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Functionalized cyclophellitols are selective glucocerebrosidase inhibitors and induce a bona fide neuropathic Gaucher model in zebrafish

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Supporting Information Placeholder

ABSTRACT: Gaucher disease is caused by inherited deficiency in glucocerebrosidase (GBA, a retaining β-glucosidase), and deficiency in GBA constitutes the largest known genetic risk factor for Parkinson's disease. In the past, animal models of Gaucher disease have been generated by treatment with the mechanism-based GBA inhibitors, conduritol B epoxide (CBE) and cyclophellitol. Both compounds however also target other retaining glycosidases, rendering generation and interpretation of such chemical knockout models complicated. Here we demonstrate that cyclophellitol derivatives carrying a bulky hydrophobic substituent at C8 are potent and selective GBA inhibitors and that an unambiguous Gaucher animal model can be readily generated by treatment of zebrafish with these.

Glucocerebrosidase (acid glucosylceramidase, GBA, E.C. 3.2.1.45,) is a lysosomal retaining β-glucosidase that belongs to the glycoside hydrolase (GH) 30 (www.cazy.org)[1] family and degrades the glycosphingolipid, glucosylceramide through a two-step Koshland double displacement mechanism (Figure 1a). Inherited deficiency in GBA causes the most common autosomal recessive lysosomal storage disorder, Gaucher disease.[2] Individuals carrying heterozygous mutations in the gene coding for GBA do not develop Gaucher disease but have a remarkable increased risk for developing Parkinson's disease (PD) and Lewy-body dementia.[3–5] Appropriate animal models linking impaired GBA functioning to Gaucher disease and Parkinson's disease are imperative both for understanding the pathophysiology of these diseases and for the development of effective treatments for these. Since complete genetic abrogation of GBA hampers animal viability due to skin permeability problems,[6] research models have been generated in the past in a chemical knockdown strategy by making use of the mechanism-based, covalent and irreversible retaining β-glucosidase inhibitor, conduritol B epoxide (CBE, **1**, Figure 1b) or its close structural analogue, cyclophellitol (**2**, Figure 1b).[7,8] One complication in the use of these compounds is their relative lack of selectivity.[9] We found that cyclophellitol **2** is unsuited for creating a reliable Gaucher animal model because it targets GBA and GBA2 with about equal efficiency.[9] On the other hand, CBE **1** exhibits some GBA selectivity but it also inhibits lysosomal α-glucosidase (GAA),[10–13] non-lysosomal glucosylceramidase (GBA2),[14,15] and lysosomal β-glucuronidase (GUSB)[16]. Effective mouse models can be generated with CBE **1** but the therapeutic window is rather narrow and varies in cellular and animal models.

Recent research from our group has revealed that functionalized cyclophellitol derivatives carrying a BODIPY substituent at C8 (cyclophellitol numbering – the primary carbon corresponding to C6 in glucose) are very potent and very selective activity-based probes (ABPs) for monitoring GBA activity *in vitro*, *in situ* and *in vivo*.[17,18] The presence of a bulky and hydrophobic substituent at this position at once proved beneficial for GBA inactivation (ABPs **3** and **4** (Figure 1c,d) proved to inhibit GBA in the nanomolar range, whereas cyclophellitol **2** is a high nanomolar to micromolar GBA inactivator) and detrimental to inhibition of other retaining β-glucosidases. Following these studies, Vocadlo and co-workers designed a set of fluorogenic substrates featuring a fluorophore at C6 of a β-glucoside the aglycon of which carried a fluorescence quencher; compounds that proved to be very selective GBA substrates *in situ*.[19] These results altogether evoked the question whether cyclophellitols bearing a simple, hydrophobic moiety at C8, such as compounds **6** and **7** (Figure 1d), would be suitable compounds for generating chemical knockdown Gaucher animal models. We here show the validity of this reasoning in the generation of a GBA-deficient *Dario rerio* zebrafish model, as revealed by the accumulation of elevated levels of the Gaucher harbinger lysolipid, glucosylsphingosine, using cyclophellitol derivatives **6** and **7**.

