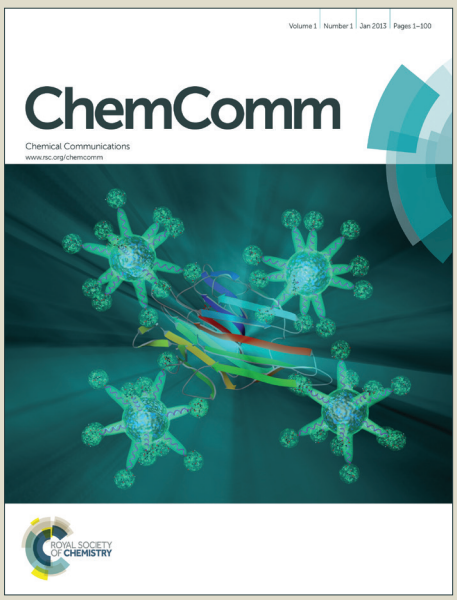
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Development of an Acid Ceramidase Activity-Based Probe

Cécile M. J. Ouairy,[a] Maria J. Ferraz,[a,b] Rolf G. Boot,[a,b] Marc P. Baggelaar,[a] Mario van der Stelt,[a] Monique Appelman,[b] Gijsbert A. van der Marel,[a] Bogdan I. Florea,[a] Johannes M. F. G. Aerts*[a,b] and Herman S. Overkleeft*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Acid ceramidase is responsible for the ultimate step in the catabolism of (glyco)sphingolipids by hydrolysis of ceramide into sphingosine and free fatty acid. Deficiency in acid ceramidase is the molecular basis of Farber disease. Here we report the synthesis and characterization of an activity-based acid ceramidase probe.

Human acid ceramidase (ACase) is a heterodimeric glycoprotein that belongs to the N-terminal nucleophile (Ntn) hydrolase family. This cysteine amidase, deficient in the lysosomal storage disorder, Farber disease, is instrumental in the final step of lysosomal degradation of (glyco)sphingolipids by hydrolysing ceramide into free fatty acid and sphingosine[1] (Fig. 1, 1). Since the identification of this enzyme half a century ago by Gatt,[2] several studies have been conducted to elucidate its processing and catalytic mechanism.[3-6] Our research focuses on the metabolism of (glyco)sphingolipids to develop new diagnostic and therapeutic strategies for application in the treatment of inherited sphingolipidoses including Gaucher disease. This recessively inherited lysosomal storage disorder is caused by a deficiency of glucocerebrosidase and results in the accumulation of its substrate glucosylceramide, most prominently in macrophages. Recently we reported that the accumulating glucosylceramide is partially deacylated to glucosylsphingosine, which is toxic and may be involved in the progress of the disease.[7] Deacylated glycosphingolipids were also previously observed in Fabry disease, another glycosphingolipidosis. In Fabry tissue, high levels of globotriaosylceramide are present in patients' plasma.[8] These findings raise the question whether acid ceramidase is the enzyme responsible for the deacylation of glycosylceramide. With the aim to establish this, and more in general to expand the repertoire of activity-based probes to include ACase, we chose to develop an activity-based ceramidase probe. We here disclose our first results in this direction by the successful development of the first (to the best of our knowledge) activity-based probe (ABP) for the *in vitro* and *in vivo* visualization of human acid ceramidase.

At the onset of our studies we considered two design strategies that might lead to the desired ceramidase ABP. In the first instance, we chose to design mimics of its natural substrate ceramide (Fig. 1, 2A, ABP **1** and **2**). Replacement of the amide in this natural substrate by a vinyl sulfone may yield a compound that reacts covalently and irreversibly with ACase, which mediates hydrolysis by an N-terminal cysteine thiol acting as a nucleophile.[6] Activity-based cysteine protease probes, which have a thiol-reactive Michael acceptor at the position occupied by the carbonyl of the scissile amide bond in the natural substrate, have proven their merits as activity-based cysteine protease probes.[9,10] At the same time, we considered carmofur as a starting point for ABP development (Fig. 1, 2B). Carmofur is a 5-fluorouracil (5-FU) releasing drug currently used in the clinic to treat colorectal cancers and has been recently reported by Piomelli and co-workers as a potent ceramidase inhibitor.[11] Probes based on both scaffolds were synthesized as summarized below (detailed information can be found in the SI).

