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1 **Dissecting Key Multivalent Processes in Glycosidase Inhibition: Insights from**
2 **Thermodynamic Modelling and Atomistic Simulations**

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33 KEYWORDS multivalency, multivalent inhibitory effect, thermodynamic modelling, molecular
34 dynamics.

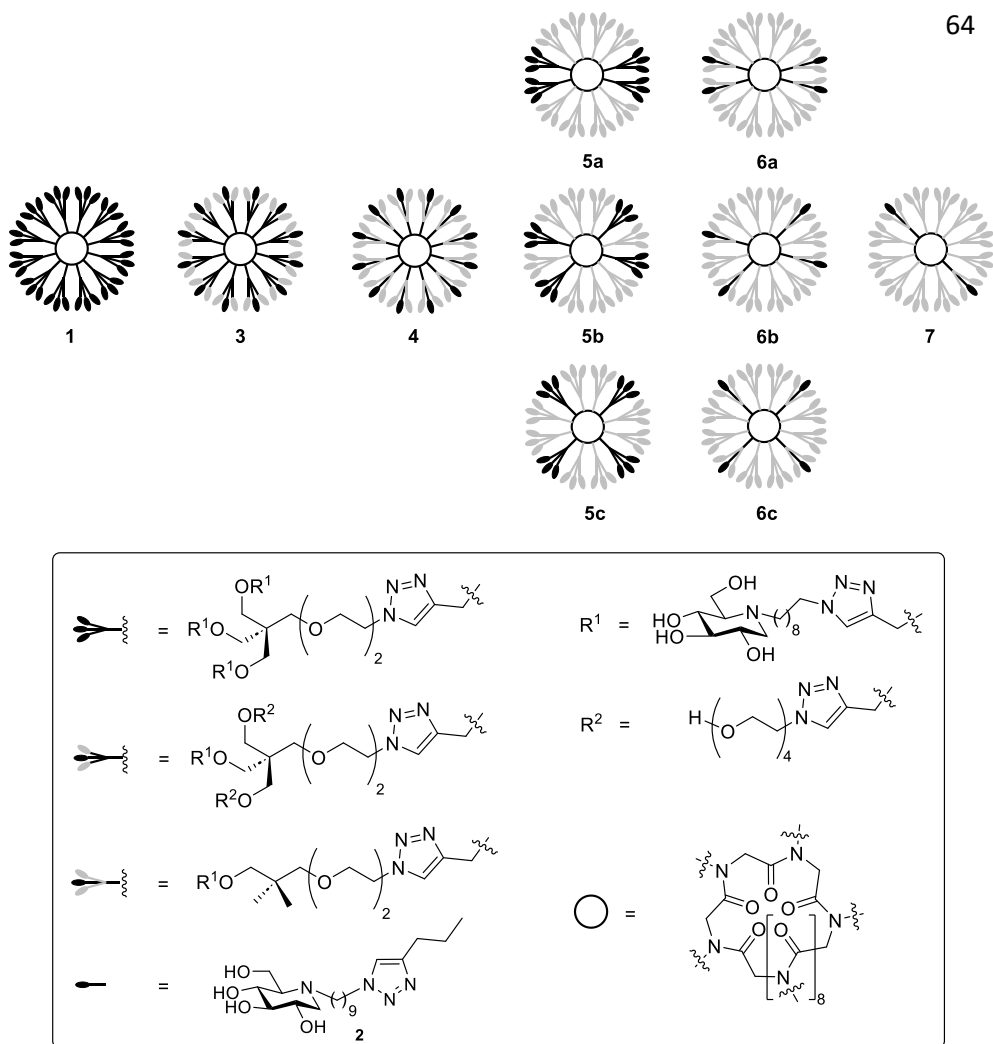
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37 **Abstract:** Multivalency represents a powerful approach to increase the inhibition potency of
38 moderate glycosidase inhibitors. Regarding the key role of catalytic glycoside hydrolysis in
39 biology, understanding the molecular mechanisms and origin of the multivalent inhibitory effect
40 is of great interest and presents a fascinating playground for theoretical studies. Our teams have
41 recently dissected key processes of multivalent glycosidase inhibition through the use of different
42 neoglycoclusters based on deoxynojirimycin (DNJ) inhitopes and a cyclopeptoid scaffold. This
43 companion article details the theoretical aspects of this former study. A thermodynamic model is
44 developed and validated, compared to literature, and extended to account for particularities of
45 the charged DNJ inhitopes.

