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Iminosugar-Dihydroazulenes as Mutant L444P Glucocerebrosidase Enhancers

Maria Giulia Davighi[+],[a] Francesca Clemente[+],[a] Emilie Sperling Andreasen,[b] Mogens Brøndsted Nielsen,[b] Camilla Matassini,*[a] Andrea Goti,[a] Amelia Morrone,[c, d] Paolo Paoli,[e] Francesca Cardona,[a] and Martina Cacciarini*[a, b]

A remarkable enhancer of human glucocerebrosidase enzyme (GCase) was identified among a set of dihydroazulene-tagged iminosugars. An unprecedented 3.9-fold increase in GCase activity was detected on fibroblasts bearing the homozygous L444P mutation, which is frequently associated with neuronopathic Gaucher forms, and which commonly results refractory to chaperone-induced refolding.

Introduction

The lysosomal glucocerebrosidase enzyme (GCase) is responsible for the hydrolysis of glucocerebrosides in the lysosomes. When the functionality of this enzyme is reduced, due to genetic mutations on *GBA1* gene, a toxic accumulation of glucocerebrosides causes the multiorgan dysfunction known as Gaucher disease (GD).[1] Currently, enzyme replacement therapy[2] and substrate reduction therapy[3,4] are available treatments for Gaucher disease, but both have disadvantages such as the high cost and the side effects, respectively. As a promising alternative, the use of small compounds acting as pharmacological chaperones (PCs) has been explored, particularly in cases where the mutations result in a misfolded GCase that retains some residual activity. The hallmark of a PC is to interact with the misfolded GCase, promoting a correct folding of the enzyme and thereby an efficient trafficking to the lysosomes, where the enzymatic activity is restored.[5–8] In recent years, the development of a PC for GCase has attracted considerable attention because of a possible correlation between GCase reduced activity and Parkinson's disease.[9–13]

Ultimately, individuals carrying *GBA1* gene mutations have a significantly increased risk of developing parkinsonism compared to the general population.[14] *GBA1* variants are classified in marginal, mild or severe, depending on the role in the development of Gaucher or Parkinson diseases and in the GCase activity reduction. Among the most recurrent variants, the N370S (p.Asn409Ser) is classified as mild and the L444P (p.Leu483Pro) as severe and neuronopathic. The latter causes a reduction of GCase activity around 80–95 % in comparison with the native GCase protein.[13] Thus, PCs for GCase represent attractive targets not only for the treatment of Gaucher disease, but also in the study of Parkinson disease.

Different approaches explored to discover effective PCs include (i) identification among GCase inhibitors, which ensures the interaction with the enzyme,[7,8,15] (ii) massive screening studies for drug repurposing, which allow to identify non-inhibitory PCs[8] and more recently (iii) development of transient covalent GCase inactivators.[16] We have extensively explored the first approach identifying several glycomimetic reversible inhibitors, which were assayed on cell lines from Gaucher patients to evaluate the enhancement of mutant GCase activity. This approach might suffer from prolonged enzyme inhibition once the enzyme-inhibitor complex is translocated in the lysosomes. To tackle this issue, we have recently described[17] photoswitchable inhibitors of GCase constituted by an enantiopure trihydroxypiperidine[18] appended to photoresponsive moieties, namely azobenzene[19] and dihydroazulene[20–21] derivatives, with the aim to benefit from the use of light as a non-invasive tool to tune its biological properties.[22–24] The trihydroxypiperidine unit was selected as the bioactive component since some derivatives have previously shown activity as PCs for GCase.[25] The photo-induced *E/Z* isomerization of azobenzene permitted an exhaustive investigation of the inhibition properties both of the "dark" and the "bright" forms, highlighting the importance of the connectivity between the bioactive unit and azobenzene.[26] Conversely, the dihydroazulene/vinylheptafulvene (DHA/VHF) systems **1**–**2** (Figure 1) showed a fast VHF-to-DHA thermal back conversion in water (6 min), allowing only

[a] *Dr. M. G. Davighi,[+] Dr. F. Clemente,[+] Dr. C. Matassini, Prof. A. Goti, Prof. F. Cardona, Prof. M. Cacciarini*
Department of Chemistry "U. Schiff", University of Florence, via della Lastruccia 3–13, 50019 Sesto F.no (FI), Italy
E-mail: camilla.matassini@unifi.it
martina.cacciarini@unifi.it

[b] *E. S. Andreasen, Prof. M. B. Nielsen, Prof. M. Cacciarini*
Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

[c] *Prof. A. Morrone*
Laboratory of Molecular Genetics of Neurometabolic Diseases, Department of Neuroscience and Medical Genetics, Meyer Children's Hospital IRCCS, Viale Pieraccini 24, 50139 Firenze, Italy

[d] *Prof. A. Morrone*
Department of Neurosciences, Psychology, Drug Research and Child Health, University of Florence, Viale Pieraccini 24, 50139 Firenze, Italy

