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Mechanistic Insight into Binding of Multivalent Pyrrolidines to α-Mannosidases


Abstract: Novel pyrrolidine-based multivalent iminosugars, synthesized by a CuAAC approach, have shown remarkable multivalent effects towards jack bean α-mannosidase and a Golgi α-mannosidase from Drosophila melanogaster, together with a good selectivity with respect to a lysosomal α-mannosidase, important for anticancer applications. STD-NMR and molecular modelling studies support a multivalent mechanism with specific interactions of the bioactive iminosugars with jack bean α-mannosidase. TEM studies suggest a binding mode involving the formation of aggregates resulting from the intermolecular cross-linked network of interactions between the multivalent inhibitors and two or more dimers of JBMan subunit heterodimers.

Introduction

During the last decades, extensive chemical efforts have been devoted to the design and synthesis of monovalent inhibitors targeting carbohydrate-processing enzymes, with only few examples in terms of marketed drugs. More recently, the concept of multivalency has emerged as an appealing and alternative strategy to the traditional lock-key approach to access new selective and potent inhibitors.¹ A specific response can be achieved by multiple interactions of the multivalent ligand with the receptors, with consequent impressive increase in the binding affinity. This phenomenon, known as “the multivalent effect”, leads to high affinity enhancement of a multivalent ligand toward a target, if compared with the monovalent counterpart.² Multivalency has been largely exploited in the field of lectins, where the presence of several carbohydrate-binding sites on the protein surface has inspired the design of a variety of glyoclusters with excellent binding affinity.³

In sharp contrast, the use of multivalent-based strategy has been less explored in glycosidase inhibition, due to the monomeric nature of many of these carbohydrate-processing enzymes. Nevertheless, affinity enhancements can also be expected for enzymes with a single active site presentation, since the high local concentration of the active moiety on the multivalent scaffold, close to the recognition site, may favor a mechanism of recapture, called “statistical rebinding”. Despite of the earliest disappointing results in glycosidase inhibition by multivalent ligands,⁴ the first example of a significant multivalent effect, observed for a trivalent deoxynojirimycin (DNJ) conjugate against the jack bean α-mannosidase (JBMan) in 2009,⁵ prompted investigations for the design of other multivalent ligands with increased valency, with the aim of reaching high levels of glycosidase affinity.⁶ Among glycosidases, α-mannosidases are the most investigated enzymes for multimeric inhibition. This has been influenced by the availability of commercial JBMan; the sequence similarities between JBMan and other two mannosidases of therapeutic interest, i.e. Golgi α-mannosidase (GMI), and lysosomal α-mannosidase (bLAM),⁷ all belonging to glycoside hydrolase (GH) 38 family; and the multimeric nature of JBMan that, in principle, can be targeted by a multivalent ligand.⁸

However, the origin of the multivalent effect is far to be unequivocally elucidated and several mechanisms have been proposed and can be used to explain the phenomenon (Figure 1). Besides the statistical rebinding effect (Figure 1a), the chelate effect can occur when the enzyme presents more than one active site (Figure 1b). In addition to the interactions of a branch of the multivalent into the active pocket, additional unspecific interactions can involve non-catalytic subsites leading to stronger interactions (Figure 1c). Alternatively, the occlusion of the active site through unspecific interactions may be plausible with more hindered ligands that are not able to enter into the active cavity (Figure 1d). Moreover, a clustering effect can occur for large size ligands, through the binding of more than one enzyme at the same time (Figure 1e), or through the formation of cross-linked networks if the enzyme possesses a multimeric nature (Figure 1f).

Supporting information for this article is given via a link at the end of the document.((Please delete this text if not appropriate))
Figure 1. Proposed binding models accounting for the multivalent effect. The interactions described may also include possible additional intermolecular contacts provided by the employed linkers.

The large and open catalytic site of GH38 α-mannosidases is optimal for a recapture mechanism (Figure 1a). Moreover, JBMan is supposed to be organized into a dimer of heterodimeric subunits, for a total weight of around 220 kDa. Each heterodimer consists of a largest subunit (66 kDa) and a smallest one (44 KDa), this latter harboring the active site. For this reason, JBMan may be suitable for a chelate effect (Figure 1b) and for the formation of aggregates involving more than one enzyme (Figure 1f). Several studies have been carried out to evaluate the stoichiometry of the binding and to prove the formation of aggregates. Isothermal Titration Calorimetry (ITC) experiments of several DNJ-based multivalent ligands revealed that the valency and the topology have no effect on the enthalpy and entropy contributions and complexes with a ratio close to 1:1 were observed.

In contrast to these results, Atomic Force Microscopy (AFM) studies of JBMan with DNJ multivalent compounds based porphyrin, calix[4]arene and cyclodextrin scaffolds clearly showed the formation of nanoassemblies of different shape and sizes depending on the compound. The formation of aggregates was also confirmed by Dynamic Light Scattering (DLS) experiments. Large aggregates of 500 nm were formed in the presence of the multimeric ligands, in contrast with a hydrodynamic diameter of JBMan alone of 10 nm.

More recently, Compain and co-workers proposed a model of binding for a 48-valent DNJ-based ligand with JBMan employing three different techniques: analytical ultracentrifugation, Transmission Electron Microscopy (TEM) and ESI-MS. The results supported the formation of aggregates ligand/JBMan with a chelation of the ligand to four active sites (one for each heterodimer).

We report herein our contribution to the field by means of a multidisciplinary integrated approach that takes advantage of the total synthesis of new multivalent pyrrolidine based inhibitors, their biological evaluation towards different mannosidases including human enzymes, TEM techniques and unprecedented NMR and molecular dynamic studies.

Results and Discussion

Synthesis

Most of the multivalent iminosugars investigated as JBMan inhibitors and reported to date bear DNJ or deoxymannojirimycin (DMJ) as the bioactive iminosugar moieties. While the influence of different scaffolds, topology and valency on JBMan inhibition has been widely investigated, the effects of differently structured iminosugars are almost unexplored. Exploiting the expertise of some of us in the total synthesis of iminosugar-based inhibitors, we decided to explore different multivalent architectures based on the natural occurring pyrrolidine iminosugar 1,4-dideoxy-1,4-imino-D-arabinitol (DAB-1). DAB-1 is known to be an active compound with a broad inhibitory spectrum towards mammalian glycosidases.