46

47 **Introduction**

48 Glycosidase inhibitors have found applications as agrochemicals and therapeutic agents, for
 49 example, to target viral infection, cancer, and genetic disorders.^{1,2} A promising strategy to increase
 50 the potency of such inhibitors is the use of multivalent clusters, molecules that cluster together
 51 multiple substrate mimicking molecular units (inhibitopes) that can bind to and thereby inhibit the
 52 corresponding glycosidase enzyme.^{3,4} The neoglycocluster **1** represents one of the most potent
 53 examples of this kind. The number of inhibitopes per cluster (= valency n) is an important parameter
 54 for the inhibitory effect. Thereby the inhibition can increase in a non-linear fashion with
 55 increasing n , *i.e.*, the increase of the valency by an order of magnitude can increase the inhibition
 56 potency by several orders of magnitude. For example, the iminosugar 1-deoxynojirimycin (DNJ)
 57 and related *N*-alkylated derivatives such as **2** are relatively modest inhibitors⁴ of the enzyme Jack
 58 Bean α -mannosidase (JB α -man): the divalent cluster **7** (Scheme 1) with two DNJ units displays an
 59 inhibition constant of 54 μ M (Table 1).^{5,6} The 36-valent cluster **1**, however, features an inhibition
 60 constant of only 1.1 nM.⁷

61
62
63



65
 66 **Scheme 1.** Simplified representation of DNJ-based cluster **1** and of its deconstructed analogues
 67 **3-7**. The molecular entities that are removed as part of the deconstruction process are shown in
 68 light grey.

69

70 Thus, the increase of the valency by a factor of 18 increases the potency by a factor of 50 000! This
 71 over-amplification of the inhibitory effect (beyond that would be expected from an analogous
 72 concentration increase) is referred to as the cluster or multivalent effect. The molecular sources
 73 of the multivalent effect are not yet fully understood. Recently, we studied by a deconstruction
 74 approach a series of new DNJ-based clusters (**3-7**) to investigate the architectural parameters that
 75 influence the multivalent effect when targeting J β -man.⁶ These clusters consist of a cyclic peptide
 76 backbone where multiple branches are attached to the backbone nitrogen atoms. Each branch
 77 comprises a flexible linker and a terminal DNJ inhitope. Under experimental conditions of the
 78 inhibition measurements (pH=5) the N-alkylated DNJ is mostly protonated (pKa = 6.7-7.1).^{8,9}
 79 Clusters **3-7** differ not only by the number of branches but also by the orientation of the branches
 80 and the chemical structures of the linkers (*unipod* or *tripod*).⁴

81

82 The enzyme J β -man (220 kDa) is a homodimer (LH)₂ bearing two active sites. Its crystal
 83 structure¹⁰ in complex with the 36-valent cluster **1** shows that the enzyme can also form dimers
 84 with four active sites; Fig. 1A shows a schematic illustration of this dimer. Each site is bound by a
 85 DNJ unit. The formation of dimers has been confirmed by analytical ultracentrifugation
 86 sedimentation velocity (AUC-SV) experiments but only for high-valency clusters **1** and **4**.⁶ It is,
 87 however, not clear if the formation of dimers is of relevance for the measurements of the
 88 inhibition potency (since these experiments are carried out with a 1000x lower (1 μ g/mL)
 89 concentration than the AUC-SV experiments (1 mg/mL).⁶

90

91 **Table 1:** Experimental⁶ and theoretical data for the studied multivalent clusters.

| Cluster | Valency n^a | $\Omega = n(n-1)^b$ | K_i (nM) ^c | rp (exp) ^d | rp (theor) ^e |
|-----------|---------------|---------------------|-------------------------|-------------------------|---------------------------|
| 1 | 36 | 1188 ^f | 1.1 | 49091 | 45362 |
| 3 | 12 | 132 | 74.0 | 730 | 970 |
| 4 | 12 | 132 | 42.0 | 1286 | 970 |
| 5a | 12 | 108 ^g | 84.0 | 643 | 794 |
| 5b | 12 | 108 ^g | 120.0 | 450 | 794 |
| 5c | 12 | 108 ^g | 97.0 | 557 | 794 |
| 6a | 4 | 12 | 3100.0 | 17 | 17 |
| 6b | 4 | 12 | 2200.0 | 25 | 17 |
| 6c | 4 | 12 | 2300.0 | 23 | 17 |
| 7 | 2 | 2 | 54000.0 | $\equiv 1$ | $\equiv 1$ |

92 ^aNumber of inhitopes. ^bDegeneracy of complex (see text). ^cExperimentally measured inhibition constants. ^dRelative
 93 inhibition potency= K_i (divalent reference **7**)/ K_i (cluster). Note that the companion study⁶ uses **2** as a monovalent
 94 reference. ^eCalculated with Eq. 10 with $p=1.5$ and the given degeneracy coefficients. ^f Reduced degeneracy: $\Omega=36 \times 33$
 95 (see text). ^g Reduced degeneracy: $\Omega=12 \times 9$.

