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Luminescence

Live-Cell Identification of Inhibitors of the Lipid Transfer Protein CERT Using Nanoluciferase Bioluminescence Resonance Energy Transfer (NanoBRET)

*Maria-Anna Kirmpaki[+], Essa M. Said[+], Fabian Schumacher, Kevin Prause, Burkhard Kleuser, and Christoph Arenz**

Abstract: A BRET system is described, in which Nanoluciferase was fused to the lipid transfer protein CERT for efficient energy transfer to a Nile red-labeled ceramide, which is either directly bound to CERT or transported to the adjacent Golgi membrane. Bulk formation of sphingomyelin, a major plasma membrane component in mammals, is dependent on CERT-mediated transfer of its predecessor ceramide. CERT is considered a promising drug target but no direct cell-based methods exist to efficiently identify inhibitors. The utility of the method was demonstrated by a library of 140 derivatives of the CERT inhibitor HPA-12. These were obtained in a combinatorial synthesis using solid-phase transacylation. Screening of the library led to six compounds that were picked and confirmed to be superior to HPA-12 in a subsequent dose-response study and also in an orthogonal lipidomics analysis.

Introduction

Lipids are structural and signaling molecules in all kingdoms of life and defined by their insolubility in water. For their proper distribution and function within different biomembranes, regulated transport processes are indispensable.[1] Sphingomyelin is one of the major mammalian plasma membrane lipids and is particularly enriched in nerve cells in general and in the myelin sheath of axons in particular.[2] It is formed within the membranes of the trans-Golgi network (TGN) by sphingomyelin synthase 1 (SMS1), which catalyzes transphosphorylation of phosphorylcholine from lecithin to ceramide (Figure 1). Ceramide however is formed within ER membranes, which makes an efficient transport of this lipid between ER and TGN membranes necessary.

The ceramide transfer protein (CERT) mediates the non-vesicular transfer of de novo synthesized or recycled ceramide from the cytosolic leaflet of the endoplasmic reticulum (ER) to trans-Golgi membranes and has been shown to be rate-limiting for sphingomyelin formation.[3] CERT is a 68 kDa cytosolic protein comprising an N-terminal PH domain, capable of binding to PtdIns4P in trans-Golgi membranes, a middle region containing a serine-rich motif (SRM), a FFAT domain, anchoring the protein to VAP-A or VAP-B of ER membranes and finally a START domain at the C-terminus that binds the substrate and is sufficient of undirected transport of ceramide in vitro (Figure 1).[4] Under standard conditions, only a fraction (~20 %) of CERT is active at membrane contact sites (MCS) between the ER and trans-Golgi, while the majority of the protein is hyperphosphorylated within the SRM, which prevents binding to trans-Golgi membranes, rendering CERT inactive and associated to ER membranes.[5] Depletion of plasma membrane sphingomyelin induces CERT dephosphorylation and an increased recruitment to MCS between ER and TGN.

Functional CERT is essential for proper embryogenesis in mice[6] and zebrafish[7] and utilized by pathogens like *chlamydia trachomatis*, apparently to ensure a supply of lipids from the host organism.[8–10] In the viral life cycle of hepatitis C virus (HCV)[11] or Herpes simplex virus (HSV)-1,[12] host CERT-mediated transfer of ceramide is important for the function of the replication machinery. Transcriptional up-regulation of CERT has been found in many cancers, while CERT depletion is associated with favorable outcomes of adjuvant chemotherapy treatment.[13] Given the fact that CERT activity is subject to strong post-transcriptional regulation and direct activity determination in living cells or cell lysates is difficult, no association data for actual CERT activities with various diseases are available to date.

Recently, reports of point mutations in the CERT1 gene associated with severe intellectual disability (ID) have attracted considerable attention. These investigations were initiated by a study that used massive exon screening to identify genetic causes of inborn ID.[14] Among the four genes

[*] M.-A. Kirmpaki,[+] E. M. Said,[+] K. Prause, C. Arenz
Institute for Chemistry, Humboldt-Universität zu Berlin, Brook-Taylor-Str. 2, 12489 Berlin, Germany
E-mail: arenzchr@hu-berlin.de

E. M. Said[+]
Chemistry Department, Faculty of Science, Suez Canal University, Ismailia 41522 (Egypt)

F. Schumacher, B. Kleuser
Freie Universität Berlin, Institute of Pharmacy, Königin-Luise-Str. 2 +4, Berlin, Germany

[+] those authors contributed equally.

