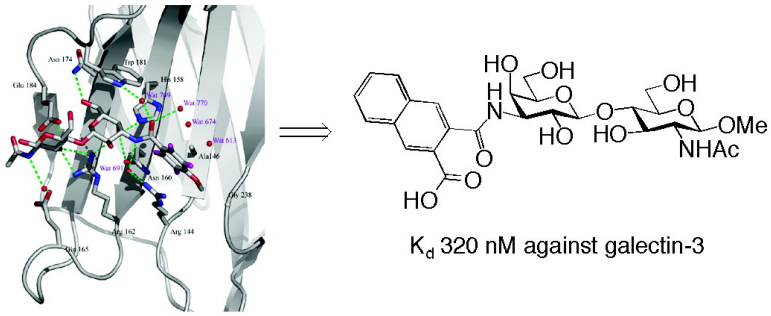
Article

Structural and Thermodynamic Studies on Cation–π Interactions in Lectin–Ligand Complexes: High-Affinity Galectin-3 Inhibitors through Fine-Tuning of an Arginine–Arene Interaction

Pernilla Srme, Pascal Arnoux, Barbro Kahl-Knutsson, Hakon Leffler, James M. Rini, and Ulf J. Nilsson

J. Am. Chem. Soc., **2005**, 127 (6), 1737-1743• DOI: 10.1021/ja043475p • Publication Date (Web): 21 January 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 11 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 01/21/2005

Structural and Thermodynamic Studies on Cation−Π Interactions in Lectin−Ligand Complexes: High-Affinity Galectin-3 Inhibitors through Fine-Tuning of an Arginine−Arene Interaction

Pernilla Sörme,[†,‡] Pascal Arnoux,[§,||] Barbro Kahl-Knutsson,[‡] Hakon Leffler,*[,‡] James M. Rini,*[,§] and Ulf J. Nilsson*[,†]

Contribution from Organic and Bioorganic Chemistry, Lund University, P. O. Box 124, SE-221 00 Lund, Sweden, Section MIG, Department of Laboratory Medicine, University of Lund, Sölvegatan 23, SE-223 62 Lund, Sweden, and Departments of Medical Genetics and Microbiology and Biochemistry, University of Toronto, Toronto, Ontario, M5S 1A8, Canada

Received October 28, 2004; E-mail: ulf.nilsson@bioorganic.lth.se

Abstract: The high-resolution X-ray crystal structures of the carbohydrate recognition domain of human galectin-3 were solved in complex with *N*-acetyllactosamine (LacNAc) and the high-affinity inhibitor, methyl 2-acetamido-2-deoxy-4-*O*-(3-deoxy-3-[4-methoxy-2,3,5,6-tetrafluorobenzamido]-β-D-galactopyranose)-β-D-glucopyranoside, to gain insight into the basis for the affinity-enhancing effect of the 4-methoxy-2,3,5,6-tetrafluorobenzamido moiety. The structures show that the side chain of Arg144 stacks against the aromatic moiety of the inhibitor, an interaction made possible by a reorientation of the side chain relative to that seen in the LacNAc complex. Based on these structures, synthesis of second generation LacNAc derivatives carrying aromatic amides at 3′-C, followed by screening with a novel fluorescence polarization assay, has led to the identification of inhibitors with further enhanced affinity for galectin-3 ($K_d \geq 320$ nM). The thermodynamic parameters describing the binding of the galectin-3 C-terminal to selected inhibitors were determined by isothermal titration calorimetry and showed that the affinity enhancements were due to favorable enthalpic contributions. These enhancements could be rationalized by the combined effects of the inhibitor aromatic structure on a cation−Π interaction and of direct interactions between the aromatic substituents and the protein. The results demonstrate that protein−ligand interactions can be significantly enhanced by the fine-tuning of arginine−arene interactions.

Introduction

The galectins are a family of proteins defined by a carbohydrate recognition domain (CRD) of about 135 amino acids with certain conserved sequence motifs and affinity for β-galactosides.[1,2] They appear to be involved in a wide variety of both extracellular (cell adhesion, induction of cell signaling including apoptosis) and intracellular activities (regulation of growth and apoptosis and RNA-splicing), although in many cases their functions remain unclear (see refs 3-10 and additional papers in ref 11). Nevertheless, the galectins have been clearly implicated in physiological and pathological mechanisms in inflammation, immunity[5,12−14] and cancer.[6,7,10,15] In addition, the modulation of galectin expression or activity has been linked to disease outcome;[5,7,16] most recently galectin-2 [17] was found to be associated with increased risk for myocardial infarction and galectin-4 [18] with inflammatory bowel disease. For these

[†] Lund University.
[‡] University of Lund.
[§] University of Toronto.
[||] Current address: CEA/Cadarache, DSV, DEVM, Laboratoire de Bioénergétique Cellulaire, 13108 Saint Paul lez Durance, France.