96

97 Obviously, the inhibition potency of these clusters depends strongly on the valency as seen when
 98 cluster **1** is deconstructed step by step (Table 1). But there is also an influence on the chemical
 99 structure of the branches, or more precisely of the linkers, as seen from the series with $n=12$:
 100 clusters **5a-c** with four *tripod* linkers feature about 1.5 times lower inhibition potencies than
 101 cluster **3** with twelve *tripod* linkers but only with one inhitope per branch. And the potency of
 102 cluster **3** is about 1.5 lower than that of cluster **4** with twelve *unipod* linkers. The importance of
 103 the linkers on the multivalent effect has been previously reported.¹¹ On the other hand, the
 104 orientation of the branches has little influence (**6a-c**). To complement this experimental work, we

105 briefly highlighted in the companion study some theoretical and simulation results.⁶ The aim of
 106 this article is to provide a greater in depth analysis of these theoretical and computational aspects.

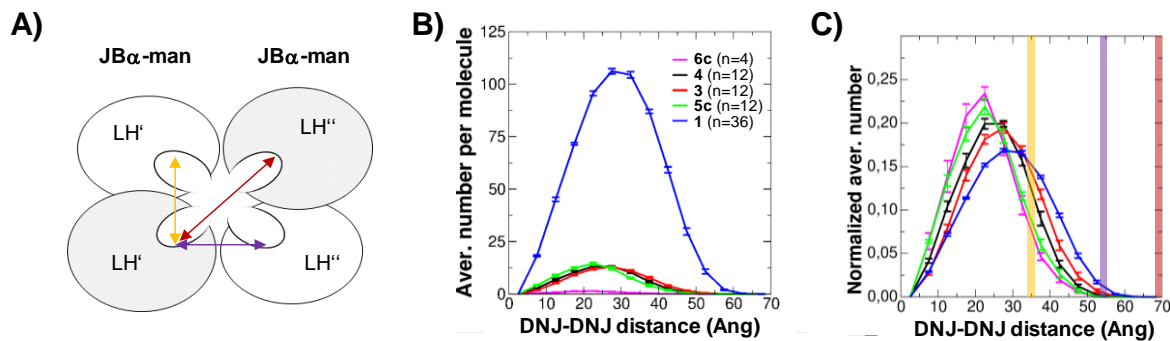
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108 Thermodynamic models can help to understand the basis of multivalency effects.¹²⁻¹⁴ Here we
 109 develop such a simplistic model with the aid of macroscopic rate constants and equilibrium
 110 constants. We only consider the formation of complexes with 1:1 stoichiometry and we assume
 111 identical chemical activity of unbound DNJ inhitopes at all steps along the binding pathway. We
 112 discuss the relationship of our approach with the more advanced approach of Kitov and Bundle.¹²
 113 We validate assumptions of the thermodynamic model using atomistic simulations than can
 114 provide valuable structural insights into the glycoclusters.^{15,16} We point out limitations of such
 115 thermodynamic models for the studied inhibitors, particularly, in the context of JBA-man
 116 dimerization (2:1 stoichiometry). We then apply an extension to account for the increasing net
 117 charge of the inhibitor with increasing valency.

118

119

120



121

122 **Figure 1:** Geometric parameters of the studied system. B) Schematic representation of dimeric
 123 JBA-man as seen in the crystal structure.¹⁰ The distances between the active sites are indicated by
 124 arrows; their values are given in subfigure C) as vertical bars (with the same color code). B)
 125 Distribution of DNJ-DNJ distances as obtained by atomistic simulations. The positions of the
 126 nitrogen atoms of the DNJ heads were used for the calculation. C) Same as A) but normalized by
 127 $n(n-1)$.

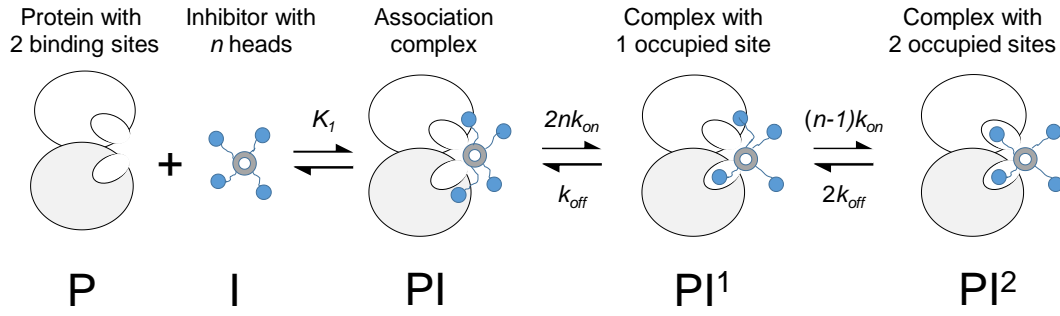
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131 Results and Discussion

