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**Targeting the CRD F-face of Human Galectin-3 and Allosterically Modulating Glycan Binding by Angiostatic PTX008 and a Structurally Optimized Derivative**

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### Calix[4]arene PTX008 is an angiostatic agent that inhibits tumor growth in mice by binding to galectin-1, a -galactoside-binding lectin. To assess the affinity profile of PTX008 for galectins, we used 15N–1H HSQC NMR spectroscopy to show that PTX008 also binds to galectin-3 (Gal-3), albeit more weakly. We identified the contact site for PTX008 on the F-face of the Gal-3 carbohydrate recognition domain. STD NMR revealed that the hydrophobic phenyl ring crown of the calixarene is the binding epitope. With this information, we performed molecular modeling of the complex to

assist in improving the rather low affinity of PTX008 for Gal-3. By removing the *N*-dimethyl alkyl chain amide groups, we produced PTX013 whose reduced alkyl chain length and polar character led to ~8-fold stronger binding than PTX008. PTX013 also binds Gal-1 more strongly than PTX008, whereas neither interact strongly, if at all, with Gal-7. In addition, PTX013, like PTX008, is an allosteric inhibitor of galectin binding to the canonical ligand lactose. This study broadens the scope for galectin targeting by calixarene-based compounds and opens the perspective for selective galectin blocking.

##### Introduction

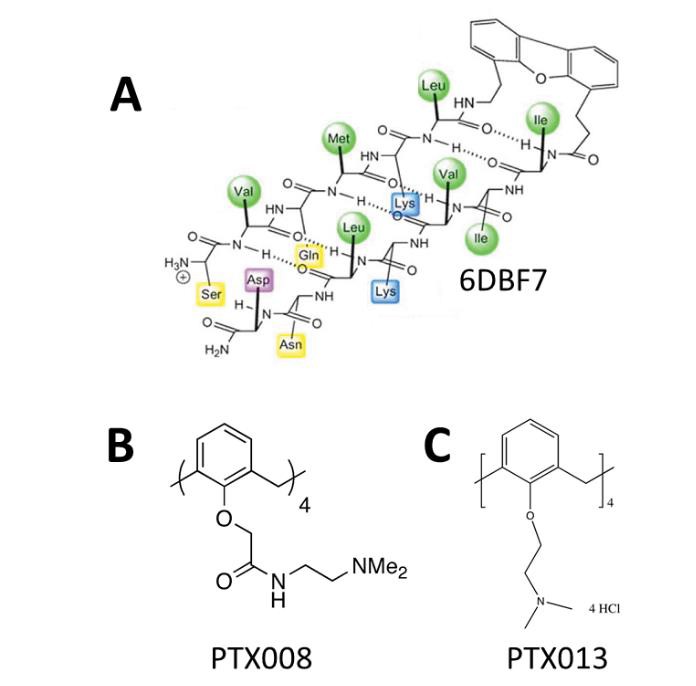
Having first been detected as hemagglutinin[1], ga(lactose-binding)lectins are now considered to form a family of multifunctional proteins.[2-5] Their broad- scale activity profile covering diverse aspects, such as cell-cell matrix recognition, growth regulation, mediator release and outside-in signaling, has attracted interest to turn insights into counter- receptor binding and post-binding effects into clinical benefit. Of note, in addition to glycans as contact partners, galectins are also being revealed to engage in specific contacts with proteins, e.g. in apoptosis[6], autophagy[7] and in immune regulation.[8] Considering the advanced status of structural analysis, e.g. by crystallography[9], efforts have been made to design galectin antagonists.[10-13]

Most galectin-targeting antagonists are ß-galactoside- based compounds that interact with the canonical site of the conserved carbohydrate binding domain (CRD). Examples of such derivatives are anomeric oxime ether derivatives of ß-galactose (*O*-galactosyl

aldoxime),[14] aryl *O*-, *S*- or Se-galactosides and - lactosides with bioinspired substitutions,[15-20] carbohydrate-based triazoles and isoxazoles,[16] 3- (1,2,3-triazol-1-yl)-1-thio-galactosides,[21], and thio- ureido *N*-acetyllactosamine derivatives.[22] Inhibitory potency and also selectivity among galectins can then be increased by exploiting spatial factors, especially display of ligands in clusters. Toward this end, a series of chemical platforms have been tested, first poly- (amidoamine)-based starburst glyco-dendrimers,[23] then, e.g. a bis-benzamido core[24] or calixarenes.[25]

