

## Targeted delivery of pharmacological chaperones for Gaucher disease to macrophages by a mannosylated cyclodextrin carrier†

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Gaucher disease (GD) is a rare monogenetic disorder leading to dysfunction of acid  $\beta$ -glucosidase ( $\beta$ -glucocerebrosidase; GCase) and accumulation of glucosylceramide in lysosomes, especially in macrophages (Gaucher cells). Many of the mutations at the origin of GD do not impair the catalytic activity of GCase, but cause misfolding and subsequent degradation by the quality control system at the endoplasmic reticulum. Pharmacological chaperones (PCs) capable of restoring the correct folding and trafficking of the endogenous mutant enzyme represent promising alternatives to the currently available enzyme replacement and substrate reduction therapies (ERT and SRT, respectively), but unfavorable biodistribution and potential side-effects remain important issues. We have now designed a strategy to enhance the controlled delivery of PCs to macrophages that exploit the formation of ternary complexes between the PC, a trivalent mannosylated  $\beta$ -cyclodextrin ( $\beta$ CD) conjugate and the macrophage mannose receptor (MMR). First, PC candidates with appropriate relative avidities towards the  $\beta$ CD cavity and the GCase active site were selected to ensure efficient transfer of the PC cargo from the host to the GCase active site. Control experiments confirmed that the  $\beta$ CD carrier was selectively recognized by mannose-specific lectins and that the corresponding PC:mannosylated  $\beta$ CD supramolecular complex retained both the chaperoning activity, as confirmed in human GD fibroblasts, and the MMR binding ability. Finally, fluorescence microscopy techniques proved targeting and cellular uptake of the PC-loaded system in macrophages. Altogether, the results support that combined cyclodextrin encapsulation and glycotargeting may improve the efficacy of PCs for GD.

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## Introduction

Lysosomal storage disorders (LSDs) are a heterogeneous group of inherited diseases caused by genetic defects affecting lysosomal catabolic enzymes.<sup>1</sup> In many cases, the mutation at the origin of the disease gives rise to a protein that cannot fold properly and is subjected to endoplasmic reticulum associated degradation (ERAD) by the quality control system of the cell. Ironically, the mutant protein is often functional, but it cannot undergo trafficking to the Golgi apparatus for maturation and then to the lysosome, which results in the abnormal accumulation of cellular debris, mostly glycosphingolipid metabolites, in different cell types and tissues.<sup>2</sup> By mechanisms that are not fully understood, such accumulation leads to a range of potentially life-threatening pathologies. Incidence of each LSD individually is rare, though altogether LSDs affect 1 in 5000 to 10 000 live births in Western countries. Gaucher disease (GD), the most prevalent LSD (about 14% of the total), is associated

with the dysfunction of  $\beta$ -glucocerebrosidase (GCase, EC no. 3.2.1.45), responsible for glucosylceramide (GlcCer) hydrolysis.<sup>3</sup> Over 300 mutations have been characterized in the gene encoding for GCase, which translates into a large array of disease manifestations, from visceromegaly in attenuated forms of type 1 GD to neurological affections in acute and subacute neuronopathic type 2 and 3 GD, and varied disease progression rates.<sup>4</sup>

The therapeutic goal towards GD, and LSDs in general, is restoring the balance between substrate influx and degradation.<sup>5</sup> Enzyme replacement therapy (ERT), in which patients are regularly supplemented with an exogenous recombinant GCase, is the main clinical treatment for GD nowadays.<sup>6</sup> Alternatively, other therapeutic approaches based on more “drugable” candidates have been investigated.<sup>7</sup> Substrate reduction therapy (SRT), based on glycosphingolipid biosynthesis inhibitors, has proven useful to reduce GlcCer influx.<sup>8</sup> Both ERT and SRT treatments address substrate accumulation but not the protein folding defects and their potential contributions to the pathophysiology of the disease.<sup>9</sup> More recently, specific ligands of the deficient enzyme capable of promoting correct folding at the ER have been shown to restore normal trafficking and, ultimately, lysosomal activity.<sup>10</sup> This so-called pharmacological chaperone therapy (PCT) constitutes a promising therapeutic paradigm for GD and many other protein folding disorders.<sup>11</sup> Somewhat counter-intuitively, competitive inhibitors of the affected enzyme, used at sub-inhibitory concentrations, can act as mutant enzyme activators through this rescuing mechanism.<sup>12</sup> In any case, the future development of PCT requires engineering small molecular entities featuring (i) highly selective affinity towards the mutant enzyme, not to disrupt parallel cellular metabolic machineries, (ii) favourable chaperoning *versus* inhibitory capabilities, (iii) membrane-diffusiveness to access cells and cellular compartments, and (iv) recognition abilities to selectively target storage affected tissues.

Recent studies have shown that bicyclic glucose mimics with amphiphilic character and GCase inhibitory properties behave as active site-directed pharmacological chaperones (PCs) for GD.<sup>13</sup> Among them, amphiphilic bicyclic nojirimycin analogues belonging to the so-called  $sp^2$ -iminosugar glycomimetic family<sup>14</sup> have proven to be able of rescuing the misfolded protein from ERAD and restoring trafficking to the lysosome,<sup>15</sup> where the catalytic activity is expressed. The efficacy of PCT for GD may benefit from strategies enhancing delivery of the chaperone to the cells that are primarily affected by glucosylceramide accumulation, namely macrophages (Gaucher cells). Site-specific delivery of recombinant glycosidases by cell membrane receptor-targeted carriers has already proven a promising strategy for improving ERT in the context of several LSDs,<sup>16</sup> but the suitability of such an approach for pharmacological chaperone delivery to macrophages remains unexplored. First, the binding affinity of the chaperone towards the carrier must be finely tuned to warrant efficient transfer to GCase. Second, the carrier must be equipped with a ligand allowing specific recognition by macrophages. Third, the PC-loaded macrophage-

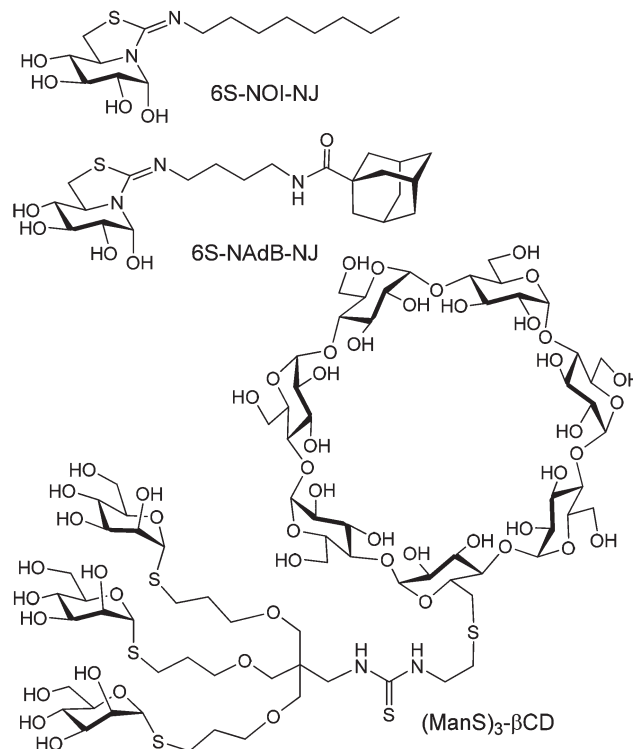


Fig. 1 Chemical structure of the  $sp^2$ -iminosugar pharmacological chaperones 6S-NOI-NJ and 6S-AdB-NJ and of the trimannosylated  $\beta$ CD carrier (ManS)<sub>3</sub>- $\beta$ CD.

targeted carrier must promote cell internalization before premature PC release.

Interestingly, native  $\beta$ -cyclodextrin (cyclomaltoheptaose;  $\beta$ CD) has been previously shown to facilitate transfer of amphiphilic  $sp^2$ -iminosugars to recombinant GCase in co-crystallization experiments.<sup>17</sup> The basket-shaped structure of CDs features a hydrophobic cavity that can accommodate guest molecules of appropriate size and improve their water solubility and bioavailability,<sup>18</sup> which has been broadly exploited in pharmaceutical technology.<sup>19</sup> The possibility to incorporate mannosyl moieties that are specifically recognized by the macrophage mannose receptor (MMR), a C-type lectin expressed at the membrane of macrophages and dendritic cells,<sup>20</sup> through selective chemical functionalization methods<sup>21</sup> further offers a good opportunity for optimization of macrophage-targeted PC:βCD complexes for enhanced PCT against GD. Herein, we have characterized the host-guest interactions of *N*'-octyl- and *N*'-[4-(adamant-1-ylcarboxamido)butyl]-iminomethylidene-6-thionojirimycin (6S-NOI-NJ and 6S-NAdB-NJ),<sup>22</sup> bearing octyl and adamantyl moieties, respectively, with the trivalent thiomannopyranosyl-tagged βCD derivative (ManS)<sub>3</sub>-βCD (Fig. 1). We have further assessed how formation of the (ManS)<sub>3</sub>-βCD:PC complexes influences both the interaction with glycosidases and the recognition by mannose-specific lectins. Our results demonstrate that (i) the chaperoning capability of the  $sp^2$ -iminosugars 6S-NOI-NJ and 6S-NAdB-NJ is preserved upon complexation with (ManS)<sub>3</sub>-βCD

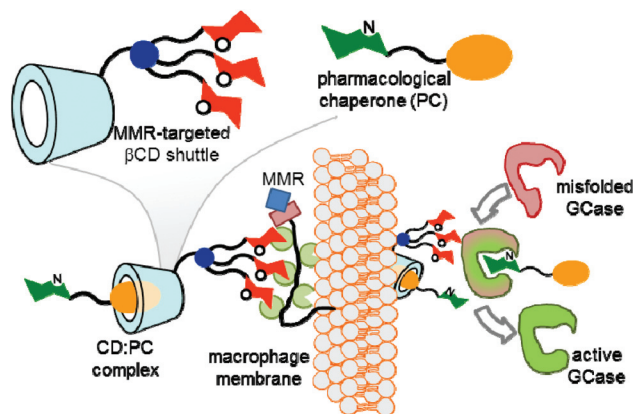


Fig. 2 Schematic representation of the strategy devised for macrophage-specific delivery of GD pharmacological chaperones by MMR-mediated internalization of their inclusion complexes with the (ManS)<sub>3</sub>-βCD carrier and further transfer to GCase.