(1) Houzelstein, D.; Goncalves, I. R.; Fadden, A. J.; Sidhu, S. S.; Cooper, D. N.; Drickamer, K.; Leffler, H.; Poirier, F. *Mol. Biol. Evol.* **2004**, *21*, 1177−1187.
(2) Leffler, H.; Carlsson, S.; Hedlund, M.; Qian, Y. *Glycoconjugate J.* **2004**, *19*, 433−440.
(3) Zick, Y.; Eisenstein, M.; Goren, R. A.; Hadari, Y. R.; Levy, Y.; Ronen, D. *Glycoconjugate J.* **2004**, *19*, 517−526.
(4) Ochieng, J.; Furtak, V.; Lukyanov, P. *Glycoconjugate J.* **2004**, *19*, 527−535.
(5) Rabinovich, G. A.; Toscano, M. A.; Ilarregui, J. M.; Rubinstein, N. *Glycoconjugate J.* **2004**, *19*, 565−573.
(6) Huflejt, M.; Leffler, H. *Glycoconjugate J.* **2004**, *20*, 247−255.
(7) Takenaka, Y.; Fukumori, T.; Raz, A. *Glycoconjugate J.* **2004**, *19*, 543−549.
(8) Patterson, R. J.; Wang, W.; Wang, J. L. *Glycoconjugate J.* **2004**, *19*, 499−506.
(9) Hsu, D. K.; Liu, F. T. *Glycoconjugate J.* **2004**, *19*, 507−515.
(10) Lahm, H.; Andre, S.; Hoeflich, A.; Kaltner, H.; Siebert, H. C.; Sordat, B.; von der Lieth, C.-W.; Wolf, E.; Gabius, H.-J. *Glycoconjugate J.* **2004**, *20*, 227−238.
(11) Leffler, H., Ed. *Glycoconjugate J.* **2004**, *19*, 433−468.
(12) Almkvist, J.; Karlsson, A. *Glycoconjugate J.* **2004**, *19*, 575−581.
(13) Hirashima, M.; Kashio, Y.; Nishi, N.; Yamauchi, A.; Imaizumi, T. A.; Kageshita, T.; Saita, N.; Nakamura, T. *Glycoconjugate J.* **2004**, *19*, 593−600.
(14) Sato, S.; Nieminen, J. *Glycoconjugate J.* **2004**, *19*, 583−591.
(15) Partridge, E. A.; Le Roy, C.; Di Guglielmo, G. M.; Pawling, J.; Cheung, P.; Granovsky, M.; Nabi, I. R.; Wrana, J. L.; Dennis, J. W. *Science* **2004**, *306*, 120−124.
(16) John, C. M.; Leffler, H.; Kahl-Knutsson, B.; Svensson, I.; Jarvis, G. A. *Clin. Cancer Res.* **2003**, *9*, 2374−2383.
(17) Ozaki, K.; Inoue, K.; Sato, H.; Iida, A.; Ohnishi, Y.; Sekine, A.; Odashiro, K.; Nobuyoshi, M.; Hori, M.; Nakamura, Y.; Tanaka, T. *Nature* **2004**, *429*, 72−75.
(18) Hokama, A.; Mizoguchi, E.; Sugimoto, K.; Shimomura, Y.; Tanaka, Y.; Yoshida, M.; Rietdijk, S. T.; de Jong, Y. P.; Snapper, S. B.; Terhorst, C.; Blumberg, R. S.; Mizoguchi, A. *Immunity* **2004**, *20*, 681−693.

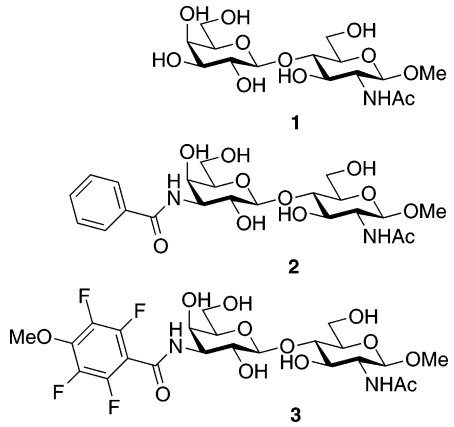


Figure 1. Structures of the parent methyl glycoside of *N*-acetyllactosamine **1** and the corresponding 3′-benzamido- and 3′-(4-methoxy-2,3,5,6-tetrafluorobenzamido) high-affinity inhibitors **2** and **3**, respectively.

reasons, galectin inhibitors would be valuable not only as tools for probing galectin function but also as lead compounds in the future development of therapeutics.

Lectin−monosaccharide complexes typically involve two to five hydrogen bonds complemented by hydrophobic and van der Waals interactions and are in general weak (K_d's in the millimolar range). Consequently, the design of small high-affinity lectin inhibitors has proven to be a considerable challenge. Since many lectins are oligomeric in nature, the most successful lectin inhibitors, to date, have been multivalent compounds designed to interact with more than one of the lectin's subunits.[19−21] Galectin-3, however, is mainly monomeric in solution, albeit in equilibrium with a lower percentage of oligomers,[22] and multivalent compounds did not seem to give higher inhibitory potency.[23] Therefore, we set out to design high-affinity inhibitors capable of tightly binding monomeric galectin-3.[24] The approach was based on the fact that the three-dimensional structure of the galectin-3 CRD (galectin-3C), in complex with LacNAc,[25] had shown that the 3-OH of the galactoside residue points toward an extension of the lactose/LacNAc binding site thought to be responsible for conferring the selectivity shown by galectin-3 for longer oligosaccharides. Chemical modifications at this position led us to the discovery[24] that benzamide substituents at LacNAc 3′-C substantially increased the affinity of galectin-3 for these compounds. One inhibitor (**3**; Figure 1), carrying a 4-methoxy-2,3,5,6-tetrafluorobenzamido group at 3′-C was shown to inhibit intact galectin-3 50 times better than the parent methyl 2-acetamido-2-deoxy-4-*O*-*β*-D-galactopyranose-*β*-D-glucopyranoside (**1**). The benzamide substitution pattern was concluded to be of critical importance, as the unsubstituted inhibitor **2** was only 15 times better than compound **1**.