Previously, we reported that the 33mer peptide anginex and its partial peptidomimetic compound 6DBF7 (Figure 1) inhibit angiogenesis and tumor growth,[26-29] and this by forming a complex with galectin-1 (Gal-1).[30] Using structure-based design, we then produced non-peptidic, calix[4]arene-based compounds that display chemical substituents to approximate amphipathic features and molecular dimensions of anginex and 6DBF7.[31] From this panel of substances, the calix[4]arene PTX008 (Figure 1, aka 0118)[11] was identified as a potent angiogenesis

inhibitor in cell-based assays and *in vivo*. PTX008 inhibits tumor angiogenesis and tumor growth in several murine tumor models.[31] Also, PTX008 displays multimodal activities in terms of inhibiting proliferation of endothelial cells and increasing leukocyte infiltration.[32] In sum, PTX008 interferes with tumor angiogenesis and vessel normalization,[33- 35] attenuates tumor escape by blocking Gal-1- dependent T cell apoptosis,[32] and inhibits metastasis by reducing tumor-endothelial cell interactions, shown to be mediated by Gal-1.[36] As a pharmaceutical agent, PTX008 (937 Da) can almost be considered a “small molecule” (i.e. < 500 Da) and should reach a therapeutic *in vivo* biodistribution. In addition, due to its aryl-crown structure, PTX008 should be chemically stable and less likely to be metabolized *in vivo* than a peptide or glycan.



**Figure 1. Chemical structures.** Structures are shown for partial peptide mimetic of anginex, named 6DBF7 (**A**), as well as calix[4]arene compounds PTX008 (**B**) and PTX013 (**C**).

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Structurally, all galectin CRDs have a -sandwich folded conformation with a 6-stranded -sheet for the canonical sugar binding S-face and an opposing 5-

values on the F-face of the CRD are emphasized by color highlights in Figure 2D. Evidently, PTX008 binds within the region of the F-face of the CRD, in which the

stranded -sheet F-face. On the molecular level,

NT is known to interact transiently.

Smaller 

PTX008 binds to the F-face of Gal-1 (KD < ~100 M) and is an allosteric inhibitor of sugar binding to this lectin.[37] The question to be answered next concerns the possibility for PTX008 binding to other members of the galectin family. After all, galectin expression has been disclosed to cover more than Gal-1 like a network,

e.g. in colon cancer,[38-40] often prominently with Gal-3 that is a versatile regulator of tumor cell apoptosis, metastasis and proliferation.[41,42] Structurally, Gal-3 consists of the canonical CRD and an N-terminal tail (NT) that is composed of nine collagen-like repeats and two serine phosphorylation sites. Here, we used HSQC NMR spectroscopy to show that PTX008 associates with the F-face of the CRD of Gal-3, albeit about an order of magnitude more weakly (50% binding ~ 1.2 mM) than to Gal-1, and also acts as an allosteric inhibitor of -galactoside binding. By identifying contact sites on both Gal-3 and PTX008, we were able to model their bound-state structure. This model directed structural alteration of PTX008 to generate calixarene PTX013 (Figure 1) that binds more strongly to Gal-3 and retains strong binding to Gal-1.

## Results and Discussion

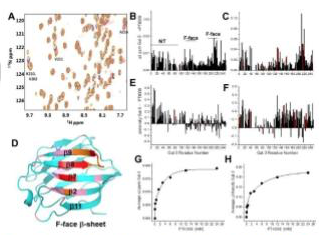
**PTX008 binds to Gal-3.** 15N-Labeled Gal-3 was tested in 1H–15N HSQC experiments in solution with increasing PTX008 concentrations. HSQC spectra (along with an expansion) obtained during the titration are overlaid in Figure 2A . The concentration-dependent effects show that while some Gal-3 resonances are significantly chemically shifted and broadened (e.g. V202, K210, V211, A216 labeled in Figure 2A), others are not,

changes are observed within the carbohydrate-binding

S-face of the CRD (residues 112-124, 136-189, and 230- 240), suggesting that PTX008 binding to the F-face appears to induce conformational and/or dynamical changes within the Gal-3 S-face as it does with Gal-1.[37]