At the onset of our studies, we sought for structural support for the design of compounds **6** and **7**. We have in the recent past synthesized Cy5-functionalized cyclophellitol **5** (unpublished) and obtained a crystal structure of human recombinant GBA soaked with this ABP (reported here). As expected (Figure 2a), the active site nucleophile (in both molecules of the asymmetric unit) had reacted with the epoxide to yield the covalently bound cyclitol in $^4C_1$-conformation, with the Cy5 moiety, via its flexible linker, clearly bound in one molecule of the asymmetric unit (the differences may reflect crystal packing constraints in a soaking experiment) accommodated by a hydrophobic pocket in GBA. Previous studies by us on the bacterial glycoside hydrolase, *Thermoanaerobacterium xylanolyticum Tx*GH116 β-glucosidase, a close homologue of human GBA2 with a conserved active site, instead showed an "inwards" position of O6 (SI Figure 2a) and a narrower and less hydrophobic pocket (SI Figure 2b) which may partially mitigate against the binding of O6-functionaised probes, thus allowing sufficient discrimination for GBA over GBA2.[20,21]

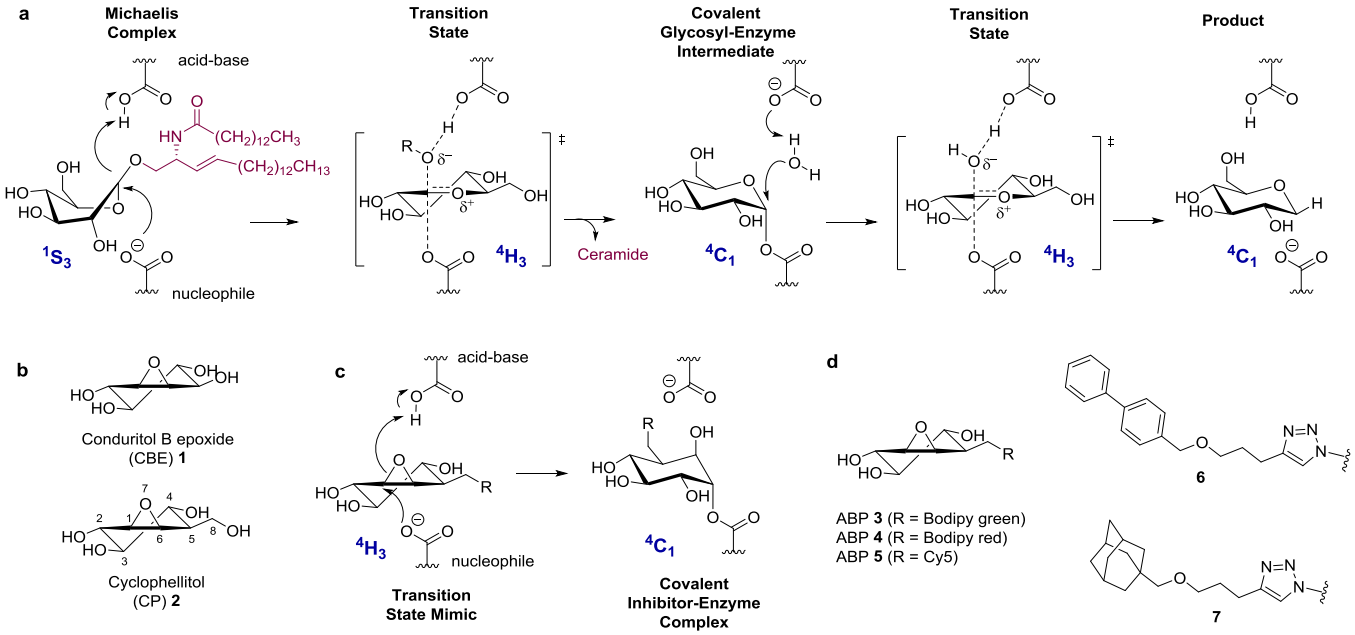

Figure 1. a) Glucocerebrosidase (GBA) hydrolyses glucosylceramide in a two-step double displacement mechanism to yield glucose and ceramide. b) Chemical structure of CBE **1** and cyclophellitol **2**. c) Mechanism-based inactivation of GBA by glucopyranoside-configured cyclitol epoxides (shown for cyclophellitol). d) Structures of C8-extended cyclophellitol derivatives used in the here-presented studies: GBA activity-based probes ABPs **3**-**5** and selective inhibitors **6** and **7** (see for the full chemical structures of ABPs **3**-**5** and **8**-**14** the Supporting Information).