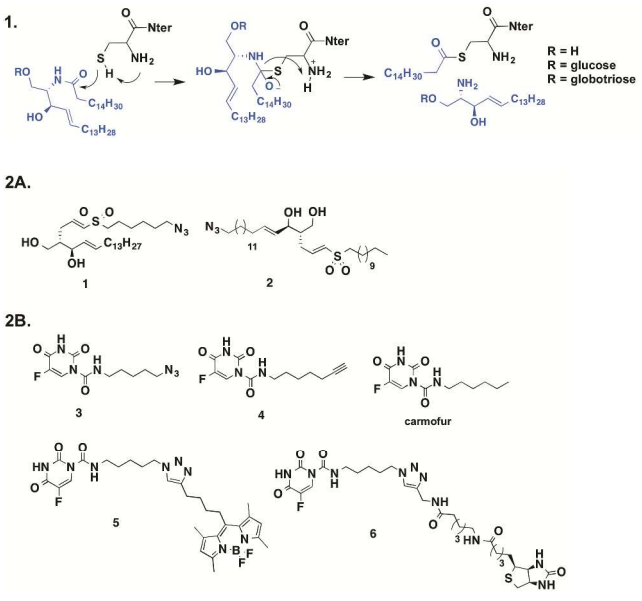
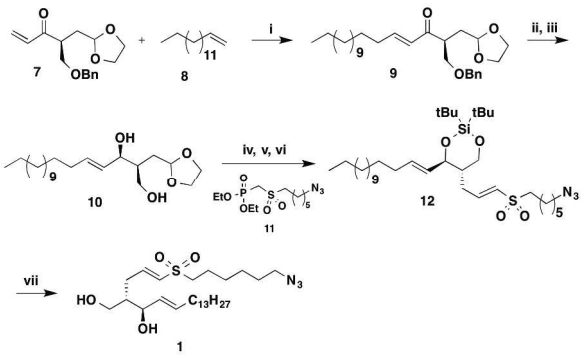


Fig. 1 (1) ACase mediated hydrolysis of (glycosyl)ceramide. (2) Structures of vinyl sulfone (A) and carmofur (B) analogues as potential activity-based acid ceramidase probes.

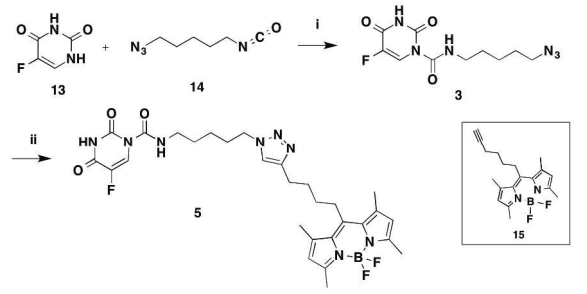
Vinyl sulfone **1** was prepared via cross-metathesis of the unsaturated ketone **7** and the long hydrophobic alkene **8** (Scheme 1). Subsequent Corey-Bakshi-Shibata reduction of alpha-beta-unsaturated ketone **9** resulted in the corresponding allylic alcohol, which was subjected to Birch reduction to obtain diol **10**. After protection of the diol function using DTBS ditriflate, removal of

the acetal followed by an HWE reaction with the ylid prepared from **11**, yielded vinyl sulfone **12**. Final deprotection using HF_3-NEt_3 afforded the desired vinyl sulfone **1** (scheme 1). A similar strategy (see details in the SI) was pursued to obtain vinyl sulfone **2**.