Binding of PTX008 (1.5 mM) also induces resonance broadening (Intensity), with the greatest increase observed for residues within the N-terminal part of the NT and differential effects observed within the CRD (Figure 2E). Negative Intensity values indicate narrower line widths and thus an increase in internal motions and/or a decrease in known NT-CRD interactions (i.e. exchange dynamics).[43] Positive

Intensity values are indicative of enhanced NT-CRD interactions, as well as the possibility of NT-PTX008 interactions when observing effects at NT residues. At an increased PTX008 concentration (15 mM), resonance broadening is increase



**Figure 2. HSQC data us ed to identify the contact region for PTX008 on full-length human Gal-3.** (**A**) HSQC spectra are overlaid in 1H–15N HSQC spectral expansions

indicating selective binding. Figure 2B presents a plot

through

15N-labeled full-length Gal-3 (80 M) titrations with PTX008 concentrations

of PTX008-induced (1.5 mM) chemical shift changes () vs. the amino acid sequence of Gal-3. The most highly shifted resonances are located within the N- terminal part of the N-terminal tail (NT) of Gal-3 (residues 1-60) and the F-face of the CRD (residues 123- 130, 190-220, and 240-250). At 15 mM PTX008, 

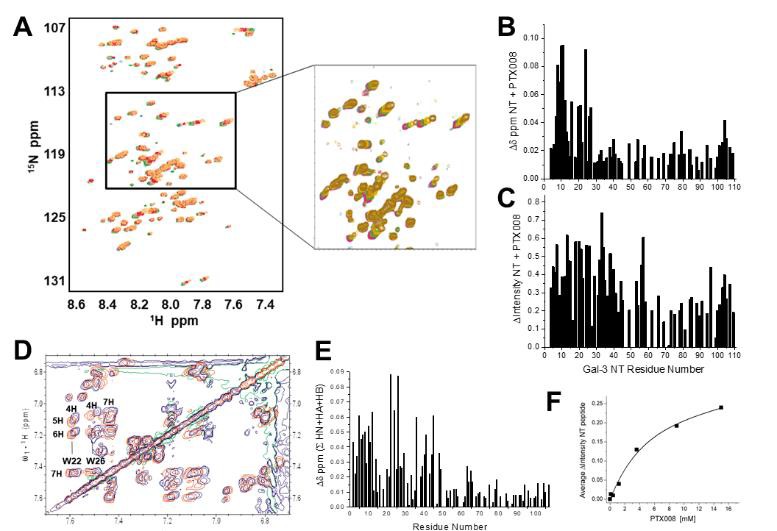
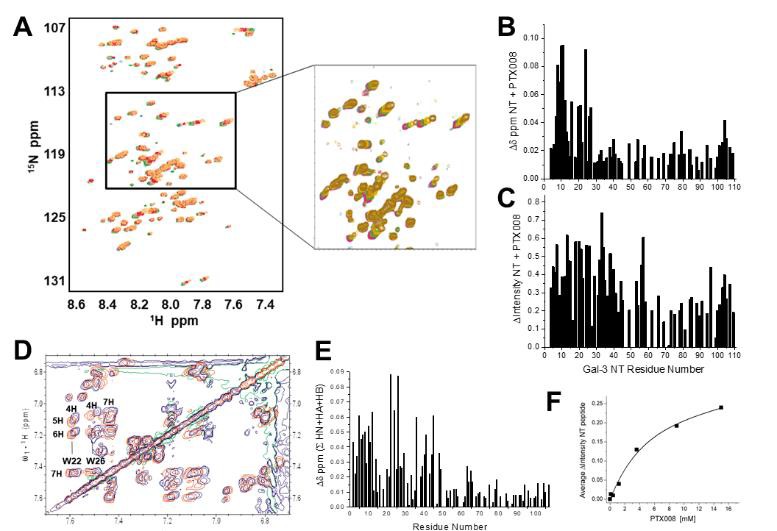
values for these residues are generally larger than those for S-face residues (Figure 2C). The largest 

