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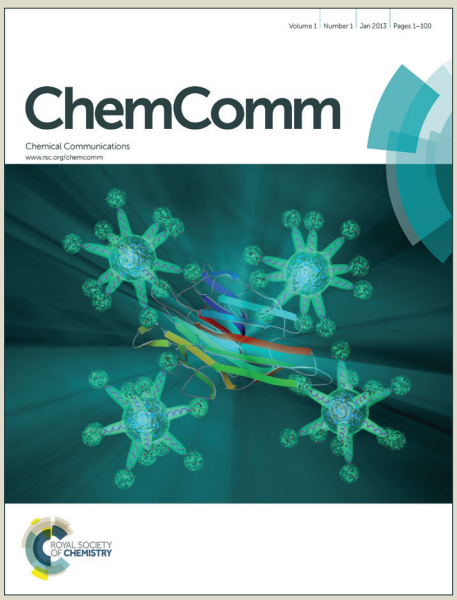

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N-alkylated Aziridines are Easily-prepared, Potent, Specific and Cell-permeable Covalent Inhibitors of Human β-glucocerebrosidase

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β-glucocerebrosidase deficiency leads to Gaucher disease and is a potential marker of Parkinson's disease. We have have identified *N*-octyl conduritol aziridine as a potent and specific covalent inactivator of GBA1 in living cells. This compound is a promising lead towards a positron emission tomography probe intended to image GBA1 activity.

Glycosidases cleave the glycosidic linkage from a sugar to release the aglycone, which itself is often a sugar, lipid, or protein. Inhibitors of glycosidases are approved therapeutics for influenza[1] and lysosomal storage disorders.[2] As well, promising inhibitors have been found to potentially treat Parkinson's disease (PD),[3] HIV/AIDS,[4] cancer[5] and lysosomal storage disorders.[2] Covalent inactivators such as mechanism-based inactivators of glycosidases have also attracted interest as potential therapeutics for influenza,[6] Gaucher disease (GD),[7] and probes of biological activity.[8]

There are four known human β-glucosidases: lysosomal glucosylceramidase (GBA1, a member of the CAZy glycoside hydrolase family GH30, www.cazy.org), non-lysosomal glucosylceramidase (GBA2, family GH116), broad-specificity β-glucosidase (GBA3, family GH1), and intestinal lactase/phlorizin hydrolase (family GH1). GD is the most prevalent of the lysosomal storage disorders, resulting from insufficient GBA1 activity causing an accumulation of the substrate glucosylceramide (Fig. 1). Standard treatment for type I (non-neuronopathic) GD is enzyme replacement therapy (ERT), in which a modified recombinant form of the GBA1 such as Cerezyme™ is intravenously administered at regular intervals.[9] It was recently shown that individuals with heterozygous mutations in GBA1 have increased risk of developing PD[10] with significant levels of the enzyme found in the Lewy bodies.[11] Reduced GBA1 activity was found in areas of the brain affected most by PD[12] observed in both early and advanced disease.[13] These studies highlight the urgent need for molecular imaging probes that can measure GBA1 activity *in vivo* to validate the enzyme as a high priority therapeutic or diagnostic target for PD.

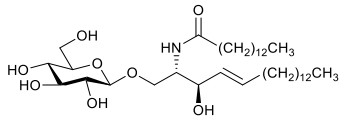

Fig. 1: Structure of glucosylceramide

The most widely used covalent inactivator of GBA1 is conduritol B-epoxide (CBE, **1**).[14] Although this simple cyclitol shows high specificity towards GBA1 over GBA2 and GBA3, it lacks an obvious location for further chemical modification to produce analogues for molecular imaging. Withers and co-workers developed 2-deoxy-2-fluoroglycosides such as **2**[8b] or by derivatives such as **3**[7] or **4**[15] bearing lipophilic leaving groups designed to mimic the ceramide in the natural substrate. However, the most potent inactivators of GBA1 reported to date are fluorescent cyclophellitol derivatives **5** and **6** developed by Overkleeft, Aerts and co-workers[8c] which were shown to be both extremely efficient and selective probes of GBA1 in living cells. The closely related probe *N*-acyl cyclophellitol aziridine **7** was also highly reactive, although it was not selective for GBA1 over GBA2 or GBA3.[8a]