Scheme 1. Synthesis of DAB-1 (2), the nonavalent compound 6 and the monovalent reference compound 5.

We recently reported the use of DAB-1 in the construction of the nonavalent 6 (Scheme 1), which showed an impressive activity towards N-acetylgalactosamine-6-sulfatase (GALNS), the enzyme deficient in the mucopolysaccharidosis Morquio A syndrome.
Taking into consideration that GALNS (like JBMan) has a dimeric nature, as revealed by its X-ray structure,\textsuperscript{18} we hypothesized that JBMan might also well accept multivalent inhibitors based on DAB-1. Nonavalent compound \textit{6} was synthesized from D-arabinose derived nitrone \textit{1}\textsuperscript{19} (Scheme 1), as previously reported.\textsuperscript{17} We synthesized the monovalent compound \textit{5} as the reference compound, bearing the same alkyl chain of a single branch of the multivalent ligands, to evaluate the enzyme affinity enhancement of the multivalent compounds. In this case, we slightly modified the published protocol, performing the copper catalyzed azide alkyne cycloaddition (CuAAC)\textsuperscript{20} with 3-butyne-1-ol directly on the deprotected azide \textit{4} instead of using the benzylated derivative \textit{8} as described in ref.17. The CuAAC reaction, performed with CuSO\textsubscript{4} (0.3 equiv.)/sodium ascorbate (0.6 equiv.), using a THF/H\textsubscript{2}O 2:1 mixture as the solvent under MW irradiation at 80 °C for 45 minutes, afforded the monovalent ligand \textit{5} in 89% yield (Scheme 1). This new strategy improved the overall synthesis of compound \textit{5} from nitrone \textit{1} (82%), and diminished the number of steps from five to three.

\begin{center}
\textbf{Scheme 2. Synthesis of the benzylated tetravalent compound \textit{10}.}
\end{center}

Initial attempts to synthesize tetravalent DAB-1 based inhibitor considered the benzylated iminosugar \textit{10}, expected to be easier to handle and purify than the corresponding deprotected derivative. Thus, the benzylated derivative \textit{8} was obtained as described in ref. 17 by nitrogen alkylation of amine \textit{7}\textsuperscript{17,21} with 1-azido-6-bromohexane (3) (Scheme 2). The CuAAC reaction of the benzylated key azido intermediate \textit{8} (4 equiv.) with the tetravalent scaffold \textit{9}\textsuperscript{22} was performed with CuSO\textsubscript{4} (0.3 equiv.)/sodium ascorbate (0.6 equiv.), using a THF/H\textsubscript{2}O 2:1 mixture as the solvent in a MW reactor at 80 °C for 45 minutes, afforded the monovalent ligand \textit{5} in 89% yield (Scheme 1). This new strategy improved the overall synthesis of compound \textit{5} from nitrone \textit{1} (82%), and diminished the number of steps from five to three.

\begin{center}
\textbf{Scheme 3. Synthesis of the tetravalent compound \textit{11} and of trivalent compound \textit{13}.}
\end{center}

Analogously, compound \textit{4} (3 equiv.) was reacted with the trivalent scaffold tris[(propargyloxy)methyl]amino-methane (12), synthesized by a three step procedure starting from tris (hydroxymethyl)aminomethane.\textsuperscript{23} The CuAAC reaction of 12 with \textit{4} (3 equiv.) was performed as previously described for the tetravalent ligand \textit{10}, affording the trivalent iminosugar \textit{13} in 48% yield (Scheme 3), after purification by FCC and size exclusion chromatography.
Biological activity evaluation

The multivalent pyrrolidine iminosugars 6, 11 and 13, together with DAB-1 (2) and the monovalent pyrrolidine 5, were evaluated toward a panel of commercial glycosidases and the results are reported in Table 1. It is worth noting that most of the multivalent pyrrolidines showed good inhibitory activity towards α-glucosidases and especially amyloglucosidase in the low µM range, but for these enzymes no multivalent effect was highlighted, since their inhibition activity is comparable to that of the monovalent reference compound 5. More interestingly, remarkable results were shown by the multivalent pyrrolidine iminosugars toward JBMan. JBMan is able to hydrolyze the terminal α-1,2, α-1,3, α-1,6 linked mannoside residues from various glycoproteins. JBMan is a retaining enzyme and like other class II α-mannosidases, it is inhibited by the iminosugar swainsonine. Significant affinity enhancement was observed towards JBMan passing from the monovalent compound 5 to higher valencies, with best results obtained for the nonavalent compound 6.

<table>
<thead>
<tr>
<th>Glycosidases</th>
<th>DAB-1 (2)</th>
<th>Monovalent 5</th>
<th>Trivalent 13</th>
<th>Tetravalent 11</th>
<th>Nonavalent 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-L-fucosidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>bovine kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-galactosidase</td>
<td>-</td>
<td>-</td>
<td>61</td>
<td>55</td>
<td>75</td>
</tr>
<tr>
<td>coffee beans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-glucosidase Saccharomyces cerevisiae</td>
<td>99 (1.2)</td>
<td>83 (153)</td>
<td>94 (37)</td>
<td>84 (198)</td>
<td>72</td>
</tr>
<tr>
<td>rice</td>
<td>85 (208)</td>
<td>24</td>
<td>72</td>
<td>72</td>
<td>59</td>
</tr>
<tr>
<td>amyloglucosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>96 (16)</td>
<td>&gt;90 (8.1)</td>
<td>98 (1.8)</td>
<td>&gt;90 (1.5)</td>
<td>&gt;90 (1.1)</td>
</tr>
<tr>
<td>β-glucosidase almonds</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-mannosidase jack bean</td>
<td>74 (376)</td>
<td>43 (1300)</td>
<td>&gt;90 (9.4)</td>
<td>&gt;90 (34)</td>
<td>&gt;90 (0.095)</td>
</tr>
<tr>
<td>β-mannosidase snail</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-N-acetylglucosaminidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- no inhibition was detected at 1 mM concentration of the compound.