[e] *Prof. P. Paoli*
Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Morgagni 50, 50134 Firenze, Italy

[+] *Equally contributed to this work.*

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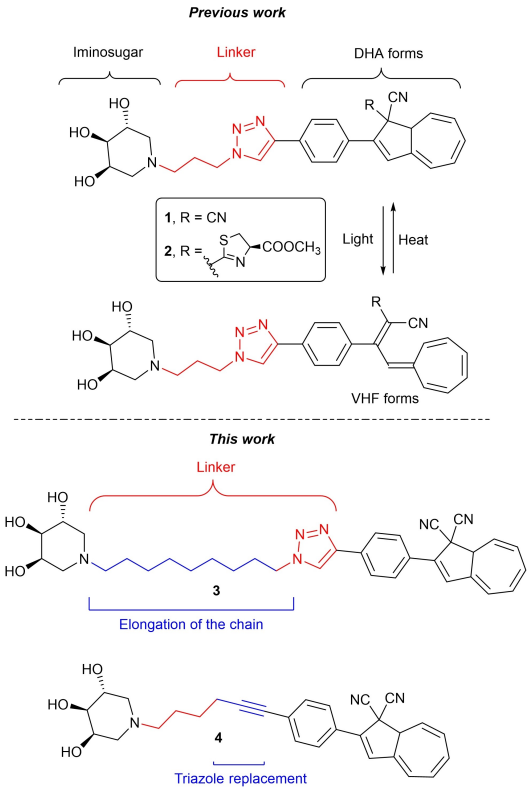


Figure 1. Dihydroazulene/Vinylheptafulvene (DHA/VHF) photoisomerization shown for systems **1**–**2** from previous work and compounds **3**–**4** synthesized and assayed in this work.

the GCase inhibitory assessment of the "dark" DHA forms and calling for a substantial redesign of the DHA *core* towards long-lived metastable VHFs.[27,28]

Herein, the GCase activity in human fibroblasts from Gaucher disease patients bearing the two most frequent mutations, N370S and L444P, co-incubated with the previously synthesized compound **1**, is examined for the first time. In addition, the synthesis and biological evaluation of compounds **3** and **4** (Figure 1) are reported to elucidate the effect of the connectivity among DHA and trihydroxypiperidine on the rescue of GCase.

Results and Discussion

Synthesis

In the design criteria for compounds **3** and **4**, the more robust DHA tag, bearing two geminal nitrile groups, such as in compound **1** was selected. The DHA moiety on derivative **2** was abandoned because of a lower GCase inhibition, with an IC_{50} of 140 μM,[18] and of a significant light-induced degradation, which prevented the GCase rescue evaluation (Table 1 and SI, Figure S27). Then, elongation of the linker between DHA and trihydroxypiperidine (compound **3**, Figure 1) and replacement of the triazole ring with a triple bond (compound **4**, Figure 1) were chosen as additional structural modifications.

Table 1. GCase inhibition in human leukocytes from healthy donors and GCase activity rescue on cell lines bearing N370S or L444P GCase mutations.[a]

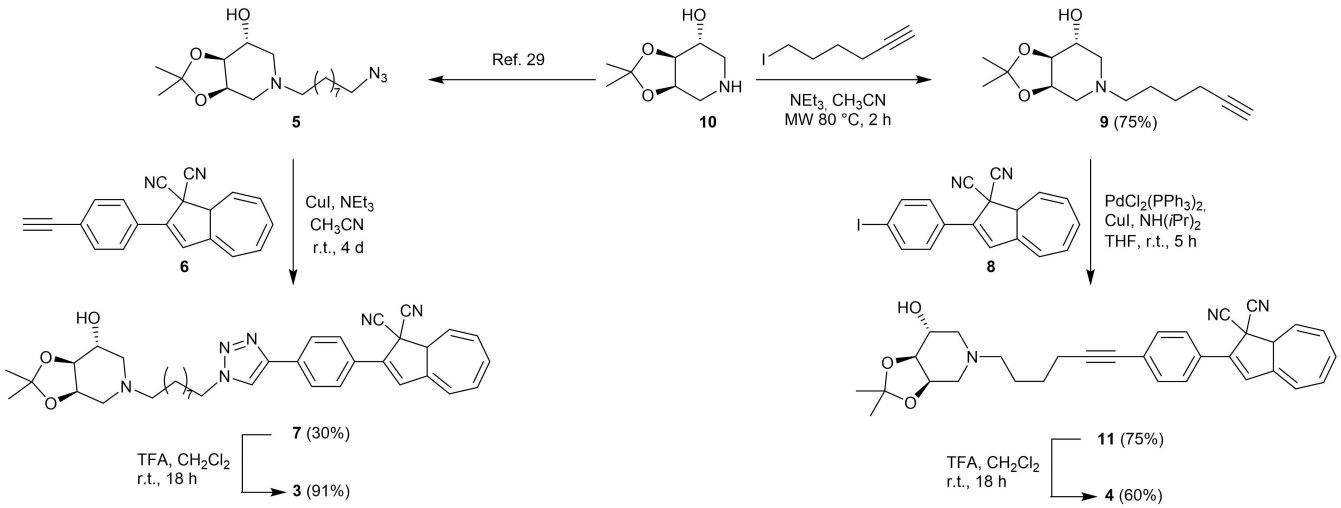
Cmpd	IC_{50} [μM][b]	N370S/RecNcil	L444P/L444P
1	22-3[18]	1.87 at 50 μM	**3.90** at 10 μM **2.80** at 1 μM
2	140 ± 34[18]	n.d.	n.d.
3	19 ± 3	1.28 at 1 μM	1.07 at 1 μM
4	31 ± 10	1.19 at 10 μM	1.19 at 10 μM
D-IFG	0.04–0.06[37]	up to **2.0** at 10 μM[37]	1.30 at 20 μM[38]
ABX	31 ± 3[39]	**2.0** at 60 μM[39]	1.2–1.3 at 50 μ[40]