Herein we report the 1.6 Å resolution crystal structure of the human galectin-3C in complex with inhibitor **3**, the first structure of a galectin-3 complex with a synthetic ligand. Moreover, we have now extended the resolution of the LacNAc complex to 1.4 Å resolution. The crystal structure of the galectin-3C−**3** complex points to the importance of an arginine−arene interaction and encouraged us to synthesize a further 56 inhibitors (**4**−**59**) consisting of LacNAc derivatives carrying aromatic amides at 3′-C. Evaluation of the galectin-3 inhibitory activity of these inhibitors (**4**−**59**) with a novel fluorescence polarization assay[26] revealed significantly improved inhibitors with K_d values as low as 320 nM. The thermodynamic parameters describing the binding of these improved inhibitors, determined by isothermal titration microcalorimetry (ITC), showed that complex formation was enthalpy-driven.

Results and Discussion

To understand the structural basis for the high affinity of compound **3** for galectin-3 and hence to obtain information to guide us in the design and synthesis of even better inhibitors, the crystal structures of galectin-3C in complex with LacNAc and **3** were solved and refined at 1.4 and 1.6 Å resolution, respectively. Although these crystals are essentially isomorphous with those reported previously,[25] the LacNAc structure was solved by the MAD phasing method using the sodium bromide quick-soak approach as a test of the ability of this method to provide high-quality experimentally determined phase information. Experimental phases were calculated to 2.0 Å resolution, resulting in very high quality electron density maps. The starting point for refinement of the complex with compound **3** was that of the galectin-3C coordinates obtained from the LacNAc complex. (See Supplementary Information for phasing and refinement statistics.)

Structure of Galectin-3 in Complex with LacNAc. Overall, the LacNAc complex refined here at 1.4 Å resolution using synchrotron data collected at 100 K agrees well with the structure refined previously at 2.1 Å resolution using data collected on a rotating anode at room temperature.[25] Nevertheless, the structure has been improved in a number of ways. First, one more residue (Pro 113) is clearly visible in the electron density at the N-terminus of the molecule. The conformation of the molecule in this region is of interest as it forms the connection to the N-terminal domain unique to galectin-3, a conformation that is not compatible with the subunit interactions found to mediate dimer formation in the canonical dimeric galectins-1 and -2.[27−29] Second, the orientations of a number of side chains have been better defined. Among those close to the binding site, the side chain of Arg144 is slightly different in that the guanidino group is rotated about the C$\delta\angle$Nϵ bond and all three nitrogen atoms are now found to form hydrogen bonds with surrounding water molecules. In addition, the side chain Arg129 is oriented in such a way that it makes a hydrogen bond with the carboxyl terminus of the molecule through its Nϵ and NH_2. Third, the number of ordered water molecules is

(19) Blixt, O.; Collins, B. E.; van den Nieuwenhof, I. M.; Crocker, P. R.; Paulson, J. C. *J. Biol. Chem.* **2003**, *278*, 31007−31019.
(20) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2002**, *124*, 14922−14933.
(21) Kitov, P. I.; Shimizu, H.; Homans, S. W.; Bundle, D. R. *J. Am. Chem. Soc.* **2003**, *125*, 3284−3294.
(22) Ahmad, N.; Gabius, H.-J.; André, S.; Kaltner, H.; Sabesan, S.; Roy, R.; Liu, B.; Macaluso, F.; Brewer, C. F. *J. Biol. Chem.* **2004**, *279*, 10841−10847.
(23) Ahmad, N.; Gabius, H.-J.; Sabesan, S.; Oscarson, S.; Brewer, C. F. *Glycobiology* **2004**, *14*, 817−825.
(24) Sörme, P.; Qian, Y.; Nyholm, P.-G.; Leffler, H.; Nilsson, U. J. *ChemBioChem* **2002**, *3*, 183−189.
(25) Seetharaman, J.; Kaningsberg, A.; Slaaby, R.; Leffler, H.; Barondes, S. H.; Rini, J. M. *J. Biol. Chem.* **1998**, *273*, 13047−13052.
(26) Sörme, P.; Kahl-Knutsson, B.; Wellmar, U.; Nilsson, U. J.; Leffler, H. *Methods Enzymol.* **2003**, *362*, 504−512.
(27) Lobsanov, Y. D.; Gitt, M. A.; Leffler, H.; Barondes, S. H.; Rini, J. M. *J. Biol. Chem.* **1993**, *268*, 27034−27038.
(28) Liao, D. I.; Kapadia, G.; Ahmed, H.; Vasta, G. R.; Herzberg, O. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1428−1432.
(29) Bourne, Y.; Bolgiano, B.; Liao, D. I.; Strecker, G.; Cantau, P.; Herzberg, O.; Feizi, T.; Cambillau, C. *Nat. Struct. Biol.* **1994**, *1*, 863−870.

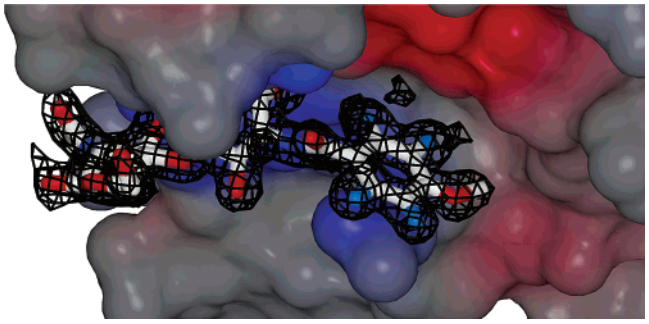


Figure 2. $2F_0-F_c$ electron density map contoured at 1σ showing the electron density associated with the inhibitor **3**. The protein surface accessible is shown and colored according to the charge (blue is positive, and red is negative).

slightly increased in the present high-resolution structure to 180, as compared to the 120 seen at lower resolution. Finally, we observed six residues in alternate conformation, namely, Ile134, Asn179, Asn222, Ser232, Ser244, and Met249. None of these residues is in contact with the LacNAc disaccharide. Excluding the atoms in alternate conformations, the root mean square deviation (RMSD) of all atoms between the low- and high-resolution structures is 0.26 Å. The high-resolution LacNAc complex structure now includes 137 residues, 180 water molecules, six residues in alternate conformations, one chloride ion, and the LacNAc disaccharide.