To quantify the multivalent effect toward JBMan, the relative inhibitory potencies (rp, given by the ratio of the IC50 values of the monovalent pyrrolidine 5 and that of the multivalent compounds) and the affinity enhancement per DAB motif (rp/n) were calculated and are reported in Table 2. For all pyrrolidine iminosugars 6, 11, and 13, a large multivalent effect was observed (rp/n >>1). The trivalent compound 13 and the tetravalent 11 showed a good multivalent effect on JBMan, with a rp/n of 46 and 10. The best result was shown by the nonavalent compound 6, with an impressive rp/n of 1520. This value is remarkable considering the data in the literature for compounds with similar valency, such as the previously reported dodecavalent DNJ-based fullerene (rp/n = 179). Curiously, the trivalent 13 is more active than 11 and this could be due to a higher similarity of 13 to nonavalent compound 6 in terms of spatial distribution of the bioactive moieties. Due to the very promising results towards JBMan, the multivalent pyrrolidine iminosugars 13, 11 and 6, as well as the monovalent compound 5, were also tested against the biological relevant class II Golgi and lysosomal α-mannosidases (GMIlb and LMα1I) from Drosophila melanogaster as a model of target enzymes in anticancer therapy and lysosomal storage disorders treatment, respectively. Indeed, reduction of the tumor growth and cell metastasis have been shown by inhibition of the Golgi α-mannosidase II by the iminosugar swainsonine, while a deficiency in lysosomal α-mannosidases is observed in the inherited disorder mannosidosis. The results are reported in Table 2.

A multivalent effect was observed towards GMIlb, since the affinity enhancement per DAB motif was higher than 1 (rp/n>1) for all the multivalent compounds and was in the range of 40 to 80. In contrast, multivalent compounds 13 and 11 were more active than 5 towards LMα1I, although no significant variation in the rp/n values was observed in dependence of the valency (rp/n values not higher than 3.5 in the best case). These results underline a remarkable selectivity of our compounds for GMIlb over LMα1I. Similar behaviour has been reported for other multivalent iminosugars based on DNJ confirming that LMα1I cannot be targeted by hindered ligands and does not take advantage of multivalent presentation of iminosugars. On the other hand, the interesting selectivity towards GMIlb over LMα1I appears particularly relevant for a selective application of the multivalent compounds in anticancer therapy, without the side effect of mannosidosis syndrome.
Finally, in agreement with the collected results towards commercial glycosidases, an interesting inhibition profile was observed with human α-mannosidase evaluated in an extract from human leukocytes. Indeed, while a 55% inhibition was observed at 5 mM inhibitor concentration for the monovalent compound 5, the multivalent compounds showed percentages of inhibition higher than 80% at 1 mM inhibitor concentration (namely, 91% inhibition for the trivalent 13, 96% inhibition for the tetravalent 11 and 80% inhibition for the nonavalent 6).

**NMR studies**

Understanding the mechanism of inhibition of multivalent ligands towards the enzymes represents an important goal in order to expand the knowledge on the protein target and to help synthetic organic chemists in the rational design of new highly active and selective inhibitors. With this aim, we decided to investigate the binding mode of our multivalent pyrrolidine iminosugars to JBMan by a combined NMR/molecular modeling protocol.

In consideration that the active site amino acids are highly conserved in GH38 α-mannosidases family, and due to the similarity in biological activity to the important therapeutic target Golgi α-mannosidase II (GMII, CG18474), JBMan has been often used as a model enzyme for studies with inhibitors.

Among the NMR techniques, STD-NMR (Saturation Transfer Difference-NMR) represents a useful experiment to characterize the binding in tightly bound ligand-protein complexes. STD-NMR technique has been used to gain new insights into the binding mechanism between oligosaccharides or glycomimetics and different receptors, including lectins and antibodies. In the field of glycosidases, STD-NMR experiments have been employed to try to understand the interactions between recombinant canine α-glucosidase and various piperidine iminosugar inhibitors, and to determine the role of the aromatic rings of carbasugar inhibitors in their interaction with α-glucosidase from Saccharomyces cerevisiae. By combining STD-NMR, τNOE and molecular modeling experiments, information about the key features in glycoside recognition of β-glucosidase from rice and β-mannosidase from barley was obtained for glucose- and mannose-configured substrates. Moreover, a combined STD-NMR/molecular modeling protocol has been applied for predicting the binding modes of two glycomimetic inhibitors to GMII.

### Table 2. Relative potency (rp) and relative potency per active unit (rp/n) of the tri-, tetra- and nonavalent pyrrolidines 13, 11 and 6 towards commercial JBMan, and recombinant GMIIb and LManII. IC₅₀ [µM] were reported.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Valency</th>
<th>JBMan rp (rp/n)</th>
<th>GMIIb IC₅₀</th>
<th>LManII rp (rp/n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>0.25</td>
<td>700 (78)</td>
<td>n.d.</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>138 (46)</td>
<td>1.4</td>
<td>125 (42)</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>38 (10)</td>
<td>0.95</td>
<td>184 (46)</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>13684 (1520)</td>
<td>0.25</td>
<td>700 (78)</td>
</tr>
</tbody>
</table>

However, to the best of our knowledge, no NMR investigation has focused on the interactions of multivalent iminosugars with either JBMan or any glycosidase to date.

In order to investigate the multivalent binding and to obtain the epitope mapping of our ligands, the monovalent pyrrolidine 5, DAB-1 (2), and the tri- 13, tetra- 11 and nonavalent 6 ligands were incubated with commercial JBMan at pH 5 and the STD-NMR spectra were acquired. The experiments were performed with different enzyme/ligand ratios depending on the type of ligand, in order to obtain an STD spectrum with good quality. Indeed, for ligands with a lower affinity, such as the monovalent 5 and DAB-1 (2), a high enzyme/ligand ratio was required (1:100 and 1:50, respectively). Good STD of the complexes with the tri- 13, tetra- 11 and nonavalent 6 were obtained with JBMan/multivalent ratios of 1:100, 1:10 and 1:10, respectively. The STD-NMR spectra were acquired at room temperature, except for the monovalent ligand, the weakest inhibitor, for which the temperature was decreased to 10 °C, to slow the off rate of the complex and maximize the saturation transfer. The obtained STD spectra of the complex with DAB-1 (2) and with the nonavalent iminosugar 6 are reported in Figure 2 (for the whole set of STD spectra, see the Supporting Information file.)
The calculation and analysis of the STD intensities of all protons in spectra of compounds 2, 5, 6, 11 and 13 allowed to map the epitope. A graphical representation is reported in Figure 3a. The highest STD (60-100% relative STD enhancement) is due to H-4 and H-3 and to the protons in the middle of the chain (H-9, H-10) for the monovalent and multivalent ligands. It is noteworthy that the same trend in the STD intensities for the protons of the pyrrolidine ring and for the chain is present in all the ligands, as shown in Figure 3b. In light of these results, it is evidenced that the pyrrolidine ring of the monovalent and multivalent ligands interacts with JBMan in the same way of DAB-1, and that the binding interactions of the multivalent iminosugars are well represented by the monovalent ligand.