[a] The best enhancement observed for compounds **1**–**4** is reported as the ratio between the activity in the presence of a given concentration and the control. [b] IC_{50} values were determined by measuring GCase activity at different concentrations of each inhibitor. n.d.: non determined.

Since elongation of the aliphatic chain to eight C atoms at the nitrogen of the trihydroxypiperidines proved to be beneficial for the interaction with GCase,[25b,c,26] the *N*-nonyl trihydroxypiperidinyl-azide **5**[29] was reacted with the acetylenic DHA **6**[30] by the copper-catalyzed azide-alkyne cycloaddition reaction,[31,32] using CuI and triethylamine in acetonitrile, to furnish **7** in 30% yield. The triazole **7** was finally deprotected with trifluoroacetic acid (TFA) in dichloromethane to give the DHA iminosugar **3** in 91% yield (Scheme 1).

Replacement of the triazole with an alkyne functionality was designed to study how the biological properties might be affected by: i) the geometry of the linker, planar (typical of the triazole ring) *vs* linear (exerted by the alkyne moiety); ii) the hydrogen bonding donor character reported for the triazole moiety, often considered for this reason a peptide bond bioisostere.[33] For a proper comparison, the number of atoms between the trihydroxypiperidinyl nitrogen and the DHA phenyl ring was maintained from **1** to **4**. In addition, the use of the alkynyl linker allowed to reduce the total synthetic pathway of compound **4** by two steps with respect to compound **1**, because the DHA unit was inserted by means of the iodo derivative **8**, which is a precursor of alkyne **6**. The synthesis was achieved by a Pd/Cu catalyzed Sonogashira reaction between trihydroxypiperidinyl-alkyne **9**, in turn synthesized by alkylation of the protected piperidine **10** with 6-iodo-1-hexyne, and the known iodo-DHA **8**[34] by using $PdCl_2(PPh_3)_2$, CuI and diisopropylamine in THF. The cross-coupling product **11** was obtained in 75% yield, and the acetonide protecting group was subsequently removed with TFA in dichloromethane to give **4** in 60% yield (Scheme 1).

Both DHAs **3** and **4** exhibited absorption maxima at 369 nm, and their photoswitching properties were followed by UV-Vis spectroscopy in water (SI, Figure S21–S26) at 25 °C. Upon irradiation at 365 nm with a LED lamp a conversion to the VHF forms was established, with maximum absorptions at 500 nm (**3**-VHF) and 496 nm (**4**-VHF). The VHF-to-DHA thermal back reactions were followed in time at 25 °C, and half-lives of 8.4 min (**3**-VHF) and 4.8 min (**4**-VHF) were obtained. Not surprisingly, the switching features of **3** and **4** in water



Scheme 1. Synthesis of compounds **3** and **4**.

resembled those found for **1** and **2** (**1**: $t_{1/2}$: 6 min; **2**: $t_{1/2}$: 4 min)[17] and for the water soluble 1,1-dicyano-2-phenyldihydroazulene derivatives reported up-to-date,[35,36] preventing also in this study to assess the biological properties on the light-irradiated compounds. In addition, evidence of a detrimental photobleaching upon light/heat cycle was found for **3** and **4** (SI, Figure S21 and S24), as for compound **2** (SI, Figure S27), while the most stable compound was **1** (SI, Figure S28).

Biological Studies

A preliminary biological screening of DHAs **3** and **4** at 1 mM towards GCase in human leukocytes from healthy donors was performed, and the IC_{50} values were determined, as shown in Table 1. Conversely, an evaluation of the corresponding light-irradiated VHFs was not possible because of the short half-lives registered in water. The results show that DHAs **3** and **4** were able to strongly bind GCase, which is visible by a potent inhibitory effect at 1 mM concentration (SI, Figure S29) with IC_{50} values of 19 μM for compound **3** and 31 μM for compound **4**. These data highlight that the occurred structural modifications did not affect the inhibitory property, in contrast to the previous finding by modifying the DHA moiety between compound **1** and **2** (22 μM vs 140 μM, respectively, Table 1). A comparison of the inhibitory activity with the two compounds which have reached the most advanced phases of clinical trials as pharmacological chaperones for GD - ambroxol (ABX) and isofagomine (D-IFG) – showed that IC_{50} values of DHAs **1**–**4** are closer to the micromolar range exerted by ABX (31 μM) (Table 1).