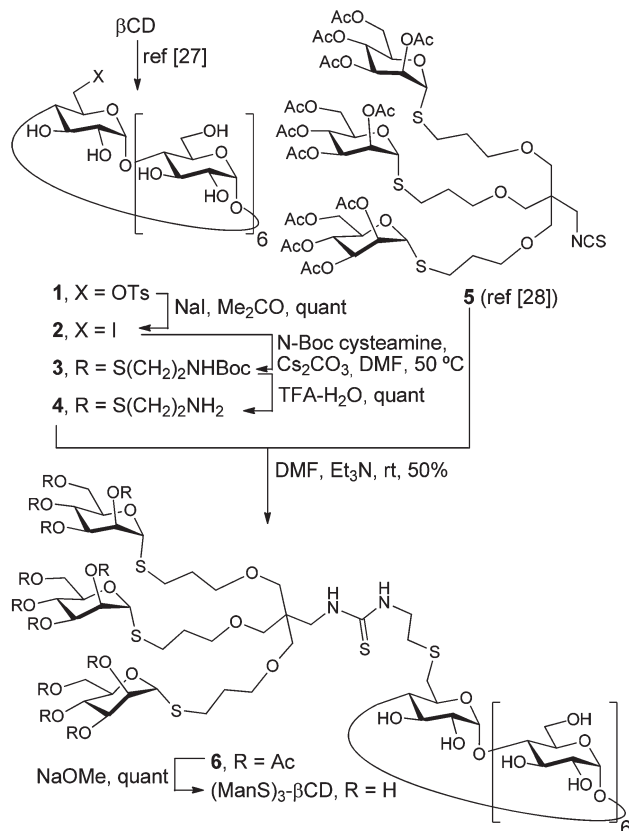
as determined in fibroblasts from GD patients and (ii) the PC-loaded βCD conjugates are internalized in macrophages, following the formation of PC:(ManS)<sub>3</sub>-βCD:MMR ternary complexes, to finally deliver the PC inside the cells (Fig. 2).

## Results and discussion

### Selection criteria for the pharmacological chaperone and cyclodextrin carrier partners

Bicyclic 5*N*,6*S*-(*N'*-alkyliminomethylidene)-6-thionojirimycin derivatives have shown a strong and selective inhibitory activity against several β-glucosidases and excellent properties as pharmacological chaperones against several GD-associated GCase mutants.<sup>15a</sup> We have chosen the *N'*-octyl and the *N'*-[4-(adamant-1-ylcarboxamido)butyl] representatives 6*S*-NOI-NJ<sup>22a</sup> and 6*S*-NADB-NJ<sup>22b</sup> in this study because the octyl and adamantyl moieties are known to exhibit a high avidity for the cavity of β-cyclodextrin in aqueous media, with association constant values (*K*<sub>as</sub>) in the range 10<sup>2</sup>–10<sup>4</sup> M<sup>−1</sup>.<sup>23</sup> Inclusion complex formation with a βCD-based carrier is then expected to ensure efficient transfer of the PC from the CD cavity to the active site of GCase. In the design of (ManS)<sub>3</sub>-βCD as the carrier partner for site-specific delivery of the selected PCs to macrophages, we kept in mind that multivalency is generally a prerequisite to elicit a biologically useful affinity in carbohydrate–protein recognition processes<sup>24</sup> and that monosubstitution of the βCD host by a mannosyl dendron is much less likely to alter the guest inclusion capabilities than polysubstitution strategies.<sup>25</sup>

The synthesis of (ManS)<sub>3</sub>-βCD has been accomplished using a modular convergent strategy that takes advantage of the high efficiency of the thiourea-forming reaction for macromolecule conjugation<sup>26</sup> (Scheme 1). First, commercial βCD was regioselectively tosylated at a single primary O-6 position by reaction with *p*-toluenesulfonyl chloride in aqueous basic medium



Scheme 1 Building block-based synthesis of the CD carrier (ManS)<sub>3</sub>-βCD.

in the presence of copper(II) (→1).<sup>27</sup> The incorporation of a cysteaminy segment was next undertaken to avoid steric constraints during the final conjugation step and to warrant accessibility of the glycoligand to molecular recognition events in the final conjugate. Nucleophilic displacement of the tosylate group in 1 by 2-(*tert*-butoxycarbonylamino)ethanethiol (*N*-Boc-cysteamine) required harsh conditions and proved troublesome, however. Alternatively, tosylate was exchanged into iodine by treatment with NaI and the resulting iodo derivative 2 was next smoothly reacted with *N*-Boc-cysteamine to furnish adduct 3. Acid-promoted carbamate removal quantitatively yielded the monocysteaminy-functionalized βCD 4. Coupling of 4 and the isothiocyanate-armed dendron 5<sup>28</sup> was conducted in DMF under Et<sub>3</sub>N catalysis at rt to give the corresponding thiourea adduct 6. Final catalytic deacetylation quantitatively furnished the target (ManS)<sub>3</sub>-βCD carrier, whose purity and structure were confirmed by mass spectrometry, NMR spectroscopy, and combustion analysis. The presence of the thiourea tether was ascertained by the <sup>13</sup>C NMR resonance at 183–180 ppm ( $\delta_{C=S}$ ) and the typical line broadening associated with restricted rotation at the pseudoamide NH–(C=S) bond at room temperature.<sup>29</sup> The unsymmetrical nature of the cyclooligosaccharide resulted in extensive signal overlapping in the <sup>1</sup>H NMR spectrum that was partially overcome by using 1D TOCSY experiments.

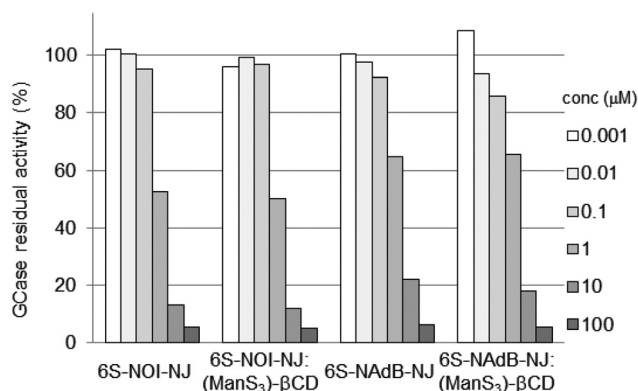
### (ManS)<sub>3</sub>-βCD:pharmacological chaperone complex formation thermodynamics

To measure the affinity of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ for the (ManS)<sub>3</sub>-βCD host, NMR titration experiments in D<sub>2</sub>O were conducted. The resonances of the βCD H-3 and H-5 protons were the most intensely affected after complex formation, supporting inclusion of the hydrophobic moieties of the PCs in the βCD cavity. Using the continuous variations method and an iterative least squares fitting procedure<sup>30</sup> the corresponding association constant ( $K_{as}$ ) values were determined to be  $399 \pm 4$  and  $1019 \pm 196 \text{ M}^{-1}$ , respectively (see the Experimental section and ESI† for details and binding isotherm figures). Titration isotherms were compatible with a 1 : 1 complex stoichiometry in both cases.

### Glycosidase inhibition capabilities of (ManS)<sub>3</sub>-βCD: pharmacological chaperone complexes

To pinpoint whether or not (ManS)<sub>3</sub>-βCD complexation alters the availability of the pharmacological chaperones to interact with glycosidases, the inhibition capabilities of 6S-NOI-NJ and 6S-NAdB-NJ free or in complex with (ManS)<sub>3</sub>-βCD were first profiled towards two commercial β-glucosidases (β-Glucases), namely β-Glucose from almonds and β-Glucose from bovine liver, belonging to the same clan as that of the human GCase (clan A).<sup>31</sup> Complexes were pre-formed by freeze-drying equimolecular mixtures of each sp<sup>2</sup>-iminosugar and the mannosylated CD carrier (ManS)<sub>3</sub>-βCD in water. The corresponding inhibition constants ( $K_i$ ) at the optimal pH of each glycosidase are shown in Table 1. In control experiments, (ManS)<sub>3</sub>-βCD alone did not affect the activities of the enzymes up to mM concentrations (data not shown). The inhibition mode, as determined by Lineweaver–Burk plots, was competitive in all cases.

The inhibitory activity of the pharmacological chaperones was not significantly altered after inclusion complex formation, with  $K_i$  values, in the μM range, that remained 2–3 orders of magnitude lower than the 6S-NOI-NJ : (ManS)<sub>3</sub>-βCD or 6S-NAdB-NJ : (ManS)<sub>3</sub>-βCD complex dissociation constants. Moreover, the percentage of enzyme inhibition for a constant concentration of 6S-NOI-NJ or 6S-NAdB-NJ was not affected by increasing concentrations of (ManS)<sub>3</sub>-βCD up to 1 : 10 ratios. These results are in agreement with fast dynamics of the equilibrium at play that facilitates the efficient transfer of the 6S-NOI-NJ and 6S-NAdB-NJ chaperones from the CD cavity to the glycosidase active site. Further experiments with human GCase confirmed this hypothesis. Thus, the



**Fig. 3** Activity of wild-type human GCase at the indicated concentrations of the PCs 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with (ManS)<sub>3</sub>-βCD. Results are expressed relative to activity of the enzyme in the absence of inhibitor (100%).

uncomplexed sp<sup>2</sup>-iminosugars and the corresponding inclusion complexes with the (ManS)<sub>3</sub>-βCD carrier were equally efficient at inhibiting glucocerebrosidase activity in cell lysates at concentrations over 0.1 μM (Fig. 3).