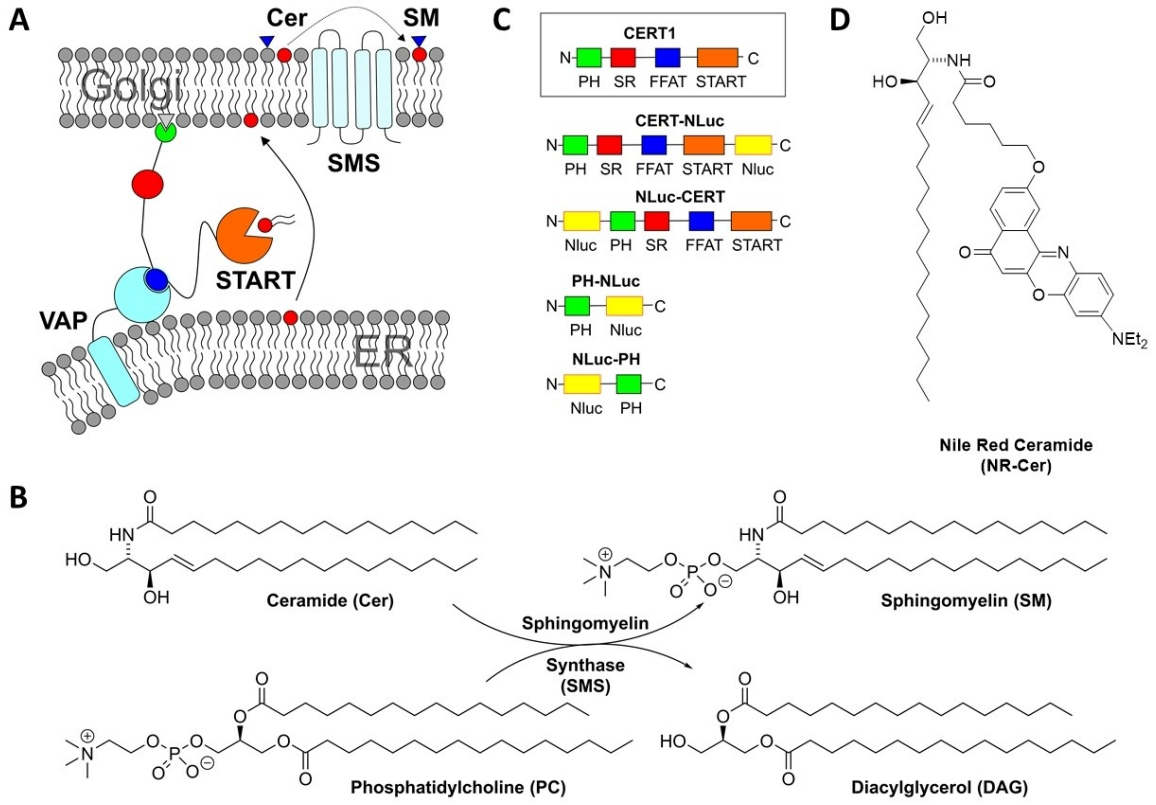


Figure 1. **A** Mechanism of CERT mediated ceramide transfer from ER to Golgi. **B** Reaction catalyzed by sphingomyelin synthase 1 (SMS1) **C** domain structure of CERT different NLuc constructs developed in this study **D** structure of Nile Red ceramide (NR-Cer).

found to be associated with ID was the CERT1 gene. Strikingly, the non-related patients expressed the same S132L variant of the CERT protein. The removed serine if phosphorylated down-regulates CERT activity. More detailed studies have shown that CERT mutations associated with ID lead indeed to CERT hyperactivity in patients.[15–17] A recently published study described a total of 31 ID patients with different CERT gain-of-function mutations.[17]

Despite these various examples, research into the role of CERT in human disease is still in its infancy. The recently discovered post-translational regulation of CERT and the growing interest in CERT inhibitors necessitate the development of valid and efficient assays for CERT activity in live cells.

Besides different in vitro assays for binding to His_6-tagged human CERT expressed from *E. coli* that have been described,[18–20] two liposomal transfer assays using recombinant CERT have been reported, one non-homogeneous, based on radioactively labeled lipids[21] and one homogeneous assay based on FRET.[22]

Cellular CERT activity is usually determined only indirectly via the (de novo synthesized) cellular sphingomyelin concentration. This can be accomplished through lipidomic measurements or feeding experiments with radiolabeled precursors.[23] An alternative with higher throughput is the determination of lysenin-mediated cytotoxicity. Lysenin is a sphingomyelin-dependent, pore-forming cytotoxin.[24] Cells with reduced sphingomyelin synthesis therefore exhibit increased resistance to lysenin. When recombinant lysenin is added to the cells, in the presence or absence of a CERT inhibitor, the actual evaluation is performed using a cytotoxicity assay. This cell-surface sphingomyelin assay, is strongly cell-type dependent and requires determination of threshold lysenin-toxicity before each assay. Moreover, expensive recombinant lysenin has to be purchased or toxin production is carried out in a safety level S2 gene laboratory. Alternatively, the concentration of ceramide and sphingomyelin can be determined by HPLC-MS-MS which is neither suitable for high throughput nor a direct CERT assay. Overall, the determination of the sphingomyelin concentration for the estimation of CERT activity must be viewed with caution, as different other factors and enzymes can influence the sphingomyelin concentration of the plasma membrane. The only method to assess CERT activity in living cells is to monitor redistribution of fluorescently labeled ceramide derivatives by confocal microscopy.[23]