Structure of Galectin-3 in Complex with Inhibitor 3. Inhibitor 3 is found to bind in the galectin-3C LacNAc binding site with the benzyl substituent in the extended site as expected (Figure 2). The benzamide nitrogen at 3′-C of the galactose residue is hydrogen-bonded to a water molecule, which is in turn hydrogen-bonded to Oδ1 of Asn160 and NH1 of Arg144 (Figure 3a,b). The benzamide carbonyl oxygen is hydrogen-bonded with two water molecules, one of which is further hydrogen-bonded to the Nε1 of Trp 181. These interactions with the amido functionality may explain why the corresponding phenyl sulfonamides or ureas at 3′-C are less efficient inhibitors.[24]

Upon binding of inhibitor **3** the only significant change in the galectin-3C structure is the conformational change observed in the side chain of Arg144. The Arg144 guanidino group, which in the complex with LacNAc is involved in a hydrogen bond with water molecules at the galectin-3C surface, is moved 3.5 Å to interact with the benzamido ring of **3** (Figure 3c,d). From being aligned along the galectin-3C surface in the complex with LacNAc, the side chain of Arg144 adopts an all-staggered conformation and points out toward the solvent in the complex with **3**. This type of ligand-induced change in conformation is an example of one of the complications associated with the structure-based approached to ligand design as recently reviewed.[30] The central carbon (Cζ) of the Arg144 guanidino group is stacked on one side of the benzamido ring at a distance of 3.5−4.2 Å from the carbon atoms of the aromatic ring. On the other side of the benzamido ring, two water molecules are found within 4.5 Å of its centroid. Additionally, one edge of the benzamido ring sits in an extended nonpolar pocket formed by the side chains of Arg144(Cβ to Cδ), Ala146(Cβ), and Asn160(Cβ). This extended nonpolar pocket thus emerged as an attractive target for the design of further optimized inhibitors as described below.

The complex with inhibitor 3 shows that the side chain of Arg144 makes what appears to be a cation−Π interaction[31] with the benzyl substituent of the inhibitor. The 4.3 Å distance seen between Nε of the arginine side chain and the centroid of the benzamido ring is in the range of what has previously been observed for arene−guanidino distances within proteins structures.[32] Cation−Π interactions have now been shown to be important in protein stability, ligand binding, and enzyme catalysis and typically, Arg side chains, Lys side chains, metal ions, or ligand cations are found to interact with the side chains of Trp, Tyr, or Phe.[33−35] Experiments designed to measure the strength of cation−Π interactions in biological systems have shown that they range from −0.4 to −2.4 kcal/mol.[35] Given that fluorinated arenes have electron-poor Π-systems (in fact, the quadrupole moment of 4-methoxy-2,3,5,6-tetrafluorobenzamide is small according to ab initio calculations) and thus form weak cation−Π interactions,[36,37] it may seem surprising that the affinity-enhancing effect of the 4-methoxy-2,3,5,6-tetrafluorobenzamido−guanidino interaction is so large. However, it has been shown that the electrostatic attraction between the cation and the permanent quadrupole moment of the Π system does not account entirely for the observed energetics in systems involving cation−Π interactions[31,35] and that van der Waals interations,[38] Π−Π interactions, charge-transfer effects, and polarization effects must also be considered. In this case, the methoxy group of **3** is positioned close to the guanidino group of Arg144, which may allow for additional electrostatic interactions that further increase the affinity.[39] Moreover, fluorinated hydrocarbons are poorly solvated in water and desolvation of the 4-methoxy-2,3,5,6-tetrafluorobenzamido moiety can be expected to contribute substantially to the affinity of **3** for galectin-3.

Synthesis of Second Generation Inhibitors. Earlier studies in natural systems[37,40] and in minimalized artificial systems in water[41] and organic solvents[39,42] have concluded that electron-withdrawing substituents (i.e. fluorine or nitro) on the aromatic component weaken while electron-donating substituents (i.e. dimethylamino) strengthen cation−Π interactions, observations that suggest that the Π-electron density of the aromatic component is of primary importance. To investigate the influence of the structure of the aromatic component of an arginine−arene cation−Π interaction in a natural system and to discover further improved inhibitors of galectin-3, we prepared 42

(30) Davis, A. M.; Teague, S. J.; Kleywegt, G. J. *Angew. Chem., Int. Ed.* **2003**, *42*, 2718−2736.

(31) Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303−1324.
(32) Flocco, M. M.; Mowbray, S. L. *J. Mol. Biol.* **1994**, *235*, 709−717.
(33) Gallivan, J. P.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9459−9464.
(34) Zacharias, N.; Dougherty, D. A. *Trends Pharm. Sci.* **2002**, *23*, 281−287.
(35) Meyer, E. A.; Castellano, R. K.; Diedrich, F. *Angew. Chem., Int. Ed.* **2003**, *42*, 1210−1250.
(36) Mecozzi, S.; West, A. P., Jr.; Dougherty, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 2307−2308.
(37) Zhong, W.; Gallivan, J. P.; Zhang, Y.; L., L.; Lester, H. A.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12088−12093.
(38) Mao, L.; Wang, Y.; Liu, Y.; Hu, X. *J. Am. Chem. Soc.* **2003**, *125*, 14216−14217.
(39) Rensing, S.; Arendt, M.; Springer, A.; Grawe, T.; Schrader, T. *J. Org. Chem.* **2001**, *66*, 5814−5821.
(40) Mu, T.-W.; Lester, H. A.; Dougherty, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 6850−6851.
(41) Ngola, S. M.; Dougherty, D. A. *J. Org. Chem.* **1998**, *63*, 4566−4567.
(42) Hunter, C. A.; Low, C. M. R.; Rotger, C.; Vinter, J. G.; Zonta, C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4873−4876.