Biphenyl-cyclophellitol **6** and adamantyl-cyclophellitol **7** were synthesized following adaptations of literature cyclophellitol syntheses (see Supporting Information for synthesis details of Cy5-cyclophellitol ABP **5** and compounds **6** and **7**) to generate superior selective GBA inhibitors for the generation of a Gaucher model zebrafish.[22,23]

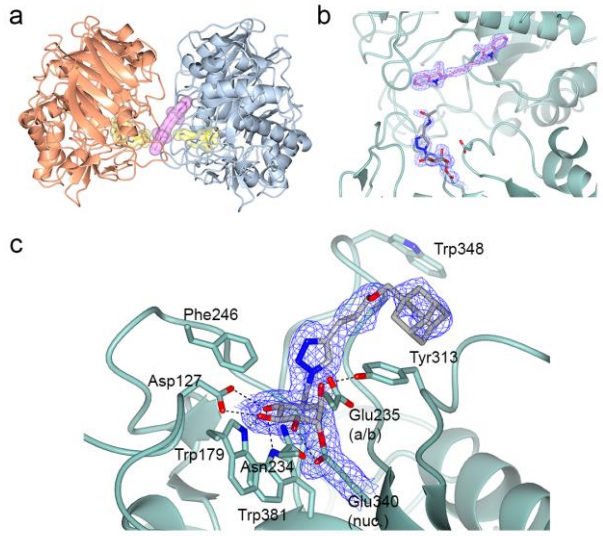

Figure 2. Structure of GBA reacted with ABP **5** and adamantyl-cyclophellitol **7**. a) GBA dimer, with the cyclophellitol and linker moiety of **5** shaded in yellow, and a single observed Cy5 in pink. b) Zoomed view of a GBA monomer reacted with ABP **5**. c) Structure of GBA with adamantyl-cyclophellitol **7**. The linker-adamantyl moiety of **7** is observed in slightly different positions in the two molecules of the asymmetric unit (PDB Code 6Q6L, Figure S2) reflecting its binding through predominantly hydrophobic interactions.

Although soaking of GBA crystals with **6** did not yield suitable structures for structural analysis (Figure S2), soaking with **7** did (Figure 2c), and again revealed binding of the hydrophobic moiety (here, the adamantane) to the same hydrophobic cavity and pocket occupied by the O6 linker on Cy5 ABP **5**. Several hydrophobic residues, including Tyr313, Phe246, and Trp348 provide the wide cavity that is able to accommodate different hydrophobic O6 substituents which is absent in other human β-glucosidases and which provides the structural basis for the inhibitory (and substrate) preferences of GBA.

We evaluated the *in vitro* activity and selectivity of compounds **6** and **7** towards GBA and the two major off-target glycosidases of CBE **1**, GBA2 and GAA, by pre-incubating the inhibitors with recombinant human GBA (rGBA, Cerezyme), human GBA2 (from lysates of GBA2 overexpressed cells), and recombinant human GAA (rGAA, Myozyme) for 3 h, followed by enzymatic activity measurement. We showed that both compound **6** and **7** were nanomolar inhibitors of rGBA (apparent IC_{50} values = 1.0 nM), which were 4,000-fold more potent than CBE **1** (apparent IC_{50} values = 4.28 μM) (Figure 3a, S3) with improved lipophilic ligand efficiencies (LipE) (Table S2). Both compounds **6** and **7** were rather inactive towards GBA2 and GAA (apparent IC_{50} values > 100 μM), similar to ABP **3** and **5** (Figure 3a, S4). When comparing their selectivity towards GBA, both compounds **6** and **7** exhibited IC_{50} ratio (GBA2/GBA and GAA/GBA) of > 100,000, thus making them 4,000-times and 200-times more selective than CBE **1** (IC_{50} ratio = 23.6 for GBA2/GBA and 444 for GAA/GBA) (Figure 3a). To evaluate the *in vivo* activity of compound **6** and **7**, compounds were added to the egg-water containing zebrafish (*Danio rerio*) embryos, and incubated for 5 days at 28 °C before subsequent homogenization and enzyme selectivity analysis by appropriate ABP labeling.[9,24] Quantification of ABP-labeled bands revealed that compounds **6** and **7** had *in vivo* apparent IC_{50} values towards GBA of 4 to 6 nM, and that they were 5- to 70- fold more potent than ABP **3** or **5** and 7,500 fold more potent than CBE **1** (Figure 3b, S5) in the zebrafish larvae. More importantly, an improved selective inactivation of