Although the cyclophellitol probes were successfully used to visualize GBA1 in cell-based fluorescence microscopy assays, clinical use of optical imaging is limited to surgical procedures due to poor tissue penetration. In contrast, positron emission tomography (PET) is a powerful and non-invasive clinically established technique that requires the injection of an ^{18}F labeled probe. Given the high sensitivity of PET requiring sub-microgram doses of probe, radioactive irreversible inhibitors have proven useful for mapping and quantifying enzyme activity in vivo since only a small fraction of the endogenous enzyme are modified to produce high contrast images and with no expected pharmacological effects.[16] Although potent irreversible PET tracers can lead to an underestimation of enzyme activity in areas of the brain where blood flow is low and enzy

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† Electronic Supplementary Information (ESI) available: Copies of the proton (^{1}H) and carbon (^{13}C) NMR spectra for all novel compounds synthesized, all enzyme kinetic data (Figures S1 – S4) and the figures for inactivation rates inside live cells (Figure S5) are available in the supporting information. See DOI: 10.1039/x0xx00000x

concentration is high, advanced kinetic modeling[17] or inhibitors with reduced trapping efficiency[18] can be used to improve image sensitivity. With this in mind, we desired a simple scaffold expected to inactivate GBA1 that is amenable to future radiolabeling. In addition, we desired an *in vitro* screening platform that could identify unlabeled leads that could later be converted into a PET tracer by conservative replacement of a C-H bond with a C-^{18}F.

We have previously converted Cerezyme (recombinant GBA1) into a tracer using an ^{18}F-labelled version of **2** and used PET for monitoring ERT in living mice.[8b] Although we could track exogenously administered enzyme in a live animal, this reagent reacted with the enzyme too slowly to be useful for imaging endogenous GBA1 *in vivo*. Withers has previously identified conduritol aziridine **8** as a covalent inactivator of the retaining β-glucosidase from *Agrobacterium* sp. (Abg).[19] We hypothesized that incorporating hydrophobic alkyl groups onto the nitrogen of the aziridine ring would mimic the lipid found on the aglycone of the natural substrate glucosylceramide, thus increasing affinity for GBA1. We were optimistic that the conduritol aziridines would show specificity towards GBA1 similar to CBE **1**, which only poorly inhibits GBA2[20] and has no effect on GBA3 or other glycosidase amilies.[21] Given the challenges associated with PET radiochemistry, the *N*-alkyl group could be used as a convenient location to incorporate a radioactive ^{18}F, producing a PET tracer through conservative replacement of an H atom for an ^{18}F. Indeed, alkylations of nitrogen atoms using ^{18}F labelled tosyl- or bromo-alkanes have been previously reported.[22] We therefore decided to probe the biological activity of this class of compounds through the preparation of three derivatives: **9**, **10**, and **11** (starting from tetrabenzyl *myo*-inositol **12**[23] as shown Scheme S1 and fully described in the Supporting Information,), which bear *N*-butyl, *N*-hexyl, and *N*-octyl groups respectively (see Fig. 2).

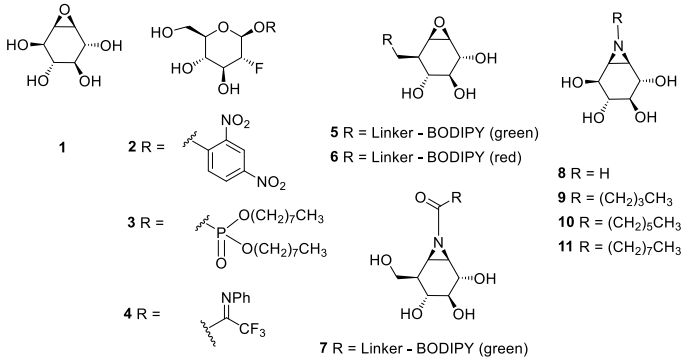

Fig. 2: Structures of selected covalent inactivators of GBA1.