Scheme 1 Reagents and conditions: (i) **8** (2 eq), Hoveyda-Grubbs second generation catalyst (5 mol%), DCM, AcOH (20 mol%), 40 °C, overnight, 78%. (ii) $BH_3.Me_2S$ (2.5 eq), (*R*)-MeCBS (1 eq), THF, 0 °C, 1 h 30 min, 78%. (iii) NH_3 (l), Li, THF, 0 °C, 82%. (iv) *t*-$Bu_2Si(OTf)_2$ (1.8 eq), 2,6-lutidine (3 eq), DMF, 0 °C, 2 h, 68%. (v) HCl (37%, aq)/THF (1:3), rt, 3 h. (vi) a) **11** (1.5 eq), NaH (1.5 eq), 0 °C then rt, 1 h; b) THF, 0 °C then rt, overnight, 34% (2 steps). (vii) HF-NEt_3 (1.9 eq), pyridine, rt, 2 h 30 min, 69%.

Fluorescent carmofur derivative **5** was synthesized by adaptation of the literature procedure towards the parent compound.[12] Reaction between 5-fluorouracil **13** and isocyanate **14** afforded carboxamide **3**, which was ligated to Bodipy-alkyne **15**[9] by Cu(I)-catalysed azide-alkyne [2+3] cycloaddition to afford the desired probe **5** (scheme 2). 2,4-Dioxopyrimidine-1-carboxamide **4** was obtained following a similar scheme (see SI for a detailed synthesis).



Scheme 2 Reagents and conditions: (i) pyridine, rt, overnight, 84%. (ii) **15** (1 eq), $CuSO_4$ (0.2 eq), sodium ascorbate (1.6 eq), sonication, 6 h, 25%.

Having the fluorescent probe **5** in hand, we assessed its effect on human acid ceramidase in extracts of Farber fibroblasts stably expressing the recombinant wild type acid ceramidase. Incubation with **5** (2.5 μM) in a citric acid buffer (pH 4.5) at 37 °C for 30 minutes followed by analysis by SDS-PAGE and fluorescence scanning revealed a fluorescent signal at approximately 40 kDa. This molecular weight corresponds to the catalytically glycosylated beta-subunit of acid ceramidase, which is separated from the alpha-subunit (13 kDa) under the reducing conditions applied (the presence of beta-mercaptoethanol).[5,6] When carmofur (10 μM) was added prior to the incubation with **5** the band was absent (figure 2A). Upon pre-incubation with carmofur derivatives **3** (10 μM) or **4** (10 μM), no band could be detected indicating that these compounds are able to compete for **5** (Fig. 2B). However upon pre-incubation with vinyl sulfone **1** or **2** (10 μM final concentration), the fluorescent signal was still detected (figure 2B). Since we obtained no indication that the ceramide mimics interacted with human acid ceramidase we focused our attention on our carmofur analogues.

A pull down experiment was performed to establish whether the observed protein in Gaucher spleen extracts corresponds to acid ceramidase. For this purpose biotinylated carmofur derivative **6** was synthetized following a sequence similar to the one depicted scheme 2. After incubation with **6** (200 μM) in a citric acid buffer (pH = 4.5) at 37 °C for 30 min, affinity purification using streptavidin beads and tryptic digestion, mass spectrometric analysis revealed five peptidic fragments corresponding to ACase. However, we were unable to identify the peptide with the catalytic cysteine residue.