To address the afore-mentioned problems, we planned to develop an easy-to-perform, meaningful cell-based assay for CERT inhibitors without having to rely on secondary readout methods such as sphingomyelin concentration. CERT mediates the spatial translocation of ceramide into the Golgi apparatus and fluorescent ceramide derivatives as Golgi stains have been known and widely used for decades.[25] Accordingly, we wanted to utilize a proximity-based method based on non-radiative energy transfer with a Golgi-anchored emitter to image the spatially defined phenomenon of

ceramide translocation and thus develop a direct way to measure CERT activity. Instead of Förster resonance energy transfer (FRET), we decided to use bioluminescence resonance energy transfer (BRET). BRET is a naturally occurring phenomenon that has been developed into a technique to study biomolecular interactions.[26,27] In contrast to FRET, the energy donor is not excited by an external radiation source, but an enzyme-catalyzed reaction produces the energy, which either becomes visible as bioluminescence or is transferred to an acceptor dye in a radiation-free process. The energy emission must be suitable to excite the acceptor and the spatial distance between emitter and acceptor must be less than 10 nm. As external excitation of the entire cell is not necessary, the process is accompanied by a minimum of background noise. One problem is that the emission of the luciferase-catalyzed reaction always overlaps with the emission of the BRET acceptor and the net BRET, i.e. the difference between the BRET emission of the acceptor and the emission in the same spectral range in the absence of the acceptor, is determined first. In high-throughput screening using BRET, there is a problem with compounds that inhibit luciferase and can therefore lower the BRET value. However, these are easy to identify when analyzing the primary data. While BRET was initially primarily used to determine protein-protein interactions (a protein in fusion with luciferase transfers energy to a protein in fusion with a fluorescent protein),[28] methods using dye-labeled small molecules as acceptors have now also been increasingly described.[29] In most cases, known ligands of extracellular receptors are linked to dyes such as fluorescein, BODIPY-FL or TAMRA, while the receptor itself is expressed as a fusion with the corresponding luciferase. This interaction can be interrupted by competitive ligands and registered by a reduction in BRET. These assays can be performed on the surface of living cells in multi-well format without further preparation. Few reports using BRET on targets within cells are available although live-cell assays appear to be particularly advantageous for targets within the cell, as they directly address the problem of cell viability and intracellular half-life.

Results and Discussion

Design of Fusion Proteins

Based on these considerations, we decided to develop a nanoBRET assay based on Nanoluciferase (NLuc) for the transfer of bioluminescence to an appropriately labeled ceramide. NLuc is a luciferase derived from a deep-sea shrimp and engineered for small size (19.1 kDa) and high stability. Its luminescence in combination with various furimazine derivatives exceeds that of other luciferases by up to 100-fold. Using the commercial pNLF1 vector system, four different NLuc constructs were assembled (Figure 1C): Two constructs contained the entire coding sequence of CERT, N-terminal (CERT-NLuc) and C-terminal (NLuc-CERT) in frame with the coding sequence for NLuc. The other two constructs consisted of CERT's PH domain only, either in N- or C-terminal position of NLuc (PH-NLuc or NLuc-PH). With the two latter variants, we wanted to test whether it might be possible to detect the localization of a correspondingly labelled ceramide in the trans-Golgi membranes and thus develop a transport assay rather than a binding assay. We were aware of the fact that the PH domain could also localize to other PtdIns4P-containing membranes, such as the plasma membrane, which would reduce the BRET efficiency of the constructs. On the other hand, the two variants with the full-length CERT could exhibit properties of both a transport and a binding assay. After cloning the respective open reading frames (ORFs) into the PNLF1-N or PNLF1-C vector respectively the constructs were transfected into HeLa cells. Interestingly, all vectors showed very high luminescence at 490 nm, in the absence of any acceptor molecule. However, the luminescence was 3- to 4-fold higher, when only the PH-domain and not the full CERT-protein was fused (Figure S1).

In the presence of fluorescent ceramides however, the mBRET value increased in the order CERT-NLuc < NLuc-PH < PH-NLuc < NLuc-CERT (not shown). Due to the superior mBRET value, suggesting an efficient energy transfer between NLuc and an NR-Cer in close vicinity, we decided to go further with the NLuc-CERT construct for assay development.