a	CBE **1** (nM)	CP **2** (nM)	ABP **3** (nM)	ABP **5** (nM)	**6** (nM)	**7** (nM)
In vitro						
rGBA	4,280 ± 500 [a]	29.6 ± 2.40 [a]	1.20 ± 0.30	3.20 ± 0.17	1.06 ± 0.19	0.96 ± 0.17
GBA2 (HEK293T lysates)	101,000 ± 20,100	29.7 ± 3.13	> 10^5	412,000 ± 10,100	> 10^5	> 10^5
rGAA	1,900,000 ± 192,000	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5
Ratio *(in vitro)*						
GBA2/ GBA	24	1	> 10^5	> 10^4	> 10^5	> 10^5
GAA/ GBA	444	> 10^3	> 10^6	> 10^6	> 10^6	> 10^6

b	CBE **1** (nM)	CP **2** (nM)	ABP **3** (nM)	ABP **5** (nM)	**6** (nM)	**7** (nM)
In vivo (Danio rerio larvae)						
GBA	4.41 x10^4 [a]	83 [a]	31.6 ± 8.88	284 ± 31.5	5.85 ± 2.44	3.94 ± 1.21
GBA2	8.90x10^5 [a]	59 [a]	> 10^4	> 10^4	> 10^4	> 10^4
GAA	9.55 x 10^6 [a]	> 10^5 [a]	> 10^4	> 10^4	> 10^4	> 10^4
Ratio *(in vivo)*						
GBA2/ GBA	22	0.714	> 316	> 35	> 1710	> 2540
GAA/ GBA	233	> 203	> 316	> 35	> 1710	> 2540

[a] Values from ref. 9

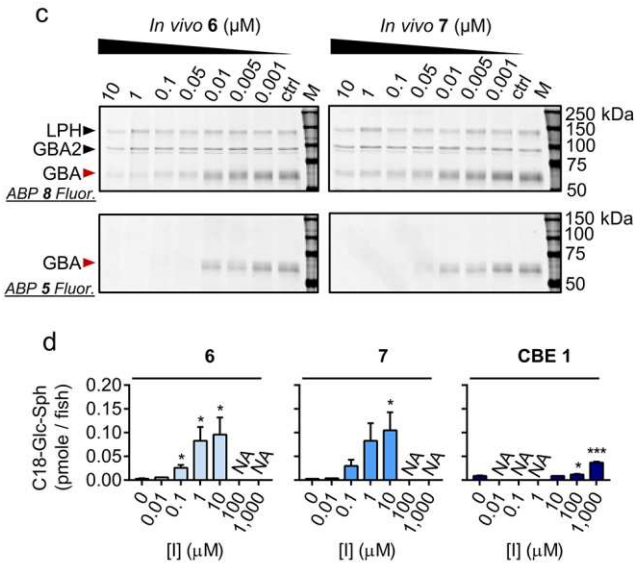

Figure 3. a) Apparent IC_{50} values for *in vitro* inhibition of GBA, GBA2 and GAA in recombinant enzymes (rGBA and rGAA) or overexpressed cell lysates (GBA2) by compounds **1**, **2**, **3**, **5**, **6** and **7**. Error ranges depict standard deviations from biological triplicates. b) Apparent IC_{50} values for i*n vivo* inhibition in 5-day treated zebrafish embryo with compounds **1**, **2**, **3**, **5**, **6** and **7**. Error ranges depict standard deviations from n = 12 to 24 individuals. c) Competitive ABPP in lysates of zebrafish treated *in vivo* with compounds **6** and **7** using broad-spectrum retaining β-glucosidase ABP **8** and selective GBA ABP **5** as read-out. d) Glucosylsphingosine levels produced in zebrafish embryos treated for five days with inhibitors **6**, **7** or CBE **1**.[9] Error ranges depict standard deviations from n = 3 individuals. N/A, not analyzed. *, $p < 0.5$; ***, $p < 0.001$.