132 *A thermodynamic non-cooperative model:* We start with a model for the inhibition of JBA-man
 133 protein (**P**, see Scheme 2) with two binding sites by a n -valent DNJ-based inhibitor (**I**). We imply
 134 that the protein **P** and inhibitor **I** form first an encounter complex **PI** before any DNJ head binds
 135 to the active site of the protein (encounter association constant K_1). We further imply k_{on} is the
 136 theoretical rate constant of DNJ inhitope binding for a monovalent inhibitor associated to a
 137 hypothetical enzyme with only one binding site; k_{off} is the corresponding rate constant for the
 138 unbinding of the DNJ inhitope. We also assume that the binding of the first DNJ inhitope, to form
 139 a singly occupied **PI**¹ complex, does not influence the binding rate of the second DNJ inhitope to
 140 form the double occupied complex **PI**² (no positive or negative cooperativity). As a result, the
 141 macroscopic rate constants for binding the first and second inhitopes are $2nk_{on}$ and $(n-1)k_{on}$,
 142 respectively. The unbinding rate constants are $2k_{off}$ (1st unbinding) and k_{off} (2nd). Note that in this
 143 model with macroscopic rate constants the species **PI**¹ and **PI**² comprise all possible ways to form
 144 a single and double occupied complex with a n -valent inhibitor.



145

146 **Scheme 2**

147

148 Thus, at equilibrium we find the following expressions:

149

150
$$\frac{[PI]}{[P][I]} = K_1$$

151

Eq. 1

152

153
$$\frac{[PI^1]}{[PI]} = \frac{2nk_{on}}{k_{off}}$$

154

Eq. 2

155
$$\frac{[PI^2]}{[PI^1]} = \frac{(n-1)k_{on}}{2k_{off}}$$

156

Eq. 3

157 The fraction of occupied binding sites is given by:

158

159
$$f = \frac{[PI^1] + 2[PI^2]}{2\{[P] + [PI^1] + [PI^2]\}}$$

160

Eq. 4

161 Under the condition $f=0.5$ (mimicking the experimental K_i measurement) we obtain:

162

163
$$\frac{[PI^2]}{[P]} = 1$$

164

Eq. 5

165 or equivalently

166
$$\frac{1}{[I]_{f=0.5}} = \frac{[PI^2]}{[P][I]}$$

167

Eq. 6

168 We note that the right-side is the definition of $K_{eq,PI2}$, *i.e.*, the equilibrium constant for the formation
 169 of the fully occupied protein (bound by two DNJ heads) and we obtain:

170

171
$$\frac{1}{[I]_{f=0.5}} = K_{eq,PI2} = n(n-1)K_1 \left(\frac{k_{on}}{k_{off}}\right)^2$$

172

Eq. 7

173 and for the relative potency rp we find:

174
$$rp = \frac{n(n-1)}{2} = \frac{\Omega}{2} \quad \text{for } n \geq 2$$

175 Eq. 8

176 when we use the case $n=2$ with $K_{eq,PI2} = 2K_1(k_{on}/k_{off})^2$ as reference.

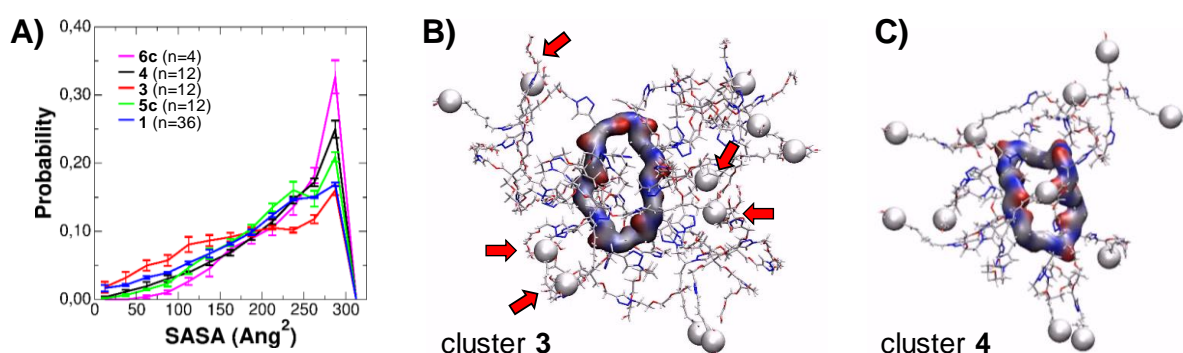
177
 178 Kitov and Bundle determined thermodynamic models for the inhibition of multisite receptor
 179 proteins by multivalent ligands.¹² These models were developed for 1:1 stoichiometry and under
 180 the assumption that the m binding sites of the receptor protein and the n branches of the inhibitor
 181 act independently and have identical binding properties. (Note that in their publication, Kitov and
 182 Bundle used the parameters m and n interchanged.) These assumptions are implicitly included in
 183 our model, too. Kitov & Bundle developed their models for different types of topologies. Our
 184 results above can be derived from the thermodynamic models of Kitov & Bundle assuming radial
 185 topology. Radial topology implies that the n branches are centrally anchored so that each ligand
 186 can interact with each binding site with the same binding strength. Again, radial topology was
 187 implicitly included in our model. Under these assumptions, Kitov & Bundle (see above) showed
 188 that for a protein with two binding sites (as in the case of JB α -man, $m=2$) the equilibrium constant
 189 for the formation of fully inhibited enzyme increases with $n(n-1)$. This $n(n-1)$ dependence results
 190 from the **degeneracy coefficient** Ω that accounts for the fact that complex designated as **PI**² are
 191 "not individual molecules but an ensemble of Ω microscopically distinguishable complexes".¹² In
 192 our model this degeneracy was incorporated into the macroscopic rate constants.