Then DHA derivatives **1**, **3** and **4** were tested in the dark on fibroblasts deriving from Gaucher disease patients bearing N370S or L444P mutations, the two most common *GBA1* mutations associated with Parkinson disease.[13] We evaluated the ability of these compounds to increase the mutant GCase activity in fibroblasts after 4 days of incubation with different concentrations compared to untreated fibroblasts. The best enhancements observed for each compound are reported in Table 1, together with reference compounds D-IFG and ABX. Poor enzymatic activity rescue was observed in both N370S and L444P cell lines after incubation with increasing concentrations of compounds **3** (1.28 and 1.07, respectively) and **4** (1.19 and 1.19, respectively). In addition, the assay highlighted the toxicity of compounds **3** and **4** at high concentrations, since the low cell viability observed hampered the measurement of GCase activity. As for compound **1**, the mutated GCase activity was enhanced by 1.87-fold at 50 μM on fibroblast carrying the N370S mutation and by a remarkable 3.90-fold at 10 μM on fibroblast with homozygous L444P mutation (Figure 2). The different results found on L444P mutated GCase for **1**, **3** and **4** highlight two essential features. Elongating the alkyl chain between triazole and DHA from 3 to 9 carbon atoms (compound **1** vs **3**) or replacing the triazole ring with an alkyne functionality (**1** vs **4**) reduce drastically the enzyme functionality rescue. Indeed, DHA-trihydroxypiperidine **1** was able to increase the enzymatic activity 2.80-fold even at a 10-fold lower concentration (1 μM) on the L444P cell line (Figure 2), which is a value already 2-fold higher than what reported for ABX (1.30-fold at 50 μM) and D-IFG (1.30-fold at 20 μM) on L444P mutated cell lines. These results on compound **1** are particularly

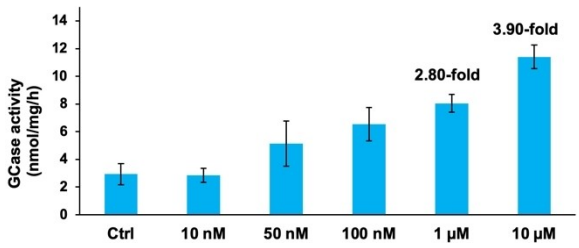


Figure 2. Influence of DHA **1** on GCase activity enhancement in human Gaucher fibroblasts with L444P mutation. The fibroblasts were incubated in the presence of compounds for four days. Ctrl: control experiment without compound.

interesting, as L444P mutation is refractory to most inhibitory chaperones, likely because the altered amino acid lies at the hydrophobic core domain, outside the catalytic pocket.[41] The comparison with the absent or minimal GCase activity rescue of the reference compounds ABX and D-IFG on L444P mutated cell lines points out the relevance of the 3.90-enhancement exerted by **1** at 10 μM.[8,42] Further studies are ongoing to confirm the suggested pharmacological chaperone-like mechanism of action following the mutant GCase trafficking to the lysosomes and the consequent substrate reduction. This latter aspect has recently emerged as an intriguing topic because reduction of glucocerebroside (the natural substrate of GCase) levels was not always observed in GD patients' fibroblasts after the incubation with GCase enhancers. Conversely, reduction of a secondary stored substrate (namely GM1-ganglioside) was recently reported, suggesting that the tracking of secondary metabolites can represent a key step for a better understanding of the pathways involved in GD.[43]

Conclusions

In summary, the synthesis and characterization of two novel DHA-appended iminosugars was presented by elongation and modification of the linker of the previously synthesized compound **1**. The biological screening on cell line bearing the L444P mutation in GCase identified a strong enhancer among the small set of DHA-trihydroxypiperidines available to-date and established the significant influence of the linker between the two components, including the importance of a triazole ring. Taking advantage of DHA's photoswitching features in the biological properties of these compounds is still an unsolved task to pursue, but in the "dark" state the DHA unit proved to be not an innocent bystander in the mutant GCase rescue, paving the way to further explore this scaffold.