### (ManS)<sub>3</sub>-βCD:pharmacological chaperone complex formation thermodynamics

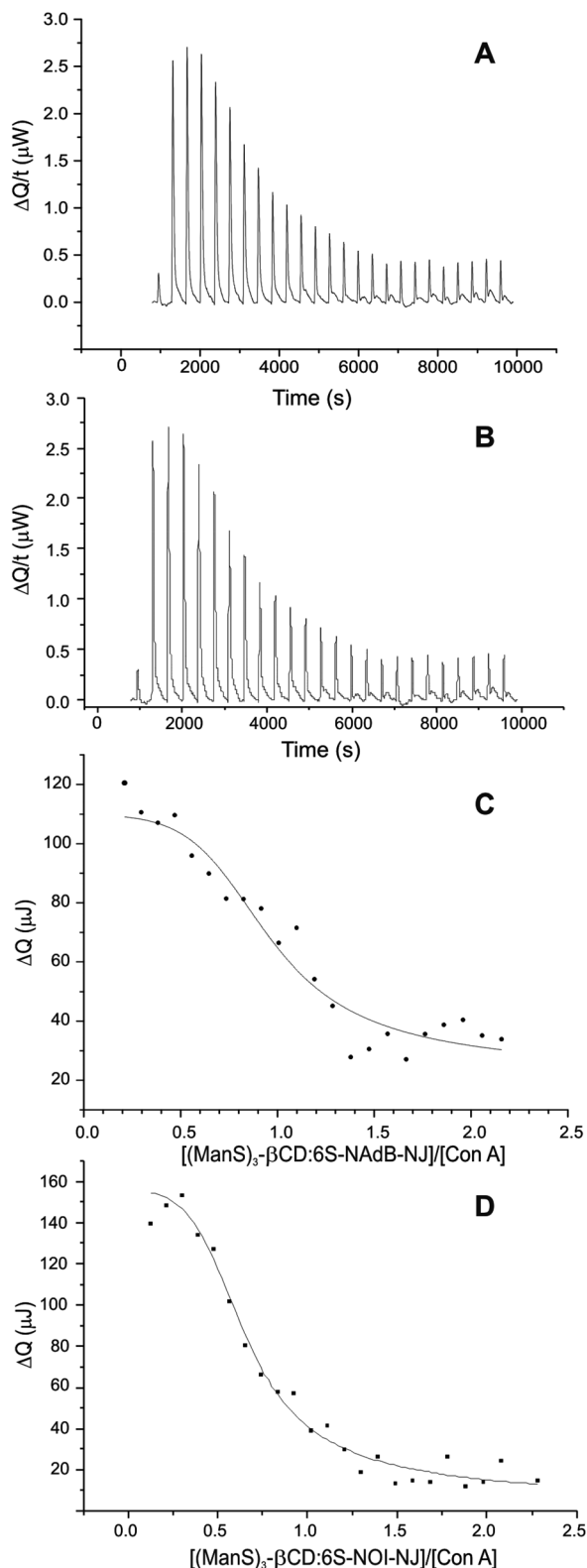
To probe the ability of the (ManS)<sub>3</sub>-βCD carrier and the corresponding complexes with the 6S-NOI-NJ and 6S-NAdB-NJ to target mannose-specific lectins, a comparative study of their binding capabilities towards the model plant lectin Concanavalin A (Con A) by isothermal titration calorimetry (ITC) was first conducted. Con A specifically recognizes α-D-mannopyranosides.<sup>32</sup> For the measurements, the unloaded carrier and the preformed 1 : 1 inclusion complexes with each PC were titrated into a solution of Con A at pH 7.4. ITC data for the binding were fitted using a single site model based on monomeric Con A. The stoichiometry ( $n$ ) determined for the complexes of any of these species with the lectin was 1 : 1 ( $n = 1$ ); *i.e.*, the mannosyl dendron in (ManS)<sub>3</sub>-βCD interacts exclusively with one unit of Con A, irrespective of being unloaded or loaded with the PC. Inclusion of the PC in the βCD cavity did not significantly affect the lectin affinity, with dissociation constants ( $K_D$ ) for the Con A : [(ManS)<sub>3</sub>-βCD : 6S-NOI-NJ] and Con A : [(ManS)<sub>3</sub>-βCD : 6S-NAdB-NJ] complexes in the 1.6–3.6 μM range (Fig. 4 and Table 2) as compared with  $2.1 \pm 0.7 \text{ μM}$  for the Con A : (ManS)<sub>3</sub>-βCD complex. These values are indicative of 20-to-80-fold affinity enhancements as compared with reported data for methyl α-D-mannopyranoside

**Table 1** Inhibition constants ( $K_i$ , μM) for 6S-NOI-NJ and 6S-NAdB-NJ and their corresponding 1 : 1 complexes with (ManS)<sub>3</sub>-βCD towards commercial glucosidases

Glucosidase (source, pH) <sup>a</sup>	6S-NOI-NJ	6S-NOI-NJ : (ManS) <sub>3</sub> -βCD	6S-NAdB-NJ	6S-NAdB-NJ : (ManS) <sub>3</sub> -βCD
β-Glucose (almond, 7.3)	$0.76 \pm 0.05$	$0.29 \pm 0.02$	$0.45 \pm 0.03$	$5.8 \pm 0.05$
β-Glucose (bovine liver, 7.3)	$3.7 \pm 0.1$	$9.9 \pm 0.1$	$68 \pm 2.0$	$33 \pm 0.02$

<sup>a</sup>  $K_i$  values were determined from the corresponding Lineweaver–Burk plots (see ESI for experimental details).





**Fig. 4** Thermograms for the titration of Con A with the 1:1 (ManS)<sub>3</sub>-βCD:6S-NAdB-NJ and (ManS)<sub>3</sub>-βCD:6S-NOI-NJ complexes (A and B, respectively) and non-linear regression fittings to the 1:1 stoichiometry model (C and D, respectively).  $\Delta Q$  represents the heat evolved after each injection of the complex.

determined by the same technique ( $K_D = 83 \mu M$ ).<sup>33</sup> Altogether the data confirm that the trivalent mannosyl ligand in the designed PC carrier (ManS)<sub>3</sub>-βCD is available to participate in lectin receptor recognition phenomena, benefitting from the multivalent effect, and that the carbohydrate-lectin interaction remains equally efficient after loading with the PC cargo.

Lectins recognizing an identical sugar epitope can quite differ structurally. Therefore, extrapolations of data on their responsiveness towards multivalent presentations of the putative ligand must be taken with care. We have made use of enzyme-linked lectin assay (ELLA) protocols, available for both Con A<sup>34</sup> and commercially available recombinant human macrophage mannose receptor (rhMMR),<sup>35</sup> to validate the results for macrophage targeting purposes. A 15-fold affinity enhancement against Con A for (ManS)<sub>3</sub>-βCD as compared to the monovalent reference methyl  $\alpha$ -D-mannopyranoside, expressed as the ratio between the concentrations required to achieve 50% inhibition ( $IC_{50}$ ) of the association of horse radish peroxidase-labelled Con A (HRP-Con A) to an immobilized ligand (yeast mannan), was determined by ELLA, in agreement with literature data for other trivalent mannosides.<sup>21a,28,36</sup> In the case of the rhMMR, the ELLA experiment afforded a (ManS)<sub>3</sub>-βCD *versus* methyl  $\alpha$ -D-mannopyranoside relative affinity enhancement significantly higher (72-fold), suggesting that the disposition of the mannosyl epitopes in the dendron is particularly appropriate to benefit from the multivalent effect in the case of this lectin. Actually, control experiments indicated that the affinity of (ManS)<sub>3</sub>-βCD towards rhMMR is analogous to that of high mannose oligosaccharides, known to be preferred ligands for this receptor in Nature,<sup>36</sup> in the same experimental setup (see ESI†).

#### Evaluation of the chaperoning capabilities of (ManS)<sub>3</sub>-βCD:6S-NOI-NJ and (ManS)<sub>3</sub>-βCD:6S-NAdB-NJ complexes in Gaucher fibroblasts

The GCase chaperoning capabilities of the sp<sup>2</sup>-iminosugars 6S-NOI-NJ and 6S-NAdB-NJ before and after complexation with (ManS)<sub>3</sub>-βCD were evaluated in three different mutant GD fibroblast lines, namely F213I/F213I (catalytic domain, neuronopathic), N370S/N370S (catalytic domain, non-neuronopathic) and L444P/L444P (non-catalytic domain, neuronopathic), as well as in wild-type fibroblasts. Cells were cultured in the absence and in the presence of different amounts of the PCs (3 and 30  $\mu M$ ) and their corresponding 1:1 complexes with (ManS)<sub>3</sub>-βCD. After 4-day incubation, GCase activity was determined by *in situ* fluorimetric measurement of the production of 4-methylumbelliferone (see ESI† for details). As observed in Fig. 5A, neither 6S-NOI-NJ, 6S-NAdB-NJ nor their complexes disrupted the activity of the wild-type enzyme, revealing that lysosomal function is not inhibited at the highest concentration tested (30  $\mu M$ ). Both 6S-NOI-NJ and 6S-NAdB-NJ enhanced the activity of GCase in a dose dependent manner, up to 3-fold, in F213I/F213I mutant fibroblasts (Fig. 5B). The non-neuronopathic N370S/N370S mutant was much less sensitive to these PCs; only 6S-NAdB-NJ at 3  $\mu M$  concentration was able to promote a significant enzyme activity

**Table 2** Thermodynamic parameters and dissociation constant ( $K_D$ ) calculated from ITC experiments for the binding of Con A to (ManS)<sub>3</sub>-βCD and to the corresponding complexes with the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ

	$\Delta G^\circ$ (kJ mol <sup>-1</sup> )	$\Delta H^\circ$ (kJ mol <sup>-1</sup> )	$T\Delta G^\circ$ (kJ mol <sup>-1</sup> )	$K_D$ (μM)
(ManS) <sub>3</sub> -βCD	-32.4 ± 1.0	-137.7 ± 10.5	-105.2 ± 10.5	2.1 ± 0.7
6S-NOI-NJ : (ManS) <sub>3</sub> -βCD	-33.0 ± 3.1	-125.3 ± 10.9	-92.3 ± 10.9	1.6 ± 0.1
6S-NAdB-NJ : (ManS) <sub>3</sub> -βCD	-31.0 ± 1.3	-105.8 ± 2.4	-74.7 ± 2.4	3.6 ± 1.5

enhancement (about 2-fold; Fig. 5C). Negligible effects were measured in the L444P/L444P mutant with either 6S-NOI-NJ or 6S-NAdB-NJ, Fig. 5D. These results are consistent with the mutation-dependent responsiveness previously encountered for this family of PCs.<sup>15a</sup> In any case, the behavior of the two PCs was largely independent of inclusion complex formation with (ManS)<sub>3</sub>-βCD, which is in agreement with a fast transfer of the PC from the βCD cavity in the carrier to the GCaase active site.