193
 194 There is an illustrative approach to rationalize the dependence of $K_{eq,PI2}$ (or equivalently of rp) on
 195 $n(n-1)$ when implying radial topology. This approach offers also a way to verify the assumption of
 196 radial topology. To do so, we rely on the fact that the free energy is a state function and the free
 197 energy of binding (or $K_{eq,PI2}$) does not depend on the chosen pathway. Let us consider the
 198 conformational selection pathway: two DNJ heads need to have the correct distance (and
 199 orientation, accessibility, etc) to be selected by the enzyme for binding to its active sites. According
 200 to the Gauss summation formula, the probability of finding such two DNJ heads is proportional to
 201 $n(n-1)/2$ if all heads are equivalent and independent from each other.

202
 203
 204
 205 *Validation of radial topology:* The branches of the studied clusters in this work are not exactly
 206 centrally anchored and the type of branches are not all identical. To probe the assumption of radial
 207 topology, we investigated the geometric properties for a subset of different clusters with the aid
 208 of full-atom molecular dynamics. We therefore determined the distribution of DNJ-DNJ distances
 209 for clusters **6c** ($n=4$), **3**, **4**, **5c** ($n=12$) and **1** ($n=36$). To be in line with the required assumptions of
 210 radial topology, the same protonated state of all DNJ heads was used (which can be considered as
 211 a low pH case). The raw distributions nicely visualize the $n(n-1)$ multivalency effect (Fig. 1B): the
 212 average number of DNJ heads at the same distance as in the protein is by factors higher for $n=36$
 213 than for $n=12$ and $n=4$.

214
 215 When normalized by $n(n-1)$ the distributions of DNJ-DNJ distances are much more similar (Fig.
 216 1C). The distributions become, however, slightly broader with increasing valency. As a result, the
 217 normalized average number of DNJ heads at a distance of ca. 35 Å (= separation of binding sites in
 218 the monomeric enzyme, yellow bar in Fig. 1C) increases with valency. On the other hand, the
 219 solvent accessibility of the inhitopes (Fig. 2A) decreases with valency. In general, it can be expected
 220 that these two compensatory trends are expected to cancel out (to some extent). There are,
 221 however, some particularities. For example, glyoclusters **3** and **4** (both $n=12$) display rather
 222 similar normalized distributions of DNJ-DNJ distances. The accessibility of the DNJ heads is,
 223 however, very different (see Figure 2A-C). Cluster **3** features 12 *tripod* branches, each with two
 224 ghost side-arms, *i.e.*, arms that are not decorated by an inhitope. These ghost side-arms shield the

225 remaining inhitope-decorated arm from the solvent (Figure 2B). In glycocluster **4** with 12 *unipod*
 226 branches these ghost-arms are not present and the accessibility of the inhitopes is substantially
 227 increased with respect to cluster **3**. This could explain a higher chemical activity of the DNJ heads
 228 and therefore a higher inhibition potency of **4** with respect to **3**. Another particularity concerns
 229 the 12-valent glycoclusters **5a-c** that feature a *tripod* branch type with three inhitope-decorated
 230 arms. Obviously, inhitopes from the same branch cannot simultaneously bind at two different sites
 231 of the receptor (that are too far away). Or, in other words, inhitopes from the same branch (*tripod*
 232 case) are closer to each other than inhitopes from different branches. This is also seen by a shift
 233 of the normalized distribution of DNJ-DNJ distances to lower values (*i.e.*, shift to the left in Figure
 234 2A) when comparing **5c** with the *unipod* cluster **4**; the latter does not suffer from such geometric
 235 constraints. Thus, the degeneracy coefficient of clusters **5a-c** is in fact only $12 \times 9 = 108$ (due to
 236 geometric constraints) and therefore reduced with respect to **4** ($12 \times 11 = 132$ without geometric
 237 constraints). This is reflected by a reduced inhibition potency. In principle, cluster **1** suffers also
 238 from these geometric constraints but the relative influence on the degeneracy coefficient is much
 239 less pronounced (36×33 with geometric constraints vs 36×35 without geometric constraints).