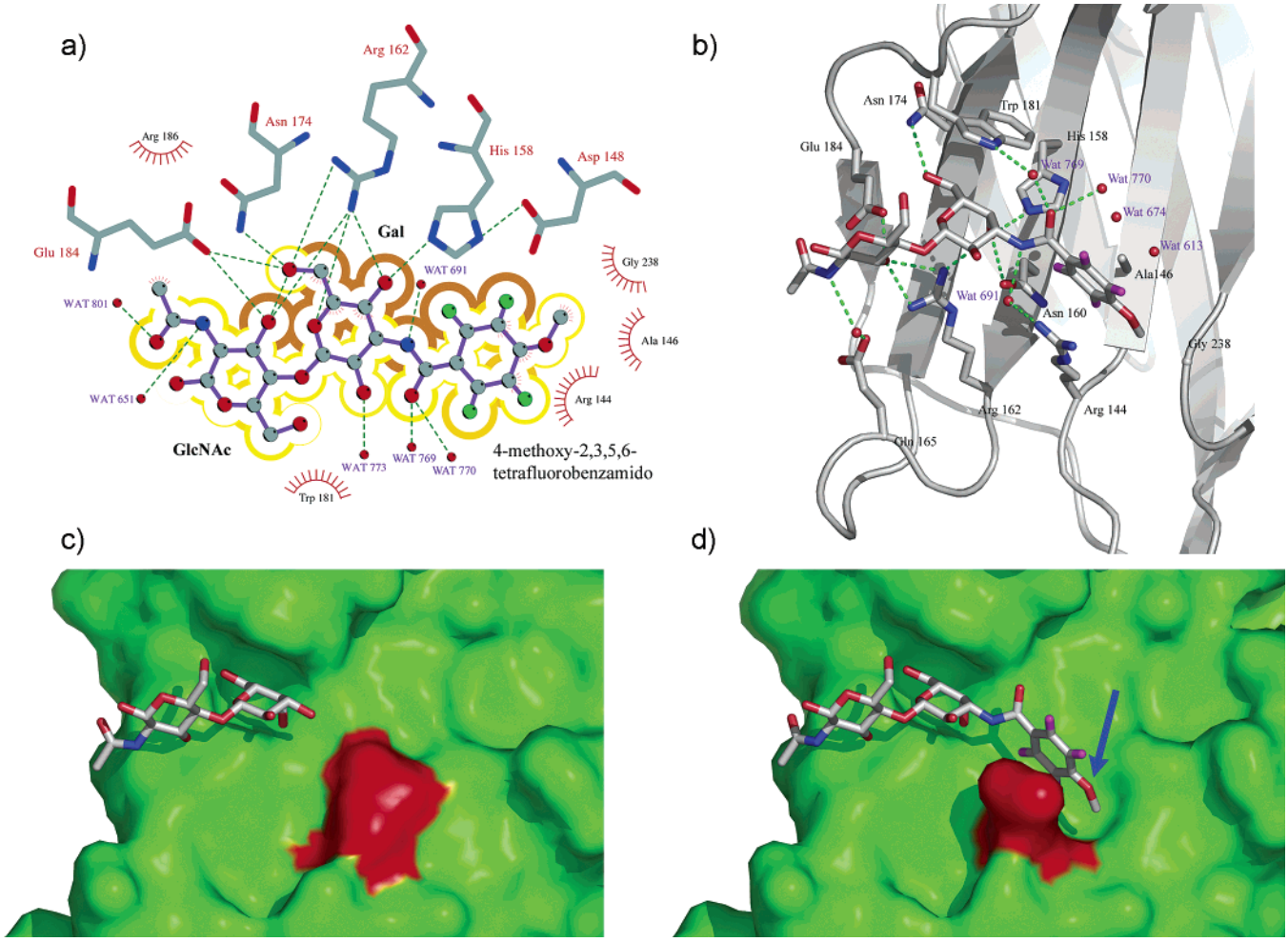


Figure 3. Interaction of the inhibitor **3** with galectin-3C. (a) Schematic of the inhibitor−protein contacts. The colored circular lines around the inhibitor indicate solvent accessibility with dark for buried and light for accessible. (b) View of selected residues and water molecules in the vicinity of the binding site. The Arg144 guanidino group (red) in the galectin-3C−LacNAc complex (c) adopts a different conformation to form a cation−Π interaction in the galectin-3C−**3** complex (d). The extended hydrophobic pocket formed by Arg144 Cβ to Cδ is indicated by a blue arrow in d.

potential LacNAc-based inhibitors all having aromatic amides at 3′-C (**4**−**46**, Figure 4) following our recently described procedure.[24]

Galectin-3 Inhibition in a Competitive Fluorescence Polarization Assay. A novel high-throughput galectin-3 solution phase assay based on fluorescence polarization[26,43] was employed to evaluate compounds **4**−**46**. Indeed, the chemical nature of the benzamido moiety strongly affects the interaction, as the inhibitory powers of **4**−**46** varied significantly. All compounds **4**−**46** were better than the unsubstituted LacNAc methyl glycoside **1** (K_d = 67 μM), and **35** was better than the unsubstituted parent benzamido derivative **2** (K_d = 6.7 μM). Although several (**15**, **24**, **32**, **34**, **37**; K_d = 0.95−1.3 μM) were similar to **3** in affinity for galectin-3, only two compounds (**41** and **44**; K_d = 0.48 and 0.32 μM, respectively) were better than **3** (K_d = 0.88 μM).

