Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *Org. Biomol. Chem.*, 2026, **24**, 2487

Design of multivalent pharmacological chaperones against Pompe disease *via* metal-free ligation

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Received 6th January 2026,
Accepted 19th February 2026

DOI: 10.1039/d6ob00021e

rsc.li/obc

We report herein the design of multivalent pharmacological chaperones (PCs), based on D- and L-deoxynojirimycin (DNJ), to treat Pompe disease (PD), a lysosomal storage disorder caused by mutations in the acid alpha-glucosidase (GAA) enzyme. Multivalent DNJ platforms were built on a pentaerythritol core by Copper Azide–Alkyne Click (CuAAC) ligation. Moreover, new phosphorus-containing dendrimers were decorated with DNJ by Strain Promoted Azide–Alkyne Click (SPAAC) reaction. Our main objective was to systematically study how the chirality and the hydrophobicity of these constructs could modulate their ability to inhibit, stabilize, and restore GAA activity. Thermal stabilization assays identified several highly effective stabilizers. Most importantly, the phosphorus-containing dendrimer **15b**, an L-*N*-undecanyl-DNJ derivative, was found to enhance GAA activity by 1.2 fold in fibroblasts from a Pompe patient at the dose of only 0.5 nM. This finding is a significant breakthrough as it represents the first report of a multivalent compound increasing GAA activity in patient cells. This novel multivalent PC, which is a very weak GAA inhibitor, offers a promising avenue for the development of effective treatments for Pompe disease.

Introduction

Lysosomal storage diseases (LSDs) are a group of metabolic disorders mostly caused by mutations of lysosomal hydrolases. The dysfunction of these proteins induces a lysosomal burden of the non-hydrolysed substrates.[1,2] Enzyme replacement therapy (ERT), which is the main therapeutic response, consists in biweekly infusions of recombinant lysosomal glycosidase to empty the lysosomal overload. Its main limitations are its ineffectiveness in treating neurological forms, its high cost and the associated immune responses. Pharmacological chaperone (PC) therapy, introduced in the early 2000s, consists in the use of protein ligands that stabilize the correct conformation of the misfolded protein by selective binding. In doing so, PCs escort the mutant protein to the lysosome where it may express its residual level of function.[3] This strategy historically took advantage of the available portfolio of high affinity competitive enzyme inhibitors. These active-site specific chaperones paradoxically allow an increase in hydrolase activity in patient cells whereas they were originally designed to inhibit their wild-type counterpart. To date, the only marketed PC is 1-deoxygalactonojirimycin (Galafold™, migalastat), which is a competitive inhibitor of α-galactosidase A used against Fabry disease.[4] However, the extent of proteins inhibition by active-site specific PCs is not directly correlated to their level of chaperoning activity. It therefore remains clinically difficult to control the balance between these two opposite effects. Non-inhibitory PCs, typically binding to an allosteric site, present the advantage of potentially activating the misfolded protein without competing with the endogenous substrate.[5] Moreover, PCs could be utilized in a combined PC-ERT therapy to reduce patient hospitalization, lower costs, and minimize the side effects associated with ERT.[6]

In the search of potent glycosidase inhibitors, the concept of multivalency has been widely explored for a diversity of enzymes.[7] Over the past decade, many multivalent iminosugars have demonstrated remarkable multivalent effects toward certain glycosidases.[8,9] This highlights that the principle of multivalency can also be applied to enzymes with a single active site, broadening its potential for innovative therapeutic strategies. Among the reported multivalent glycosidase inhibitors, some addressed lysosomal enzymes.[9,10] In 2012, a breakthrough was brought by Compain and Coll. with the first report of multivalent iminosugars targeting glucocerebrosidase (GCase, E.C.3.2.1.45), the lysosomal enzyme implied in the Gaucher disease, the highest prevalent LSD.[11] An heptavalent

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deoxynojirimycin (DNJ) built on a cyclodextrin scaffold showed a high multivalent inhibitory effect (rp/n = 28, rp = relative potency and n = valency) towards GCase. For this glycosidase, several mechanisms may contribute to the multivalent effect,[9] including clustering or cross-linking effects—since previous studies have suggested that GCase exists as a dimeric or tetrameric structure[12]—as well as subsite-binding effects. Moreover, Compain and Coll. demonstrated for the first time that this multivalent inhibitor also improved the enzyme activity in N370S human fibroblasts (1.5-fold increase at 10 μM). Thus, multivalency was not only valuable for developing glycosidase inhibitors but was also relevant for accessing pharmacological chaperones. Several examples of multivalent PCs of GCase have latter on been reported by Compain *et al.*[13,14] and other research groups.[15–20] To our knowledge, multivalent PCs have been described for only two other lysosomal enzymes: alpha-galactosidase[19] and alpha-mannosidase,[21] which are involved in Fabry disease and alpha-mannosidosis, respectively. Recently, we described original poly(phosphorhydrazone) dendrimers bearing monofluorocyclooctyne units on their periphery.[15,16] Thanks to Strain Promoted Azide–Alkyne Click (SPAAC) reaction, we synthesized multivalent DNJs[15] and multivalent hydroxypyrrolidines.[16] Most of these constructs were found to act as potent GCase inhibitors and were able to increase the GCase activity of fibroblasts from Gaucher patients with N370S mutation. During this work, we noticed with interest that the synthesized multivalent DNJs were better inhibitors of acid alpha-glucosidase (GAA, EC 3.2.1.20) than of GCase. In particular, multivalent compounds **1** and **2** displayed sub-micromolar inhibition of GAA (Fig. 1).[15] This finding caught our attention since GAA is the enzyme implied in Pompe disease (PD).