Next, we tested aziridines **9-11** as time-dependent inactivators of GBA1 using a continuous substrate release assay.[8c, 24] As shown in the supporting information (Fig. S1 – S3), the *N*-alkyl aziridines inactivated GBA1 in a concentration-, time-, pH- and temperature-dependent manner with inactivator efficiencies (k_i/K_i) comparable to the best inactivators reported to date (see Table 1). Encouragingly, the inactivation rates far exceeded those of ^{11}C-deprenyl, a covalent inactivator already established as a PET tracer for imaging monoamine oxidase in humans.[16a] As expected, the length of the alkyl chain correlated inversely with the K_i value, with measured values of 2400 nM, 50 nM and 4.8 nM for **9**, **10**, and **11** respectively. This suggests that the alkyl chains effectively mimic the natural ceramide aglycone. Incubation of near-equimolar amounts of GBA1 with **9**, **10**, or **11** showed that inhibition was not resulting from a highly reactive contaminant present in very small quantities, which can be problematic when synthesizing novel inhibitors.[25] In addition, the GBA1-inactivator complex was very stable as no return of catalytic activity was observed over a 12-hour incubation, within the 2 hour time frame required for PET imaging.

Table 1– Kinetic parameters for selected covalent inactivators towards GBA1.

	k_i (min^{-1})	K_i (μM)	k_i/K_i (μM^{-1}min^{-1})	Ref
1	0.59	140	0.00042	20
2	0.030	0.0025	0.000012	8b
3	-	-	0.098	7
5	0.127	0.007	17.76	8c
6	0.208	0.008	25.10	8c
9	0.14 ± 0.01	2.4 ± 0.3	0.058 ± 0.016	Herein
10	0.14 ± 0.018	0.050 ± 0.008	2.8 ± 1.3	Herein
11	0.12 ± 0.014	0.0048 ± 0.00075	25 ± 3.4	Herein

Since compound **11** showed the greatest efficiency as an inactivator of recombinant GBA1, we evaluated its ability to inhibit GBA1 in living HeLa cervical cancer and fibroblast cells (J2/3T3). HeLa cells were chosen because they were readily available, easy to work with, and likely to possess all three of GBA1, GBA2, and GBA3 activities. Fibroblasts were chosen because these cells have only GBA1 and GBA2, and little to no expression of GBA3.[21] To evaluate the specificity of compound **11** as an inactivator of β-glucosidase activity relative to other glycosidases, cell lysates prepared from HeLa cells treated with **11** or CBE **1** were assayed for other glycosidase activities. As shown in Fig. 3a), only total β-glucosidase activity showed any significant decrease in enzyme activity, clearly demonstrating that both aziridine **11** and CBE **1** are highly specific inactivators of β-glucosidase activity. To quantify specificity of the aziridine inhibitor towards GBA1 over GBA3, we purchased recombinant human GBA3 and measured k_i/K_i values for **11**. Consistent with CBE **1**, conduritol aziridine **11** weakly inactivated GBA3 with k_i = 0.13± 0.006 min^{-1}, K_i = 5.4 ± 0.8 μM and k_i/K_i = 0.024 ± 0.8 μM^{-1}min^{-1} (Fig. S4). Interestingly, *N*-acylaziridine cyclophellitols efficiently labeled GBA3.[8a] We next evaluated the specificity of **11** towards GBA1 over GBA2 by examining the time-dependent loss of β-glucosidase activity from lysates prepared from living Hela and fibroblast cells. We chose to measure residual β-glucosidase activity from cell lysates rather than prepare a fluorescent derivative of **11** because incorporation of a large and bulky fluorophore onto the aziridine scaffold would certainly influence inhibitor efficiency, selectivity and/or cell permeability of these compounds. Therefore, in vitro screening using fluorescent probes would not accurately reflect the selectivity of a potential PET tracer where only an ^{18}F atom would be installed on the *N*-alkyl chain. As shown in Fig. 3b), detergent extracted cell lysates prepared from both cell lines showed a rapid decrease in β-glucosidase activity in a time-dependent fashion. The high efficiency of **11** as a β-glucosidase inactivator inside live cells was evident as less than 50% of initial activity remained after ~45 minutes.

