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Photoswitchable inhibitors of human β-glucocerebrosidase†

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Light-switchable inhibitors of the enzyme β-glucocerebrosidase (GCase) have been developed by anchoring a specific azasugar to a dihydroazulene or an azobenzene responsive moiety. Their inhibitory effect towards human GCase, before and after irradiation are reported, and the effect on thermal denaturation of recombinant GCase and cytotoxicity were studied on selected candidates.

The incorporation of photochromic linkers or scaffolds into biologically active compounds has been investigated in recent years as a tool to tune biomolecular interactions by light.[1] An attractive photo-controlled strategy consists in promoting prodrug activation by photocleavable moieties and is applied to activate compounds locally, at the pathological site, to increase their selectivity towards a specific target thus limiting adverse side-effects.[2] In this work, a less invasive approach has been investigated by the exploitation of an appropriate combination of light, time and temperature to modulate the degree of isomerization between two switching states, in order to stabilize or destabilize the specific conformation of a bioactive compound (an inhibitor) towards its biological target (an enzyme) and tune its biological properties.[3] Two photoresponsive moieties were explored (Fig. 1).

The dihydroazulene/vinylheptafulvene couple (DHA-Ph/VHF-Ph, Fig. 1a) is a photo/thermoswitch, whose DHA form is quantitatively converted by light-induced ring-opening into VHF, which in turn can undergo a reversal ring-closure to DHA under thermal[4] or Lewis acid catalysed conditions.[5] It has been extensively investigated in the context of energy storage and molecular solar thermal systems,[6] but it has never been studied as ligand for photo-controlled bioactivity purposes. Replacement of one nitrile with alternative functional groups was reported to have a substantial impact on the VHF half-life,

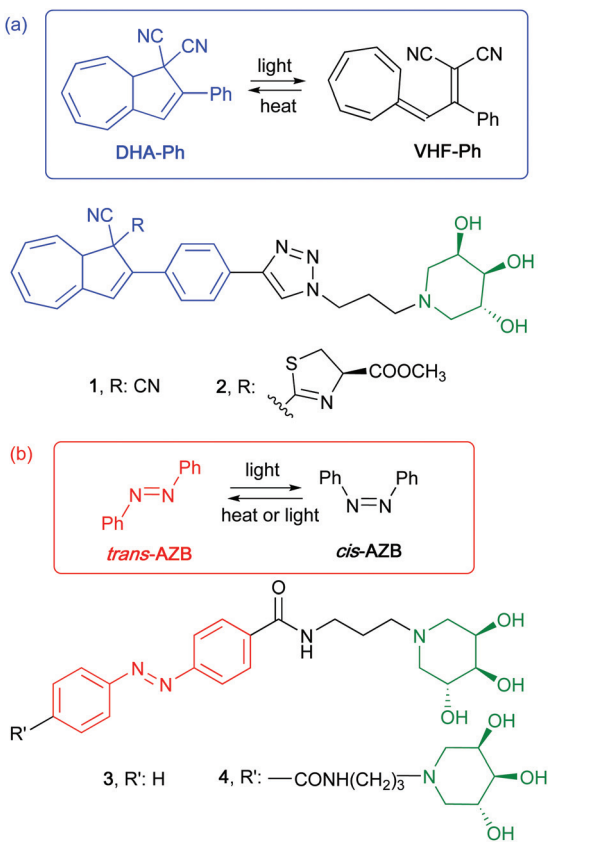

Fig. 1 Photoswitches incorporated into bioactive trihydroxypiperidine presented in this paper. In the separated boxes, DHA/VHF and *trans*/*cis* AZB couples are shown.

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hence on the VHF-to-DHA thermal back reaction (TBR), making it a promising time-tunable photoswitch.[7]

The azobenzene moiety (*trans*-AZB/*cis*-AZB, Fig. 1b) has been widely used for different photopharmacology applications, with some limitations mainly associated with the use of UV light.[8] The AZB system is characterized by a drastic change of the geometry and hence of the dipole moment of the two species, from the *trans* linear form to the *cis* bent form. However, in most cases UV-light irradiation of the stable *trans* isomer produces a *cis*/*trans* mixture, regulated by a photostationary state (PSS), whose ratio is specific for every structure depending on the substituents on the phenyl rings.[8b,c] The back isomerization of the AZB system occurs thermally and photochemically.[8]