Test of Different Labeled Ceramides as BRET Acceptors

To test different ceramides as possible BRET acceptors, cells were transfected with NLuc-CERT and split into 96 well plates the next day. After another overnight incubation, Nano-Glo Vivazine live cell substrate was added, followed by different ceramides. NBD-ceramide is the best studied fluorescent ceramide and has been reported to be a vital Golgi stain, four decades ago.[25] The NBD-dye exhibits the best fluorescence in a non-polar environment and the absorption spectrum overlaps almost perfectly with the emission of the NLuc, both peaking around 460 nm. A disadvantage, however, is that at the emission peak of NBD at around 538 nm the emission of NLuc is very high and about 2/3 of its maximum. BODIPY-TR-ceramide is an intermediate available in our laboratory for the synthesis of a sphingomyelinase probe[30] and both absorbance and emission peaks are clearly red-shifted compared to the NBD, but with a lower Stokes shift. Nile red ceramide was developed by us previously and seemed ideal for this application.[31] Although the absorption is clearly red-shifted to that of NBD, one third of the maximum absorption is still achieved at 460 nm. On the other hand, the Stokes shift is significantly larger than that of the BODIPY-TR and the broad emission peak ensures that significantly more than a third of the maximum emission intensity is recorded even at 650 nm. Comparison of the three ceramides as BRET acceptors showed that NR-Cer was slightly superior over NBD-Cer at 590 nm, a difference that was more pronounced at 616 nm and 665 nm, respectively. In contrast, BODIPY-TR-Cer yielded negative net-BRET values at 590 nm (probably due to absorption of residual NLuc luminescence), low netBRET at 616 nm and performed best at 645 nm. However, even at 645 nm, netBRET was only 10–20 % of that seen for NR-Cer. Finally,

NR-Cer and a read out at 616 nm was chosen for the following BRET experiments. In the presence of NR-Ceramide, the luminescence at 616 nm is significantly higher compared to the control with the CERT-fused NLuc alone. Conversely, in presence of NR-Cer, there is a slightly reduced luminescence at 460 nm, suggesting that a fraction of the Nluc reaction energy is transferred to NR-Cer, which is in the immediate vicinity. As Nile Red proved to be the best of the dyes tested, it is worth noting that to our knowledge this is the first report to date of the use of Nile Red as an acceptor in a nanoBRET assay.

The suitability of the NLuc-CERT fusion as a donor in combination with NR-Cer as an acceptor for conducting live cell CERT assays was confirmed by titration with NR-Cer, whereby the mBRET value increased with increasing concentrations of NR-Cer. Moreover, BRET was decreased dose-dependently in presence of HPA-12, a ceramide analog CERT inhibitor. Interestingly, the decrease in mBRET promoted by HPA-12 could be reversed by higher concentrations of NR-Cer, obviously reflecting the fact that HPA-12 is a ceramide analogue and competitive inhibitor of CERT (Figure 2A).

As NR-Cer used in this study is labeled in its fatty acid part, it was tested whether it might be subject to cleavage by the lysosomal acid ceramidase. This could lead to a reduction in effective substrate concentration and thus less BRET efficiency. However, cells pre-treated with the acid ceramidase inhibitor SACLAC[32] showed only slightly elevated mBRET values (Figure 2B). This effect was not significant, suggesting that either the majority of NR-Cer is not taken up via endocytosis or that the translocation from the endolysosomal compartments to the Golgi occurs faster than acid ceramidase- mediated degradation.

Mechanistic Studies

Due to the superior BRET, the full-length N-NLuc-CERT-C fusion protein was selected for further experiments, being aware that two mechanisms of action are plausible for this construct. Since we found a strong binding of NR-Cer to the recombinant CERT in previous studies,[22] a "receptor-like mechanism" in which the BRET donor signal is directly transferred to the substrate bound by CERT is conceivable. On the other hand, assuming that NLuc fused to CERT is

