. Bai, A.; Szulc, Z. M.; Bielawski, J.; Mayroo, N.; Liu, X.; Norris, J.; Hannun, Y. A.; Bielawska, A. *Bioorg. Med. Chem.* **2009**, *17*, 1840.
28. Liu, J.; Antoon, J. W.; Ponnapakkam, A.; Beckman, B. S.; Foroozesh, M. *Bioorg. Med. Chem.* **2010**, *18*, 5316.
29. Gatt, S.; Dagan, A. *Chem. Phys. Lipids* **2012**, *165*, 462.
30. Kolter, T.; van Echten-Deckert, G.; Sandhoff, K. *Tetrahedron* **1994**, *50*, 13425.
31. Proksch, D.; Klein, J. J.; Arenz, C. *J. Lipids* **2011**, *2011*, 971618.
32. Gennari, C.; Nestler, H. P.; Salom, B.; Still, W. C. *Angew. Chem., Int. Ed* **1995**, *34*, 1765.
33. Schulze, H.; Schepers, U.; Sandhoff, K. *Biol. Chem.* **2007**, *388*, 1333.