from zero (green contours) to 24 mM (brown contours). (**B-F**) HSQC chemical shift (**B,C**)

and resonance broadening (**E,F**) maps are shown for PTX008 binding to Gal-3 at PTX008 concentrations of 1.5 mM (**B,E**) and 15 mM (**C,F**). (**D**) The crystal structure of the Gal-3 CRD (PDB 1A3K) is presented with the F-face forward and the -strands labeled. The most perturbed resonances (sequences) are highlighted in red (> 2SD above average), orange (1SD to 2SD above average) and pink (average to 1SD above average), with all others in cyan (< average). (**G,H**) PTX008 titration curves are shown for Gal-3 as (**G**) and Intensity (**H**) values averaged over all residues vs. total concentration of PTX008 for residues within the CRD (Figure 2F).

NMR is sensitive to a range of chemical exchange regimes on the chemical shift time scale from “slow” exchange in which KD values are sub-M and individual

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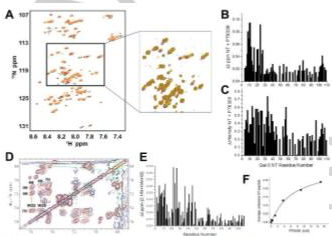


resonances are observed for each exchanging state to “fast” exchange in which KD values are generally > ~3

M when the on-rate kon is diffusion limited.[44] “Intermediary” exchange is observed when KD values fall in between fast and slow exchange and is reflected in NMR spectra by resonance broadening and minimally shifted signals. Unlike binding events that occur in the fast exchange regime, averaging between bound and free states in the intermediate exchange regime is inefficient, and thus one cannot accurately determine KD values. For PTX008 binding to Gal-3,  values are relatively small and resonance broadening (Intensity) is significant, suggesting that this molecular interplay occurs in the intermediate exchange regime. However, plots of and Intensity**F** (averaged over all amino acids of Gal-3) vs. the PTX008 concentration (Figure 2G,H, respectively) show that the 50% change occurs at ~1.2 mM. Even though this value should indicate that PTX008 binds to Gal-3 in fast exchange, NMR spectra show characteristics of intermediate exchange. This in turn indicates that either the PTX008 binding on rate (kD) is slower than expected for fast exchange or PTX008 binding to the Gal-3 F-face is “fuzzy” in which this ligand is not locked into a single, defined binding mode and thus could cause a spread in NMR signals.

Since we know that the NT can transiently fold onto the F-face of the CRD,[43] it appears that PTX008 may bind to the NT in this conformer of the Gal-3, an event that tends to displace the NT from the CRD. To assess whether PTX008 can indeed bind to the NT independent of the presence of the CRD, we used uniformly 15N-labeled NT peptide (residues 1-113) and performed an HSQC titration with PTX008 as shown in Figure 3A, along with an expanded region of the HSQC signals. These data suggest that PTX008 and NT can associate in the absence of the CRD. and Intensity values plotted vs. the NT sequence (Figure 3B,C) indicate that most of the changes are observed within NT residues 1-50. In order to acquire corroborating evidence for the site of interaction, we prepared this section of the NT peptide (residues 1-50) at natural abundance of 15N and acquired 1H TOCSY data with it in the presence of PTX008. In this experiment, we observed that the aromatic systems of the two

tryptophan moieties within this peptide (W22 and W26) are significantly perturbed (Figure 3D), indicating that PTX008 interacts with that region of the NT. Figure 3E presents the plot of values summed over all 1H NH, Hand Hresonances vs. the NT sequence. Changes are greatest for residues from F4 to A13, W22 to W26, and Y36 to Y45, results that are consistent with those obtained by titration with the 15N-labeled NT peptide shown in Figure 3B. In this regard, we conclude that PTX008 interacts directly with the N-terminal region of the Gal-3 NT. Figure 3F plots the average  values vs. the PTX008 concentration, with fits extrapolating the 100% change to 0.27 ppm such that the 50% effect falls at ~ 4 mM.