PD, also known as glycogen storage disease type II, is a LSD characterized by mutations in the gene encoding GAA. It results in an abnormal glycogen accumulation in the lysosomes of the heart, muscles, and other cells.[22] PD displays a broad phenotypic spectrum ranging from severe infantile-onset to milder later-onset forms. Most PD patients suffer however from progressive muscle hypotonia and respiratory failure. To date, ERT is the first and only treatment approved (Alglucosidase marketed as Lumizyme in U.S.A. and Myozyme elsewhere, as well as Avalglucosidase Alfa, Cipaglucosidase Alfa) and is administered regularly in one- to two-week intervals for life to prevent the accumulation of glycogen. Several active site-specific PCs of GAA have been described,[23] most often belonging to sugar[24–26] or iminosugar families, such as DNJ[27–29] or *N*-Butyl DNJ (NB-DNJ).[30] In 2023, a combination therapy (Pombiliti), consisting in Cipaglucosidase Alfa plus the NB-DNJ PC, was approved by FDA to treat certain adults with late-onset Pompe disease. In this case, PC allowed the stabilization of the recombinant enzyme at neutral pH in circulating blood which led to an enhancement of the GAA activity in the patients lysosomes.[31] Non-inhibitory PCs have also been reported,[23] such as *N*-acetylcysteine and L-NB-DNJ.[32,33] Allosteric binding sites of NAC have been identified by a co-crystal structure,[34] whereas mechanism of action of L-NB-DNJ has not yet been identified.[33] Regarding multivalent compounds, only Compain and C. have evaluated certain of their DNJ-cyclodextrin conjugates for GAA inhibition and observed a weak multivalent effect.[11,15] To date, no multivalent PC of GAA has been identified.

Prompted by the preliminary results obtained with **1** and **2**, we conducted an in-depth study of the effect of multivalent DNJs on GAA inhibition, enzyme thermal stabilization, and restoration of cellular enzyme activity. For this purpose, we relied on two multivalent scaffolds: pentaerythritol-based dendrimers and a new family of phosphorus-containing dendrimers in which the clickable monofluorocyclooctyne unit is replaced by an azadibenzocyclooctyne (ADIBO), known to have a greater reactivity toward SPAAC chemistry (compounds **3**-G0 and **4**-G1, Fig. 2).[35] L or D *N*-substituted DNJs were grafted on the surface of these dendrimers, either through an aliphatic or a pegylated spacer, in order to study the influence of hydrophobicity and chirality.

Results and discussion

Chemical synthesis

Preparation of clickable phosphorus dendrimers. First, the phenol-ADIBO derivative **5** was readily accessed from commercially available ADIBO amine and 4-hydroxy benzenepropanoic acid according to an EDC-promoted coupling reaction.[36,37] The clickable dendrimer of generation 0 (G0) **3**-G0 was prepared by nucleophilic substitution of chlorine atoms of hexachlorocyclotriphophazene (**6**) under basic conditions (Scheme 1) and unreacted phenol-ADIBO compound was easily removed by washings in diethyl ether. Similarly, the clickable dendrimer of generation 1 (G1) **4**-G_1 was obtained from the generation 1 polythiophosphoramidate (PTPAm) dendrimer **7**.[36,37] The grafting of derivative **5** onto the dendrimer's surface was easily monitored by means of ^{31}P NMR (see SI) since the phosphorus atoms of the phosphazene ring resonate as a broad signal centered in the 7.7–7.8 ppm region, whereas the thiophosphoramidate moiety resonates as a typical singlet centered on 68.5 ppm.

Click reactions with ω-azido-DNJs. To determine the multivalent effect, analysed in terms of the relative inhibition potency (rp/n), the monovalent reference compounds **9a–c** and **10a–b** were prepared (Scheme 2). D-DNJ derivatives **8a** and **8c**, with an undecanyl or a PEG spacer respectively, were prepared following already reported strategy (see SI).[38] The synthesis of L-DNJ according to the procedure described by D'Alonzo *et al.* remained unsuccessful in our hand despite several attemps.[33] However, a more recent synthetic route allowed the successful synthesis of L-DNJ coupled with the ω-azido undecanyl spacer.[39] CuAAC reactions between pentyne and azido derivatives **8a–c** were performed under microwaves irradiation in the presence of $CuSO_4$ and sodium ascorbate. Copper salts were removed by repeated washing with a saturated EDTA aqueous solution. Ammonia deprotection gave monovalent compounds