241 **Figure 2.** Solvent accessibility of DNJ inhitopes. A) Probability distribution of solvent accessible
 242 surface area (SASA) of the DNJ heads as calculated with a solvent probe radius of 2.4 Ang. B)
 243 Typical snapshot from the molecular dynamics simulation of cluster **3**. The backbone is shown as
 244 surface, the bonds of the branches as thin sticks and the nitrogen atoms of the DNJ heads as white
 245 balls. The arrows indicate inhitopes that are shielded from the side-arms. C) Same as subfigure B)
 246 but for cluster **4**.

248
 249
 250
 251 *Limitations of the non-cooperative thermodynamic model.* For the binding of the studied inhibitors
 252 to a single J β -man molecule the radial topology assumption might be valid to some extent due to
 253 compensation effects. When comparing the experimental relative inhibition potencies with those
 254 calculated by Eq.7 (Fig. 3A), we realize, however, that the current thermodynamic model does not
 255 capture the full extent of the multivalent effect. In fact, the experimental *rp* values show an
 256 approximate $n^{3.5}$ -dependency for large values of *n* Eq. 7 provides only a n^2 -dependency. There are
 257 two potential explanations for this discrepancy:

- 258
- 259 1) Possibility to form complexes with 2:1 stoichiometry (2 J β -man receptors:1
 260 multivalent inhibitor). These complexes feature four receptor binding sites which leads
 261 to higher order terms, *i.e.*, additional n^3 - and n^4 -terms, to properly describe the *n*-
 262 dependency of the degeneracy coefficient.¹² If 2:1 complexes were responsible for the
 263 $n^{3.5}$ -like dependency of *rp*, it would imply that the fraction of formed 2:1 complexes
 264 should increase with the valency. Indeed, high-valency clusters **1** and **4** are the only
 265 clusters of the series that have been found to form 2:1 complexes.⁶ On the other hand,
 266 both clusters feature a rather similar fraction of 2:1 complexes.⁶ A significantly larger
 267 fraction of 2:1 complexes would be expected for 36-valent cluster **1** in comparison to

268 12-valent cluster **4** (even if the geometric constraints of cluster **1** are taken into
269 account).

270
271 It should also be noted that cluster **4** is the only 12-valent cluster that forms a 2:1
272 complex. In the case of 12-valent cluster **3** the solvent accessibility and therefore the
273 chemical activity of the DNJ heads is probably too low to form such a 2:1 complex. Note
274 that a reduced chemical activity of the inhitope impacts the formation of a fully-inhibited
275 2:1 complex by the power of 4 ($\sim k_{on}^4$). In the case of the 12-valent clusters **5a-c** the
276 degeneracy coefficient is significantly reduced due to geometric constraints (see above).
277 The degeneracy coefficient for a fully-inhibited 2:1 complex for cluster **4** is
278 $12 \times 11 \times 10 \times 9 = 11880$ while for clusters **5a-c** it is only $12 \times 9 \times 6 \times 3 = 1944$.

279
280 Finally, the experimental conditions are different for the measurement of the inhibition
281 constant and the fraction of 2:1 complexes. It can therefore not be concluded with
282 certainty that the formation of complexes with 2:1 stoichiometry is responsible for the
283 $n^{3.5}$ -like dependency of rp .

284
285 2) Increasing favorable electrostatic interactions with increasing inhitope valency. At
286 pH=5.5 the *apo* J β α -man features an overall negative charge of about $-6.6 \pm 0.2e$ as
287 obtained from continuous constant-pH molecular dynamics simulations.¹⁷ The
288 inhibitors, however, are charged positively. For example, **6c** (n=4) and **5c** (n=12) are
289 charged $+2.4 \pm 0.3e$ and $+5.4 \pm 0.3e$, respectively, due to the partial protonation of the DNJ
290 inhitopes. Due to their opposite charge, the enzyme and the neoglycoclusters attract
291 each other, and the attractive force increases with increasing valency. At pH=4, on the
292 other hand, the charge of the enzyme is $9.4 \pm 0.1e$. (The isoelectric point of this enzyme
293 is at about pH=5). The charge of the clusters **6c** and **5c** is $+3.2 \pm 0.2e$ and $+8.1 \pm 0.3e$,
294 respectively. Thus, at pH=4 repulsive electrostatic interactions are expected between the
295 positively charged enzyme and the positively charged inhibitor and this repulsion would
296 increase with increasing valency, *i.e.*, with increasing positive charge.