### Macrophage-targeting and delivery capabilities of (ManS)<sub>3</sub>-βCD:pharmacological chaperone complexes

To confirm the potential of the (ManS)<sub>3</sub>-βCD carrier to target the MMR in macrophages, the ability to adhere to resident peritoneal macrophages (mice) was first probed *in vitro*. Adhesion was quantified using a method adapted from that reported by Muller and Schuber<sup>37</sup> that takes advantage of the strong enhancement in the fluorescent intensity of 6-*p*-toluidino-2-naphthalenesulfonic acid (TNS) upon inclusion in the hydrophobic cavity of βCD and βCD derivatives to form 1:1 complexes.<sup>21a</sup> The cells were incubated with different concentrations of the 1:1 TNS:(ManS)<sub>3</sub>-βCD complex at 4 °C to prevent phagocytosis and the amount of complex associated with the cell membrane was determined fluorimetrically. As denoted in Fig. 6A, fluorescence levels increased upon incubation of the macrophages with the TNS:(ManS)<sub>3</sub>-βCD complex in a dose dependent manner, while the non-targeted TNS:βCD complex, used as a control, did only produce background fluorescence levels. Interestingly, TNS:(ManS)<sub>3</sub>-βCD adhesion to macrophages was gradually inhibited in the presence of increasing concentrations of uncomplexed (ManS)<sub>3</sub>-βCD or the corresponding 1:1 (ManS)<sub>3</sub>-βCD:6S-NOI-NJ and (ManS)<sub>3</sub>-βCD:6S-NAdB-NJ complexes with very similar efficiencies (Fig. 6B, IC<sub>50</sub> 42, 39 and 36 μM, respectively). This result supports that the carrier and the corresponding PC or TNS complexes compete for the same receptor at the surface of macrophages and is in agreement with the involvement of the MMR in the adhesion process.

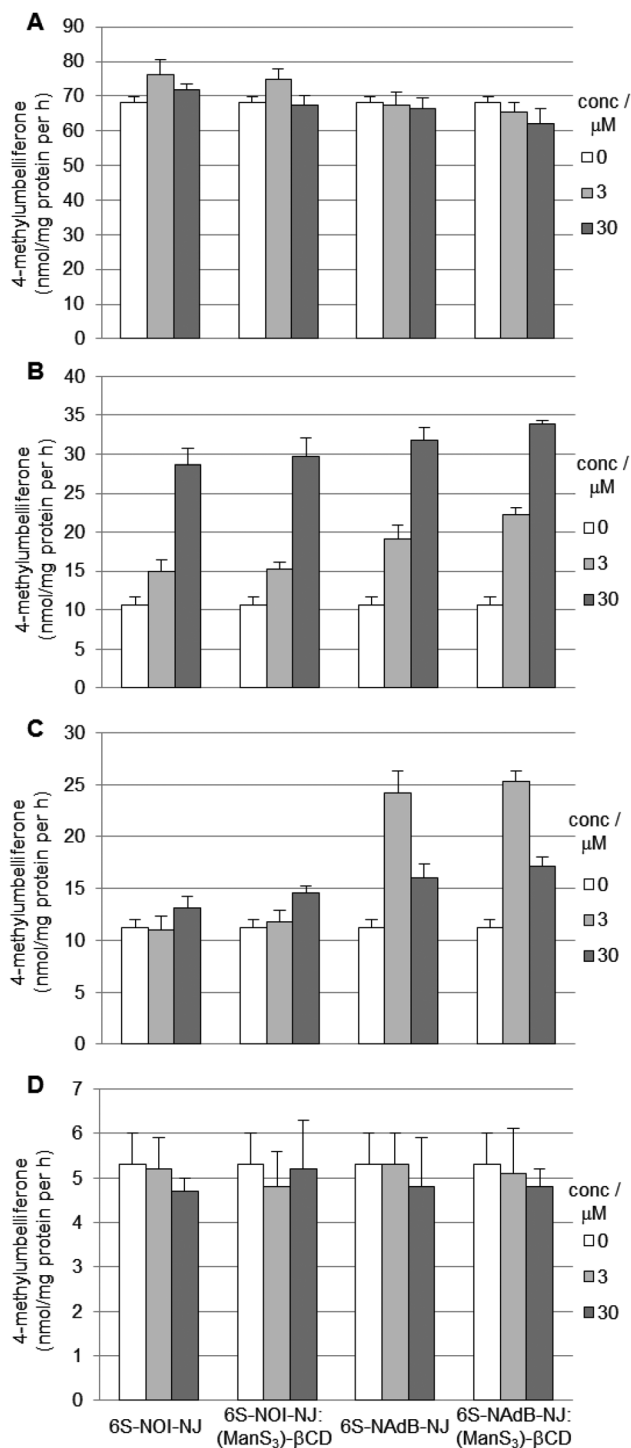
We have previously used a covalently attached dansyl tag to trace sp<sup>2</sup>-iminosugar internalization in fibroblasts.<sup>15b</sup> However, the presence of the relatively big fluorescent moiety may significantly alter the membrane-crossing and diffusion properties of the molecule as compared with the unlabelled chaperone, limiting the scope of the conclusions. In order to ascertain whether or not the PC:(ManS)<sub>3</sub>-βCD complexes are internalized in macrophages following MMR binding, we have instead devised competitive assays using the adamantane-equipped dansyl fluorescent probe 7 and the mannosyl

derivative 8 (Fig. 7; see ESI† for synthetic details) as a model βCD guest and an MMR ligand, respectively. Experiments were conducted in macrophage-like cells differentiated from THP-1 human monocytes, monitoring by 3D fluorescence microscopy. These cells mimic many characteristic features of human primary macrophages, including MMR expression.

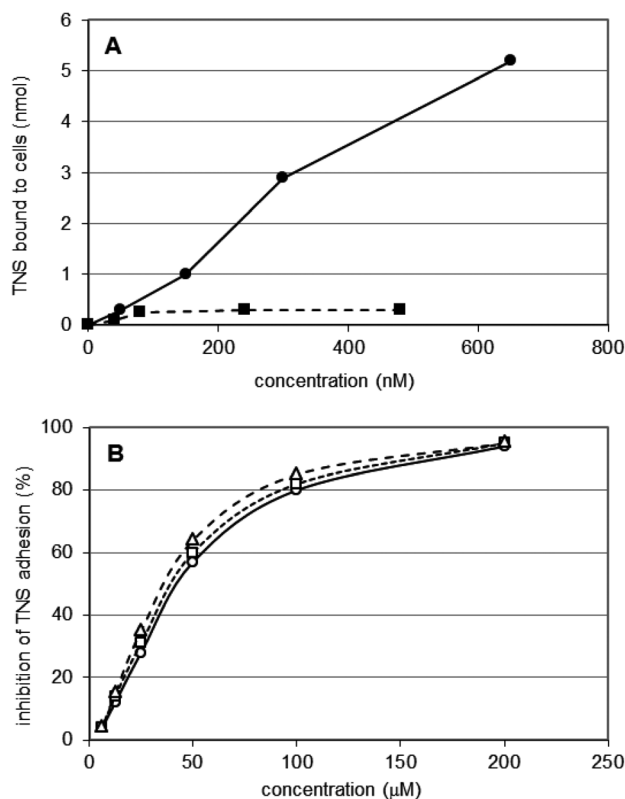
In control experiments, incubation of the cells with the reference compound 8 (400 μM) produced an intense green fluorescence in the cytoplasm that was virtually abolished by excess yeast mannan (data not shown), in agreement with MMR-mediated internalization of the conjugate. A virtually identical result was obtained when 8 was replaced by the 1:1 (ManS)<sub>3</sub>-βCD:7 complex at the same concentration (Fig. 8, row A), but not when uncomplexed 7 was incubated with the cells under identical conditions, strongly supporting that the (ManS)<sub>3</sub>-βCD carrier promotes internalization of the included guest upon binding to the MMR. In parallel experiments, an excess of the sp<sup>2</sup>-iminosugar 6S-NAdB-NJ did not affect internalization of 8, discarding the direct interaction of the chaperone with the MMR. However, in the case of the 1:1 (ManS)<sub>3</sub>-βCD:7 complex the fluorescence intensity significantly decreased upon mixing with increasing amounts of 6S-NAdB-NJ prior to incubation of the cells (Fig. 8, rows B and C). This is consistent with the partial displacement of the fluorescent probe 7 from the carrier by the pharmacological chaperone and the subsequent internalization of the non-fluorescent (ManS)<sub>3</sub>-βCD:6S-NAdB-NJ complex through the MMR-mediated route. Accordingly, internalization of the (ManS)<sub>3</sub>-βCD:7 complex was also abolished in the presence of excess of yeast mannan (Fig. 8, row D). A quantitative representation of the relative intracellular fluorescent intensities for the above experiments is shown in Fig. 8E. With the intrinsic limitations of any indirect method, the ensemble of results support the hypothesis that (ManS)<sub>3</sub>-βCD specifically interacts with the MMR through the trivalent mannosyl antenna and that this interaction elicits internalization of the corresponding inclusion complexes with PCs into the macrophages.

## Conclusions

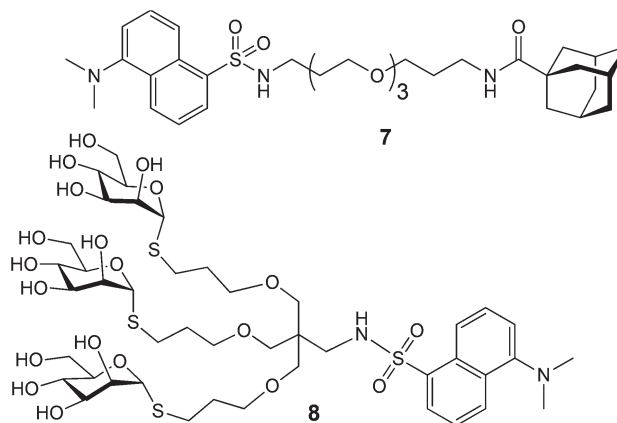
The present study provides a proof of concept of the suitability of the βCD-mannosyl dendron conjugate (ManS)<sub>3</sub>-βCD as a macrophage-targeted delivery system for sp<sup>2</sup>-iminosugar-type amphiphilic pharmacological chaperones against Gaucher disease. The monosubstituted but multivalent conjugate structure of the carrier prototype has been purposely conceived to keep the inclusion capabilities towards the hydrophobic



**Fig. 5** Chaperone activity of 6S-NOI-NJ and 6S-NAdB-NJ and their corresponding 1:1 complexes with  $(\text{ManS})_3\text{-}\beta\text{CD}$  on wild type (A) and mutant GCases (B, F213I/F213I; C, N370S/N370S; D, L444P/L444P) in fibroblasts (*in situ* cell enzyme assay). Cells were cultured for four days in the absence or presence of increasing concentrations of the compounds. Lysosomal GCase activity was estimated in intact cells by measuring the rate of production of 4-methylumbelliferone (see ESI† for details).