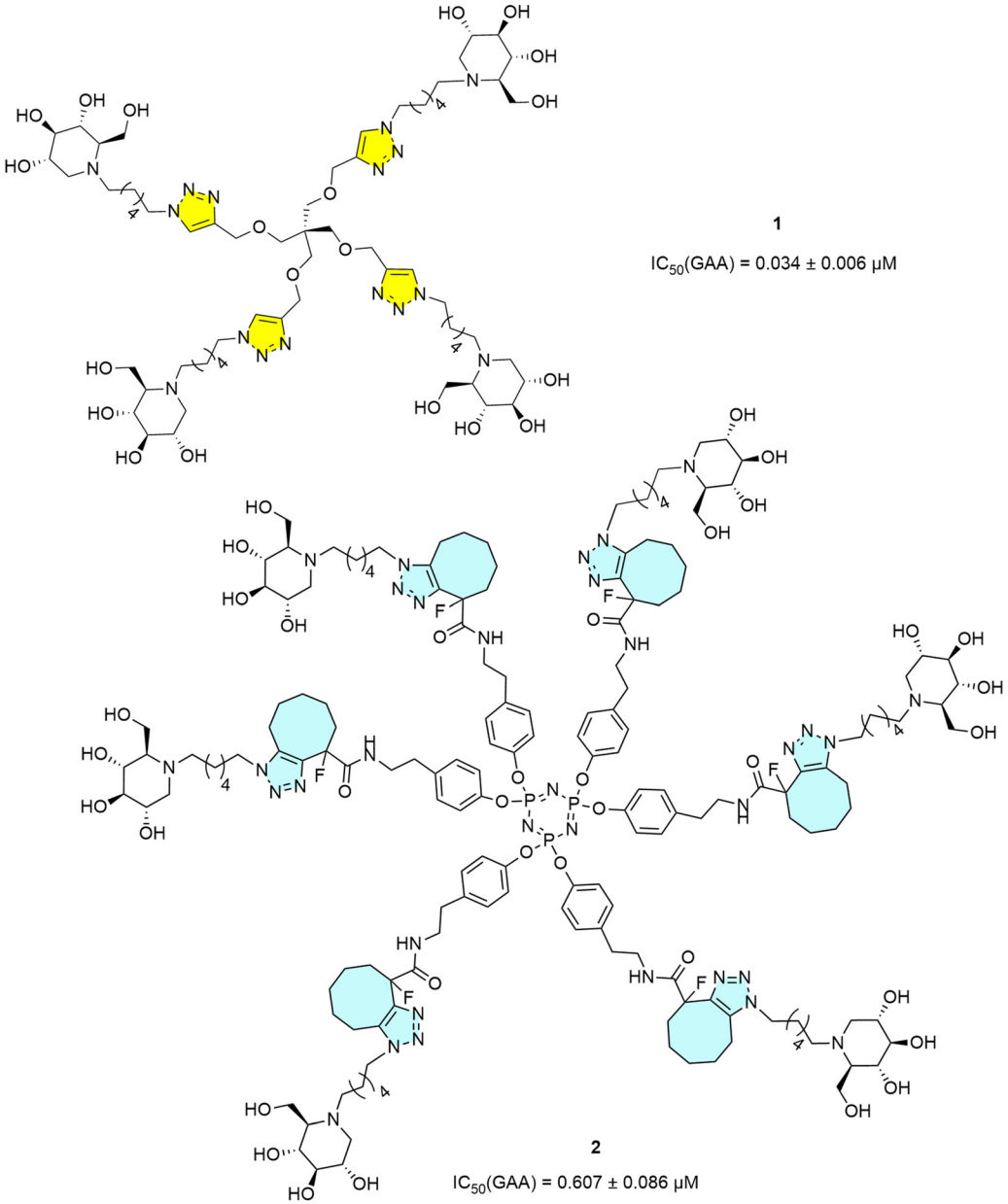


Fig. 1 D-DNJ based iminosugars reported by us in previous work.[15]

9a–c with moderate to low yields over two steps due to the formation of oxidation by-products, as previously described.[40]

Stirring of DNJ derivatives **8a** and **8b** with commercial azadibenzocyclooctyne amine in a methanol/THF mixture at 45 °C followed by ammonia deprotection give SPAAC products **10a** and **10b** as an inseparable 2 : 3 mixture of regioisomeric cycloadducts with 98% and 53% yield, respectively. The same sequence was applied to obtain multivalent compounds by CuAAC and SPAAC reactions. Thus, the three clickable DNJ derivatives **8a–c** were grafted under microwaves on tetravalent and hexavalent pentaerythritol-based dendrimers **11** and **13** prepared following reported procedures (Scheme 3).[41,42] Deprotection with aqueous ammonia led to desired compounds **12a–c** and **14a–c** with high yields. DNJ derivatives **8a** and **8b** were grafted on hexavalent and dodecavalent phosphorus dendrimers **3**-G0 and **4**-G1 by means of a simple stirring in methanol/THF. After purification, deprotection led to the desired compounds **15a–b** and **16a–b**, isolated as a mixture of regioisomers with good yields over the two steps (Scheme 4).

Enzymatic assays

Inhibition of *rh*GAA. The synthesized mono- and multivalent compounds were evaluated for their inhibition of commercially available recombinant human GAA (*rh*GAA) (Table 1). 4-Methylumbelliferyl-α-D-glucopyranoside was used as a fluorogenic GAA substrate to release fluorescent 4-methyl-