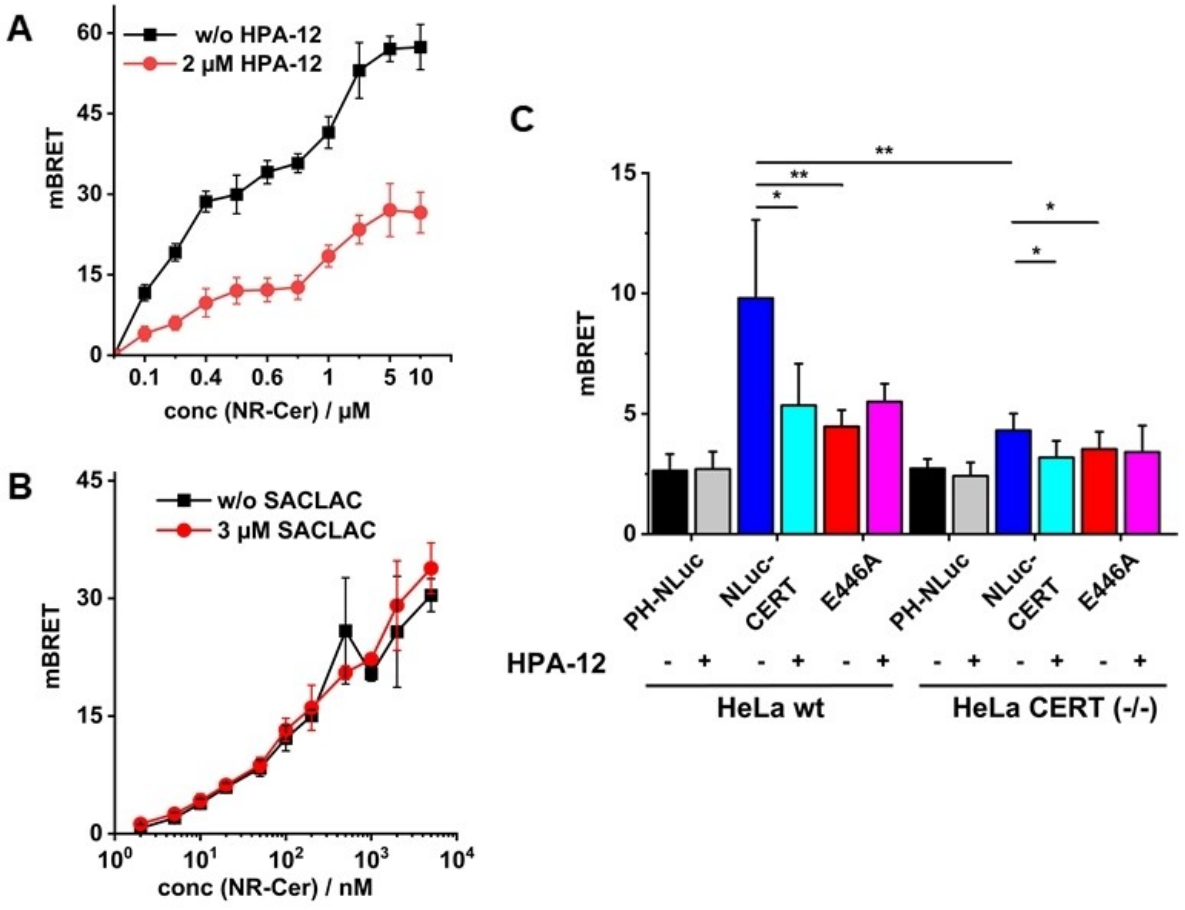


Figure 2. Mechanistic studies. **A** mBRET increases gradually with increased NR-Cer concentration and HPA-12 inhibition is partially rescued by NR-Cer **B** pre-treatment of cells with the acid ceramidase inhibitor SACLAC has no effect on BRET efficiency **C** mBRET in presence of 0.2 µM NR-Cer for different constructs with or without HPA-12 and in HeLa cells with or without natural CERT expression. Error bars depict standard deviation. Significance is assessed by student's t-test * = $p < 0.05$; ** $p < 0.01$.

directly associated with the trans- Golgi, an energy transfer to NR-Cer transferred to the adjacent membrane could also be possible. The latter contribution can be described as a partial "transfer assay". We reasoned that in the case of a receptor-like mechanism, BRET would have to be lowered if the construct was provided with mutations affecting the ceramide-binding START domain of the final protein. However, these mutations should only partially affect the transfer of NR-Cer into trans-Golgi membrane, as the cells used have an intact CERT protein.

Indeed, when the NLuc-fusion of the E446A variant of CERT with a reduced ceramide binding capacity was used for the BRET experiment, the mBRET value was dramatically reduced (Figure 2C). The BRET signal did not decrease further in the presence of HPA-12, suggesting that binding of NR-Cer to NLuc-CERT in a receptor-like fashion significantly contributes to the BRET signal observed. To test the possibility of a transfer-assay mechanism, the same plasmids were expressed in HeLa cells deficient in CERT (HeLa CERT (−/−)). Surprisingly, in comparison to expression in cells having natural CERT expression, also the BRET signal of the non-mutated NLuc-CERT fusion was significantly reduced. This suggests that also the transfer of NR-Cer to the TGN, mediated by the natural CERT present in HeLa cells contributes to the BRET signal observed. Interestingly, presence of HPA-12 further decreased BRET. Taken together, these results indicate a likely dual mechanism of the BRET assay developed. This idea was further supported by the fact that also in HeLa CERT (−/−) cells the E446A variant showed again a significantly reduced BRET, compared to the non-mutated NLuc-CERT. Furthermore, a double mutant (E446A/Y553F) with two amino acid changes responsible for ceramide binding showed even less BRET signal and two mutants (S132L and S135P) reported to cause CERT gain of function showed significantly increased BRET in both, HeLa and HeLa (CERT−/−) cells (data not shown).

Synthesis of a Library of HPA-12 Anaogues

Once the live cell assay for CERT had been established, its usefulness for quantitative screening was to be demonstrated in practice. Towards this end, a combinatorial synthesis of HPA-12 derivatives was envisioned to determine their cellular activity and at the same time to test the newly developed assay in a practical application.