To unambiguously confirm the validity of **5** as APB and establish its binding to the ACase nucleophile, we mutated the catalytic cysteine to a serine (ASAH1-C122S) and stably expressed this in Farber fibroblasts lacking endogenous acid ceramidase. After incubation with **5** (2.5 or 1 μM) and SDS-Page, the fluorescent signal was absent in extracts expressing this mutant form of acid ceramidase (figure 2B) while prominently present in cells expressing the wild type enzyme. These data underscore that ACase belongs to the Ntn-hydrolase family with a cysteine residue as the active site nucleophile.[6,14-16]

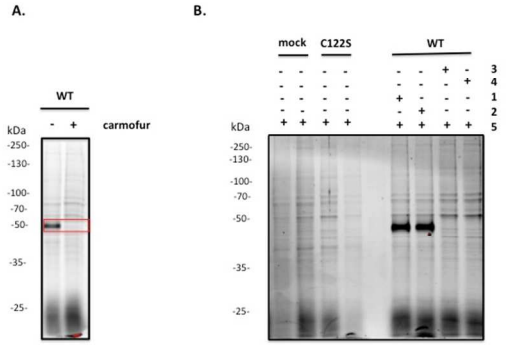


Fig 2 A) Labeling of ASAH1 in Farber fibroblasts stably expressing WT ASAH1 using ABP **5** (2.5 μM). Competition experiment performed with carmofur (10 μM). B) ABPP using **5** (2.5 μM and 1 μM) with different ASAH1 constructs at the same protein concentration (0.47 $mg.mL^{-1}$). Competition experiment with **1** (10 μM), **2** (10 μM), **3** (10 μM) and **4** (10 μM) using WT ASAH1 constructs at the same protein concentration (0.47 $mg.mL^{-1}$).

As the next objective we set out to demonstrate the use of ABP

5 to identify acid ceramidase in tissues homogenates. For this purpose, we took human spleen from healthy controls and Gaucher patients. When the homogenate was treated with ABP **5** (2.5 μM) for 1 h at 37 °C a band at the same height as previously reported was observed (Fig. 3). Pre-incubation with carmofur (10 μM) blocked enzymatic activity in both cases. These data showed that endogenous levels of acid ceramidase can be detected with **5** and thus validate this probe as an effective activity-based acid ceramidase probe. Of note, in extracts derived from a spleen of a type 1 Gaucher patient a relative large quantity of active acid ceramidase molecules was detected as compared to normal spleens.

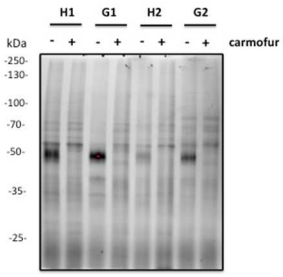


Figure 3 ABPP using **5** (2.5 μM) and competition with carmofur (10 μM) in tissues (healthy and Gaucher spleen) at the same protein concentration (1.6 $mg.mL^{-1}$).

In conclusion, we have developed compounds **5** and **6** as the first ABPs that efficiently target acid ceramidase. Using the antineoplastic drug carmofur we have been able to develop a 5-FU analogue to visualize (with **5**) and identify (with **6**) the desired lysosomal enzyme. Unlike some cysteine protease inhibitors and ABPs,[17-19] the vinyl sulfone moiety is not reacting with the active-site cysteine in the case of ceramidase mimics, at least when introduced into ceramide analogues. One could envision the introduction of alternative electrophilic traps in the ceramide scaffold and that have intrinsic reactivity towards cysteine thiols (alternative Michael acceptors, halomethyl ketones, epoxyketones). We have not studied this because endogenous acid ceramidase from healthy and Gaucher spleen tissue can be detected with carmofur-based ABPs. We found that the activity of the enzyme appeared to be considerably higher in Gaucher disease patients compared to tissue from healthy individuals. Our findings thus represent a good starting point in the study of this important enzyme and we are currently investigating the implications of ceramidase in the onset and progression of Gaucher disease and Farber disease.