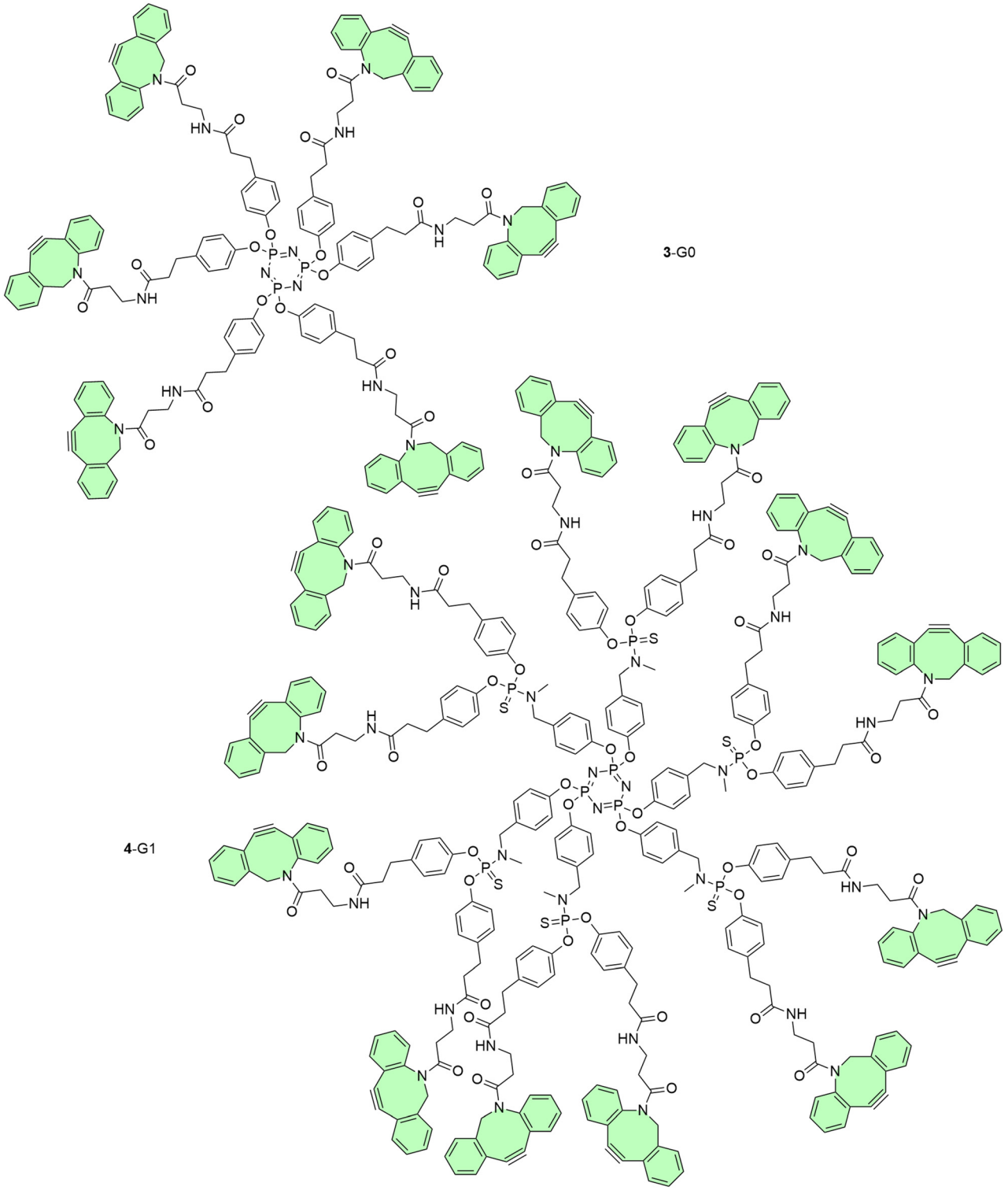
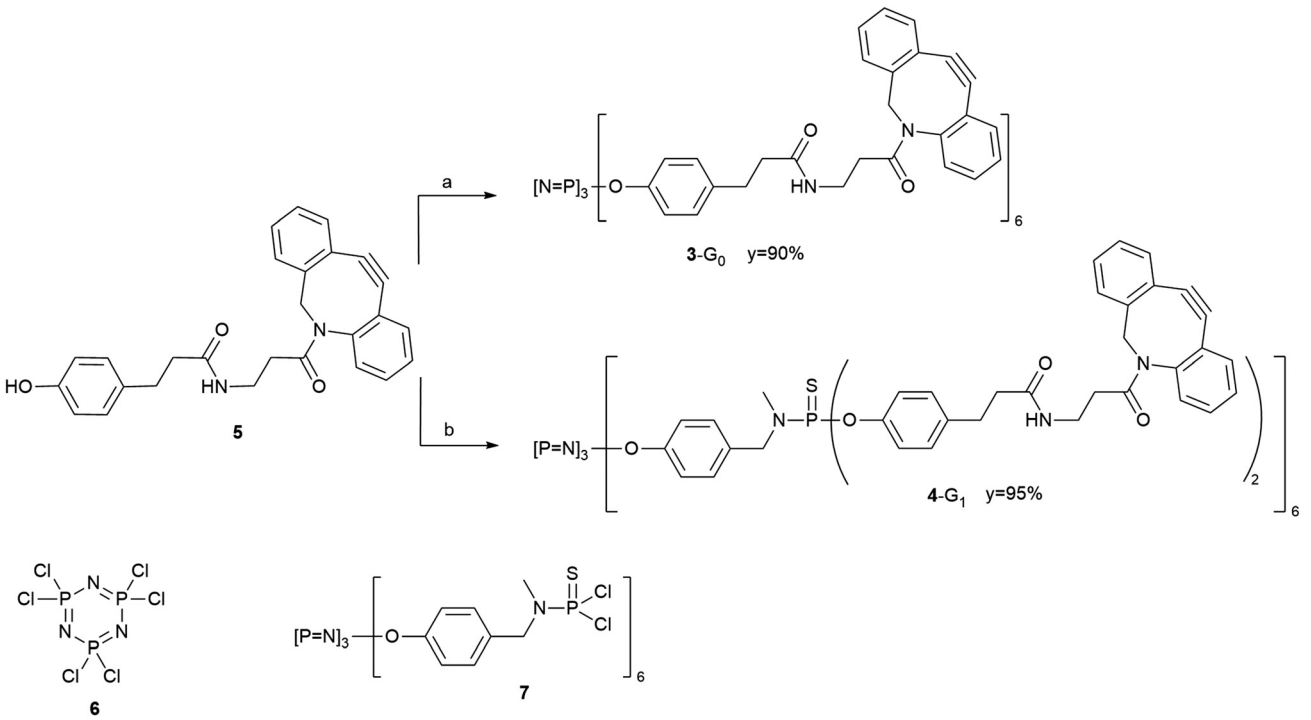


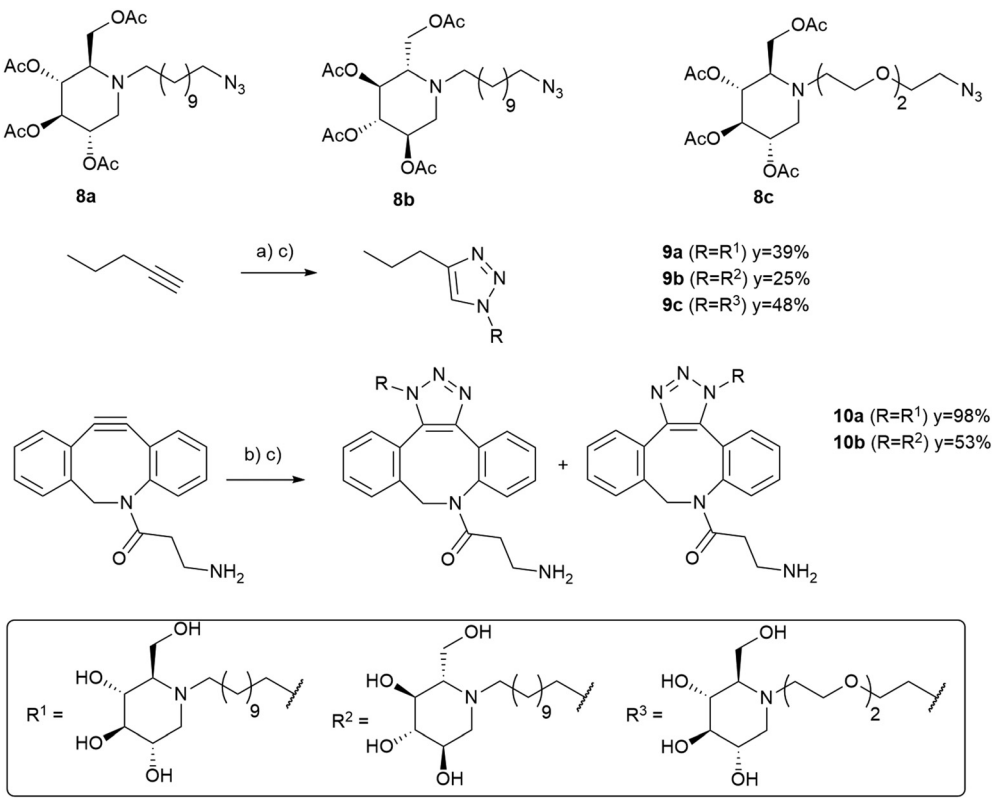
Fig. 2 Phosphorus dendrimers for SPAAC cycloaddition described in this work.

umbelliferone (λ_{ex} = 360 nm and λ_{em} = 455 nm) upon enzymatic hydrolysis.