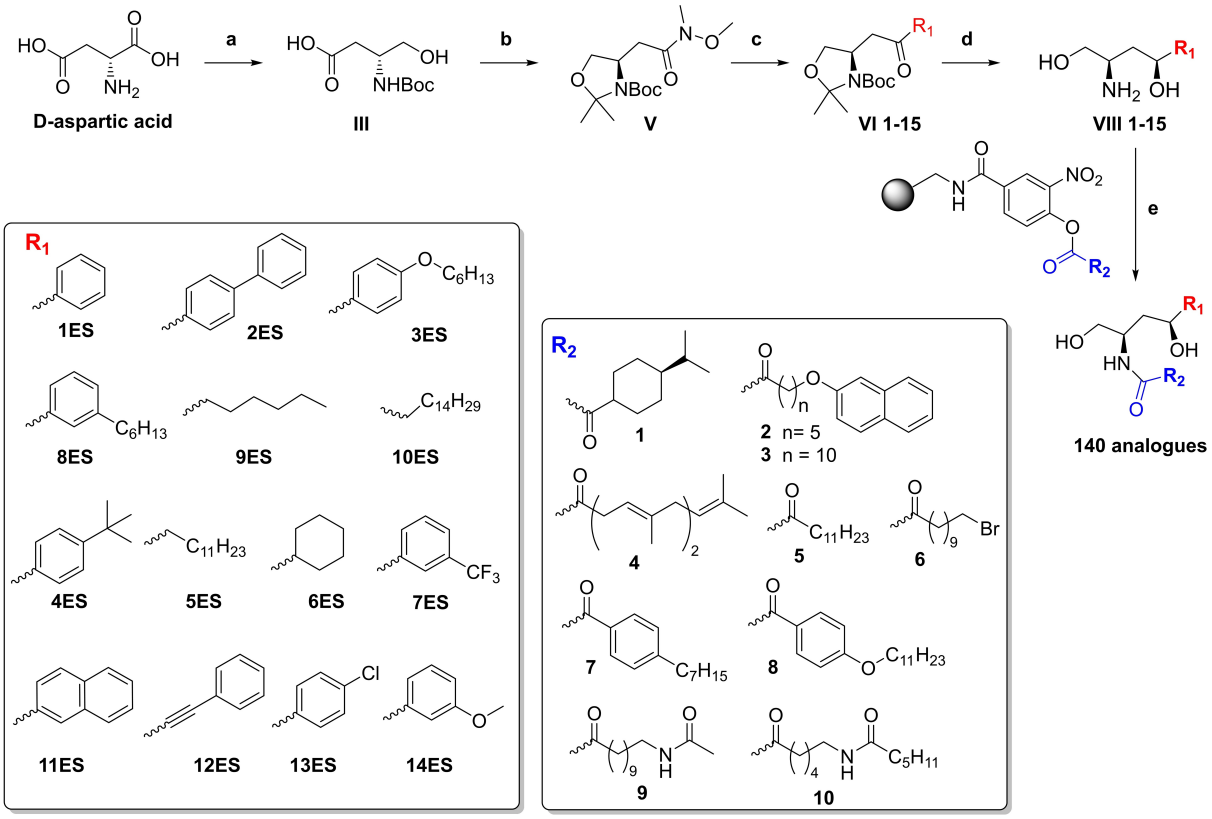
So far, only a few CERT inhibitors have been described, among which the ceramide analog HPA-12 clearly stands out due to its rather high potency. HPA-12 was originally discovered due to its ability to suppress SM biosynthesis without affecting the two sphingomyelin synthases.[23] Notably, its stereochemistry was revised after a first in-depth synthetic study.[33] The compound has also been shown to be active in vivo and is able to cross the blood–brain barrier in mice, albeit with a very short half-life.[34] A few derivatives of HPA-12 have been described in various synthetic studies and increases in binding affinity of up to 3 orders of magnitude have been observed in binding studies using recombinant CERT or its START domain.[19] Neither has the potential for novel HPA-12 derivatives been fully exploited nor have such compounds been evaluated in live cells.

HPA-12 is a ceramide analog. This means that an amphiphilic chiral dihydroxyamine is acylated with a fatty acid. For the dihydroxyamine part, a novel and efficient synthetic approach was developed starting from D-aspartic acid, which was converted to the corresponding acid anhydride **II** after Boc protection (Scheme 1 and S1). Reduction of the acid anhydride **II** with $NaBH_4$ and subsequent reaction with 2,2 dimethoxy propane successfully furnished the N-protected L-β-homoserine **IV**. The carboxylic acid group of the latter was converted into its Weinreb amide analogue **V**. Reaction with a series of organometallic agents (either organolithium or Grignard reagent) provided the corresponding ketones **VI 1–15** which were subjected to stereoselective reduction using L-Selectride to yield 15 different protected amines **VII 1–15**. After deprotection, trans-acylation with solid-phase bound activated esters[35,36] furnished a combinatorial library of 140 HPA-12 analogues.