Experimental Section

Synthesis and Characterization of Compounds 3–4

General Methods

Commercial reagents were used as received. All reactions were carried out under magnetic stirring and monitored by TLC on 0.25 mm silica gel plates (Merck F254). Column chromatography purification was carried out on Silica Gel 60 (32–63 μm) or on silica gel (230–400 mesh, Merck). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Gemini 200 MHz, a Varian Mercury 400 MHz or on a Varian INOVA 400 MHz instrument at 25 °C. ^{1}H-NMR and ^{13}C-NMR spectra were referenced against the residual solvent signal. Integrals are in accordance with assignments, coupling constants are given in Hz. For detailed peak assignments 2D spectra were measured (COSY, HSQC). For practical reasons the assignment of H and C atoms in NMR characterizations reflects the numbering of chemical structures in the Supporting Information. IR spectra were recorded with a IRAffinity-1S SHIMADZU or IRAffinity-1 SHIMADZU system spectrophotometers. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter. High Resolution Mass spectra (HRMS) were recorded with an ESP-MALDI-FT-ICR spectrometer equipped with a 7 T magnet (calibration of the instrument was done with NaTFA cluster ions) using Electrospray Ionization. Spectroscopic measurements were performed in a 1 cm path length quartz cuvette. UV-vis absorption spectra were recorded on a Varian Cary 50 UV-Vis spectrophotometer equipped with a Peltier heat exchange unit by scanning wavelengths from 800 to 200 nm at a rate of 600 nm/min. Photoswitching experiments were performed using ThorLabs M365L2 LED lamp, 360 mW, 700 mA mounted LED lamp with a variable focus lens with brightness mode at 5% ~34 mA. The brightness mode was controlled by an THORSLABS DC4100 4-channel LED driver equipped with a DC-4100HUB connector. Reaction and purification of light sensitive materials were performed shading the flask or the column with aluminium foil.

Compound 7. To a nitrogen-degassed solution of alkyne **6**[30] (74 mg, 0.27 mmol) in CH_3CN (9 mL), the azide **5**[29] (78 mg, 0.23 mmol), CuI (59 mg, 0.31 mmol), and degassed NEt_3 (63 μL, 0.46 mmol) were added. The mixture was stirred in darkness at room temperature for 4 days until the disappearance of starting material **5** was assessed by a TLC control (EtP:AcOEt 1:1). The mixture was concentrated under vacuum and subsequently the crude was treated with 'Quadrasil MP®' resin keeping the mixture under stirring at room temperature in the minimum amount of MeOH for 16 hours (1 g of resin for each mmol of copper). The crude was purified by FCC in the dark (SiO_2, gradient eluent from EtP:AcOEt 1:10 to AcOEt) to give 42 mg of **7** (0.07 mmol, 30%) as a bright orange waxy solid and indistinguishable mixture of diastereoisomers. **7**: R_f 0.11 (EtP:AcOEt 1:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.91 (d, J = 8.2 Hz, AB system, 2H, Ar), 7.81 (s, 1H, triazole), 7.77 (d, J = 8.2 Hz, AB system, 2H, Ar), 6.90 (s, 1H, H-3'), 6.58–6.51 (m, 1H), 6.49–6.41 (m, 1H), 6.35–6.25 (m, 2H), 5.80 (dd, J = 3.6, 10.1 Hz, 1H, H-8'), 4.38 (t, J = 7.1 Hz, 2H, CH_2-15), 4.27 (m, 1H, H-3), 4.03 (t, J = 4.4 Hz, 1H, H-4), 3.95–3.89 (m, 1H, H-5), 3.78 (br s, 1H, H-8a'), 2.95–2.76 (m, 1H, OH), 2.75–2.67 (m, 1H, Ha-2), 2.59–2.51 (m, 1H, Ha-6), 2.49–2.42 (m, 1H, Hb-6), 2.41–2.29 (m, 3H, Hb-2, CH_2-7), 1.99–1.86 (m, 2H, CH_2-14), 1.48 (s, 3H, Me), 1.46–1.38 (m, 2H, CH_2-8), 1.36–1.21 (m, 13H, CH_2-9, CH_2-10, CH_2-11, CH_2-12, CH_2-13, Me). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 146.6, 139.6, 138.7, 132.4 (C-3'), 132.3, 131.0 (2 C), 130.0, 127.8, 126.8 (2 C), 126.3 (2 C), 121.2, 120.1 (triazole), 119.5 (C-8'), 115.2 (C≡N), 112.8 (C≡N), 109.3 (O<u>C</u>$(CH_3)_2$), 77.0 (C-4), 72.2 (C-3), 67.6 (C-5), 57.8 (C-7), 55.9 (C-2), 55.6 (C-6), 51.2 (C-8a'), 50.6 (C-15), 45.2 (C-1'), 30.3 (C-14), 29.4, 29.3, 29.0, 28.4, 27.3, 26.8, 26.5 (2 C). HRMS (ESP^+) calcd for $C_{37}H_{44}N_6O_3$ $[M+H]^+$: m/z = 621.35477; found 621.35514. IR (neat): ν = 2929, 2855, 2160, 1613, 1459, 1436, 1379, 1242, 1218, 1142, 1056, 973, 911, 840 cm^{-1}.