Notes and references

[a] *Leiden Institute of Chemistry, Leiden University, P. O. Box 9502, 2300 RA Leiden, The Netherlands; Tel: +31715274342; E-mail:* h.s.overkleeft@chem.leidenuniv.nl *or j.m.f.g.aerts@lic.leidenuniv.nl*
[b] *Department of Medical Biochemistry, Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105 AS Amsterdam, The Netherlands*

† Electronic Supplementary Information (ESI) available: Experimental and analytical details on the synthesis of the compounds, and experimental biochemical procedures. See DOI: 10.1039/b000000x/

1 C. R. Gault, L. M. Obeid and Y. A. Hannun, *Adv. Exp. Med. Biol.* 2010, **688**, 1.
2 S. Gatt, *J. Biol. Chem.* 1963, **238**, 3131.
3 K. Bernardo, R. Wurhithz, T. Zenk, R. J. Desnick, K. Ferlinz, E. H. Schuchman and K. Sandhoff, *J. Biol. Chem.* 1995, **270**, 11098.
4 X. He, N. Okino, R. A. D. S. G. Dhami, H. Schulze, K. Sandhoff and E. H. Schuchman, *J. Biol. Chem.* 2003, **278**, 32978.
5 H. Schulze, U. Schepers, K. Sandhoff, *Biol. Chem.* 2007, **388**, 1333.
6 N. Shtraizent, J.-H. Park, C. He, R. Shalgi and E. H. Schuchman, *J. Biol. Chem.* 2008, **283**, 11253.
7 N. Dekker, L. van Dussen, C. E. M. Hollak, H. Overkleeft, S. Scheij, K. Ghauharali, M. J. van Breemen, M. F. Ferraz, J. E. M. Groener, M. Maas, F. A. Wijburg, D. Speijer, A. Tylki-Szymanska, P. K. Mistry, R. G. Boot and J. M. Aerts, *Blood* 2011, **118**, e118.
8 J. M. Aerts, J. E. Groener, S. Kuiper, W. E. Donker-Koopman, A. Strijland, R. Ottenhoff, C. van Roomen, M. Mirzaian, G. E. Wijnburg, G. E. Linthorst, A. C. Veder, S. M. Rombach, J. Cox-Brinkman, P. Somerhaju, R. G. Boot, C. E. M. Hollak, R. O. Brady and N. J. Poorthuis, *Proc. Natl. Acad. Sci. U.S.A.* 2008, **105**, 2812.
9 M. J. Evans and B. F. Cravatt, *Chem. Rev.* 2006, **106**, 3279.
10 D. Kato, K. M. Boatright, A. B. Berger, T. Nazif, G. Blum, C. Ryan, K. A. H. Chehade, G. S. Salvesen and M. Bogyo, *Nat. Chem. Biol.* 2005, **1**, 33.
11 N. Realini, C. Solorzano, C. Pagliuca, D. Pizzirani, A. Armirotti, R. Luciani, M. P. Costi, T. Bandiera and D. Piomelli, *Scientific Reports* 2013, **3**, 1035.
12 D. Pizzirani, C. Pagliuca, N. Realini, D. Branduardi, G. Bottegoni, M. Mor, F. Fabio Bertozzi, R. Scarpelli, D. Piomelli and T. Bandiera, *J. Med. Chem.* 2013, **56**, 3518.
13 M. Verdoes, U. Hillaert, B. I. Florea, M. Sae-Heng, M. D. P. Risseeuw, D. V. Filippov, G. A. van der Marel and H. S. Overkleeft, *Bioorg. Med. Chem. Lett.* 2007, **17**, 6169.
14 C. Oinonen, J. Rouvinen, *Protein Sci.* 2000, **9**, 2329.
15 J. Pei, N. V. Grishin, *Protein Sci.* 2003, **12**, 1131.
16 S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman, *Nucleic Acid Res.* 1997, 25.
17 J. T. Palmer, D. Rasnick, J. L. Klaus and D. Bromme, *J. Med. Chem.* 1995, **38**, 3193.
18 M. D. Mertens, J. Schmitz, M. Horn, N. Furtmann, J. Bajorath, M. Mares and M. Gütschow, *ChemBioChem* 2014, **15**, 955.
19 L. Mendieta, A. Pic, T. Tarrag, M. Teixid, M. Castillo, L. Rafecas, A. Moyano and E. Giralt, *ChemMedChem* 2010, **5**, 1556.