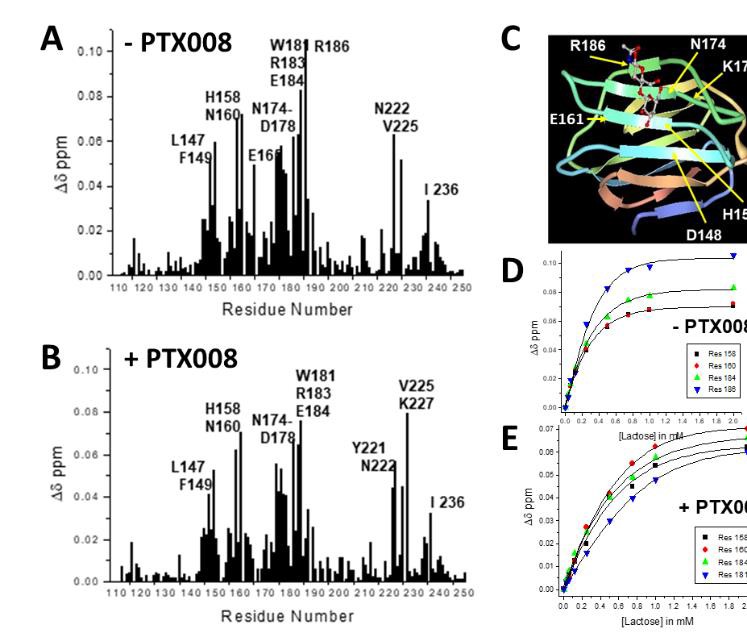
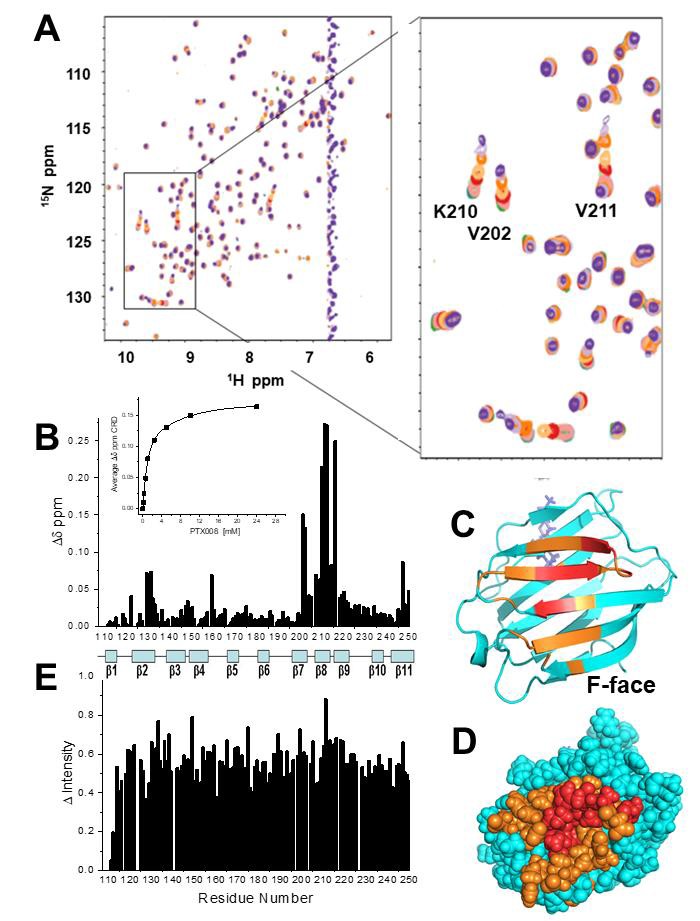