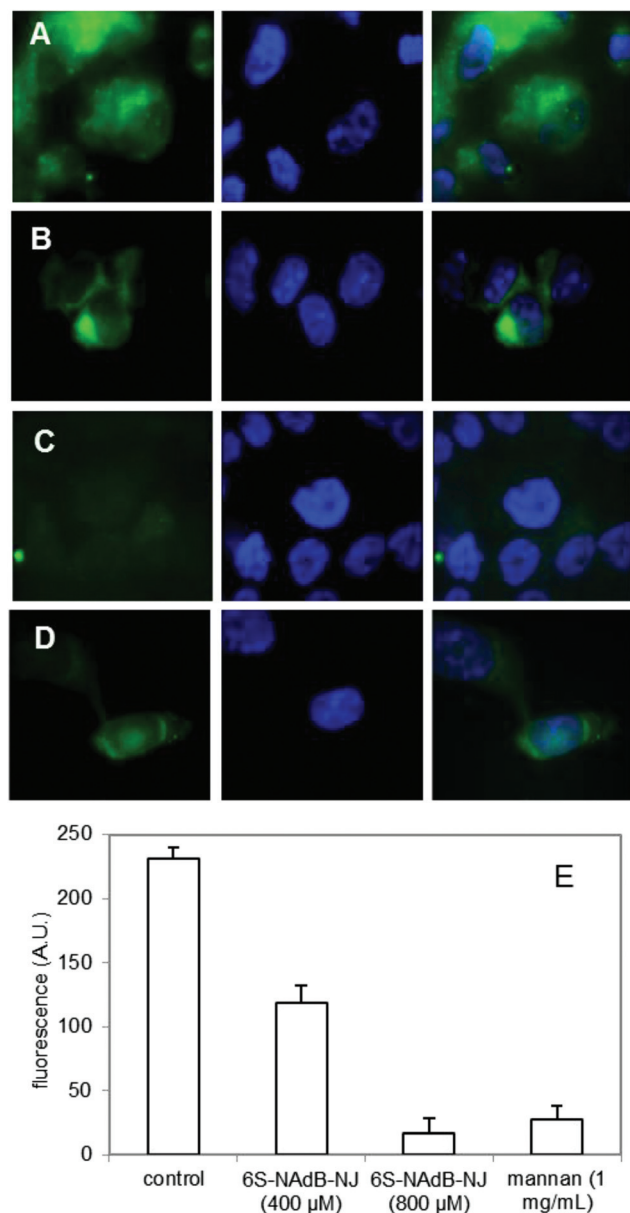


**Fig. 6** (A) Fluorimetrically determined adhesion of 1:1 TNS: $(\text{ManS})_3\text{-}\beta\text{CD}$  (filled line, ●) and TNS: $\beta\text{CD}$  (slashed line, <) complexes to the cell membrane of mice macrophages and (B) competitive membrane-bound TNS displacement assay using  $(\text{ManS})_3\text{-}\beta\text{CD}$  (filled line, ○), and the 1:1 6S-NOI-NJ: $(\text{ManS})_3\text{-}\beta\text{CD}$  (dotted line, □) and 6S-NAdB-NJ: $(\text{ManS})_3\text{-}\beta\text{CD}$  (slashed line, Δ) complexes.



**Fig. 7** Structure of dansylated probes **7** and **8** used for confocal microscopy monitoring of macrophage targeting and cellular uptake of PC:CD complexes.

moieties of the PC candidates, 6S-NOI-NJ and 6S-NAdB-NJ while warranting efficient mannose-specific lectin recognition abilities. Both hypotheses have been first demonstrated for commercial  $\beta$ -glucosidases and Con A lectin and further validated in human  $\beta$ -glucocerebrosidase and human MMR (recombinant). Complexation of the pharmacological



**Fig. 8** Fluorescence microscopy pictures (3D projections) of THP-1 human monocytic cells differentiated into macrophage-like cells incubated with (ManS)<sub>3</sub>-βCD:7 complex (400 μM) alone (row A) and in the presence of 6S-NAdB-NJ (400 or 800 μM, rows B and C, respectively) or yeast mannan (1 mg mL<sup>-1</sup>, row D). The fluorescence of probe 7, the nuclear staining reagent Hoechst 33342, and the merged pictures are represented in the left, center, and right columns, respectively. Acquired z planes were separated by 0.3 μm, and an average of 50 planes was taken for each cell. Panel (E) quantitates the intracellular fluorescence after treatment with (ManS)<sub>3</sub>-βCD:7 complex (400 μM) alone (control) and in the presence of 6S-NAdB-NJ (400 or 800 μM) or yeast mannan (1 mg mL<sup>-1</sup>). Identical circular regions of interest (ROIs) comprising 200 cells were taken for fluorescence quantification.

chaperones by (ManS)<sub>3</sub>-βCD preserves their chaperoning potential. The corresponding PC complexes specifically recognize the MMR at the surface of macrophages, the cell type that is mostly affected in GD patients, and this recognition phenomenon elicits macrophage internalization. The

combination of macrophage targeting abilities and efficient PC transfer to GCase may provide a means for improving the therapeutic outcome of pharmacological chaperone treatments for Gaucher disease and warrants further *in vivo* evaluation.

## Experimental

### General methods

6<sup>1</sup>-O-*p*-Toluenesulfonylcyclomaltoheptaose (**1**)<sup>27</sup> and 2,2,2-tris-[5-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]-ethyl isothiocyanate (**5**)<sup>28</sup> were obtained according to literature procedures. Iminosugars 6S-NOI-NJ<sup>22a</sup> and 6S-NAdB-NJ<sup>22b</sup> were synthesized according to literature procedures. The synthesis of dansylated derivatives **7** and **8** is described in the ESI.† Optical rotations were measured at 20 ± 2 °C in 1 dm tubes on a Jasco P-2000 polarimeter. UV spectra were recorded in 1 cm tubes on a Jasco V-630 spectrophotometer. <sup>1</sup>H (and <sup>13</sup>C NMR) spectra were recorded at 500 (125.7) MHz with Bruker 500 DRX magnet. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 F254 Merck with visualization by UV light and by charring with ethanolic 10% H<sub>2</sub>SO<sub>4</sub> and 0.1% ninhydrin. Column chromatography was carried out on Silica Gel 60. ESI mass spectra were recorded on a Bruker Daltonics Esquire6000™ ion-trap mass spectrometer. Elemental analyses were carried out at the Instituto de Investigaciones Químicas (Sevilla, Spain).

**6<sup>1</sup>-Deoxy-6<sup>1</sup>-iodocyclomaltoheptaose (2).**<sup>38</sup> To a solution of monotosylated βCD derivative **1** (1.0 g, 0.76 mmol) in DMF (18 mL) NaI (0.57 g, 3.8 mmol, 5 eq.) was added. The mixture was stirred at 80 °C overnight and then the solvent was removed under reduced pressure. The resulting residue was stirred in a mixture of <sup>t</sup>BuOH-EtOH-H<sub>2</sub>O (5 : 4 : 3, 10 mL), filtrated and washed with cold EtOH (10 mL), DCM (10 mL) and acetone (10 mL), and dried to yield compound **2** in 90% yield (0.87 g). *R*<sub>f</sub> = 0.18 (6 : 3 : 1 MeCN-H<sub>2</sub>O-NH<sub>4</sub>OH); [*α*]<sub>D</sub> = +128.7 (*c* 0.75, MeOH); <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>, 313 K): δ 5.72–5.60 (m, 13H, OH-2<sup>I-VII</sup>, OH-3<sup>II-VII</sup>), 5.56 (d, 1H, *J*<sub>OH,3</sub> = 2.3 Hz, OH-3<sup>I</sup>), 4.89–4.83 (m, 6H, H-1<sup>I-VII</sup>), 4.88 (d, 1H, *J*<sub>1,2</sub> = 3.7 Hz, H-1<sup>I</sup>), 4.57–4.43 (m, 6H, OH-6<sup>II-VII</sup>), 3.80–3.51 (m, 24H, H-3<sup>II-VII</sup>, H-5<sup>II-VII</sup>, H-6<sup>II-VII</sup>), 3.70 (m, 1H, H-5<sup>I</sup>), 3.63 (bd, 1H, *J*<sub>6a,6b</sub> = 11.3 Hz, H-6a<sup>I</sup>), 3.54 (bd, 1H, *J*<sub>5,6a</sub> = 3.0 Hz, H-6b<sup>I</sup>), 3.43 (m, 1H, H-3<sup>I</sup>), 3.45–3.26 (m, 12H, H-2<sup>II-VII</sup>, H-4<sup>II-VII</sup>), 3.33 (m, 1H, H-2<sup>I</sup>), 3.20 (t, 1H, *J*<sub>3,4</sub> = *J*<sub>4,5</sub> = 8.9 Hz, H-4<sup>I</sup>); <sup>13</sup>C NMR (125.7 MHz, DMSO-*d*<sub>6</sub>, 313 K): δ 102.7–102.1 (C-1<sup>I-VII</sup>), 86.5 (C-4<sup>I</sup>), 82.3–81.9 (C-4<sup>II-VII</sup>), 73.6–72.4 (C-2<sup>I-VII</sup>, C-3<sup>II-VII</sup>, C-5<sup>I-VII</sup>), 70.0 (C-3<sup>I</sup>), 60.6–60.4 (C-6<sup>II-VII</sup>), 10.0 (C-6<sup>I</sup>); ESIMS: *m/z* 1279.7 [*M* + Cl]<sup>−</sup>. Anal. Calcd for C<sub>42</sub>H<sub>69</sub>IO<sub>34</sub>: C, 40.52; H, 5.59; found: C, 40.13; H, 5.62.