297
298 If these electrostatic interactions played indeed a major role for the multivalent effect,
299 then lowering of the pH from 5.5 to 4 would display a stronger impact on the inhibition
300 potency for high valencies than for low valencies. To test this hypothesis, inhibition
301 constants of two representative compounds have been measured at pH 4.⁶ K_i of the
302 tetravalent cluster **6c** increases by a factor of about 80 from $2.3 \mu\text{M}$ (pH 5.5) to $184 \mu\text{M}$
303 (pH 4). On the other hand, K_i of the 12-valent **5c** increases by a factor of more than 400
304 from 97 nM (pH 5.5) to $42 \mu\text{M}$ (pH 4). Indeed, the cluster with the higher valency
305 displays the stronger decrease in inhibition potency. As a consequence we decided to
306 develop an extension of the thermodynamic model that empirically includes
307 electrostatic effects.

308
309
310
311 *Extension to charged inhitopes:* It is well known that electrostatics play a crucial role for the
312 associations of macromolecules (in particular for the parameter K_1 of Eq. 7). Usually the binding
313 affinity is linearly correlated to the product of the total charge of the two interacting molecules. It
314 has been shown by MC simulations, however, that for oppositely charged macromolecules in polar
315 solvents this linear correlation breaks for larger net charges.¹⁸ By keeping the charge of one
316 macromolecule fixed at a certain negative value and increasing the positive charge of the other
317 molecule, the binding affinity displayed a logarithmic-like behavior. We can therefore propose the
318 following dependence of K_1 on n :

319

320
$$K_1 \approx K_1' n^p$$

 321 Eq. 9

322 and we obtain:

323
$$\frac{1}{[I]_{f=0.5}} \approx n^{1+p}(n-1) K_1' \left(\frac{k_{on}}{k_{off}}\right)^2 = n^p \Omega K_1' \left(\frac{k_{on}}{k_{off}}\right)^2$$

 324 Eq. 10

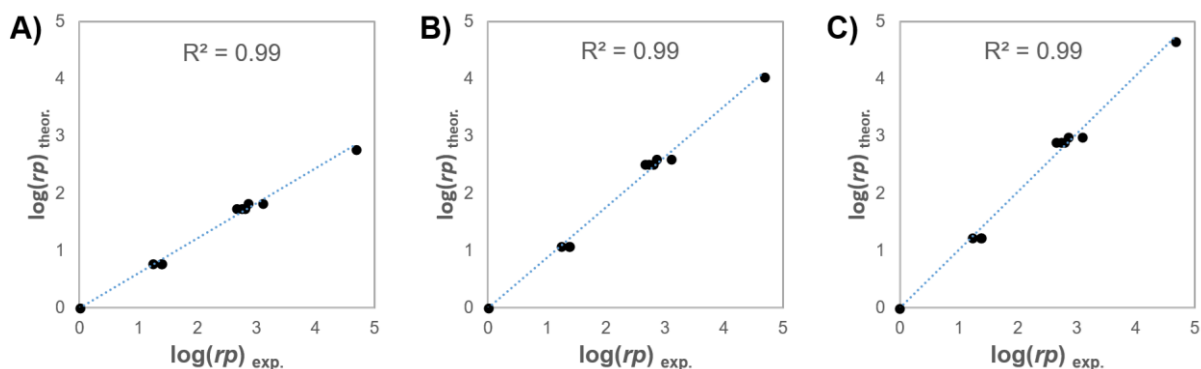
325 Where K_1' is the association constant of the encounter complex for a monovalent ligand and p is a
 326 parameter of the studied system (*i.e.*, enzyme & type of inhitope). This parameter may also depend
 327 on the experimental conditions (*e.g.* pH, see below). With $p=0$ we recover Eq. 7. For $p=1$ we can
 328 derive the following simple expression for the relative inhibition potency:

329
$$rp \approx \frac{n^2(n-1)}{4} = \frac{n\Omega}{4} \quad \text{for } n \geq 2$$

 330 Eq. 11

332 Here again the divalent cluster **7** was used as reference for the calculation of rp . Fig. 3B shows the
 333 correlation between Eq. 11 and the experimental rp values. Already this simple model provides an
 334 excellent description for the dependence of rp on n . A slightly better regression slope (=closer to
 335 unity) is obtained with $p=1.5$ (Fig. 3C)⁶ as used in the companion study.⁶

336
 337
 338



339 **Figure 3:** Correlation of experiment (abscissa) and theoretical model (ordinate) based on Eq.7
 340 (A), Eq. 11 (B) and Eq. 10 with $p=1.5$ (C). For clusters **1** and **5a-c** we used a reduced degeneracy
 341 coefficient (see text). The main difference between these graphs is the increasing slope from 0.61
 342 (A) to 0.88 (B) and 1.01 (C).
 343

344
 345
 346
 347 **Conclusion**

348 Simplistic thermodynamic models can be applied to understand in parts the multivalent effect of
 349 JBA-man inhibition by neoglycoclusters. Microscopic structural differences such as the inhitope
 350 distributions and solvent accessibility can lead, however, to deviations from such simplistic
 351 models. An extension to the model has been proposed for charged inhitopes to take into account
 352 electrostatic contributions to the binding affinity. In summary, the studied multivalent inhibitors
 353 of this work and the herein developed extended thermodynamic model are unique in the sense
 354 that they open up a new dimension (electrostatics) in addition to the standard statistical
 355 (degeneracy) dimension. We anticipate that this approach can be applied to other protein targets
 356 and thereby pave the way to the design of new and more targeted multivalent inhibitors.