If it is assumed that the aromatic moieties of the good inhibitors stack against the Arg144 guanidino group in a manner similar to that of **3**, conclusions can be drawn concerning the effects of the arene structure and substitution pattern on the cation−Π interaction. In general, we see that para- and meta-substitution of the benzamide is beneficial, while ortho-substitution is detrimental to the interaction with galectin-3. Fluorination (**4**−**7**) of the aromatic ring results in inhibitors whose affinities are worse than the parent unsubstituted benzamide **2**.[24] This is probably due to a decrease in the Π-electron density in the fluorinated benzamides and thus a decrease in the cation−Π interaction strength. As a consequence, the *p*-methoxy group of **3** can be concluded to be essential for high affinity.

Methoxy-substitution at the meta-position is beneficial; the *m*-methoxy group of **9** can be expected to form favorable direct (oxygen) electrostatic interactions with the guanidino group of Arg144, as well as to fill the cleft formed by Arg144 Cβ to Cδ. Presumably *p*-methoxy (**10**) is somewhat less efficient than *m*-methoxy (**9**) at making favorable direct interactions due to the increased distance from the guanidino group and the cleft formed by Arg144 Cβ to Cδ. *o*-Methoxy-substitution (**8**) gives inhibitory power worse than no substitution (**2**). The observation that two *o*-methoxy substituents (**12**) are detrimental to galectin-3 binding is not unexpected, since, upon stacking of the Arg144 guanidino group onto the aromatic ring, one or the other of the two methoxy groups would interfere with the protein surface. The observation that the introduction of two *m*-methoxy groups (**15**) leads to an activity surpassing that of **3** seemed surprising at first, because if **15** forms a complex with galectin-3

(43) Sörme, P.; Kahl-Knutsson, B.; Huflejt, M.; Nilsson, U. J.; Leffler, H. *Anal. Biochem.* **2004**, *334*, 36−47.

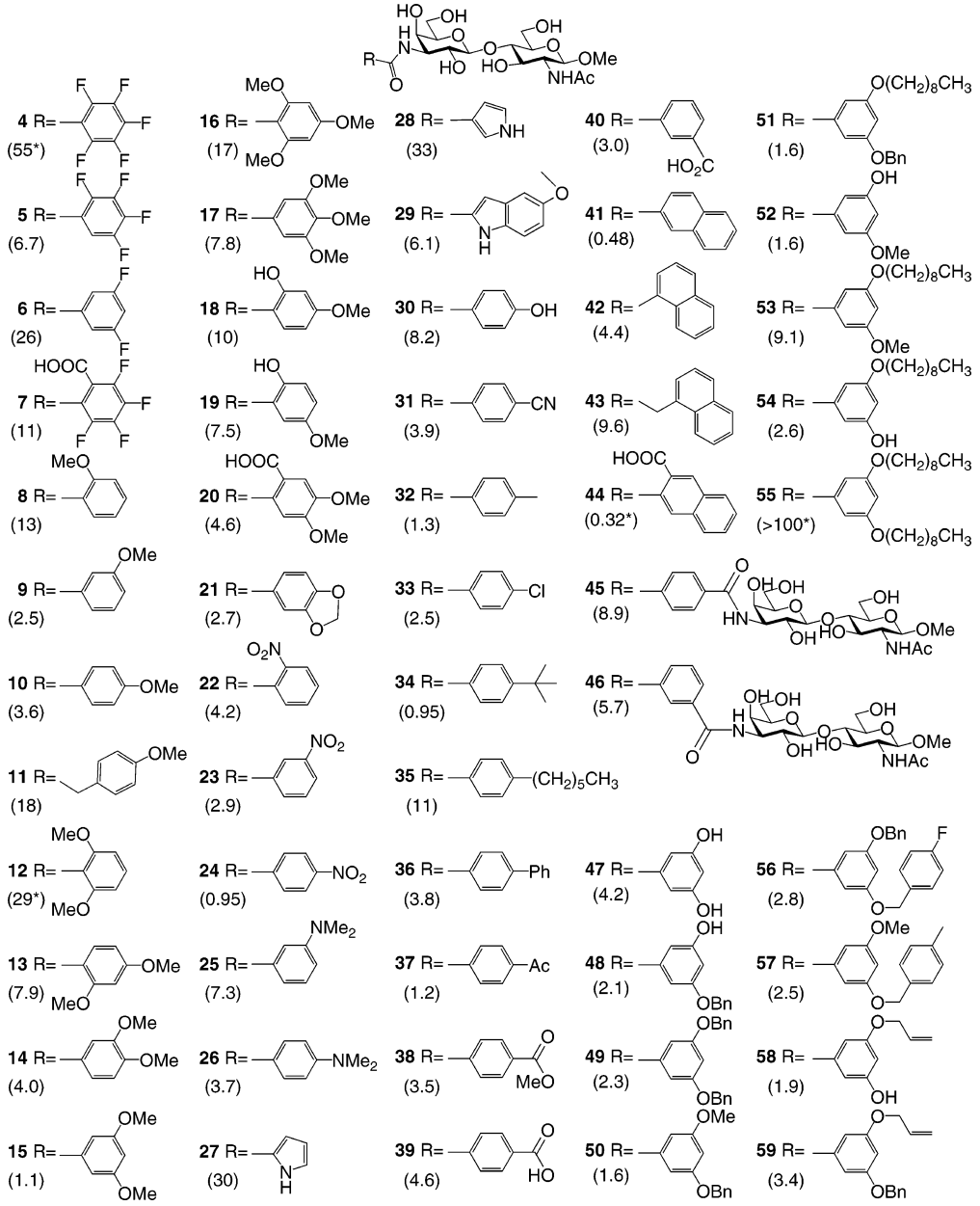


Figure 4. Synthetic LacNAc derivatives (**4**−**59**) carrying aromatic amides at 3′-C. K_d values (μM) as determined by a competitive fluorescence polarization assay were determined at 8 μM inhibitor concentration and are shown in brackets. *K_d for **44** had to be determined at 1.6 μM concentration of **44** because of its high affinity, and the K_d for **4**, **12**, and **55** had to be determined at 40 μM concentration because of their low affinities for galectin-3.