**6<sup>1</sup>-[2-(*tert*-Butoxycarbonylamino)ethylthio]cyclomaltoheptaose (3).** To a solution of **2** (1.42 g, 1.14 mmol) and Cs<sub>2</sub>CO<sub>3</sub> (0.48 g, 1.48 mmol, 1.3 eq.) in dry DMF (18 mL), under an Ar atmosphere, *tert*-butyl *N*-(2-mercaptoethyl)carbamate (250 μL, 1.48 mmol, 1.3 eq.) was added. The suspension was heated at



75 °C for 3 h and the solvent was reduced until 1/3 volume. The resulting residue was poured into acetone (150 mL), filtered and purified by column chromatography (6 : 3 : 1 MeCN–H<sub>2</sub>O–NH<sub>4</sub>OH) to give **3** in 67% yield (0.98 g); *R*<sub>f</sub> = 0.47 (6 : 3 : 1 MeCN–H<sub>2</sub>O–NH<sub>4</sub>OH); [ $\alpha$ ]<sub>D</sub> = +115.3 (*c* 1.0 in DMSO); <sup>1</sup>H NMR (500 MHz, 9 : 1 DMSO-*d*<sub>6</sub>–D<sub>2</sub>O, 333 K):  $\delta$  4.88–4.82 (m, 6H, H-1<sup>II-VII</sup>), 4.84 (d, 1H, *J*<sub>1,2</sub> = 3.0 Hz, H-1<sup>I</sup>), 3.86–3.59 (m, 24H, H-3<sup>II-VII</sup>, H-5<sup>II-VII</sup>, H-6<sup>II-VII</sup>), 3.82 (bt, 1H, *J*<sub>4,5</sub> = *J*<sub>5,6a</sub> = 8.6 Hz, H-5<sup>I</sup>), 3.65 (t, 1H, *J*<sub>2,3</sub> = 8.6 Hz, H-3<sup>I</sup>), 3.42–3.26 (m, 12H, H-2<sup>II-VII</sup>, H-4<sup>II-VII</sup>), 3.34 (dd, 1H, H-2<sup>I</sup>), 3.26 (t, 1H, H-4<sup>I</sup>), 3.08 (m, 2H, CH<sub>2</sub>N<sub>Cyst</sub>), 3.07 (bd, 1H, H-6a<sup>I</sup>), 2.71 (m, 1H, H-6b<sup>I</sup>), 2.64 (m, 2H, CH<sub>2</sub>S<sub>Cyst</sub>), 1.43 (bs, 9H, CMe<sub>3</sub>); <sup>13</sup>C NMR (100.6 MHz, 9 : 1 DMSO-*d*<sub>6</sub>–D<sub>2</sub>O):  $\delta$  156.0 (CO), 102.9–101.2 (C-1<sup>I-VII</sup>), 86.7 (C-4<sup>I</sup>), 82.3–82.0 (C-4<sup>II-VII</sup>), 78.6 (C<sub>q</sub>), 73.5–72.4 (C-2<sup>I-VII</sup>, C-3<sup>I-VII</sup>, C-5<sup>I-VII</sup>), 60.6–60.2 (C-6<sup>II-VII</sup>), 43.0 (CH<sub>2</sub>N<sub>Cyst</sub>), 39.9 (CH<sub>2</sub>S<sub>Cyst</sub>, C-6<sup>I</sup>), 29.0 (CMe<sub>3</sub>); ESIMS: *m/z* 1316.9 [M + Na]<sup>+</sup>. Anal. Calcd for C<sub>49</sub>H<sub>83</sub>NO<sub>36</sub>S: C 45.47, H 6.46, N 1.08. Found: C 45.31, H 6.46, N 0.96.

#### 6<sup>I</sup>-(2-Aminoethylthio)cyclomaltoheptaose hydrochloride (**4**).<sup>39</sup>

Compound **3** (94 mg, 72  $\mu$ mol) was treated with a 1 : 1 TFA–water mixture (3 mL) at rt for 1 h. The solvents were then evaporated under reduced pressure and acid traces were removed by co-evaporating with water several times. The residue was finally dissolved in diluted aq. HCl and freeze-dried to quantitatively furnish compound **4** as hydrochloride salt (89 mg). [ $\alpha$ ]<sub>D</sub> = +113.6 (*c* 0.9, H<sub>2</sub>O); <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O):  $\delta$  5.04 (d, 1H, *J*<sub>1,2</sub> = 3.7 Hz, H-1<sup>II</sup>), 4.99 (d, 1H, *J*<sub>1,2</sub> = 3.7 Hz, H-1<sup>I</sup>), 5.00–4.97 (m, 5H, H-1<sup>III-VII</sup>), 3.95 (bt, 1H, *J*<sub>4,5</sub> = 9.4 Hz, H-5<sup>I</sup>), 3.91–3.72 (m, 20H, H-3<sup>III-VII</sup>, H-5<sup>III-VII</sup>, H-6<sup>III-VII</sup>), 3.86 (t, 1H, *J*<sub>2,3</sub> = *J*<sub>3,4</sub> = 9.4 Hz, H-3<sup>II</sup>), 3.85 (t, 1H, *J*<sub>2,3</sub> = *J*<sub>3,4</sub> = 9.4 Hz, H-3<sup>I</sup>), 3.78 (m, 2H, H-6<sup>II</sup>), 3.77 (m, 1H, H-5<sup>II</sup>), 3.61–3.44 (m, 12H, H-2<sup>III-VII</sup>, H-4<sup>III-VII</sup>), 3.58 (dd, 1H, H-2<sup>II</sup>), 3.57 (m, 2H, H-2<sup>I</sup>, H-4<sup>I</sup>), 3.47 (t, 1H, *J*<sub>4,5</sub> = 9.4 Hz, H-4<sup>II</sup>), 3.16 (t, 2H, <sup>3</sup>*J*<sub>H,H</sub> = 6.4 Hz, CH<sub>2</sub>N<sub>Cyst</sub>), 3.07 (dd, 1H, *J*<sub>5,6a</sub> = 2.9 Hz, *J*<sub>6a,6b</sub> = 14.0 Hz, H-6a<sup>I</sup>), 2.91 (dd, 1H, *J*<sub>5,6a</sub> = 6.5 Hz, H-6b<sup>I</sup>), 2.86 (t, 2H, CH<sub>2</sub>S<sub>Cyst</sub>); <sup>13</sup>C NMR (125.7 MHz, D<sub>2</sub>O):  $\delta$  101.9–101.7 (C-1<sup>I-VII</sup>), 83.8 (C-4<sup>I</sup>), 81.3–81.1 (C-4<sup>II-VII</sup>), 73.0–71.1 (C-2<sup>I-VII</sup>, C-3<sup>I-VII</sup>, C-5<sup>I-VII</sup>), 60.5–60.2 (C-6<sup>II-VII</sup>), 38.4 (C-6<sup>I</sup>), 32.5 (CH<sub>2</sub>N<sub>Cyst</sub>), 29.7 (CH<sub>2</sub>S<sub>Cyst</sub>); ESIMS: *m/z* 1194.8 [M – Cl]<sup>+</sup>.

6<sup>I</sup>-[2-[N'-[2,2,2-Tris[5-(2,3,4,6-tetra-*O*-acetyl- $\alpha$ -D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]cyclomaltoheptaose (**6**). To a solution of **4** (94 mg, 72  $\mu$ mol) and Et<sub>3</sub>N (20  $\mu$ L, 144  $\mu$ mol, 2 eq.) in dry DMF (1 mL), a solution of **5** in dry DMF (1 mL) was added. The reaction mixture was stirred at room temperature for 72 h preserving the pH at 8–9 with Et<sub>3</sub>N. The reaction mixture was then concentrated and the residue was purified by column chromatography (MeCN  $\rightarrow$  3 : 1 MeCN–H<sub>2</sub>O) to give **6** in 50% yield (93 mg). *R*<sub>f</sub> = 0.66 (6 : 3 : 1 MeCN–H<sub>2</sub>O–NH<sub>4</sub>OH); [ $\alpha$ ]<sub>D</sub> = +57.8 (*c* 1.0, MeOH); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD, 313 K):  $\delta$  5.39 (bs, 3H, H-1<sub>Man</sub>), 5.36 (d, 3H, *J*<sub>1,2</sub> = 1.6 Hz, *J*<sub>2,3</sub> = 2.8 Hz, H-2<sub>Man</sub>), 5.30 (t, 3H, *J*<sub>3,4</sub> = *J*<sub>4,5</sub> = 9.8 Hz, H-4<sub>Man</sub>), 5.23 (dd, 3H, H-3<sub>Man</sub>), 5.04 (d, 1H, *J*<sub>1,2</sub> = 3.7 Hz, H-1<sup>II</sup>), 5.01–4.98 (m, 5H, H-1<sup>III-VII</sup>), 5.00 (d, 1H, *J*<sub>1,2</sub> = 3.3 Hz, H-1<sup>I</sup>), 4.41 (ddd, 3H, *J*<sub>5,6a</sub> = 4.9 Hz, *J*<sub>5,6b</sub> = 2.1 Hz, H-5<sub>Man</sub>), 4.30 (dd, 3H, *J*<sub>6a,6b</sub> = 12.1 Hz, *J*<sub>5,6a</sub> = 5.2 Hz, H-6a<sub>Man</sub>), 4.16 (dd, 3H, *J*<sub>5,6b</sub> = 5.2 Hz, H-6b<sub>Man</sub>), 3.97 (ddd, 1H, *J*<sub>4,5</sub> = 9.3 Hz, *J*<sub>5,6a</sub> =