These data show the existence of specific interactions of the ligands with JBMan, which presumably take place within the enzyme active site. This is also in agreement with the inhibition mode found for the whole set of compounds, showing competitive or mixed type inhibition in all cases (see the Supporting Information file).

These experiments suggest that a recognition mechanism exclusively based on non-specific interactions of the multivalent ligands with other parts of the enzyme can be discarded (binding mode (d) of Figure 1). Conversely, a binding mode involving both active site and non-catalytic subsites cannot be completely excluded (binding mode (c) of Figure 1).

To expand our knowledge on the conformation in solution of the pyrrolidine ring of the ligands in their free and JBMan-bound states, 2D-NOESY and tr-NOESY were acquired. The obtained results were compared to those expected for the computed conformation of the monovalent compound 5. From the analysis of the acquired 2D-NOESY of the free ligands (see Supporting Information file), we observed the presence of the same cross-peaks in the monovalent (5) and multivalent ligands (13, 11 and 6). This evidence suggests, as expected, that the pyrrolidine ring maintains the same conformation in all the ligands. Moreover, we observed the presence of a strong NOE effect between H-3 and H-4 in the five-membered ring in all compounds, and a small J coupling between these two protons in the ‘H-NMR spectra (H-3 and H-4 appear as narrow signals). These experimental results, together with the analysis of the pyrrolidine conformations of the monovalent 5 generated by the computational program Maestro 10.7, suggested that the most stable conformation in solution is the °E. This pyrrolidine conformation was then used as input for molecular dynamics (MD) simulations of the monovalent ligand, as shown in Figure 4a. The tr-NOESY experiment of the tetravalent ligand 11 with the enzyme was recorded with a ratio JBMan:tetravalent = 1:10 in order to obtain the conformation of the bound pyrrolidine, (see Supporting Information file). The inversion of the cross-peak sign of the pyrrolidine ring is representative of the detection of the bound conformation, indicating that the ligand is bound to the protein. The presence of the strong NOE effect between H-3 and H-4 in the tr-NOESY, accounted for a major °E conformation also in the bound state. Interestingly, the disappearance of the NOEs between CH₂-6 and H-3 was observed in the tr-NOESY, suggesting a change in the orientation of the 6-CH₂OH when passing from the free to the bound form. Unfortunately, we did not find the correct conditions to acquire the tr-NOESY of the other ligands. Nevertheless, on the basis of the STD-NMR observations, it is highly plausible that the same °E geometries were also kept in their bound forms.

**Molecular dynamics (MD) studies**

To support our experimental data and to propose a binding mode of the ligands into the active site of the α-mannosidase, a molecular modeling approach was performed. The X-ray crystal structure of JBMan is not available yet. Nevertheless, the complete primary sequence, recently reported by Kumar et al, was used to check the homology between JBMan and other α-mannosidases. In particular, the sequence alignment was performed with two α-mannosidases with two described X-ray crystal structures, the lysosomal α-mannosidase from *Bos taurus* (bovine, bLAM⁶) and the Golgi α-mannosidase II from *Drosophila melanogaster* (fruit fly, GMII).⁶ Despite the low percentage of identity for the complete sequences, a significant homology was found for the active site regions. This region of JBMan showed with GMII a 61% identity and 86% of similarity. For bLAM, a 93% of identity and 100% of similarity was found (the sequence alignment is reported in the Supporting Information file). Moreover, by comparing these three different mannosidase sequences, it is noteworthy that the active site aminoacids⁶ His23, Asp25, Trp28, Tyr35, Asp145 (the catalytic residue), Arg170, Tyr210, Asp268, Phe269, Tyr325, Trp333, His385, His386, Asp387, Thr392 and Tyr625 are highly conserved in all the mannosidases. In addition, by superimposing the crystal structures of GMII and of bLAM, the same 3D orientation of the active site residues is maintained (see Supporting Information file). Indeed, many of these residues are involved in the interactions with the substrate and with the inhibitors in the GMII. Their presence in the other mannosidases also explains the broad inhibitory activity of swainsonine for all the class II α-mannosidases. The excellent observed match at the catalytic site (see below) allowed us to validate the use of the
available crystal structures of the GManII-inhibitor complexes as structural templates for the molecular dynamics simulations. This approach was also backed up by the biological tests described above, which showed a good affinity of the multivalent pyrrolidines towards GManII but not LManII.

The comparison of the binding modes of different competitive inhibitors with pyrrolidine, indolizidine and salicinol structures to GManII have been reported by Mol tessier et al.34 This analysis evidenced the importance of the hydroxyl groups in the interaction with the zinc ion into the active site of the enzyme. In particular, we chose the complexes of GManII with salicinol (PDB 1TQS)35 and the dihydroxypyrrolidine iminosugar reported by Vogel and co-workers36 (PDB 2F18) as models, due to the structure similarity with our ligand (see Supporting Information file). On the basis of these models, we then built the starting geometries for the possible binding modes of the monovalent pyrrolidine 5 (in the 4E conformation) with GManII. A manual docking approach was first employed, using the Discovery Studio 4.5 software, by superimposing the endocyclic heteroatoms and the hydroxy groups interacting with the zinc ion with those of our ligand (see Supporting Information file). MD simulations of all the generated complexes were run with Amber 12 for 20 ns, to evaluate if the hypothesized binding modes are possible in solution and to deduce the most likely one. The results of the MDs are reported in the Supporting Information file. The analysis of STD NMR results and some relevant structural features indicated that the most plausible mode is that reported in Figure 4a (Mode 1), obtained employing the GManII-dihydroxypyrrolidine iminosugar complex as initial model. First, we compared the expected STDs for this complex, generated by using the CORCEMA-ST matlab scripts,37 with the experimental ones. A very good agreement between the predicted and experimental STD values was observed (Figure 4b), strongly supporting the choice of this mode versus others (see Supporting Information file).