structurally similar to that of **3**, then one of the methoxy groups would point more or less into bulk solution. However, the presence of two symmetrically placed methoxy groups would, of course, be entropically favorable, because two degenerate conformers of **15** would interact with galectin-3 with the same enthalpic contribution. Compound **17** also carries two *m*-methoxy groups, in addition to a *p*-methoxy group, but it is significantly less active than **15**. However, in 1,2,3-trisubstituted benzene rings, the methoxy groups are typically arranged alternatively up and down relative to the benzene ring, a geometry that would result in a steric clash in a galectin-3−**17** complex structurally similar to that of galectin-3−**3**. This hypothesis is further supported by the fact that **21** is a slightly better inhibitor than **14**. In **21** the methylene bridge between the *m*- and *p*-oxygens can adopt an orientation similar to the methyl group of **9**.

Taken together, simply introducing an electron-donating methoxy functionality on an aromatic partner in an arene−guanidino interaction does not automatically lead to enhanced interaction. The direct electrostatic and steric interactions of the introduced substituent with the protein surface can overshadow electron-donating effects and have to be taken into consideration, as do the effects of the substituent on ligand conformational preferences. In the small arginine receptors studied by Schrader, for example, it has been suggested that the direct electrostatic interactions between the aromatic methoxy group and the arginine guanidino group were more important than the cation−Π interactions in stabilizing the complexes.[39] Their hypothesis was supported by electrostatic potential calculations, which gave similar results with benzene and anisole.

The electron-donating dimethylamino-substituent in the para-position (**26**) only marginally improves the inhibitor affinity.

This result may be explained by the fact that, compared to the *p*-methoxy compound (**10**) discussed above, it is sterically less complementary to the hydrophobic cleft formed by Arg144 Cβ to Cδ. *m*-Dimethylamino substition as in compound **25** does not affect the inhibitory power, presumably because such a substituent would have to be positioned away from the Arg144 side chain for steric reasons. Furthermore, the higher degree of protonation of the dimethylamino substituent at near-neutral pH, as compared to the methoxy, negatively influences both a direct electrostatic interaction with the guanidino group and the indirect interaction via electron donation to the benzene ring. Unprotonated dimethylamino-substitution has been shown to enhance cation−Π interactions in an artificial model system in chloroform.[42]

Among the other substituents investigated, only *p*-nitro (**24**), *p*-methyl (**32**), *p*-*tert*-butyl (**34**), and *p*-acetyl (**37**) give rise to high-affinity inhibitors. The nitro-substituent of **24** extends the Π-system of the benzamido group closer to the Arg144 residue, thus leading to an improved interaction with the Arg144 guanidino group. However, the possibility that the nitro group forms direct bidentate interactions with the Arg144 guanidino group in a structurally different fashion cannot be excluded. Surprisingly, *p*-carboxy substitution (**39**) results in a rather small affinity enhancement, although it can be regarded as an isostere of **24**.

Among the larger aromatic systems, 2-naphthamides proved to be exceptionally good at enhancing the affinity for galectin-3. The 2-naphthamido derivatives **41** and **44** are well-suited to forming a cation−Π interaction with the Arg144 guanidino group *and* to fill the hydrophobic cleft lined by Arg144 Cβ−Cδ. Large and more polarizable Π-systems typically form strong cation−Π interactions.[35,39] The *o*-carboxy substituent of **44** has only a small affinity-enhancing effect, but it may be useful as a means of increasing the solubility of the inhibitors. The heteroaromatic amides, exemplified by the indol **29** and pyrroles **27** and **28**, are surprisingly poor even though indol (i.e. Trp) is common in protein cation−Π interactions and pyrrole is an electron-rich Π-system. This suggests that the benzamide−Arg144 interaction is indeed specific and very sensitive to the exact positioning of the Π-system, a conclusion supported by the observation that phenylacetamide **11**, 1-naphthamide **42**, and naphthylacetamide **43** are less efficient inhibitors.

The K_d of 320 nM for **44** is more than 2 orders of magnitude better than that of the parent LacNAc **1** (K_d = 67 μM) and represents an unusually high affinity for a monovalent ligand−lectin interaction. The excellent inhibitory power of the dimethoxy-substituted **15** suggested the synthesis of even further optimized inhibitors by means of replacing the methoxy with other alkoxy groups. The synthesis of 13 derivatives (**47**−**59**) did indeed show that 3,5-dialkoxy-substitution of the benzamide was beneficial for complex formation as 11 of them were better inhibitors than the unsubstituted reference compound **2**. However, none of them was better than the 3,5-methoxy-substituted benzamide **15**.