**Figure 3. HSQC data of binding of PTX008 to the NT of Gal-3.** (**A**) HSQC spectra are overlaid through the PTX008 titration with 15N-labeled NT (100 M) from Gal-3 (residues 1-113). PTX008 concentrations range from zero (green contours) to 15 mM (dark yellow contours). An expanded region from these HSQC spectra is shown with PTX008 concentrations from zero (red contours) to 15 mM (brown contours). (**B**) An HSQC chemical shift map is shown for PTX008 binding to the free NT. (**C**) An HSQC resonance broadening map is shown for PTX008 binding to the NT. (**D**) 1H TOCSY spectra present the aromatic resonance region of the unlabeled NT (residues 1-113) in the absence (red contours) and presence (blue contours) of 15 mM PTX008. W22 and W26 side chain Nring resonances are labeled. (**E**) values (= [(1H)2]1/2) are shown as the sum of values for NH, H, and Hresonances, and plotted vs. the sequence of the NT (residues 1-113). (**F**) A titration curve (total PTX008 concentration) is shown for sequence-averaged Intensity values for PTX008 binding to Gal-3 NT residues (1-113).

Next, we investigated whether PTX008 can bind to the Gal-3 CRD independent of its NT. For this, we used the 15N-labeled CRD of Gal-3 (no NT present) and measured shifts in HSQC titrations with PTX008 (Figure 4A). HSQC spectral expansions exemplify that three resonances within the CRD F-face (i.e. V202, K210, and V211) are significantly shifted and broadened during the titration.

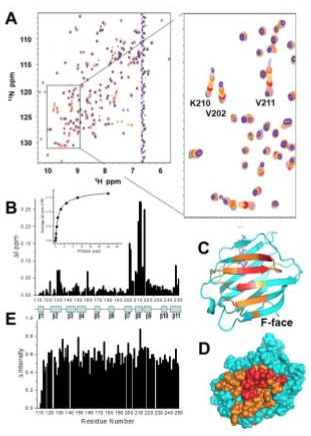
values for all amino acids of the CRD are plotted vs. the Gal-3 CRD sequence (Figure 4B), disclosing that effects are greatest for F-face residues as highlighted

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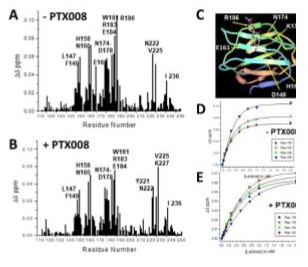
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on the structure of the CRD (Figure 4C,D). This in turn defines the PTX008 binding epitope on Gal-3 that is essentially the same as that observed with full-length Gal-3 (Figure 2D). Note also that resonance broadening (Intensity, Figure 4E) is greater than that measured with full-length Gal-3 (Figure 2E,F). The insert to Figure 4B documents the relation between values, averaged over all residues, and the concentration of PTX008. The 50% change occurs at about 1.5 mM, which is nearly the same as for PTX008 interactions with the CRD in full-length Gal-3 (Figure 2G,H).



**Figure 4. HSQC data of binding of the PTX008 to the CRD of Gal-3.** (**A**) HSQC spectra are overlaid in 1H-15N HSQC spectral expansions for the titration of 15N-labeled Gal-3 CRD (80 M) with PTX008 concentrations from zero (green contours) to 24 mM (purple contours). An expanded region from these HSQC spectra is shown with PTX008 concentrations ranging from zero (green contours) to 15 mM (purple contours). (**B**) An HSQC chemical shift map is shown for PTX008 binding to the CRD of Gal-3. The insert to **B** shows a PTX008 titration curve for Gal-3 CRD values averaged over all residues vs. the concentration of PTX008. The total PTX008 concentration is shown on the x- axis in these plots. (**C**) The crystal structure of Gal-3 CRD (PDB 1A3K) is shown with the F-face forward. The most perturbed resonances (sequences) are highlighted in red (> 2SD above average), orange (1SD to 2SD above average) and cyan (< average). (**D**) The same orientation shown in **C** is given as a space filling model using the same color coding to define the most probable binding site for PTX008. (**E**) An HSQC resonance broadening map is shown for PTX008 binding to the Gal-3 CRD.