![Figure 4](image)

Figure 4. The complex of GManII with the monovalent pyrrolidine 5. (a) The selected binding mode (Mode 1), deriving from the analysis of the MD simulations. The monovalent ligand is presented in green and the zinc ion in magenta. The non-covalent interactions are represented with the green dotted lines, and the coordination to the zinc with the red lines. (b) comparison of the predicted and experimental STD intensities.

The analysis of the non-covalent interactions during the MD permitted to deduce the presence of an electrostatic interaction of the positively charged endocyclic nitrogen atom with the catalytic residue Asp204, maintained over 95% of the MD time. The role of this residue in the hydrolytic activity of the enzyme has been demonstrated by Howard et al.8b Indeed, its interaction with the endocyclic nitrogen of the ligands is a common feature of the competitive inhibitors of mannosidase enzymes. Two stable hydrogen bonds between O-4 of the monovalent ligand and Asp92, and between O-6 and Asp472 were also observed (100% of MD time). Moreover, in this binding mode, the O-4 and O-6 of the monovalent ligand coordinate the zinc ion, leading to its hexacoordination, which involves also His90, His471, Asp92 and Asp204. In particular, we noted that the CH2OH group in position 6 of ligand 5 rotates during the initial steps of the MD to afford the chelate zinc coordination. This result is in agreement with the experimentally observed change in the NOE interactions of H-6 in the tr-NOESY spectrum.

On the basis of these results for the monomer species, a MD simulation of the trivalent ligand 13, introduced into the active site in the binding mode 1 (Figure 5a) was also carried out. The aim was to verify whether the same interactions were possible for a multivalent ligand, where the steric hindrance may play an important role. A MD simulation of 10 ns was run and analyzed. The results showed that hexacoordination at zinc was kept during the MD, in agreement with the MD results of the monovalent ligand 5. Moreover, the same stable non-covalent interactions were observed and are shown in Figure 5b. These theoretical results are compatible with the same epitope mapping observed in the STD-NMR experiments for the monovalent and multivalent ligands, derived from the specific interaction of the pyrrolidine ring into the α-mannosidase active site. On the basis of these results, we can predict that multivalent ligands bearing longer alkyl chains may behave as more active inhibitors due to their easy entrance into the deeper active site of GManII as shown in Figure 5a.

![Figure 5](image)

Figure 5. (a) The complex of GManII with the trivalent ligand 13. (b) the binding mode 1 of 13 into the active site. The zinc coordination and the non-covalent interactions are shown with red lines and dotted green lines, respectively.

**TEM studies**

To obtain insights into the binding modes explaining the large multivalent effect observed, the JBMan-ligand complexes were also analyzed by Transmission Electron Microscopy (TEM) with negative staining. JBMan is a high molecular-weight metalloenzyme of about 220 KDa composed of two pairs of subunits of molecular weight of 66 and 44 KDa, respectively.
and is visible by TEM. For the mannosidase alone, the image clearly showed particles of approximately 10x20 nm (Figure 6a) matching the size of one dimer of JBMan subunit heterodimers, as previously reported. The complex of JBMan with the tetra- and nonavalent ligands (11 and 6) were analyzed by TEM (Figures 6b and 6c), after incubation of 1 hour at room temperature. In both complexes, several different arrangements were observed, involving two or more enzyme particles. Focusing on the size and shape of the aggregates, an “S” shape arrangement frequently appeared. These aggregates with the “S” shape may correspond to the interaction of one inhibitor with the active site of two different dimers of JBMan subunit heterodimers, as presented in the cartoon representation (Figure 6d). In other words, the “S” shape originates from the interaction of two molecules of inhibitor with three dimers of JBMan subunit heterodimers. This hypothesis seems highly plausible, since the maximum length of 3-4 nm of the ligands (measured with Discovery 4.5) limits its simultaneous binding to two different active sites of the same dimer (length of the JBMan particle of 20 nm), in contrast to the large 48-valent DJN ligand of Compain and co-workers. Consequently, to explain this particular arrangement of the particles, we suggest that the formation of aggregates derives from interactions of the ligand with two active sites of two different dimers of JBMan subunit heterodimers. However, it is worth noting that the dimensions of the ligands strongly affect the type and size of the JBMan aggregates.

Available glycosidases have highlighted a remarkable multivalent effect towards JBMan, with a highest rp/n = 1520 for the nonavalent compound 6. Moreover, the profile of inhibition was evaluated towards the biologically relevant LManII and GMIIb, showing maintenance of the multivalent effect with GMIIb, together with an interesting selectivity towards GMIIb over LManII. These observations open the way to the therapeutic application of these multivalent compounds as selective inhibitors of Golgi α-mannosidases. To investigate the binding mode of the multivalent inhibitors to α-mannosidases, a combined experimental/theoretical STD-NMR/MD analysis has also been carried out. This protocol allowed to assess that a specific interaction of these molecules into the glycosidase active site takes place. Finally, TEM studies shed light on the multivalent binding mode, showing the formation of ligand-JBMan aggregates, probably by an intermolecular cross-linking mechanism. The results of this integrated and multidisciplinary approach highlight key aspects for the design of new more active inhibitors. Indeed, we presume that both the length of the alkyl chain and the global size of the multivalent ligand may affect enzyme affinity and influence the type of aggregation between the ligands and the enzyme molecules. Work is underway in our laboratories in order to prove these hypotheses.

Figure 6. Electron microscopy images obtained for JBMan with negative staining. Representative areas show top, bottom, side and views. The scale bars drawn on each of the three micrographs are 50 nm long. (a) TEM picture of JBMan alone, (b) TEM for JBMan incubated for 1 hour with the tetravalent ligand 11, and with the nonavalent ligand 6. The aggregates are shown in the green squares. (d) Cartoon representation of the interaction of the tetravalent ligand with JBMan to explain the “S” shape of the aggregates.