Identification and Characterization of Improved HPA-12 Derivatives

Next, it was investigated whether the compound collection contained derivatives with improved activity compared to HPA-12 and the entire library was tested at a concentration of 200 nM in living HeLa cells in 96 well plates in a single determination. At this concentration, HPA-12 showed a moderate but significant inhibition of the BRET signal of about 40 %. Remarkably, cell-based assay yielded a total of 26 derivatives – or almost 20 % of the compounds tested – showing higher reduction of BRET compared to HPA-12 (Figure 3A). Next, the 6 best compounds were picked and tested in a dose-response assay in triplicates (Figure 3C). Interestingly, among these best hits was also a compound that had been described in an earlier study and proven to be the best binder of the START domain with an EC_{50} value of 1.5 nM (HPA-12: 4100 nM), but no cellular activity was provided.[19] Indeed, all 6 compounds picked were confirmed to be significantly more potent than HPA-12. The IC_{50} values ranged from 10 nM to 47 nM compared to 350 nM for HPA-12 (Figure 3).

To ensure that these results are not assay-specific, but correspond to the actual inhibition of the compounds on cellular CERT activity, the compounds were analyzed in an independent experiment. For this purpose, HeLa cells were treated with 1 μM HPA-12 or one of the new derivatives and a lipidome analysis was performed after 24 h, which is currently the state of the art, despite the potential shortcomings mentioned above. The analysis included sphingomyelin, ceramide and hexosylceramides as well as lactosylceramides. While it was expected that sphingomyelin would be reduced, the inhibition of CERT should also lead to an increase in ceramides and especially hexosylceramides. In fact, the sphingomyelin/ceramide ratios were highly significantly reduced in presence of HPA-12. Strikingly, all derivatives that showed higher potency in the new assay also showed a significantly more pronounced reduction of the SM/



Scheme 1. Synthesis of the HPA-12 analogue library. Reagents and conditions: **a** 1) Boc_2O, 1 M NaOH, quant.; 2) EDCI, DCM, 69%; 3) $NaBH_4$, THF, r.t. **b** 1) DMP, acetone, BF_3OEt_2 2) EDCI, DMH, NMM, DCM, 43% over 3 steps **c** R_1MgBr or R_1Li. **d** 1) L-Selectride 2) AcCl in MeOH. **e** shaking with 10-fold excess of resin loaded active ester. DMP: 2,2-Dimethoxypropane, DMH: *N,O*-Dimethylhydroxylamin -hydrochlorid, NMM: N-Methylmorpholine.

Cer ratio compared to HPA-12 in the lipidomic assay, although the change in the lipidome was already highly significant in the presence of HPA-12. The strong effects of the compounds in cells also show that the sensitivity of the BRET assay, which can be adjusted within certain limits by the concentration of NR-Cer used, is meaningful for the activity of the compounds in the lipidomic assay.

Conclusions

The ceramide transfer protein CERT is an important representative of a group of lipid transfer proteins whose importance for cellular lipid homeostasis is only just being recognized. To date, there is no direct and valid cell-based method to identify inhibitors for CERT and similar lipid transfer proteins at reasonable effort. This task is becoming all the more urgent as the role of CERT as a potential target molecule for infectious diseases, cancer or intellectual disability becomes increasingly clear. The proximity-based method presented here makes use of NLuc fused to CERT to quantitatively and highly sensitively identify CERT inhibitors in live cell assays. With HPA-12, a ceramide analog is available as a potent inhibitor, which is even able to cross the blood–brain barrier. Expression of the NLuc-CERT reporter carrying the E446A mutation, which reduces the ability to bind ceramide, results in a sharp decrease in the BRET signal, suggesting that the assay functions largely through energy transfer to NR-Cer bound directly to the START domain of CERT.

However, from a medicinal chemistry perspective, allosteric inhibitors are of particular interest because they are generally more specific and are likely to have greater metabolic stability. Due to the lack of standards for this type of inhibitor, this study could not definitively demonstrate that the newly developed assay can identify inhibitors other than competitive ones.

However, since the expression of the NLuc-CERT reporter is also dramatically reduced in HeLa cells lacking natural CERT expression, it can be assumed that the NR-Cer transported into the membrane region adjacent to the NLuc fusion can act as a BRET acceptor and contribute significantly to the generated signal.

NLuc has proven to be a superior tool in screening campaigns, as it is practically free of cellular autofluorescence and eliminates numerous sources of error in conventional screening assays. Inhibitors of NLuc can be immediately recognized as “false positives”, since not only the acceptor fluorescence is reduced, but above all the bioluminescence of NLuc itself.