Thermodynamic Studies on Galectin-3: Inhibitor Complexes. To further characterize the underlying basis for the affinity-enhancing effect of the 3′-benzamido-substitution of LacNAc, three of the best inhibitors (**15**, **24**, and **32**), with suitably high water-solubility, were selected for ITC experiments. Only a few thermodynamic studies on galectin−ligand interactions have been described in the literature,[23,44−49] and the present study is the first to analyze small synthetic inhibitors with galectin-3C. Notably, the best monovalent ligand described, to date, is the blood group A tetrasaccharide with K_d = 72 μM (as compared to 191 μM for LacNAc).[47] The isothermal titration microcalorimetry experiments were performed with galectin-3C to avoid the possibility that the N-terminal domain of intact galectin-3 might mediate higher order oligomers.[22,50] In addition, galectin-3C is considerably more soluble than intact galectin-3. The methyl glycoside of *N*-acetyllactosamine **1** was included in the experiments as a reference ligand; it was found to have K_d = 82 μM, a value lower than that obtained with reducing *N*-acetyllactosamine and intact galectin-3 [47] (K_d = 191 μM). It should also be noted that the K_d values obtained for the various compounds by ITC are approximately 5-fold higher than those obtained by fluorescence polarization (the relative affinities are essentially identical). Although the basis for this difference is not known, the fluorescence polarization experiments were performed on intact galectin-3 where effects arising from the N-terminal domain—such as oligomerization—may be important.

Table 1. Thermodynamic Parameters for the Interactions between Galectin-3C and Selected Inhibitors

	1	15	24	32
ΔG (kcal/mol)	−5.6	−7.2	−7.2	−7.2
ΔH (kcal/mol)	−9.6 ± 0.6	−11.2 ± 0.1	−11.7 ± 0.1	−12.0 ± 0.1
$T\Delta S$ ((kcal/mol)/K)	−4.0	−4.0	−4.5	−4.8
K_d (μM)	84.0 ± 10.0	5.0 ± 0.3	4.8 ± 0.3	5.7 ± 0.4

The optimized inhibitors **15**, **24**, and **32** exhibited K_d values of 5.0, 4.8, and 5.7 μM, respectively, in the ITC experiments, an approximate 15-fold increase in affinity compared to the parent methyl glycoside of *N*-acetyllactosamine **1** (84 μM). These increases correspond to a change in binding free energy ($\Delta\Delta G$) of approximately 1.7 kcal/mol, a value that agrees well with that observed for cation−Π interactions in other biological systems.[35,37] As shown in Table 1, these changes are a direct reflection of changes in the enthalpy of binding ($\Delta\Delta H$ between 1.6 and 2.4 kcal/mol). In two cases, the favorable enthalpy changes are offset somewhat by unfavorable changes in entropy[51−53] relative to compound **1** (i.e. **24** and **32** with $\Delta\Delta S$ = 0.5 and 0.8 kcal/mol, respectively). Interestingly, the *m*,*m*-dimethoxy-substituted benzamido derivative **15** displays no entropy penalty relative to the parent LacNAc **1**.

Conclusions

Analysis of high-resolution crystallographic data, together with synthesis and evaluation of a large panel of benzamido derivatives and ITC measurements, provides important information about inhibitor−galectin-3 interactions, which is essential

(44) Gupta, D.; Moonjae, C.; Cummings, R. D.; Brewer, C. F. *Biochemistry* **1996**, *35*, 15236−15243.
(45) Schwarz, F. P.; Ahmed, H.; Bianchet, M. A.; Amzel, L. M.; Vasta, G. R. *Biochemistry* **1998**, *37*, 5867−5877.
(46) Bharadwaj, S.; Kaltner, H.; Korchagina, E. Y.; Bovin, N. V.; Gabius, H.-J.; Surolia, A. *Biochim. Biophys. Acta* **1999**, *1472*, 191−196.
(47) Bachhawat-Sikder, K.; Thomas, C. J.; Surolia, A. *FEBS Lett.* **2001**, *500*, 75−79.
(48) Ahmad, N.; Gabius, H.-J.; Kaltner, H.; André, S.; Kuwabara, I.; Liu, F.-T. *Can. J. Chem.* **2002**, *80*, 1096−1104.
(49) Brewer, C. F. *Glycoconjugate J.* **2004**, *19*, 459−465.
(50) Brewer, C. F. *Biochim. Biophys. Acta* **2002**, *1572*, 255−262.
(51) Toone, E. J. *Curr. Opin. Struct. Biol.* **1994**, *4*, 719−728.
(52) Lemieux, R. U. *Acc. Chem. Res.* **1996**, *29*, 373−380.
(53) Gohlke, H.; Klebe, G. *Angew. Chem., Int. Ed.* **2002**, *41*, 2644−2676.

for the development of inhibitors with higher affinity and specificity. In particular, an Arg144−arene stacking that involves a cation−Π interaction appears to be of importance for high affinity, which suggests that exploitation and fine-tuning of cation−Π interactions may very well emerge as a more general strategy toward the enhancement of protein−ligand interactions. However, the present work also reenforces the fact that direct interactions of the arene substituents with the protein surface are also very important in the inhibitor design process. In addition, the synthesis of potent specific monovalent inhibitors of galectin-3, as described herein, provides valuable research tools as well as an advance toward the goal of developing galectin-based therapeutic drugs.

Acknowledgment. This project was partly supported by a grant from the Association pour la Recherche sur le Cancer (France), as well as grants from the program "Glycoconjugates in Biological Systems" sponsored by the Swedish Strategic Research Foundation, the Swedish Research Council, the Swedish Medical Research Council (Grant No. 12165), and the Medical Research Council (Canada)/Canadian Institutes of Health Research.

Supporting Information Available: Data collection and refinement statistics for galectin-3−ligand complexes, experimental procedures, NMR and HRMS-FAB data for compounds **4**−**59**, synthesis and NMR data for 3,5-dialkoxybenzoic acids, and ^{1}H NMR spectra of **4**−**59** (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

JA043475P