2.3 Hz, *J*<sub>5,6b</sub> = 6.6 Hz, H-5<sup>I</sup>), 3.92–3.84 (m, 17H, H-3<sup>III-VII</sup>, H-6<sup>II-VII</sup>), 3.88 (t, 1H, *J*<sub>3,4</sub> = 9.5 Hz, H-3<sup>II</sup>), 3.87 (t, 1H, *J*<sub>2,3</sub> = *J*<sub>3,4</sub> = 9.3 Hz, H-3<sup>I</sup>), 3.81–3.76 (m, 6H, H-5<sup>II-VII</sup>), 3.73 (m, 4H, CH<sub>2</sub>N, CH<sub>2</sub>NH<sub>Cyst</sub>), 3.58 (2d, 6H, <sup>3</sup>*J*<sub>H,H</sub> = 6.0 Hz, H-3<sub>Pent</sub>), 3.54 (dd, 1H, H-2<sup>I</sup>), 3.54 (dd, 1H, *J*<sub>1,2</sub> = 9.5 Hz, H-2<sup>II</sup>), 3.56–5.49 (m, 11H, H-2<sup>III-VII</sup>, H-4<sup>II-VII</sup>), 3.53 (m, 1H, H-4<sup>I</sup>), 3.47 (m, 8H, CH<sub>2</sub>NH, H-1<sub>Pent</sub>), 3.18 (dd, 1H, *J*<sub>6a,6b</sub> = 12.8 Hz, H-6a<sup>I</sup>), 2.94 (dd, 1H, H-6b<sup>I</sup>), 2.88 (t, 2H, <sup>3</sup>*J*<sub>H,H</sub> = 7.2 Hz, CH<sub>2</sub>S<sub>Cyst</sub>), 2.87–2.78 (m, 6H, H-5<sub>Pent</sub>), 2.17–1.98 (4 s, 36H, MeCO), 1.96 (m, 6H, H-4<sub>Pent</sub>); <sup>13</sup>C NMR (125.7 MHz, CD<sub>3</sub>OD, 313 K):  $\delta$  182.0 (CS), 170.9–170.1 (CO), 102.5–102.2 (C-1<sup>I-VII</sup>), 84.6 (C-4<sup>I</sup>), 82.6 (C-1<sub>Man</sub>), 81.8–81.7 (C-4<sup>II-VII</sup>), 73.4–73.2 (C-3<sup>I-VII</sup>), 72.9 (C-5<sup>I-VII</sup>), 72.5–72.3 (C-2<sup>I-VII</sup>), 71.1 (C-2<sub>Man</sub>), 69.8 (C-3<sub>Man</sub>), 69.5 (C-3<sub>Pent</sub>), 69.1 (C-5<sub>Man</sub>), 66.3 (C-4<sub>Man</sub>), 62.4 (C-6<sub>Man</sub>), 60.8–60.6 (C-6<sup>II-VII</sup>, C-1<sub>Pent</sub>), 44.3 (CH<sub>2</sub>N, CH<sub>2</sub>N<sub>Cyst</sub>), 33.3 (C-6<sup>I</sup>), 32.1 (CH<sub>2</sub>S<sub>Cyst</sub>), 29.3 (C-4<sub>Pent</sub>), 28.0 (C-5<sub>Pent</sub>), 19.4–19.1 (MeCO); ESIMS: *m/z* 1314.8 [M + 2 Na]<sup>2+</sup>. Anal. Calcd for C<sub>101</sub>H<sub>158</sub>N<sub>2</sub>O<sub>64</sub>S<sub>5</sub>: C, 46.93; H, 6.16; N, 1.08; S, 6.20; found: C, 47.11; H, 6.34; N, 0.89; S, 5.87.

6<sup>I</sup>-[2-[N'-[2,2,2-Tris[5- $\alpha$ -D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]cyclomaltoheptaose ((ManS)<sub>3</sub>- $\beta$ CD). The trivalent mannosyl carrier (ManS)<sub>3</sub>- $\beta$ CD was obtained by treating a solution of compound **6** (59 mg, 23  $\mu$ mol) in dry MeOH (3 mL) with methanolic NaOMe (1 mL, 27  $\mu$ L, 0.1 eq.) at rt for 1 h. The reaction mixture was neutralized with Amberlite IR-120 (H<sup>+</sup>) ion exchange resin, the resin filtered-off and the solvent evaporated under reduced pressure to yield (ManS)<sub>3</sub>- $\beta$ CD in 97% yield (46 mg). [ $\alpha$ ]<sub>D</sub> = +27.5 (*c* 1.0, H<sub>2</sub>O); UV (H<sub>2</sub>O)  $\lambda$ <sub>max</sub> = 262 nm ( $\epsilon$ <sub>mM</sub> = 2308); <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O, 323 K):  $\delta$  5.53 (bs, 3H, H-1<sub>Man</sub>), 5.33 (m, 6H, H-1<sup>II-VII</sup>), 5.29 (d, 1H, *J*<sub>1,2</sub> = 3.0 Hz, H-1<sup>I</sup>), 4.29 (bs, 3H, H-2<sub>Man</sub>), 4.19–3.99 (m, 31H, H-3<sup>II-VII</sup>, H-5<sup>II-VII</sup>, H-6<sup>II-VII</sup>, H-1<sub>Pent</sub>, H-3<sub>Man</sub>, H-4<sub>Man</sub>, H-5<sub>Man</sub>, H-6<sub>Man</sub>), 4.10 (t, 1H, *J*<sub>2,3</sub> = *J*<sub>3,4</sub> = 9.1 Hz, H-3<sup>I</sup>), 4.09 (m, 1H, H-5<sup>I</sup>), 3.97–3.85 (m, 12H, H-2<sup>II-VII</sup>, H-4<sup>II-VII</sup>), 3.93 (m, 2H, CH<sub>2</sub>N<sub>Cyst</sub>), 3.90 (dd, 1H, H-2<sup>I</sup>), 3.76 (t, 6H, <sup>3</sup>*J*<sub>H,H</sub> = 5.4 Hz, H-3<sub>Pent</sub>), 3.75 (t, 1H, H-4<sup>I</sup>), 3.65 (s, 2H, CH<sub>2</sub>NH), 3.45 (bd, 1H, *J*<sub>6a,6b</sub> = 11.2 Hz, H-6a<sup>I</sup>), 3.15 (t, 2H, <sup>3</sup>*J*<sub>H,H</sub> = 6.5 Hz, CH<sub>2</sub>S<sub>Cyst</sub>), 3.07 (dd, 1H, *J*<sub>5,6b</sub> = 7.3 Hz, H-6b<sup>I</sup>), 2.99–2.87 (m, 6H, H-5<sub>Pent</sub>), 2.14 (m, 6H, H-4<sub>Pent</sub>); <sup>13</sup>C NMR (125.7 MHz, D<sub>2</sub>O, 323 K):  $\delta$  182.3 (CS), 102.6–102.1 (C-1<sup>I-VII</sup>), 85.3 (C-1<sub>Man</sub>), 85.0 (C-4<sup>I</sup>), 81.3–80.9 (C-4<sup>II-VII</sup>), 73.6–71.4 (C-2<sub>Man</sub>, C-3<sub>Man</sub>, C-5<sub>Man</sub>, C-2<sup>I-VII</sup>, C-3<sup>I-VII</sup>, C-5<sup>I-VII</sup>), 70.4 (C-3<sub>Pent</sub>), 67.2 (C-4<sub>Man</sub>), 61.5 (C-1<sub>Pent</sub>), 60.5–60.2 (C-6<sup>II-VII</sup>), 44.7 (CH<sub>2</sub>N, CH<sub>2</sub>N<sub>Cyst</sub>), 34.2 (C-6<sup>I</sup>), 33.8 (CH<sub>2</sub>S<sub>Cyst</sub>), 29.3 (C-4<sub>Pent</sub>), 28.0 (C-5<sub>Pent</sub>); ESIMS: *m/z* 1062.3 [M + 2 Na]<sup>2+</sup>. Anal. Calcd for C<sub>77</sub>H<sub>134</sub>N<sub>2</sub>O<sub>52</sub>S<sub>5</sub>: C, 44.46; H, 6.49; N, 1.35; S, 7.71; found: C, 44.25, H, 6.22; N, 1.03; S, 7.42.

#### NMR titration experiments

Association constants (*K*<sub>as</sub>) were determined in D<sub>2</sub>O at 313 K by measuring the proton chemical shift variations in the <sup>1</sup>H NMR spectra of a solution of the  $\beta$ CD derivative (ManS)<sub>3</sub>- $\beta$ CD in the presence of increasing amounts of the corresponding iminosugar (6S-NOI-NJ or 6S-NAD<sub>B</sub>-NJ). The chemical shifts of the diagnostic signals obtained at 10–11 different CD:iminosugar concentration ratios were used in an iterative least-squares fitting procedure. For a detailed description, see ESI<sup>†</sup>

### Isothermal titration calorimetry (ITC)

ITC experiments were performed in a multichannel thermal activity monitor (TAM) isothermal heat conduction micro-calorimeter (Thermometric AB 2277/201, Järfälla, Sweden) equipped with a 1.1 mL titration vessel. The calorimeter was thermostated at  $25 \pm 0.5$  °C. The vessel was loaded with 0.8 mL of protein solution using a Hamilton syringe, thermostated at 25 °C and continuously stirred at 60 rpm. The CD derivative 3 was injected by a computer controlled syringe pump (Hamilton Microlab M). Injections were made over a period of 10 s with intervals of 6 min. The experiment was monitored and analyzed using Digitam 4.1 software (Thermometric). To minimize dilution artifacts, the ligand was dissolved in the same dialysis buffer as the protein. The errors are provided by the software from the best fit of the experimental data to the model of equal and independent sites, and correspond to the standard deviation in the fitting of the curves. A separate experiment was performed to determine the heat of dilution of the ligand in the dialysis buffer. Reported data are the mean of two measurements made with different protein preparations; experiments were repeated more than twice if the obtained parameters were not congruent.

### Inhibition assays against commercial glycosidases

Inhibition constant ( $K_i$ ) values were determined by spectrophotometrically measuring the residual hydrolytic activities of the  $\beta$ -glucosidases (from bovine liver or almonds; Sigma) against *p*-nitrophenyl  $\beta$ -D-glucopyranoside. Each assay was performed in phosphate buffer at the optimal pH for the enzymes. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of iminosugar or iminosugar:CD complex (prepared by freeze-drying equimolecular mixtures of each partner). The mixture was incubated for 10–30 min at 37 °C and the reaction was quenched by addition of 1 M  $\text{Na}_2\text{CO}_3$ . Reaction times were appropriate to obtain 10–20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 nm. Approximate values of  $K_i$  were determined using a fixed concentration of a substrate (around the  $K_M$  value for the different  $\beta$ -glucosidases) and various concentrations of an inhibitor. Full  $K_i$  determinations and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis. Representative examples of the Lineweaver–Burk plots, with typical profile for competitive inhibition mode, are shown in the ESI (Fig. S11–S13†).