Compound 3. A solution of **7** (36 mg, 0.06 mmol) in DCM (1 mL) was left stirring with CF_3COOH (6 drops) in darkness at room temperature for 18 h until the disappearance of starting material **7** was assessed by a TLC control (DCM:MeOH:NH_4OH (6%) 10:1:0.1). Then, the crude mixture was concentrated and the crude residue was purified by FCC in the dark (SiO_2, DCM:MeOH:NH_4OH (6%) 10:1:0.1) to give 31 mg of **3** (0.05 mmol, 91%) as a yellow waxy solid and indistinguishable mixture of diastereoisomers. **3**: *Rf* 0.18 (DCM:MeOH:NH_4OH (6%) 10:1:0.1). ^{1}H-NMR (400 MHz, CD_3OD) δ ppm: 8.39 (s, 1H, triazole), 7.95 (d, J = 8.5 Hz, AB system, 2H, Ar), 7.86 (d, J = 8.5 Hz, AB system, 2H, Ar), 7.19 (s, 1H, H-3'), 6.61–6.55 (m, 1H), 6.51–6.40 (m, 2H), 6.35–6.28 (m, 1H), 5.79 (dd, J = 3.7, 10.2 Hz, 1H, H-8'), 4.43 (t, J = 7.1 Hz, 2H, CH_2-15), 3.89 (m, 1H, H-3), 3.82–3.75 (m, 2H, H-5, H-8a'), 3.43–3.35 (m, 1H, H-4), 2.85–2.68 (m, 2H, Ha-2, Ha-6), 2.41–2.29 (m, 2H, CH_2-7), 2.29–2.21 (m, 1H, Hb-2), 2.14–2.00 (m, 1H, Hb-6), 1.99–1.89 (m, 2H, CH_2-14), 1.53–1.42 (m, 2H, CH_2-8), 1.38–1.22 (m, 10H, CH_2-9, CH_2-10, CH_2-11, CH_2-12, CH_2-13). ^{13}C-NMR (100 MHz, CD_3OD) δ ppm: 147.7, 140.5, 140.2, 134.3 (C-3'), 133.1, 132.1, 131.8, 131.6, 128.8, 128.0 (2 C), 127.2 (2 C), 122.8

(triazole), 122.8, 120.6 (C-8′), 116.5 (C≡N), 114.2 (C≡N), 75.2 (C-4), 69.5 (C-5), 69.1 (C-3), 59.2 (C-7), 58.2 (C-6), 57.5 (C-2), 52.5 (C-8a′), 51.6 (C-15), 46.5 (C-1′), 31.2, 30.5, 30.4, 30.0, 28.5, 27.5 (2 C). HRMS (ESP^+) calcd for $C_{34}H_{40}N_6O_3$ $[M+H]^+$: $m/z = 581.32347$; found 581.32326. IR (neat): $\nu = 3324$, 2911, 2850, 2812, 1654, 1468, 1352, 1227, 1064, 982, 834 cm^{-1}. UV-Vis (water/5% dmso): $\lambda_{max\text{-}DHA}$/nm, (ε/M^{-1} cm^{-1}) = 369 (16300), $\lambda_{max\text{-}VHF}$/nm, (ε/M^{-1} cm^{-1}) = 500 (11100).

Compound 9. A solution of piperidine **10**[18a] (100 mg, 0.58 mmol), 6-Iodo-1-hexyne (99 μL, 0.75 mmol) and NEt_3 (120 μL, 0.87 mmol) in CH_3CN (3 mL) was stirred under microwave irradiation at 80 °C for 2 h, until the disappearance of starting material **10** was assessed by a TLC control (DCM:MeOH:NH_4OH (6%) 5:1:0.1). The reaction mixture was concentrated and then the crude was purified by FCC (SiO_2, DCM:MeOH:NH_4OH (6%) 50:1:0.1) to afford 110 mg of **9** (0.43 mmol, 75%) as a pale-yellow oil. **9**: *Rf* 0.38 (DCM:MeOH:NH_4OH (6%) 20:1:0.1). $[\alpha]_D^{23} = +$ 20.36 (c = 0.55, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 4.29 (m, 1H, H-3), 4.05 (t, J = 4.4 Hz, 1H, H-4), 3.97–3.90 (m, 1H, H-5), 2.76 (dd, J = 6.1, 11.7 Hz, 1H, Ha-2), 2.60–2.54 (m, 1H, Ha-6), 2.53–2.46 (m, 1H, Hb-6), 2.42–2.33 (m, 3H, Hb-2, CH_2-7), 2.20 (td, 2.6, 7.0, 13.4 Hz, 2H, CH_2-10), 1.95 (t, J = 2.6 Hz, 1H, H-12), 1.64–1.51 (m, 4H, CH_2-8, CH_2-9), 1.50 (s, 3H, Me), 1.35 (s, 3H, Me). ^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 109.5 (O<u>C</u>$(CH_3)_2$), 84.3 (C-11), 76.9 (C-4), 72.1 (C-3), 68.7 (C-12), 67.6 (C-5), 57.2 (C-7), 56.0 (C-2), 55.6 (C-6), 28.4, 26.5, 26.3, 26.0, 18.4 (C-10). HRMS (ESP^+) calcd for $C_{14}H_{23}NO_3$ [$(M+H)^+$]: $m/z = 254.17507$; found 254.17495. IR ($CHCl_3$): $\nu = 3593$, 3499, 3306, 3005, 2992, 2943, 2826, 1464, 1381, 1246, 1200, 1146, 1059, 935 cm^{-1}.