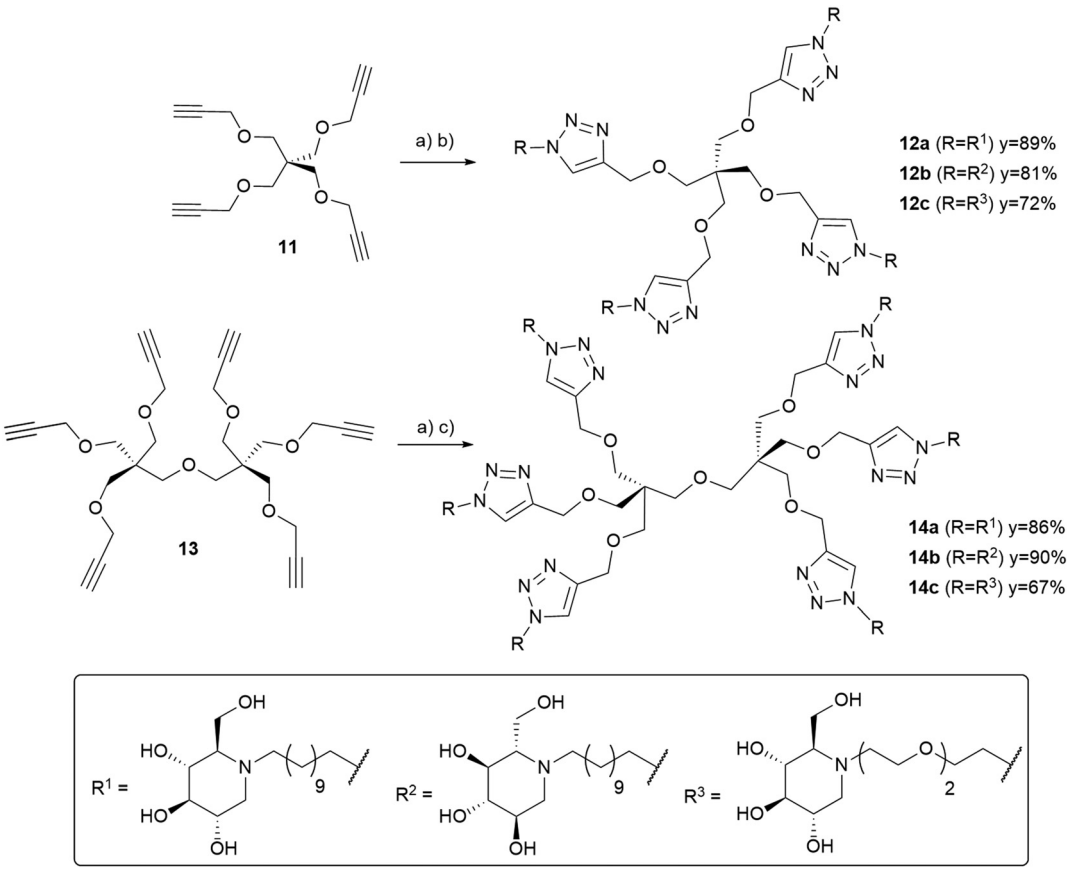
As expected, the dendrimers decorated with L-*N*-undecanyl-DNJ did not, or only weakly, inhibit GAA. Conversely, all the multivalent compounds bearing D-*N*-undecanyl-DNJ (compounds **9a**, **10a**, **12a**, **14a**, **15a** and **16a**) were GAA inhibitors with IC_{50} values between 0.0105 μM and 0.401 μM. This confirms that GAA inhibition is sensitive to chirality, as observed earlier.[33] However, multivalent D-DNJs with a PEG spacer (compounds **9c**, **12c** and **14c**) were poor inhibitors of GAA,



Scheme 1 Synthesis of clickable dendrimers **3**-G0 and **4**-G1 resulting from the grafting of **5** on hexachlorocyclotriphosphazene (**6**) and generation 1 PTPam dendrimer **7**. (a) **6**, Cs_2CO_3, overnight, THF (b) **7**, Cs_2CO_3, overnight, THF.



Scheme 2 Syntheses of monovalent compounds. (a) Azido derivative (**8a**, **8b** or **8c**), $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF/H_2O, MW, 80 °C, 30 min. (b) Azido derivative (**8a** or **8b**), MeOH/THF, 45 °C, 3 h. (c) NH_4OH, 2M, CH_3OH.



Scheme 3 (a) Azido derivative **8a**, **8b** or **8c**, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF/H_2O, MW, 80 °C, 30 min. (b) NH_4OH, 2M, CH_3OH.