Interestingly, ~20% of overall β-glucosidase activity remained in the detergent-extracted lysates prepared from fibroblast and ~35% from HeLa cell lines after prolonged incubation times at 50 nM of **11**. The persistent β-glucosidase activity observed in the lysates cannot be due

to GBA3 since taurocholate, included in the lysate assay buffer, strongly inhibits this enzyme.[21] To determine if GBA2 was responsible for the majority of the persistent residual activity, detergent extracted lysates prepared from Hela and fibroblasts were assayed at pH 4.2. At this pH, GBA1 remains highly active (~60% activity relative to pH = 5.5)[8c] while the non-lysosomal GBA2 is almost completely inactive.[20] As shown in Fig. 3c), when lysates prepared from HeLa or fibroblast cells treated with aziridine **11** (300 nM) and assayed at pH 4.2, the vast majority of the residual β-glucosidase activity was abolished in both cell lines indicating that the residual activity was likely from GBA2. We then took lysates prepared from HeLa and fibroblast cells incubated with aziridine **11** and treated those samples with 2.5 mM of CBE **1** for 30 minutes. Compound **1**, a moderately efficient inactivator of GBA1, was recently shown to also inactivate GBA2 (albeit less efficiently, with k_i/K_i = 0.000021 μM^{-1} min^{-1})[20] while not affecting GBA3.[21] As expected, treatment with **1** completely abolished all residual β-glucosidase activity in fibroblast cells and the vast majority in HeLa cells demonstrating that GBA2 is the enzyme responsible for the persistent β-glucosidase activity. Thus, it was concluded that conduritol aziridine **11** shows high selectivity for GBA1 over GBA2/GBA3 meaning that ^{18}F-labeled versions of **11,** used at tracer concentrations typical for PET, would have sufficient specificity for selectively imaging GBA1.

In order to be useful as a PET tracer for imaging enzymes in the brain, the aziridines must show rapid cell permeability through either passive diffusion or active transport. To rule out that endocytosis is the primary mode of entry into HeLa cells, apparent IC_{50} values both 37 and 18 ºC were determined. As shown in Fig. 3d), HeLa cells incubated with aziridine **11** displayed an apparent IC_{50} value of 0.023 ± 0.003 μM at 37 ºC with a slight increase to 0.10 ± 0.02 μM at 18 ºC. This indicates that one of passive diffusion or active transport must be the main mechanism of cellular entry, because endocytosis ceases at 18 ºC and below. The increase in the IC_{50} value can likely be attributed to a measured decrease in the GBA1 inactivation rates of **11** at lower temperatures (See Fig. S3 and Table S1).

To obtain a quantitative measure of inactivator efficiency that could be used to evaluate covalent inhibitors as potential PET probe candidates, a second order rate constant of inactivation (k_i/K_i) was determined for living HeLa and fibroblast cells using aziridine **11**. Determining the inactivation rates towards intracellular GBA1 would reflect inhibition under natural conditions and is a combination of the time required for the compound to pass through cellular membranes and inactivate the target enzyme. This is important for ranking PET probe candidates as both diffusion and inactivation rates are important for a potential tracer intended to enter the brain. Similar experiments using live hepatic cells have been used to predict drug-drug interactions *in vivo* by quantifying the rates of irreversible inhibition