### Lysosomal enzyme activity assay

Lysosomal enzyme activities in cell lysates were determined as described previously.<sup>40</sup> Briefly, cells were scraped in ice-cold 0.1% Triton X-100 in water. After centrifugation (6000 rpm for 15 min at 4 °C) to remove insoluble materials, protein concentrations were determined using a protein assay rapid kit (Wako, Tokyo, Japan). The lysates were incubated at 37 °C with

the corresponding 4-methylumbelliferyl  $\beta$ -D-glucopyranoside solution in 0.1 M citrate buffer (pH 4). The liberated 4-methylumbelliferone was measured with a fluorescence plate reader (excitation 340 nm; emission 460 nm; Infinite F500, TECAN Japan, Kawasaki, Japan). For enzyme inhibition assay, cell lysates from normal skin fibroblasts were mixed with the 4-methylumbelliferyl  $\beta$ -D-glucopyranoside substrates in the absence or presence of increasing concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1:1 complexes with the mannosylated carrier (ManS)<sub>3</sub>- $\beta$ CD.

### Cell culture and GCase activity enhancement assay

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium–10% foetal bovine serum at 37 °C under a humidified atmosphere containing 5%  $\text{CO}_2$ . One control cell line (H37) and three lines of GD cells that carried the GlcCer-ase mutations F213I/F213I, L444P/L444P, and N370S/N370S, respectively, were used.<sup>40</sup> The culture medium was replaced every 2 d with fresh medium. For enzyme activity enhancement assay, cells were cultured in the presence of different concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1:1 complexes with the mannosylated carrier (ManS)<sub>3</sub>- $\beta$ CD for 5 days and harvested by scraping. Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

### Enzyme-linked lectin assays (ELLA)

Nunc-Inmuno plates (MaxiSorp™) were coated overnight with yeast mannan at 100  $\mu\text{L}$  per well diluted from a stock solution of 10  $\mu\text{g mL}^{-1}$  in 10 mM phosphate buffer saline (PBS, pH 7.3 containing 0.1 mM  $\text{Ca}^{2+}$  and 0.1 mM  $\text{Mn}^{2+}$ ) at rt. The wells were then washed three times with 300  $\mu\text{L}$  of washing buffer (containing 0.05% (v/v) Tween 20) (PBST). The washing procedure was repeated after each of the incubations throughout the assay. The wells were then blocked with 150  $\mu\text{L}$  per well of 1% BSA/PBS for 1 h at 37 °C.

For determination of Con A binding affinity, the wells were filled with 100  $\mu\text{L}$  of serial dilutions of peroxidase labeled Con A from  $10^{-1}$  to  $10^{-5}$   $\text{mg mL}^{-1}$  in PBS, and incubated at 37 °C for 1 h. The plates were washed and 50  $\mu\text{L}$  per well of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (0.25  $\text{mg mL}^{-1}$ ) in citrate buffer (0.2 M, pH 4.0 with 0.015%  $\text{H}_2\text{O}_2$ ) was added. The reaction was stopped after 20 min by adding 50  $\mu\text{L}$  per well of 1 M  $\text{H}_2\text{SO}_4$  and the absorbances were measured at 415 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. In order to carry out the inhibition experiments, each inhibitor was added in a serial of 2-fold dilutions (60  $\mu\text{L}$  per well) in PBS with 60  $\mu\text{L}$  of the desired lectin–peroxidase conjugate concentration on Nunclon™ (Delta) microtiter plates and incubated for 1 h at 37 °C. The above solutions (100  $\mu\text{L}$ ) were then transferred to the mannan-coated microplates, which were incubated for 1 h at 37 °C. The

plates were washed and the ABTS substrate was added (50  $\mu\text{L}$  per well). Color development was stopped after 20 min and the absorbances were measured.

For determination of recombinant human MMR (rhMMR) binding affinity, the wells were filled with 100  $\mu\text{L}$  of serial dilutions of rhMMR from a 10 mg  $\text{mL}^{-1}$  stock solution in PBS (pH 7.3 containing 0.1 mM  $\text{Ca}^{2+}$  and 0.1 mM  $\text{Mn}^{2+}$ ), and incubated at 37  $^{\circ}\text{C}$  for 1 h. The plates were washed three times with PBST as described above and 100  $\mu\text{L}$  of a solution of biotinylated anti-human MMR antibody (0.2 mg  $\text{mL}^{-1}$ ; R&D Systems) in PBS was added in each well, and the plates were further incubated for 1 h at 37  $^{\circ}\text{C}$ . The complex NeutrAvidin®-biotinylated HRP was preformed separately by successively adding to Tris buffer (9.6 mL, 50 mM, pH 7.6) a solution of NeutrAvidin® (100  $\mu\text{g mL}^{-1}$  in Tris buffer, 1.2 mL; Thermo Scientific) and a solution of biotin-conjugated HRP (25  $\mu\text{g mL}^{-1}$  in Tris buffer, 1.2 mL; Thermo Scientific). The mixture was shaken for 30 min at rt and the solution was immediately transferred into the plates (60  $\mu\text{L}$  per well). After 1 h at 37  $^{\circ}\text{C}$ , these plates were washed twice with Tris (250  $\mu\text{L}$  per well) and ABTS (0.25 mg  $\text{mL}^{-1}$ , 50  $\mu\text{L}$  per well) in citrate buffer (0.2 M, pH 4.0 with 0.015%  $\text{H}_2\text{O}_2$ ) was added. After 5 min at rt, the optical density was measured at 415 nm. Blank wells were processed with anti-human MMR antibody as well as NeutrAvidin®-biotinylated HRP. The concentration of rhMMR that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. For the competitive lectin binding inhibition experiment, (ManS) $_3$ - $\beta$ CD was mixed in a serial of 2-fold dilutions (60 mL per well) in HEPES buffer (20 mM, pH 7.4) with 60 mL of the appropriate rhMMR concentration in PBS buffer on Nunclon® (Delta) microtitre plates and incubated for 1 h at 37  $^{\circ}\text{C}$ . The above solutions (100  $\mu\text{L}$ ) were then transferred to the mannan-coated titer plates, which were incubated for 1 h at 37  $^{\circ}\text{C}$ . The plates were washed and the solution of biotinylated anti-human MMR antibody in PBS (100  $\mu\text{L}$ ) was added in each well, and the plates were further incubated for 1 h at 37  $^{\circ}\text{C}$ . Then the NeutrAvidin® solution was transferred into the plates (60  $\mu\text{L}$  per well). After 1 h at 37  $^{\circ}\text{C}$ , these plates were washed twice with Tris (250  $\mu\text{L}$  per well) and ABTS was added (50  $\mu\text{L}$  per well). Optical density at 415 nm was determined after 5 min.

The percentage of inhibition was calculated as follows:

$$\% \text{ Inhibition} = [A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}] / A_{(\text{no inhibitor})} \times 100$$

Results in triplicate were used for plotting the inhibition curves for each individual ELLA experiment. Typically, the  $\text{IC}_{50}$  values (concentration required to achieve 50% inhibition of the lectin association to the coating polysaccharide) obtained from several independently performed tests were in the range of  $\pm 15\%$ . Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible. The inhibition for methyl  $\alpha$ -D-glucopyranoside were included as positive and negative controls, respectively.

## Determination of mice macrophage adhesion

For evaluation of the interaction with macrophages, the procedure reported by Muller and Schuber<sup>37</sup> for mannosylated liposomes was adapted. Briefly, resident peritoneal macrophages were obtained from female Balb/c mice (6 to 8 weeks old) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% decomplemented fetal calf serum (FCS) containing heparin (5 U  $\text{mL}^{-1}$ ). The cell number was adjusted to  $10^6$  cells  $\text{mL}^{-1}$  and the suspension was plated (final volume 1 mL) in multiwell plates. After 2 h under a humidified atmosphere of 5%  $\text{CO}_2$  in air (final pH 7.4), non-adherent cells were eliminated by rinsing the dishes three times with PBS. The adherent cells, 24 h after their isolation, were fed with fresh serum-less DMEM and incubated with different amounts of TNS:(ManS) $_3$ - $\beta$ CD complex. TNS: $\beta$ CD complex was used as control. After the incubation time, the medium was pipetted-off and the cells washed four times with cold PBS (4  $^{\circ}\text{C}$ ). TNS associated with the cells was determined fluorimetrically (JASCO fluorimeter, model FP-715, excitation at 308 nm and monitoring emission at 443 nm) after cell digestion in 1 mL PBS containing 0.1% of emulphogene BC-720, and scraped with a rubber policeman. Standard fluorescence curves were established under the same conditions with aliquots of the initial TNS:(ManS) $_3$ - $\beta$ CD and TNS: $\beta$ CD preparations in order to correlate the measured fluorescence intensity with the amount of CD derivative. Experiments were performed in duplicate, and results did not differ more than 5%.

## Human macrophage internalization monitoring by fluorescence microscopy

THP-1 human monocytic cells were cultured in RPMI medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37  $^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. Cells were seeded onto 6-well plates at  $1.5 \times 3 \times 10^6$  cells per well. THP-1 monocytic cells were differentiated into macrophage-like cells by phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) incubation at a final concentration of 100 ng  $\text{mL}^{-1}$  for 3 d and it was followed by 1 d in PMA-free medium before treatments.

THP-1 macrophages were grown on 1 mm (Goldseal No. 1) glass coverslips for 24 h in RPMI containing 10% fetal bovine serum. After treatment, cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min, and permeabilized in 0.1% saponin for 5 min. For nuclei staining, glass coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33342 (1 mg  $\text{mL}^{-1}$ ) and washed with PBS (three 5 min washes). Finally, the coverslips were mounted onto microscope slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). 3D projections were performed using a Delta-Vision system (Applied Precision; Issaquah, WA) with an Olympus IX-71 microscope (Shinjuku, Japan) with 100 $\times$  objective/1.35 NA and filters set for DAPI, fluorescein isothiocyanate provided by Applied Precision. Acquired z planes were separated by 0.3  $\mu\text{m}$ , and an



average of 50 planes was taken for each cell. The 3D stacks were subjected to Quick Projection using the Softworx software. This allows confirming that the fluorescence is located inside the cell and not just in the cell membrane. Quantification of fluorescence signal was performed in 200 cells using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

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