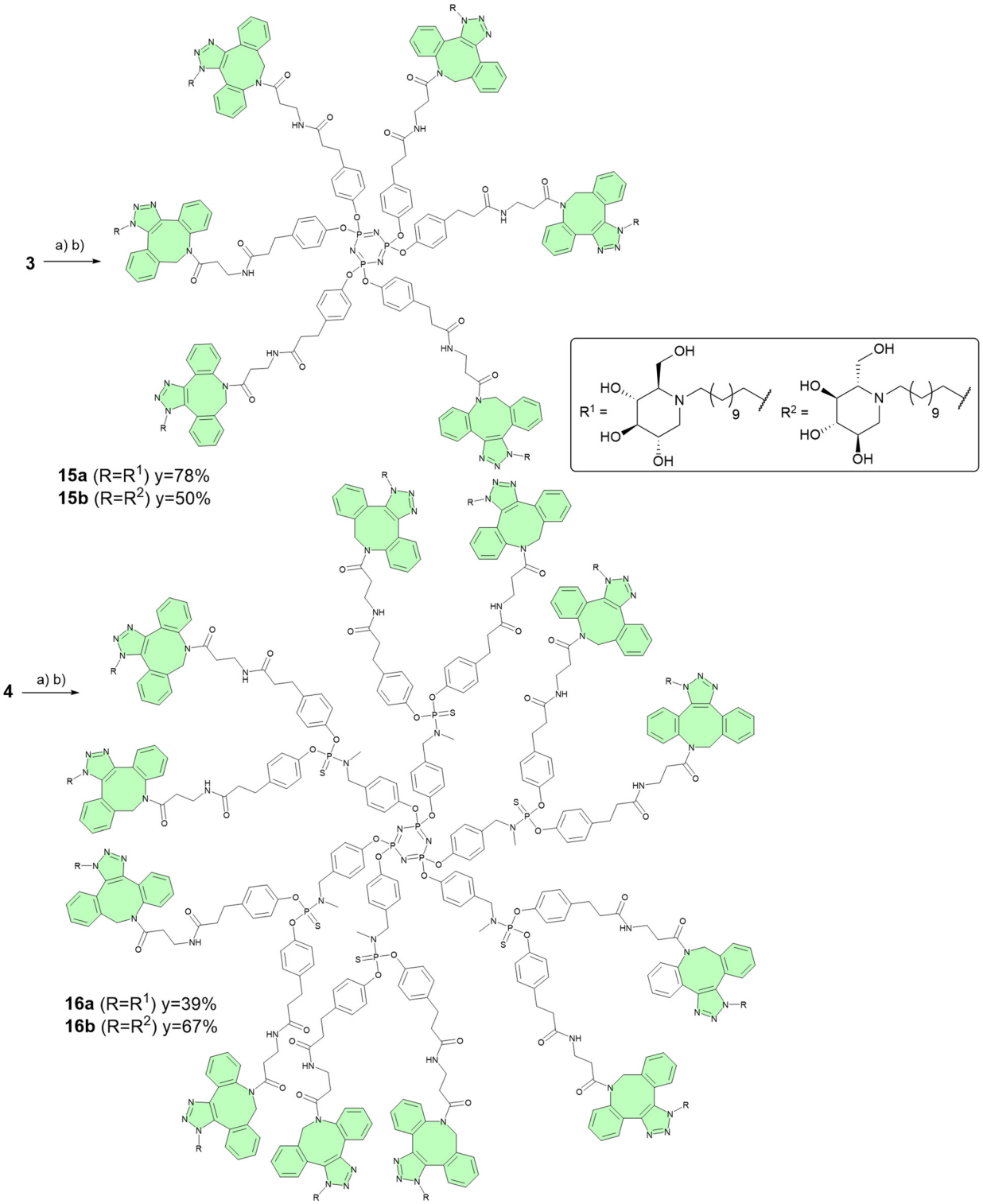
suggesting that a hydrophilic chain is detrimental to the inhibition of GAA. Regarding the dendrimers **12a** and **14a** built on a pentaerythritol core, the elongation of the alkyl chain tended to have a slightly favourable effect on inhibition when compared to our previous results.[15] Furthermore, moderate multivalent were observed for these compounds effects (1.8 and 5.7 rp/n values for **12a** and **14a**, respectively). The phosphorus-containing dendrimers **15a** and **16a** presenting D-*N*-undecanyl-DNJ were also inhibitors with IC_{50} values of the same order of magnitude as those previously described with monofluorocyclooctatriazole units (compounds **1** and **2**, Fig. 1).[15] However, no multivalent effect was observed with the phosphorus dendrimers and, more generally, it seems that the inhibition is less significant for dendrimers of this family.

Thermal stabilization of *rh*GAA. Differential Scanning Fluorimetry (DSF) was used to monitor the thermal denaturation of *rh*GAA in the absence or presence of PC candidates in McIlvaine buffer (pH = 7.4). To mimic the conditions under which a PC stabilizes GAA *in vivo*, a neutral pH was selected to reproduce non-lysosomal conditions. The melting temperature (T_m) is the inflection point of the sigmoidal melting curve and enhancement of T_m indicates an increase of global stability. In the absence of any PC, a T_m of 63.5 ± 0.3 °C was determined for *rh*GAA, in accordance with previously reported values.[43] D-NN-DNJ, selected as reference PC, induced a stabilization of 12.0 °C at 25 μM in our experimental conditions (Table 1).

At 25 μM, all the tested D-DNJ multivalent derivatives (except **16a** and **14c**) increased the global stability of the *rh*GAA with a ΔT_m between 10.8 and 17.4 °C. For L-DNJ derivatives, only **15b** induced an enhancement in T_m of 12.3 °C at 25 μM. However, when tested at 250 μM, the monovalent reference **10b** produced a significant shift of 8.1 °C while **12b**, **14b** and **16b** still did not induce any thermal stabilizing effect (see SI). These results indicate that no clear relationship exists between the thermal stabilizing effect and the inhibitory potency of *rh*GAA as observed by others before.[29] Compounds **12a**, **14a**, **15a** and **15b**, which induced the best stabilising effect at 25 μM, were selected to be assessed as PC *in cellulo*. In the search of non-inhibitory PC, compound **15b**, which only weakly inhibit GAA at up to 1 mM, was particularly promising.

Cell assays

The effect of the four compounds **12a**, **14a**, **15a** and **15b** was evaluated on mutant GAA activity in cultured fibroblasts derived from two adult PD patients (Table 2). The mutation c.-32-13T > G carried by these patients, frequently reported in cases of late-onset PD, occurs in 40% to 90% of the cases, with a predominance among Caucasian populations.[44] Residual activity (RA) was assessed in these cell lines and amounts to



Scheme 4 (a) Azido derivative (**8a** or **8b**), MeOH/THF, 45 °C, 3 h. (b) NH_4OH, 2M, CH_3OH (only one regioisomer is represented on dendrimers for clarity purposes).