Compound 11. A degassed solution of compound **8**[34] (67 mg, 0.18 mmol) in (*i*-Pr)$_2$NH (68 μL) and dry THF (3.4 mL), was added to a mixture of compound **9** (89 mg, 0.35 mmol), $PdCl_2(PPh_3)_2$ (6 mg, 0.01 mmol) and CuI (3 mg, 0.018 mmol) and the mixture was stirred in darkness for 5 hours at room temperature until the disappearance of starting material **8** was assessed by a TLC control (DCM:MeOH:NH_4OH (6%) 20:1:0.1). The reaction mixture was concentrated and then the crude was purified by FCC in the dark (SiO_2, DCM:MeOH:NH_4OH (6%) 20:1:0.1) to give 67 mg of **11** (0.13 mmol, 75%) as a red oil and indistinguishable mixture of diastereoisomers. **11**: *Rf* 0.28 (DCM:MeOH:NH_4OH (6%) 20:1:0.1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.65 (d, J = 8.4 Hz, AB system, 2H, Ar), 7.47 (d, J = 8.3 Hz, AB system, 2H, Ar), 6.87 (s, 1H, H-3′), 6.61–6.52 (m, 1H), 6.51–6.43 (m, 1H), 6.38–6.26 (m, 2H), 5.81 (dd, J = 3.8, 10.2 Hz, 1H, H-8′), 4.31 (m, 1H, H-3), 4.07 (t, J = 4.4 Hz, 1H, H-4), 3.99–3.92 (m, 1H, H-5), 3.81–3.75 (m, 1H, H-8a′), 2.79 (dd, J = 6.1, 11.8 Hz, 1H, Ha-2), 2.62–2.57 (m, 1H, Ha-6), 2.56–2.50 (m, 1H, Hb-6), 2.50–2.43 (m, 4H, CH_2-7, CH_2-10), 2.42–2.36 (m, 1H, Hb-2), 1.73–1.56 (m, 4H, CH_2-8, CH_2-9), 1.51 (s, 3H, Me), 1.36 (s, 3H, Me). ^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 139.6, 138.7, 132.6 (C-3′), 132.4 (2 C), 131.1, 131.0, 129.5, 127.8, 126.1 (2 C), 125.8, 121.3, 119.5 (C-8′), 115.2 (C≡N), 112.7 (C≡N), 109.5 (O<u>C</u>$(CH_3)_2$), 93.1 (C-12), 80.6 (C-11), 76.8 (C-4), 72.1 (C-3), 67.6 (C-5), 57.3 (C-7), 56.0 (C-2), 55.5 (C-6), 51.2 (C-8a′), 45.1 (C-1′), 28.4 (Me), 26.5 (2 C, C-8, C-9), 26.2 (Me), 19.6 (C-10). HRMS (ESP^+) calcd for $C_{32}H_{33}N_3O_3$ $[M+H]^+$: $m/z = 508.25947$; found 508.25909. IR (neat): $\nu = 3410$, 2931, 2814, 2223, 1509, 1466, 1456, 1438, 1379, 1243, 1217, 1143, 1120, 1054 cm^{-1}.

Compound 4. A solution of **11** (55 mg, 0.11 mmol) in DCM (2 mL) was left stirring with CF_3COOH (14 drops) in darkness at room temperature for 18 h until the disappearance of starting material **11** was assessed by a TLC control (DCM:MeOH:NH_4OH (6%) 10:1:0.1). Then, the crude mixture was concentrated and the crude residue was purified by FCC in the dark (SiO_2, DCM:MeOH:NH_4OH (6%) 10:1:0.1) to give 30 mg of **4** (0.06 mmol, 60%) as an orange waxy solid and indistinguishable mixture of diastereoisomers. **4**: *Rf* 0.17 (DCM:MeOH:NH_4OH (6%) 10:1:0.1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.65 (d, J = 8.6 Hz, AB system, 2H, Ar), 7.46 (d, J = 8.6 Hz, AB system, 2H, Ar), 6.88 (s, 1H, H-3′), 6.61–6.52 (m, 1H), 6.50–6.43 (m, 1H), 6.36–6.26 (m, 2H), 5.80 (dd, J = 3.7, 10.4 Hz, 1H, H-8′), 3.93 (br s, 1H, H-3), 3.86–3.75 (m, 2H, H-5, H-8a′), 3.43–3.28 (m, 1H, H-4), 3.06–2.97 (m, 1H, Ha-6), 2.96–2.84 (m, 1H, Ha-2), 2.72–2.55 (m, 3H, OH), 2.51–2.40 (m, 4H, CH_2-7, CH_2-10), 2.34–2.22 (m, 1H, Hb-6), 2.09–1.93 (m, 1H, Hb-2), 1.72–1.53 (m, 4H, CH_2-8, CH_2-9). ^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 139.6, 138.7, 132.7 (C-3′), 132.4 (2 C), 131.2, 131.0, 129.5, 127.8, 126.1 (2 C), 125.8, 121.4, 119.6 (C-8′), 115.2 (C≡N), 112.7 (C≡N), 110.6 (interference), 93.0 (C-12), 80.7 (C-11), 77.4 (C-4), 69.5 (C-5), 68.5 (C-3), 57.3, 57.2, 57.0 (C-7), 51.2 (C-8a′), 45.1 (C-1′), 26.5, 26.2, 19.6 (C-10). HRMS (ESP^+) calcd for $C_{29}H_{29}N_3O_3$ $[M+H]^+$: $m/z = 468.22817$; found 468.22792. IR (neat): $\nu = 3437$, 3207, 2913, 2812, 2216, 1509, 1470, 1353, 1329, 1119, 1081, 1053, 1022, 1006, 910, 833 cm^{-1}. UV-Vis (water/5% dmso): $\lambda_{max\text{-}DHA}$/nm, (ε/M^{-1} cm^{-1}) = 368 (24200); $\lambda_{max\text{-}VHF}$/nm, (ε/M^{-1} cm^{-1}) = 500 nm (24900).