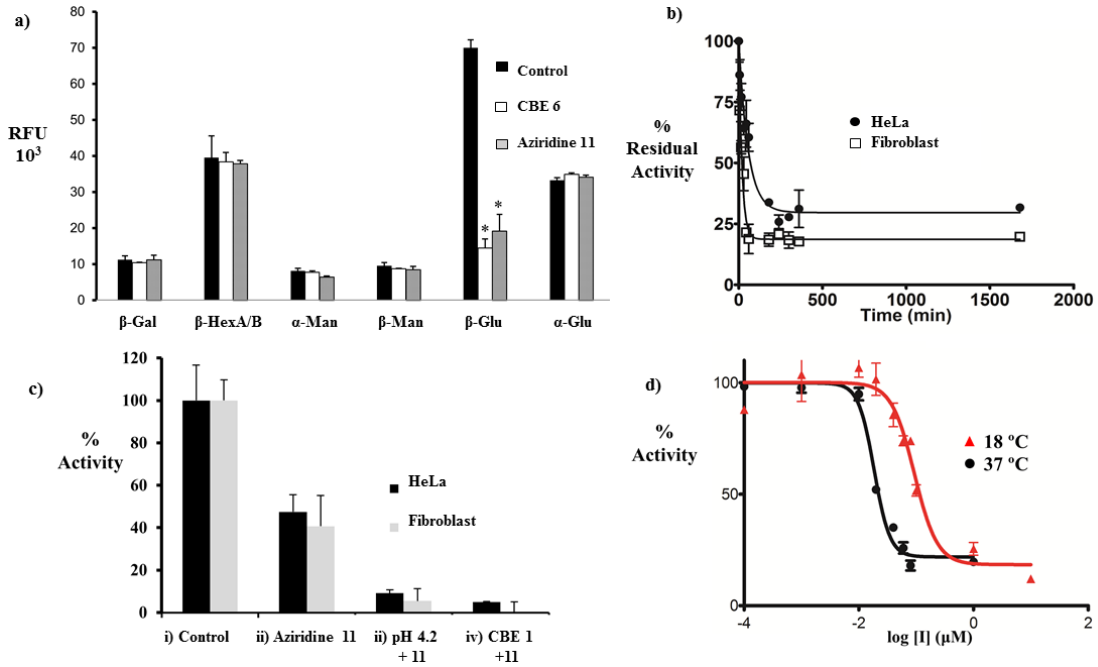

Fig. 3: **a)** Measured fluorescence of residual glycosidase activity after overnight cell treatment of CBE **1** (50 μM) or aziridine **11** (1 μM). Commercially available fluorescent substrates were used to assay for β-galactosidase (β-Gal), β-hexosaminidase A and B (β-HexA/B), α-mannosidase (α-Man), β-mannosidase (β-Man), β-glucosidase (β-Glu), and α-glucosidase (α-Glu) activities. Comparisons between inhibitor treated lysates and control were done using one way analysis of variance (ANOVA) followed by Fisher LSD post-hoc tests (SigmaStat software, Systat, Chicago, IL, USA). Significance was accepted at $p \leq 0.05$, * denotes significantly different from control. **b)** Residual β-glucosidase activity as a function of time at pH 5.5 for detergent-extracted lysates prepared from fibroblast (open squares) or HeLa cervical cancer (filled circles) cells treated with 50 nM aziridine **11**. **c)** The relative β-glucosidase activity in HeLa (black bars) and fibroblast (grey bars) cells. Conditions are as follows: i) untreated cells with lysates assayed at pH 5.5 ii) cells treated with aziridine **11** (300 nM) for 2 hours with lysates assayed at pH 5.5 iii) cells treated with aziridine **11** (300 nM) for 2 hours with lysates assayed at pH 4.2, and iv) cells treated with aziridine **11** (300 nM) for 2 hours with lysates treated with 2.5 mM of **1** then assayed at pH 5.5. **d)** Residual β-glucosidase activity vs log of concentration in lysates prepared from HeLa cells treated with aziridine **11** at 37 ºC (solid circles) and 18 ºC (red triangles). All data represents the mean values ± s.d. of at least 2 independent trials.

of P_{450} enzymes of early drug candidates.[26] As shown in Fig. S5, aziridine **11** maintained a high rate of inactivation of β-glucosidase activity inside living HeLa and fibroblast cells with measured k_i/K_i = 7.0 ± 1.1 $\mu M^{-1}min^{-1}$and k_i/K_i = 17 ± 7 $\mu M^{-1}min^{-1}$ respectively. Such high rates of inactivation inside live cells indicates that intracellular accumulation was rapid and that ^{18}F-labeled derivatives of **11** may be potentially suitable for *in vivo* PET imaging of GBA1 activity.

In conclusion, three novel conduritol aziridines bearing *N*-butyl, *N*-hexyl, and *N*-octyl chains were synthesized using a straightforward seven-step procedure starting from *myo*-inositol. All three compounds were tested in vitro as covalent inactivators of human GBA1. The hydrophobic alkyl chains appended to the amine were intended to mimic the lipid aglycone found in the natural glycolipid substrate for GBA1 and indeed, all three proved to be excellent covalent inactivators of GBA1. The best of the three compounds, *N*-octyl-conduritol aziridine **11**, was an efficient inhibitor towards GBA1 at low nanomolar concentrations. Moreover, it was shown to be a membrane permeable, highly efficient and selective inactivator of intracellular GBA1 suggesting that **11** could be a valuable tool for studying the enzyme in disease promoting processes such as Parkinson's or Gaucher disease. In addition, compounds of this type represent promising leads for the development of ^{18}F-labelled derivatives modified on the *N*-alkyl group that may prove useful as PET imaging probes for studying ERT and Gaucher disease, as well as evaluating GBA1 as an early diagnostic marker of Parkinson's.

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