In this communication we report the synthesis, characterization and biological studies towards a human enzyme of clinical relevance of molecular switches **1** and **2** (DHA-based) as well as **3** and **4** (AZB-based), which are tethered to a selected azasugar (Fig. 1). A specific trihydroxypiperidine (TriHP) has been chosen as the active compound since some of its derivatives[9] (Fig. 2) are known to act as pharmacological chaperones (PCs) for Gaucher disease.[10] This genetic metabolic disorder is caused by misfolding-derived deficient activity of β-glucocerebrosidase (GCase), the enzyme responsible for the hydrolysis of glucosylceramide to ceramide and glucose.[11] The consequent progressive accumulation of glucosylceramide in the lysosomes ultimately leads to severe organ dysfunctions.

PCs represent an innovative therapeutic option for this pathology because they are reversible inhibitors of the mutated enzyme that, when administered at sub-inhibitory concentration, impart a rescue of the enzyme residual activity.[12] The PC binds the misfolded enzyme in the endoplasmic reticulum (ER), improves its folding and promotes its trafficking to the lysosome, where the PC is replaced by the enzyme natural substrate. Therefore, an ideal PC should have a strong affinity to the target enzyme in the endoplasmic reticulum and a lower binding in the lysosome, allowing easy PC–enzyme complex dissociation. This feature has been recently achieved exploiting the difference in pH between the ER (pH = 7) and the lysosome (pH = 5), by preparing pH-sensitive inhibitors that undergo cleavage and consequent self-inactivation in the lysosome.[12b] We reasoned that self-inactivation might be addressed also with inhibitor temporal structural changes induced by light/heat cycles, hence the incorporation of a photoswitchable unit to the bioactive compound. Ideally, the irradiated/metastable form of the photo-responsive PC should have higher affinity with the enzyme than the non-irradiated species, so that the thermal back reaction of the photoswitch would induce self-inactivation upon reaching the lysosome, maximizing the chaperone *vs.* inhibitor behavior. To achieve this goal, a perfect spatiotemporal control of the switch transformation and a marked difference in affinity of the two forms of the switchable system are crucial issues to be tackled.

The most biologically promising trihydroxypiperidine (TriHP) warhead has been chosen[9] and the length of the spacer between the photo-sensitive unit and the TriHP has been carefully selected on the basis of previous GCase inhibition data, taking into account the presence of two hydrophobic pockets in the enzyme, which calls for a C8-C12 spacer.[9c] Hence, TriHP was linked to the DHA scaffold by copper-catalysed azide–alkyne cycloaddition (Scheme 1a), by reacting *para*-acetylene-2-phenyl-DHA **6** with the protected azido TriHP **5**[13] in the presence of CuI and triethylamine in acetonitrile. The resulting triazole **7** was ultimately deprotected with TFA in dichloromethane to give DHA derivative **1** in 71% yield, as a mixture of two diastereoisomers. Then, **1** was reacted in the dark with L-cysteine-methyl ester[7b] to give **2** in 38% yield. Next, the TriHP amine **8**, obtained by reduction of **5** (see ESI†), was reacted with mono-acylchloride-AZB **9** or bis-acylchloride-AZB **10** to provide compounds **11** and **12**, which were deprotected with TFA in CH_2Cl_2 to give **3** (>95% *trans*) and **4** (100% *trans*), in 73% and 88% yield, respectively (Scheme 1b). AZB has been used here as a suitable scaffold to insert one or two units of TriHP, therefore furnishing also a divalent structure, which could enhance the biological affinity.[14]

Preliminarily to their biological tests, the photoswitching properties of compounds **1–4** have been assessed. Irradiation at 365 nm (for **1** and **2**) or 340 nm (for **3** and **4**) of an aqueous solution of each photoswitch converted DHAs fully into VHFs,