GBA was achieved with both compounds **6** and **7**. At a concentration of 0.1-10 μM of compound **6** or **7**, ABP labeling of GBA with broad-spectrum retaining β-glucosidase ABP **8** (Figure S1) and GBA specific ABP **5** was abrogated (Figure 3c), while other enzymes such as GBA2, LPH (Figure 3c) or GAA, ER α-glucosidase GANAB, and lysosomal β-glucuronidase GUSB (Figure S6a, S6b) were not affected.

At 0.1-10 μM of inhibitor **6** or **7**, we also observed 10- to 30-fold elevation in the level of glucosylsphingosine (GlcSph), which is known to be formed by acid ceramidase-mediated conversion of accumulating GlcCer in lysosomes.[25,26] Therefore, this observation also strongly points to *in vivo* inactivation of lysosomal GBA. For comparison, reaching similar GlcSph levels in the zebrafish with CBE required 1,000 to 10,000-fold higher concentration in comparison with compounds **6** or **7** (Figure 3d), concentrations at which GBA2 and GAA may also be targeted (Figure 3b). Finally, we investigated the brain permeability of these new inhibitors - a crucial feature for their future application in the study of neuropathic Gaucher disease and Parkinson's disease. Adult zebrafish of 3 months' age were treated with DMSO, ABP **3** or compound **7** (1,6 nmol/fish, approximately 4 μmol/kg) administrated via food intake, and after 16 h brains and other organs were isolated, homogenized, and analyzed by ABP labeling using ABP **5** (GBA), ABP **8** (GBA2 + GBA), ABP **11** (GAA at pH 4.0 and ER α-glucosidase GANAB at pH 7.0), and ABP **13** (lysosomal β-glucuronidase GUSB) (Figure S1). Labeling of brain homogenate of adult zebrafish with ABP **5** resulted in considerable GBA labeling in control and ABP **3**-treated fish, but no labeling in brain homogenates from fish treated with compound **7** (Figure 4). Labeling by the broad-spectrum β-glucosidase ABP **8** showed that GBA2 was not a target of compound **7**, nor was the lower running band (48 kDa), which we hypothesize to be the cytosolic β-glucosidase, GBA3. We noted that the expression level of this protein is likely variable among individual fish, as 4 out of 6 fishes in the control group lacked this band. In the visceral organs (both liver and spleen), both ABP **3** and compound **7** selectively abrogated GBA, while not affecting the labeling on other tested glycosidases (Figure S7).

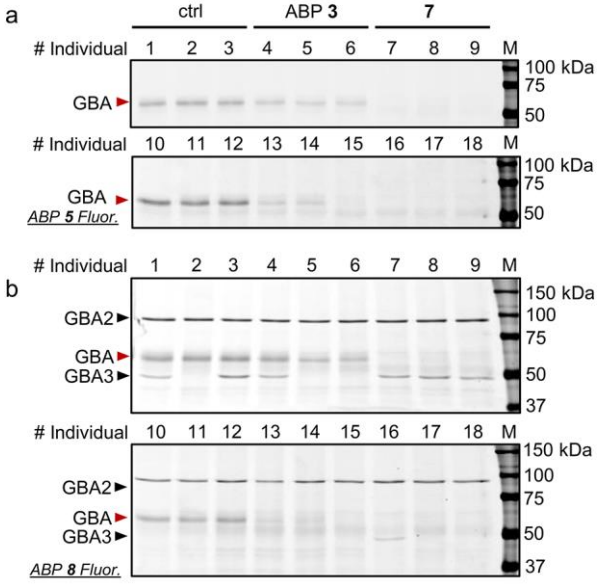

Figure 4. *In vivo* targets of ABP **3** and **7** in brains of adult zebrafish. Competitive ABPP in adult zebrafish homogenates with selective GBA ABP **5** (a) or broad-spectrum retaining β-glucosidase ABP **8** (b) as read-out.

To summarize, crystallographic studies aided the rational design of novel cyclophellitol analogues **6** and **7**, which turned out to be very potent and selective GBA inhibitors, also in zebrafish embryos

and adult zebrafish (GBA2/GBA inhibition ratio > 1,000). Compound **7**, that also completely block GBA activity in the brain, is in our opinion superior to CBE **1** and CP **2** for generating GBA deficiency on demand in zebrafish, thus to create zebrafish models for neuropathic Gaucher, to assist research in the context of neuropathic GD and PD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Experimental data and procedures, crystallographic data and synthesis (PDF).

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Notes

The authors declare no competing financial interests.

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