357 **Methods**

358 *General:* Simulations with implicit solvation model were carried out with the program CHARMM,¹⁹
359 version c45b2, on CPU Intel Xeon Gold 6126. Explicit-water simulations were performed with
360 OpenMM,²⁰ version 7.4, on GPU Nvidia RTX2080 with mixed precision. Continuous constant pH-
361 simulations were carried out with the OpenMM-interface[ref] of CHARMM c45b2 on GPU Nvidia
362 GTX1080. Input files for the enzyme (including two Zn²⁺ ions and four glycan modifications) were
363 obtained from the CHARMM-GUI server²¹ with the PDB Reader module using the PDB entry 6B9P
364 and the force field CHARMM36m. Parameters of the zinc (II) ions were those of Stote and
365 Karplus.²² Topologies & parameters of the neoglycoclusters were also obtained from the
366 CHARMM-GUI server using the Ligand Reader module (CGenFF²³ parameters). All DNJ heads were
367 constructed in their protonated state.

368

369

370 *Molecular dynamics simulation of neoglycoclusters:* For each cluster we generated with CHARMM
371 four different starting structures by simulated annealing using the implicit solvation model
372 FACTS²⁴ and its recommended simulation parameters. The initial model from CHARMM-GUI was
373 first heated to 450 K within 1ns and then cooled down to 298.15K within 10ns using Langevin
374 dynamics. This procedure was repeated four times with different initial velocities (ISEED) leading
375 to four different starting structures for the subsequent simulations with OpenMM. Each structure
376 was solvated in a cubic box of TIP3P water molecules (side length 5 nm) with periodic boundary
377 conditions. Non-bonded interactions were cut off at 0.9 nm. Long range electrostatic interactions
378 were treated by Particle-Mesh Ewald. Covalent hydrogen bonds were kept constrained. Equation
379 of motion was integrated with OpenMM's Langevin integrator at constant temperature (298.15
380 K). After a short equilibration of 10 ns in the NVT ensemble using a time step of 1 fs we performed
381 300 ns of MD in the CPT ensemble (1 atm) using a time step of 2 fs. The last 200 ns were used for
382 analysis purposes, *i.e.*, 100 ns served as equilibration. Each MD simulation (originating from a
383 different starting structure) was first separately analyzed, and then mean values were obtained
384 by averaging over the four separate analyses of each neoglycocluster. Error bars correspond to
385 standard error of the mean.

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388 *Constant pH-Simulations of neoglycoclusters and JB α -man enzyme:* Similar to the explicit-water
389 simulations we created different starting structures (5 in total) for the enzyme and the clusters
390 **5c** and **6c** using the same simulated annealing protocol. Simulations were carried out, however,
391 with a continuous constant pH method at pH-values of 4.0 and 5.5 using the GBSW solvation model
392 with the approach of Brooks and coworkers.²⁵ Standard settings were used for the GBSW model
393 (*i.e.*, ionic strength and surface tension coefficient were kept at zero). In the case of the enzyme a
394 restraining force (CONS HARM) was applied to the non-hydrogen protein & zinc atoms during the
395 simulated annealing runs (force constants of 10.0 and 1.0 kcal/mol/Ang² for the backbone/Zn²⁺
396 and side-chains, respectively) to avoid unfolding of the enzyme. For the DNJ inhitope we used a
397 pK_a of 6.94 as known from the literature (N-butyl-DNJ, miglustat).⁹ Following a calibration
398 procedure,²⁶ the continuous-pH parameters A and B were set to -60.3 and 0.453, respectively, for
399 the DNJ inhitope. Parameter BARR was set to 1.75. For the amino-acid groups of the enzyme we
400 used the standard pK_a values and continuous-pH parameters of the GBSW solvation model as
401 provided in the CHARMM distribution. Each starting structure was then equilibrated for 5 ns at
402 T=298.15K before a production run of 5 ns occurred. The restraining forces were reduced by a
403 factor of 10 with respect to the simulated annealing simulations. For each simulation the mean
404 charge of the cluster or the enzyme was obtained by averaging over all snapshots (saved every
405 500 ps). Then mean values and standard errors of the mean were obtained by averaging over the
406 five simulations (corresponding to the five different starting structures).

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