Conclusions

A multidisciplinary approach has been employed to study a new variety of glycosidase inhibitors. Novel tri-, tetra- and nonavalent pyrrolidine iminosugars (13, 11 and 6) have been synthesized exploiting the CuAAC cycloaddition of an azido iminosugar intermediate with multivalency alkene scaffolds. The monovalent reference compound 5 has also been prepared for comparison. Biological inhibition assays towards a panel of commercially available glycosidases have highlighted a remarkable multivalent effect towards JBMan, with a highest rp/n = 1520 for the nonavalent compound 6. Moreover, the profile of inhibition was evaluated towards the biologically relevant LManII and GMIIb, showing maintenance of the multivalent effect with GMIIb, together with an interesting selectivity towards GMIIb over LManII. These observations open the way to the therapeutic application of these multivalent compounds as selective inhibitors of Golgi α-mannosidases. To investigate the binding mode of the multivalent inhibitors to α-mannosidases, a combined experimental/theoretical STD-NMR/MD analysis has also been carried out. This protocol allowed to assess that a specific interaction of these molecules into the glycosidase active site takes place. Finally, TEM studies shed light on the multivalent binding mode, showing the formation of ligand-JBMan aggregates, probably by an intermolecular cross-linking mechanism. The results of this integrated and multidisciplinary approach highlight key aspects for the design of new more active inhibitors. Indeed, we presume that both the length of the alkyl chain and the global size of the multivalent ligand may affect enzyme affinity and influence the type of aggregation between the ligands and the enzyme molecules. Work is underway in our laboratories in order to prove these hypotheses.

Experimental Section.

Synthesis.

General methods: Commercial reagents were used as received. All reactions were carried out under magnetic stirring and monitored by TLC on 0.25 mm silica gel plates (Merck F254). Column chromatographies were carried out on Silica Gel 60 (32-63 µm) or on silica gel (230-400 mesh, Merck). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. 1H NMR spectra were recorded on a Varian Mercury-400 or on a Varian INOVA 400 instrument at 25 °C. 13C NMR spectra were recorded on a Varian Gemini-200 or on a Varian Gemini-300. Chemical shifts are reported relative to TMS (1H: δ = 0.00 ppm) and CDCl3 (13C: δ = 77.0 ppm). Integrals are in accordance with assignments, coupling constants are given in Hz. For detailed peak assignments 2D spectra were measured (COSY, HSQC, NOESY, and NOE as necessary). Small scale microwave assisted syntheses were carried out in a CEM Discover microwave apparatus for synthesis with an open reaction vessel and an external surface sensor. IR spectra were recorded with a BX FT-IR Perkin-Elmer System spectrophotometer. ESI-MS spectra were recorded with a Thermo Scientific™ LCQ Fleet Ion Trap Mass Spectrometer. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter.

Synthesis of monovalent compound 5: To a solution of 4 (80 mg, 0.31 mmol) in 6 ml of 2:1 THF/H2O, CuSO4 (30 mol%, 15 mg, 0.09 mmol), sodium ascorbate (60 mol%, 37 mg, 0.19 mmol) and 3-butyln-1-ol (28 µL, 0.27 mmol) were added. The reaction mixture was stirred in microwave at 80 °C for 45 min, until a TLC analysis (CH2Cl2/MeOH 6:1 + 1% v/v 6% NH4OH) showed the disappearance of the starting material (Rf = 0.29) and the formation of a new product (Rf = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by FCC (CH2Cl2/MeOH 1:1 + 1% v/v 6% NH4OH) affording pure 5 (Rf = 0.24, 91 mg, 0.28 mmol, 89% yield) as a yellow oil. [α]D23 = -15.7 (c = 0.21 in MeOH); 1H-NMR (400 MHz, D2O): δ = 7.69 (s, 1H, H-Trizole), 4.26 (t, J = 6.8 Hz, 2H, H-12), 4.04-4.03 (m, 1H, H-4), 3.85-3.83 (m, 1H, H-3), 3.72 (t, J = 6.5 Hz, 2H, CH2OH), 3.64 (d, J = 6.4 Hz, 2H, H-6), 3.04 (d, J = 12.2 Hz, 1H, Ha-5), 2.36-2.76 (m, 2H, Ha-5 and Ha-7), 2.80 (t, J = 6.4 Hz, 2H, CH2CH2OH), 2.68-2.62 (m, 1H, H-2),
2.45-2.39 (m, 1H, H-7), 1.76 (quint, 3J(H,H)=6.4 Hz, 2H-11), 1.46-1.32 (m, 2H, H-8), 1.24-1.08 (m, 4H, H-9 and H-10); 13C-NMR (50 MHz, D2O): δ = 145.2 (s, C-Triazole), 123.8 (d, C-Triazole), 78.3 (d, C-3), 75.0 (d, C-4), 72.8 (d, C-2), 60.6 (t, CH2OH), 60.5 (t, C-6), 58.4 (t, C-5), 55.6 (t, C-7), 50.2 (t, C-12), 29.1 (t, C-11), 27.8 (t, CH2CH2O), 25.9, 25.8, 25.2 (t, 3C, C-8, C-9 and C-10); MS (ESI): m/z 329.17 [M+Na]+ (94); 351.10 [M+Na]+ (100); elemental analysis calcul (%) for C36H3N6O10Si (628.41): C 54.66, H 5.87, N 17.09.