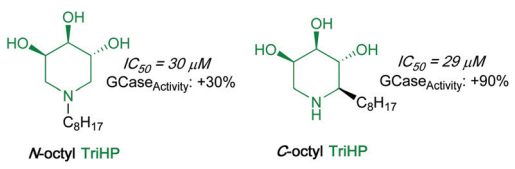
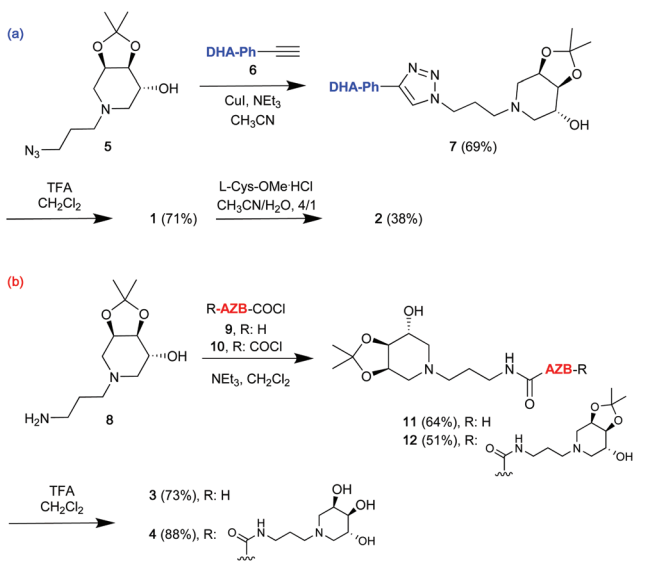


Fig. 2 Previously investigated TriHPs with corresponding IC_{50} and GCase activity enhancement.



Scheme 1 Synthesis of DHA-based compounds **1** and **2** (a) and AZB-based compounds **3** and **4** (b).

while AZBs reached a PSS constituted by a *cis*/*trans* isomeric mixture, with prevalence of the *cis* isomer. UV-visible and ^{1}H-NMR spectroscopies were used to determine the half-life time of the metastable species (VHF or *cis*-AZB derivative) and the isomeric ratios after irradiation and after 1.5 h of thermal relaxation at r.t. (Table 1).

The measurements were conducted in water in the presence of dimethylsulfoxide (2–4%) to reproduce the standard conditions of the biological studies. The DHA derivatives **1** and **2** were irradiated in a UV cuvette (concentration *ca.* 10^{-5} M) at 365 nm for up to 6 min, which is a time range known to induce a full transformation into the corresponding VHFs, and the spectra showed neat isosbestic points, indicating that only two species are involved (see ESI†). The decay of absorbance of VHF followed a first-order kinetics and allowed to calculate for system **1** a VHF-to-DHA half-life of 6 min in water (with 2% DMSO) at 25 °C, which is in line with the data for the only other water-soluble DHA derivative reported to date.[15] Polar media are known to decrease the VHF half-life because of a polar transition state involved in the 10-π-electrocyclic ring-closure.[16] Surprisingly, switching studies on system **2** provided a VHF half-life of a similar magnitude (4 min), contrary to expectation following the introduction of the thiazoline ring. Indeed, replacement of one nitrile with a thiazoline group has previously shown to be able to prevent indefinitely the thermal back reaction (TBR) in acetonitrile.[7b] Even considering the strong dependency of VHF-to-DHA transformation on solvent polarity (the higher the polarity, the faster the TBR), this example suggests a levelling effect of water on the TBR, independent from the specific substituent, and prompts for further studies. UV-visible absorption spectra of compound **3** upon irradiation at 340 nm showed a decrease of the characteristic absorbance at 325 nm and the evolution of a broad shoulder, typical of the *cis* isomer, at 428 nm. ^{1}H-NMR spectroscopy was used to establish the PSS distribution of 88% *cis* isomer, reached after 3 h irradiation at 340 nm of the solution for the biological tests. Keeping the sample in the dark at r.t. for 1.5 h resulted in 85% *cis* isomer, and this content remained quite constant within the following 18 h (81% *cis*), indicating a slow thermal reversion and verifying prevalence of *cis* isomer in the timeframe of the biological studies. Similarly, *trans*-**4** was irradiated for 3 h at 340 nm giving a PSS mixture of 68% *cis*-AZB by ^{1}H-NMR spectroscopy. Leaving the NMR sample at room temperature in the dark overnight furnished a mixture with 63% *cis*-AZB. The thermal relaxation of system **4** proved to occur in more than 3 days.