4.9% for patient 1 and 9.0% for patient 2 (Table 2). Miglitol at 1 μM was used as a positive control.[26,34] Cells were incubated in the presence of 0.5 nM to 50 nM of tested compound for 48 h with six replicates and the GAA activity was compared to that obtained in untreated cells to determine the fold increase (Fig. 3). The choice of these very low concentrations was based on preliminary tests (not shown) at 400 nM where only decrease in enzyme activity was observed with D-undecanyl-

Table 1 IC_{50} values (μM) and thermodynamic stability of *rh*GAA at pH 7.4 in presence of compounds measured by DSF

	Cpd	Valency	IC_{50} (μM)	[cpd] = 25 μM	
				T_m (°C)	ΔT_m [a] (°C)
Derivatives of D-DNJ	Miglitol		1.137 ± 0.226	ND	0.4[b] (ref. 26)
	NN-DNJ		0.4215 (ref. 47)	75.5	12.0
	9a	1	0.240 ± 0.037	ND	ND
	12a	4	0.0105 ± 0.0006 (rp/n = 5.7)	80.9	**17.4**
	14a	6	0.0229 ± 0.0014 (rp/n = 1.8)	79.7	**16.2**
	10a	1	0.0329 ± 0.0046	74.3	10.8
	15a	6	0.0379 ± 0.0048	76.4	**12.9**
	16a	12	0.401 ± 0.026	63.8	0.3
	9c	1	0.469 ± 0.028	ND	ND
	12c	4	0.168 ± 0.011	75.2	11.7
	14c	6	0.733 ± 0.059	63.5	—
Derivatives of L-DNJ	**9b**	1	18.0% at 1 mM	ND	ND
	12b	4	95.2 ± 25.0	62.6	—
	14b	6	25.6 ± 3.4	63.5	—
	10b	1	25.8 ± 3.1	63.5	—
	15b	6	17.2% at 1 mM	75.8	**12.3**
	16b	12	NI	63.5	—

ND: not determined. [a] ΔT_m is the difference between the values of T_m measured in the presence of compound and T_m of *rh*GAA alone (T_m (*rh*GAA) = 63.5 ± 0.3 °C at pH 7.4). [b] Assessed at 30 μM. The four-best stabilizing ΔT_m are highlighted.

Table 2 Genotype of PD patients

Patient ID	Mutation allele 1	Mutation allele 2	% GAA RA[a]
Patient 1	c.-32-13T > G	c.2237G > A	4.9%
Patient 2	c.-32-13T > G	c.1076-1G > C and c.2065G > A	9.0%

[a] RA: residual activity. The residual activity percentage corresponds to the ratio between the GAA activity measured in patient fibroblasts and that measured in healthy control fibroblasts.

DNJ dendrimers. In patient 1's cells, all the compounds induced a decrease in GAA activity, with the most significant decrease observed for compound **14a** at 50 nM. Only miglitol led to a 1.2-fold increase in GAA activity. For **14a** and **15b**, the decrease in GAA activity revealed to be dose-dependent. This observation is surprising for **15b**, since it is was shown to be a very weak inhibitor of the enzyme. This might be explained by the potential lysosomal accumulation of these multivalent lipophilic amines due to the density of positive charges at their surface once in the acidic lysosomal environment.[45] In patient 2's cells, similar trends were observed, with a dose effect for **14a** and **15b**. Remarkably, at the very low concen-

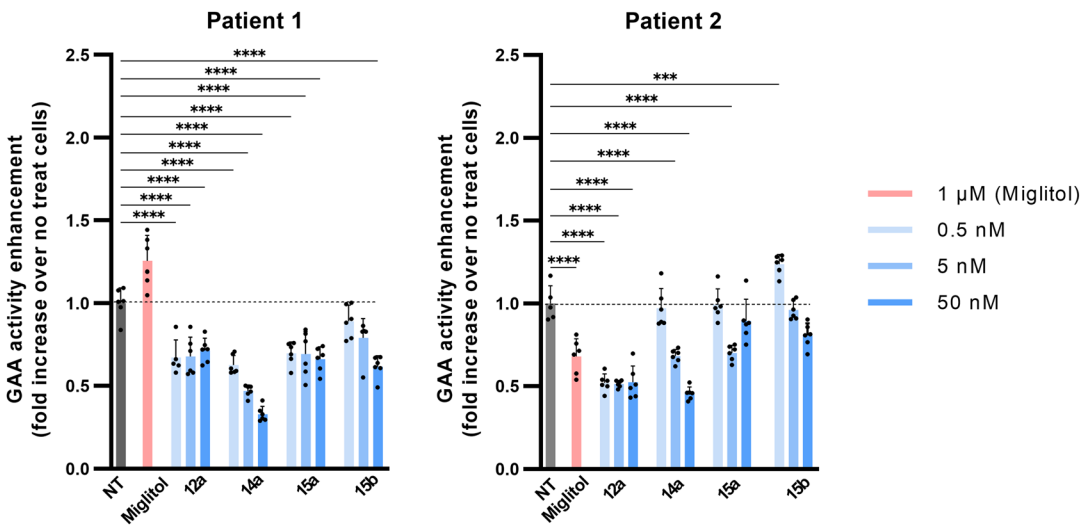


Fig. 3 Effect of compounds **12a**, **14a**, **15a** and **16b** on GAA activity enhancement reported as the ratio between the GAA activity in presence of compound and the GAA activity without compound. Each value represents the mean ± SEM (N = 6). NT: no treatment. Statistical significance was determined using Student's t-test: *** $p \leq 0.001$ and **** $p \leq 0.0001$.

tration of 0.5 nM, **15b** induces this time an enhancement in enzymatic activity by a factor of 1.2. Even though this effect is weak, it is however statistically significant and might be therapeutically relevant in cells with sufficient residual enzyme activity such as that of patient 2.[46] Thus, compound **15b** induces a decrease in GAA activity in patient 1's cells but an increase in patient 2's cells, indicating an allele-dependent pharmacological chaperone effect. This dependence is also noted with miglitol. These observations highlight that the balance between inhibitory and chaperone activity of iminosugars is highly sensitive to the nature of the second mutant GAA allele. It is thus the first time that a multivalent compound is shown to allow the increase of GAA activity in Pompe patient cells. This result is particularly interesting since **15b** is a ligand of the GAA protein and a weak inhibitor of the enzyme (17.2% inhibition at 1 mM). However, these very encouraging results need to be confirmed in fibroblasts of patients carrying other mutations.