Biochemical Characterization

Inhibitory activity towards human GCase from leukocyte homogenates. All experiments on biological materials were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. In keeping with ethical guidelines, all blood and cell samples were obtained for storage and analyzed only after written informed consent of the patients (and/or their family members) was obtained, using a form approved by the local Ethics Committee (Codice Protocollo: Lysolate "Late -onset Lysosomal Storage Disorders (LSDs) in the differential diagnosis of neurodegenerative diseases: development of new diagnostic procedures and focus on potential pharmacological chaperones (PCs). Project ID code: 16774_bio, 5 May 2020, Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Area Vasta Centro, Florence, Italy). Control and patient samples were anonymized and used only for research purposes.

Compounds **3** and **4** were screened towards GCase in leukocytes isolated from healthy donors (controls). Isolated leukocytes were disrupted by sonication, and a micro-BCA protein assay kit (Sigma–Aldrich) was used to determine the total protein amount for the enzymatic assay, according to the manufacturer instructions. Enzyme activity was measured in a flat-bottomed 96-well plate. Compound solution (3 μL), 4.29 μg/μL leukocytes homogenate (7 μL), and substrate 4-methylumbelliferyl-β-D-glucoside (3.33 mM, 20 μL, Sigma–Aldrich) in citrate/phosphate buffer (0.1:0.2, M/M, pH 5.8) containing sodium taurocholate (0.3%) and Triton X-100 (0.15%) were incubated for 1 h at 37 °C. The reaction was stopped by addition of sodium carbonate (200 μL; 0.5 M, pH 10.7) containing Triton X-100 (0.0025%), and the fluorescence of 4-methylumbelliferone released by GCase activity was measured in SpectraMax M2 microplate reader (λex = 365 nm, λem = 435 nm; Molecular Devices). For each compound a blank composed by a water solution containing 0.2% of bovine serum albumin (BSA), inhibitor and substrate (called "inhibitor blank") was tested and compared with the experiment blank, composed by BSA and substrate. No "inhibitor blank" differs from the experiment blank, demonstrating that the inhibitors do not interfere with the fluorescence of the hydrolyzed substrate. Percentage of GCase inhibition is given with respect to the control (without compound). Data are mean ± SD (n = 3).

IC_{50} determination. The IC_{50} values of inhibitors **3** and **4** against GCase were determined by measuring the initial hydrolysis rate with 4-methylumbelliferyl-β-D-glucoside (3.33 mM). Data obtained were fitted to the following equation using the Origin Microcal program (see SI for further details).

GCase Activity Assays on Cell Lines

The effect of compounds **1**, **3** and **4** on mutated GCase activity was evaluated in Gaucher patients' cells fibroblasts with the N370S/RecNcil and L444P/L444P mutations. Gaucher disease patients' cells were obtained from the "Cell line and DNA Biobank from patients affected by Genetic Diseases" (Gaslini Hospital, Genova, Italy).

The cell passage number of fibroblasts is between 12 to 24. Fibroblasts cells (25×10^4) were seeded in T25 flasks with DMEM supplemented with fetal bovine serum (10%), penicillin/streptomycin (1%), and glutamine (1%) and incubated at 37 °C with 5% CO_2 for 24 h. The medium was removed, and fresh medium containing the compounds was added to the cells and left for 4 days. The medium was removed, and the cells were washed with PBS and detached with trypsin to obtain cell pellets, which were washed four times with PBS, frozen and lysed by sonication in water. Enzyme activity was measured as reported above. Reported data are mean ± S.D. (n = 2).

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Conflict of Interests

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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