All new compounds **1–4** were assayed as GCase inhibitors in human leukocytes from healthy donors (Table 2), together with **13** [9a,14] and **14–16**, which were synthesized and evaluated as control compounds (Fig. 3). The photoswitchable systems showed to be good GCase inhibitors with 88–100% inhibition at 1 mM. As a general trend, all the calculated IC_{50} values lay in the micromolar range thus showing that the introduction of the photoswitchable moieties does not hamper the inhibition of GCase enzyme. Among the not irradiated compounds, **1** and *trans*-**3** showed the strongest inhibition with IC_{50} of 22 and 15 μM, respectively. These values are in line with IC_{50} of 30 and 29 μM observed for *N*- and *C*-octyl TriHPs (Fig. 2).[9] Therein the aliphatic chain occupies a hydrophobic pocket in the GCase active site,[9c,17,18] thus playing a pivotal role in the interaction with the enzyme, as observed also for alkylated trihydroxypyrrolidines.[19] Systems **1–2** were initially tested only in the dark (>97% DHA form, entries 1 and 3), because of the very fast thermal back reaction. Tentative experiments with constant irradiation during the enzymatic test were performed, but were infeasible, giving unreliable data (see ESI†). To gain insights on the effect of VHF form on inhibition, system **1** was irradiated for 30 min and 60 min, before the incubation with the enzyme. During these attempts, we observed that the DMSO amount used to prepare the solution of **1** for the enzymatic test is critical for the stability of the system upon irradiation. Hence, we decreased it from 50% to 10% DMSO in water, and entries 1–2 have been evaluated with this optimized

Table 1 Half-lives determined in 2–4% DMSO in water at 25 °C (if not otherwise stated) by UV-Vis measurements. Isomeric ratios determined by ^{1}H-NMR spectroscopy at 400 MHz or by UV-Vis spectroscopy after irradiation and after thermal relaxation at r.t

System	Ratio after irradiation	$t_{1/2}^{TBR}$	After 1.5 h
1 [a]	>97%VHF	6 min	>97% DHA
2 [a]	>97% VHF	4 min, 1 min[b]	>97% DHA
3 [c]	88% *cis*	>1 d	85% *cis*
4 [c]	68% *cis*	>3 d	63% *cis*[d]

[a] Irradiation at 365 nm, ratio evaluated by UV-Vis spectra and by comparison with previous results.[7b] [b] At 37 °C. [c] Irradiation at 340 nm, ratio evaluated by ^{1}H-NMR spectroscopy. [d] After 18 h.

Table 2 Percentage inhibition of GCase in human leukocytes extracts incubated with compounds (1 mM) and corresponding IC_{50} values

Entry	System	GCase inhib. [%]	IC_{50} [μM]
1	DHA-**1**	100	22 ± 3
2	DHA/VHF-**1** [a]	100	13 ± 0.2
3	DHA-**2**	88	140 ± 34
4	*trans*-**3**	100	15 ± 1
5	PSS-**3** (88% *cis*)	100	70 ± 3
6	*trans*-**4**	95	35 ± 4
7	PSS-**4** (68% *cis*)	100	30 ± 2
8	**13**	21 [9a]	>1000
9	**14**	12	>1000
10	**15**	6	>1000
11	**16**	18	>1000

[a] Solution of **1** irradiated for 60 min before incubation.

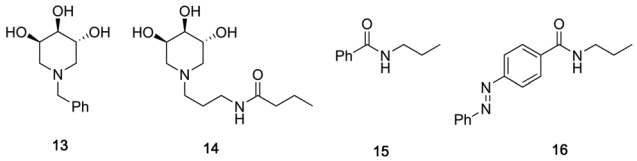


Fig. 3 Structures of control compounds.

condition. IC_{50} values of **1** with 50% or 10% DMSO laid within the error range (see ESI†). An IC_{50} value lower than for the sole DHA-**1** was observed after 60 min irradiation (entry 2 *vs.* 1). As for entry 2, the DHA/VHF ratio could not be determined, therefore this encouraging IC_{50} data does not bring to effective conclusion and calls for a modification of the DHA scaffold to increase the TBR.