Synthesis of benzylated tetratetral 10: To a solution of 8 (104 mg, 0.196 mmol) in 3 mL of a 2:1 THF/H2O mixture, CuSO4 (30 mol%, 2 mg, 0.015 mmol), sodium ascorbate (60 mol%, 6 mg, 0.03 mmol) and 9 (14 mg, 0.049 mmol) were added. The reaction mixture was heated in MW reactor at 80 °C for 45 min, until TLC analysis (PE:AcOEt 3:1) showed disappearance of the starting material (Rf = 0.59) and formation of a new product (Rf = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (MeOH/CH2Cl2/NaHCO3 4:3:1) and Size Exclusion Chromatography Sephacryd LH-20 (eluting with H2O) affording pure 13 (102 mg, 0.11 mmol, 48%) as a yellow oil. Rf = 0.79 (MeOH/CH2Cl2/NaHCO3 4:3:1) [α]D25 = +5.7 (c = 0.21, MeOH); 1H-NMR (500 MHz, D2O) δ ppm: 7.82 (s, 3H; H-Triazole), 4.43 (s, 3H; OCH2Triazole), 4.25 (t, 3J(H,H)=6.8 Hz, 6H; H-12), 4.09-0.48 (m, 3H; H-4), 3.88 (t, 3J(H,H)=2.9 Hz, 3H; H-3), 3.70-3.68 (m, 6H; H-6), 3.31 (s, 3H; CH2O), 3.21-3.18 (m, 3H; H-5a), 3.00-2.87 (m, 3H; H-7a), 2.98 (dd, 3J(H,H)=11.7 Hz, 3J(H,H)=4.9 Hz, 3H; H-5b), 2.89 (q, J = 5.2 Hz, 3H; H-2), 2.65-2.58 (m, 3H; H-7b), 1.73 (quint, 3J(H,H)=7.3 Hz, 6H; H-11), 1.48-1.38 (m, 1H; H-8), 1.22-1.06 (m, 12H; H-9 and H-10); 13C-NMR (50 MHz, D2O) δ ppm: 143.9 (s, 3H; C-Triazole), 124.7 (d, 3C; C-Triazole), 77.4 (d, 3C; C-3), 74.5 (d, 3C; C-4), 76.3 (d, 3C; C-2), 69.7 (t, 3C; CH2O), 63.6 (t, 3C; CH2CH2O), 59.7 (3C; C-6), 58.4 (t, 3C; C-5), 56.5 (s; CCHO), 55.9 (t, 3C; C-7), 50.2 (t, 3C; C-12), 29.1 (t, 3C; C-11), 25.5 (3C; C-8), 25.4, 25.1 (t, 6C; C-9 and C-10); MS (ESI): m/z calcul (%) for C46H39N10O12SilNa+: 1032.63 [M+Na]+; found 1032.75; elemental analysis calcul (%) for C36H3N6O10Si (1010.23): C 54.69, H 8.28, N 18.02; found C 54.48, H 7.93, N 17.84.

Biological tests

Inhibition studies with commercial enzymes. The percentage of inhibition towards the corresponding glycosidase was determined in the presence of 1 mM of the inhibitor on the well. Each enzymatic assay (final volume 0.12 mL) contains 0.01 to 0.5 units/mL of the enzyme and 10 mM aqueous solution of the appropriate p-nitrophenyl glycopyranoside (substrate) buffered to the optimal pH of the enzyme (citric/phosphate buffer solutions). Enzyme and inhibitor were preincubated for 5 min at rt, and the reaction started by addition of the substrate. After 20 min of incubation at 37 °C, the reaction was stopped by addition of 0.1 mL of sodium borate solution (pH 9.8). The p-nitrophenol formed was measured by visible absorption spectroscopy at 405 nm. Under these conditions, the p-nitrophenolase released led to optical densities linear with both reaction time and concentration of the enzyme. For the best inhibitors, the IC50 value (concentration of inhibitor required for 50% inhibition of enzyme activity) was determined from plots of % inhibition versus inhibitor concentration. Each experiment (% IC50) is performed by duplicate and the average value is given.

Enzyme assay of recombinant GMIB and LManI. Mannosidase activity of enzyme preparations were measured using p-nitrophenyl-a-D-mannopyranoside (pNP-Man; Sigma) as a substrate at 2 mM concentration (by dilution from a 100 mM stock in dimethylsulfoxide) in 50 mM acetate at optimal pH of the enzymes (GMIB at pH 6.0 and LManI at pH 5.2) and 0.5 μL of the enzyme in a total volume of 50 μL for 1–2 h at 37 °C. GMIB was assayed in the presence of 0.5 mM CoCl2. The inhibitor was preincubated with the enzyme in the buffer for 5 min at room temperature and the reaction was started by addition of the substrate. The reactions were terminated with two volumes (0.1 mL) of 0.5 M sodium carbonate and the production of p-nitrophenol was measured at 405 nm using a multimode reader Mithras LB943 (Berthold Technologies). The representative IC50 plot and the average results of at least three independent experiments made in duplicate are presented.

Inhibition studies with human α-mannosidase. The enzymatic activity was evaluated by setting up the test in triplicate in a flat-bottomed 96 well plate. Incubation: 3 μL of inhibitor solution, 7 μL of leukocytes homogenate from healty donor (0.3 mg/mL) and 20 μL of substrate solution (methylumbelliferyl-α-D-mannopiranoside) in phosphate/citrate buffer (0.2 M/0.1 M, pH 4.0) for 1 h at 37 °C. At the end of the incubation the reaction is stopped by addition of 200 μL of stop buffer (NaHCO3:NaOAc 0.5M pH 7.0 + Triton X-100 0.025%). Measurement: After adding to the plate the points of the standard curve with free methylumbellifere the fluorescence is measured in SpectraMax M2 microplate fluorescence is
measured in SpectraMax M2 microplate reader (Molecular-Devices) using an excitation wavelength of 365 nm and an emission wavelength of 435 nm. The percentage of α-mannosidase inhibition is given with respect to the control (without inhibitors).