Conclusion

New polythiophosphoramidate dendrimers grafted with 6 or 12 copies of a clickable dibenzocyclooctyne motif have been prepared. A strain-promoted alkyne–azide cycloaddition (SPAAC) enabled the straightforward synthesis of multivalent phosphorus-containing dendrimers functionalized on the surface with D- or L-DNJ pharmacophores. Additionally, a copper-catalyzed click reaction allowed the preparation of tetra- and hexavalent L- or D-DNJ derivatives based on a pentaerythritol core. As expected, dendrimers decorated with L-*N*-undecanyl-DNJ showed little or no GAA inhibition, whereas those grafted with D-*N*-undecanyl-DNJ were inhibitors. Weak multivalent effects were observed with tetra- and hexavalent dendrimers **12a** and **14a**. Compounds **12a**, **14a**, **15a** and **15b** which exhibited the highest thermal stabilizing effect at 25 μM according to DSF were selected to be assessed as PCs in patient fibroblasts. The phosphorus-containing dendrimer **15b** presenting L-*N*-undecanyl-DNJ induced a small but significant enhancement in GAA activity. This effect is remarkable because it is the first time that a multivalent ligand is observed to induce an increase in GAA activity *in cellulo*. Crucially, this compound displays only very weak enzyme inhibitory properties. Current investigations are underway to fully elucidate the mechanism of action of this dendrimer. Future work will involve extending these studies to include fibroblasts derived from patients carrying diverse GAA mutations to comprehensively assess its broad applicability.

Experimental

Synthetic chemistry

General procedure for CuAAC under MW conditions. A solution of $CuSO_4{\cdot}5H_2O$ (0.3 eq. per alkyne) and L-ascorbic acid sodium salt (3 eq. per Cu^{II}) in water ($[Cu^{II}]$ = 0.05 M) were added to a solution of alkyne (1 eq.) and azide derivate (1.1 eq. per alkyne) in DMF ([Alkyne] = 0.01 M). The mixture was stirred and heated under microwave irradiation at 80 °C and 23 W. Then, the mixture was diluted in EtOAc, washed with saturated EDTA until the aqueous layer is colorless. The organic layer is dried over Na_2SO_4 and concentrated to give the desired product.

General procedure for deacetylation. A solution of NH_4OH (2M in CH_3OH, 130 eq.) was added to a solution of peracetylated multivalent cluster (1 eq.) in CH_3OH (55.5 ml $mmol^{-1}$). The resulting mixture was stirred under nitrogen at r.t. overnight. The resulting deacetylated cluster was obtained by co-evaporation with toluene and CH_3OH.

General procedure for SPAAC reaction. THF, CH_3OH and water were first degassed under argon. Then the monovalent cyclooctyne (1.3 eq.) or dendrimer derivative (0.7 eq.) and azide (1 eq.) were dissolved in a THF/CH_3OH (1 : 1, [Alkyne] = 0.01 M) before adding water (1/3 of the final volume). The mixture was stirred at 40 °C at 1200 rpm.

Inhibition assays on *rh*GAA

Recombinant human alpha-Glucosidase acid/GAA (8329-GH), purchased from R&D was used in the inhibition studies. The used substrate 4-methylumbelliferyl-α-D-glucopyranoside[47] was purchased from Sigma-Aldrich. Briefly, enzyme solutions (10 μL from a stock solution containing 2.2 μg mL^{-1}) in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (100 mM sodium citrate and 200 mM sodium phosphate buffer, pH 5.2) were incubated at 37 °C without (control) or with inhibitor at a final volume of 50 μL for 30 min. After addition of 25 μL of 4-methylumbelliferyl-α-D-glucopyranoside (4.7 mM, McIlvaine buffer pH 4.6), the samples were incubated at 37 °C for 15 min.

Enzymatic reactions were stopped by the addition of aliquots (100 μL) of glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined with a FLUOstar microplate reader (BMG Labtech) at 355 nm (excitation) and 460 nm (emission).

Assay of GAA activity on human fibroblasts

Primary cultured skin fibroblasts isolated from patients with Pompe disease were obtained from the Clinical Chemistry Department of Toulouse University Hospital and supplied by Prof. T. Levade (INSERM UMR1037, Cancer Research Center of Toulouse). All experiments were conducted in accordance with the French rules and institute policies on ethics and human material usage and were approved by the CODECOH Ethics Committee of the Softmat laboratory (approval number DC-2020-4322), recognized by the "Ministère de l'enseignement supérieur, de la recherche et de l'innovation". Written informed consent was obtained from all participants for molecular genetic analyses and cell culture studies. The human fibroblasts were immortalized after transfection with a plasmid encoding the SV40 large T antigen. Cells were routinely cultured in DMEM medium supplemented with 10% inactivated fetal calf serum and 1% antibiotics. Protocol to assess

cell GAA activity in 48-well plates was adapted from https://doi.org/10.1016/j.bmc.2022.117129. 30 000 fibroblasts were seeded in 48-well plates and incubated for 48 h with compounds to be tested. Then, cells were washed twice with cold PBS. Cell lysates were obtained directly in the well by adding 50 µl of 0.1% Triton X100, and stored for 48 h at −80 °C before protein quantification by BCA Assay (Pierce). GAA enzyme activity was determined on cell lysates in sodium acetate buffer pH 5.6 using 4-methylumbelliferyl-α-D-glucopyranoside (Sigma-Aldrich, St Louis, MO, USA) as substrate.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

The data that support the findings of this study are available in the published article and its supplementary information (SI). Supplementary information: synthesis and characterization, NMR spectra for new compounds, mass spectra for **15a** and **16a**, IC_{50} and DSF curves. See DOI: https://doi.org/10.1039/d6ob00021e.

Acknowledgements

We thank Pr. T. Levade (Institut National de la Santé et de la Recherche Médicale (INSERM) UMR1037, Centre de Recherches en Cancérologie de Toulouse (CRCT)) for his help and his valuable guidance throughout this work. We also acknowledge the French Ministry of Higher Education and Research for funding Dr Guichot's PhD fellowship.

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