In principle, other proteins that can anchor NLuc in the membranes of the TGN would be worthy of constructing a

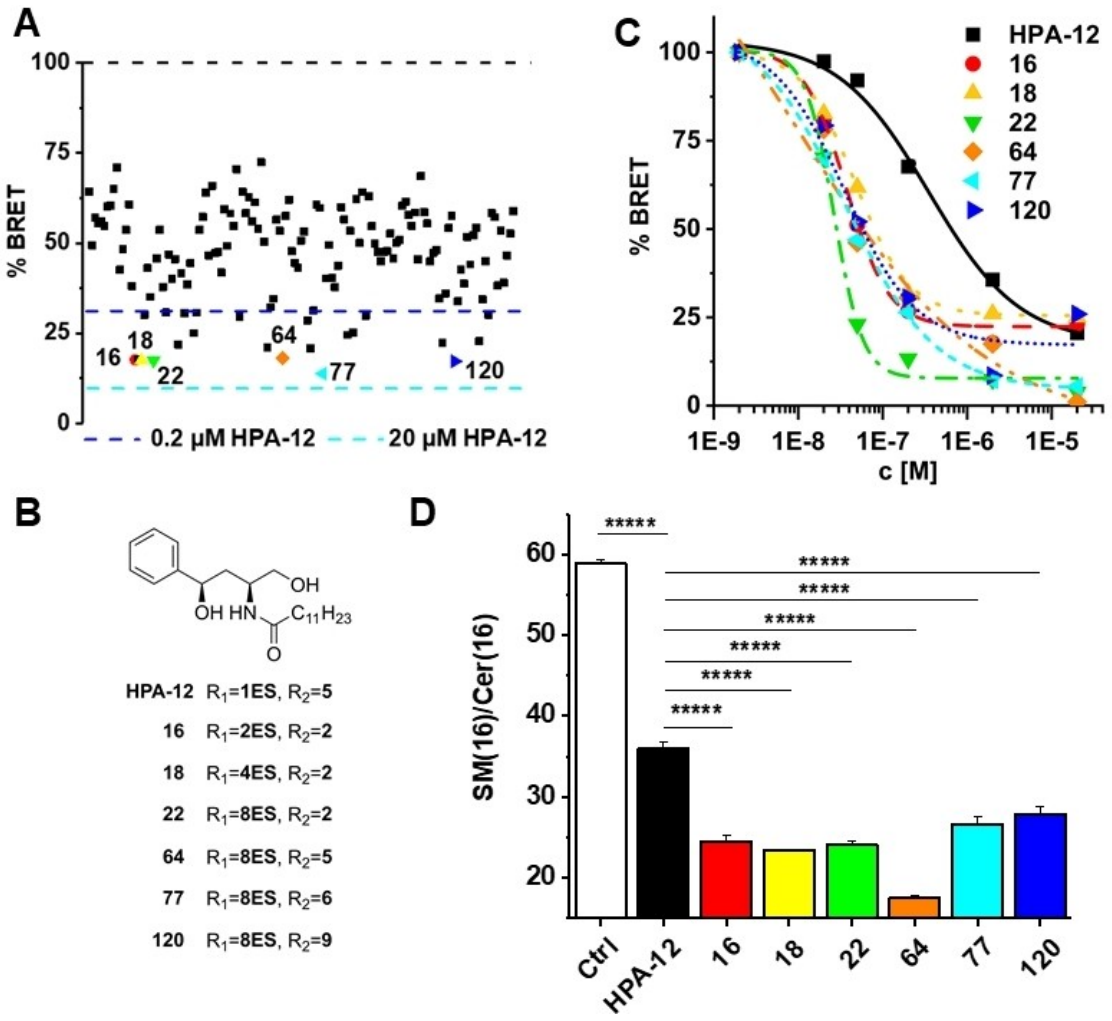


Figure 3. Screening and evaluation of the HPA-12 analogue library. **A** Screening result of 140 compounds at 0.2 µM concentration in comparison to untreated (100% BRET) or **HPA-12** at 0.2 µM (blue dashed line) or 20 µM. Concentration of **NR-Cer** = 0.2 µM. **B** Structural codes for HPA-12 and six selected hits. **C** Dose response curves for selected hits, fitted to Hill's equitation. Error bars are omitted for clarity. **D** Ratios of cellular SM (16:0) and Cer (16:0) determined by quantitative HPLC-MS-MS evaluation. Error bars depict standard deviation. Four asteriscs: $p < 1\ E^{-04}$; five asteriscs $p < 1\ E^{-05}$ determined by student's t-test.

potential "transfer-only" assay. The advantage of the assay presented here and its assumed dual mechanism is at least a very robust BRET signal.

Indeed, lipid transfer proteins have only recently been intensively investigated and inhibitors for their study are lacking. Certainly, the method presented here can be adapted to other lipid transfer proteins and their cargoes this study will provide a blueprint for similar studies with other lipid transfer proteins.

Supporting Information

The authors have cited additional references within the Supporting Information (Ref. [37–43]).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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