**NMR experiments**

All NMR experiments were recorded on a 800 MHz AVANCE-III Bruker spectrometer equipped with standard triple-channel probe. All NMR spectra were processed with the software TopSpin. The samples for NMR experiments were prepared in 100 mM Acetate buffer pH 5.0 with 5 mM ZnCl₂ in D₂O. Typical concentrations and buffer conditions were 2 mM and 100 mM acetate buffer pH 5.0 with 5 mM ZnCl₂ in D₂O. The Jack Bean α-Mannosidase (JBMan) was purchased from Sigma Aldrich as an ammonium sulfate suspension and the buffer was changed to 100 mM sodium acetate, 5 mM ZnCl₂ pH 5 in D₂O by centrifugation through a 10 kDa cut-off Amicon filter. NMR tubes with 3 mm diameter were used. The samples for the STD experiments were prepared using ligand/ligand ratios varying from 1:10 to 1:100, with protein concentration of 20-30 μM. The STD-NMR spectra were acquired at the temperature of 283 K for the free ligand and 298 K for DAB and the multivalent ligands. The proton water signals were suppressed with an excitation sculpting module with gradients. Selective saturation of the protein resonances (on-resonance spectrum) was performed by irradiating at 0.15 ppm, using a series of 30 ms PC9 pulses, for a total saturation time of 2.0 s. A spin-lock filter was used to remove the NMR signals of the protein background. The reference spectrum (off-resonance) was obtained by irradiation at 9 100 ppm. To determine the epitope mapping of each ligand, the STD intensities were calculated with the program TopSpin, using the off-resonance spectrum as reference and, then, were normalized by attributing the value of 100 % to the ligand signal with the highest response. The STD signal of the methylene group near the triazole (CH₂-12) was affected by the water suppression and so, not considered in the analysis. Samples for the 2D-NOESY and tNOESY experiments were prepared in acetate buffer pH 5 in D₂O. 2D-NOESY experiments were acquired at 2 mM ligand concentration. tNOESY experiments were performed at 30 μM protein concentration with protein/ligand molar ratios of 1:1. Spectra were obtained with the standard NOESY sequences using mixing times of 400 ms for the free ligand and 200 ms for the tNOESY experiments.

**Computational methods**

**Conformational search.** An ensemble of low-energy conformers of ligand 5 was generated using a molecular mechanics conformational search protocol in MacroModel. Ligand 5 was submitted to a conformational search with a low gradient convergence threshold (0.05) in 2500 steps. The mixed torsional/low-mode sampling method and the MM2 force field in water were used, as integrated in the MAESTRO (Schrodinger) suite of programs.

**MDs with Amber 12.** The complexes of the monovalent ligand 5 and GMII (PDB codes 2F18 and 1TQ5), obtained by manual docking with Discovery Studio 4.5, were adjusted with Antechamber, adding the van der Waals parameters, employing the GAFF and FF12SB force field model and parametrized, introducing the Van der Waals parameters in LEAP program. The starting 3D structures were placed into a 12 Å cubic box of explicit TIP3P waters, and six sodium counterions were added to neutralize the systems. Before starting the real molecular dynamics (MD) simulations with AMBER 12, two consecutive minimizations were performed: (a) involving only the water molecules and ions, keeping the protein complex fixed and (b) involving the entire system. After the minimization, the system was gradually heated from 100 to 300 K over a timescale of 20 ps with restriction on the complex atoms. Then, the system was equilibrated during 100 ps at 300 K. The equilibrated structures were the starting points for the final MD simulations (20 ns) recorded at constant temperature (300 K) and pressure (1 atm) using a NPT ensemble with periodic boundary conditions. A total of 20,000,000 molecular dynamics steps were run with a time step of 1 fs per step. Coordinates and energy values were recorded every 1000 steps (2 ps) for a total of 20,000 MD models, to ensure that the simulations evolved toward a stable plateau. A detailed analysis of each MD trajectory (root main-square deviation, rmsd, H-bond occupancies and oxygen-Zn distances) was accomplished using the cpptraj module included in AmberTools 12 package. The complex of the trivalent ligand 13 and GMII was built in the same way of the Mode 1 of the monovalent ligand 5. A molecular dynamic of 10 ns was run with Amber 12 and analysed, as previously described for the monovalent complexes.

**CORCEMA-ST calculations.** A representative geometry of Mode 1 (frame 10000) was selected from the previously described molecular dynamic simulations and the entire system was minimized running 2500 steps without restraints with Amber 12, as described before. The minimized structure was used as pdb-entry in CORCEMA-ST matlab calculations. Polar protons in ligand and protein were removed and all aminoacids within 10 Å around the ligand were considered. In agreement with the experimental STD-NMR conditions, the protein concentration was kept to 20 μM and the ratio of monovalent:protein to 100:1. The correlation time (τc) was set to 0.510⁻⁶ s⁻¹ for the monovalent ligand in the free state and to 12110⁻⁶ s⁻¹ for the bound state (estimated following an empirical approximation that employs the formula r_nMW/2400, where r_n represents the ratio of the protein and MW the protein molecular weight), since the monovalent ligand 5 is a weak mannosidase inhibitor with the Ki in the mM range, Ktot value was set to 10⁵. The Kbinding value was set to 10⁵ L/mmol⁻¹s⁻¹, assuming a diffusion controlled kinetic model, and the saturation time to 2 s as used in the NMR experiments. To calculate the theoretical STD the irradiation onto Ilc, Leu, and Val methyl groups was considered as an approximation to the experimental irradiation around 0 ppm.

**TEM : Transmission Electron Microscopy with negative staining**

First, overnight dialysis of JBMan in 100 mM buffer acetate pH 5.0 with 5 mM ZnCl₂ was performed. The protein was then diluted to a 0.0087 mg/mL concentration. The ligand was then added to the JBMan solution to obtain an enzyme : ligand = 1:3 molar ratio and incubated for 1 h. The solution was finally diluted to a final concentration of enzyme of 0.005 mg/mL. The samples were applied to glow discharge carbon-coated copper grids and stained with 2% (w/v) uranyl acetate. Micrographs were taken at low radiation dose on a Jeol JEM-1230 LaB₆ transmission electron microscope operated at 100 kV and equipped with an Orius SC1000 CCDcamera. Micrographs were taken using a nominal magnification of 30,000 (2.3 Å/pixel) per sample.

**Acknowledgements**

This work was supported by the Italian Ministry of Health/Regione Toscana (grant Ricerca Finalizzata RF-2011-02347694 to FC) and by MIUR-Italy (PRIN 2010-2011, 2010L9SH3K 006 to AG). Ente Cassa di Risparmio di Firenze is acknowledged for a fellowship to GD (grant N° 2014/0303). CM thanks Accademia dei Lincei/Fondazione Donnegan for a fellowship. We also thank MINECO (Spain) for financial support through grants CTQ2015-64597-C2-1-P, CTQ2012-31247, CTQ2016-77270-R and one Juan de la Cierva contract to AG.

**Keywords:** multivalency • mannosidase • DAB-1 • pyrrolidine iminosugars • NMR studies