Albeit the introduction of a benzyl ring at nitrogen in the TriHP skeleton was known to dramatically reduce the affinity towards GCase (**13**, entry 7),[9a] the azobenzene moiety of *trans*-**3** positively contribute to inhibition. Irradiating *trans*-**3** and *trans*-**4** to accumulate the *cis*-isomer and generate PSS-**3** (88% *cis*) and PSS-**4** (68% *cis*) increased the IC_{50} for the monovalent inhibitor PSS-**3** up to 70 μM (entry 5 *vs.* 4), while it did not alter the IC_{50} value of the divalent inhibitor PSS-**4** (entry 7 *vs.* 6). Monovalent compound **3** showed a remarkable inhibitory activity difference (4.7-fold) upon switching from *trans*- to *cis*-isomer, analogously to what was reported for an azobenzene-based β-D-thiogalactoside derivative recently proposed as β-galactosidase photoswitchable inhibitor.[19] Conversely, compound **4** seems to be unaffected by photo-conformational changes, likely because its divalent nature involves chelating or clustering effects favoured by GCase dimerization,[20] which may represent the driving force of the inhibition. To better investigate the structural basis of *trans*-**3** interaction with the enzyme, control systems **14**, **15** and **16** were synthesized and evaluated (Fig. 3). Their negligible inhibitory activity towards GCase (entries 9–11) demonstrated that neither the *N*-substitution of the TriHP with a chain containing an amide moiety (**14**) nor a simple *N*-alkyl benzamide (**15**) is sufficient to strongly inhibit GCase, suggesting the key role played by the azobenzene moiety. Lack of inhibition by compound **16** proved the assumed importance of the azasugar moiety. Then, the mechanism of action of *trans*-**3** has been studied. Unlike previously investigated TriHPs, which are competitive GCase inhibitors,[9c] a non-competitive type inhibition was observed for *trans*-**3** (see ESI†). A careful analysis of the literature showed that the pharmacological chaperone L-isofagomine was also reported to be a non-competitive GCase inhibitor.[21] In order to mimic the stabilization effect made by PCs in cells on misfolded enzymes, *trans*-**3** was tested in a thermal denaturation experiment using recombinant GCase. Recovery of GCase activity was measured at 48 °C in the absence (control, ctrl) and in the presence of increasing concentrations of *trans*-**3** at different incubation times (Fig. 4). Compound *trans*-**3** was found to stabilize GCase at all the tested concentrations after 20 and 40 min (blue and red bars), and remarkable recovery of GCase activity (up to 1.6-fold) was still observed at 50 and 100 μM after 1 h incubation (green bars). These data clearly indicate that *trans*-**3** is a good candidate as pharmacological chaperone. In parallel, a cytotoxicity evaluation was carried out for compounds **1**, *trans*-**3** and *trans*-**4** using wild type (WT) fibroblasts (see ESI†). DHA **1** showed a good cell viability (>90%) up to 50 μM. In the AZB family, *trans*-**3** was only slightly more cytotoxic, while *trans*-**4** showed almost 100% cell viability even at 100 μM and after 48 h.

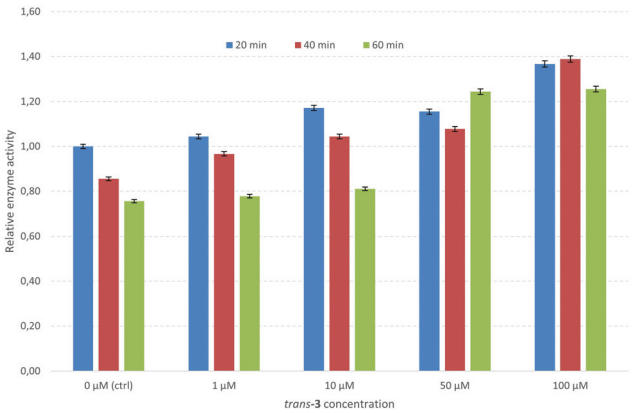


Fig. 4 Stabilization of recombinant human GCase by heat inactivation with *trans*-**3**. Relative enzyme activity after thermal denaturation (48 °C) for 20, 40 or 60 min at indicated inhibitor concentrations *vs.* the corresponding assay at 37 °C. Data are mean ± RSD (n = 3).

Conclusions

In summary, water-soluble photoswitchable human GCase inhibitors were synthesized bearing a specific azasugar and a light sensitive unit (DHA or AZB). DHA systems showed a TBR in aqueous media too fast in the timeframe of the biological test, limiting the evaluation of the inhibition activity of the irradiated forms, but the encouraging IC_{50} value and the low toxicity exerted by **1** after 48 h stimulate the development of modified DHAs for further studies. As for the AZB derivatives, **3** showed remarkable inhibitory activity change by irradiation, the *trans* form turning out more active than the *cis*. Interestingly, a non-competitive inhibition mechanism was established for *trans*-**3** together with preliminary indication of a PC behavior, assessed by GCase stabilization under thermal denaturation conditions.

Conflicts of interest

There are no conflicts to declare.

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