**Effect of PTX008 on carbohydrate binding of Gal-3.** To assess the effect of PTX008 on carbohydrate binding to Gal-3, we acquired 15N–1H HSQC spectra with 15N-



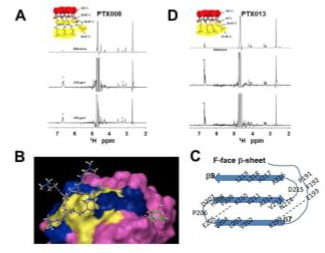
**Figure 5. Effect of PTX008 on binding of lactose to Gal-3.** (**A**,**B**) Chemical shift maps are shown for lactose (maximum used vis-à-vis zero concentration) binding to the Gal- 3 CRD (40 M) in the absence (**A**) and presence (**B**) of 10 mM PTX008. (**C**) The crystal structure of the Gal-3 CRD (PDB 1A3K) is presented with the S-face forward and the positions of some key lactose binding residues indicated. The stick structure of bound lactose is shown in gray. (**D,E**) Lactose titration curves are shown for four amino acid residues that have some of largest chemical shift changes (H158, N160, E184, R186).

labeled full-length Gal-3 and its CRD (in the presence of a saturating amount of PTX008) as a function of increasing lactose concentration. Figure 5A,B document plots of maximal values vs. the Gal-3 CRD sequence. In the absence of PTX008, the most shifted residues (Figure 5A) are those in the canonical lactose- binding site (see corresponding HSQC data on dimeric galectins Gal-1 and Gal-7).[45,46] The profile changes somewhat when PTX008 is bound to the CRD F-face (Figure 5B). In this instance, a few residues within the lactose-binding site (Figure 5C) show significantly modified values (compare Figures 5A and 5B). Whereas values for lactose-binding residues E161 and R186 are nearly gone, those for Y221 and K227 at the F-face of the CRD are highly increased. Interestingly, the main chain NH and CH groups of R186 are almost on the F-side of the CRD, near the edge, whereas the R186 side change sweeps around the edge to reach the S-face. In this regard, PTX008 binds to the F-face near R186, perhaps explaining the effect. Figure 5D,E show plots of values for four of the most shifted CRD resonances (H158, 160, W181, 184). Since lactose binding occurs in the fast exchange regime on the chemical shift time scale, relatively accurate KD values

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could be calculated: 170 M in the absence of PTX008 and 350 M in the presence of PTX008. In this regard, binding of lactose to Gal-3 is about 2-fold less with PTX008-bound Gal-3, an effect that is allosterically induced.



**Figure 6. STD NMR spectroscopy spectra on PTX008 and PTX013 when binding galectins and structure of the complex.** STD NMR spectroscopical experiments were performed with PTX008 (**A**) or PTX013 (**D**) in the presence of 30 μM of Gal-1 or Gal-3. (**A,D**) The top panels show the off-resonance reference NMR spectra. The middle panels show the STD spectra (scaled up) for Gal-1, and the bottom panels show the STD spectra (scaled up) for Gal-3. Asterisks indicate peaks receiving comparatively higher degrees of magnetization. At the top of each STD spectroscopy data set, the chemical structures of PTX008 (**A**) or PTX013 (**D**) are shown, with epitope mapping indicating STD intensities for each signal relative to STD intensities with highlights in red indicating 100% effect, orange for 40% to 60% effect, and yellow circles for < 40% effect. (**B**) Space-filling model showing the PTX008 binding site on the F-face of the Gal- 3 CRD crystal structure (PDB 1A3K). Yellow indicates the primary binding residues, and blue the secondary ones. The energy-minimized structure of PTX008 bound to the Gal- 3-binding epitope is shown as a stick structure in gray. A molecule of lactose bound to the canonical site of Gal-3 is shown as a stick model in green. (**C**) The three -strands (7, 8, and 9) on the F-face of Gal-3 to which PTX008 binds are drawn, with some of the key binding residues labeled.

**Optimization of PTX008.** Even though our HSQC Gal-3 titrations with PTX008 do not provide for a precise determination of KD values, PTX008 binds Gal-3 more weakly than it does Gal-1, disclosing selectivity between these two galectins. These data suggest that there is considerable room for improvement when targeting Gal-3. With this aim in mind, we needed to identify the PTX008 epitope that is in contact with Gal-

3 and computational modeling of the complex to optimize PTX008 binding to Gal-3. In terms of identifying the binding epitope on PTX008, Figure 6A shows saturation transfer difference (STD) NMR data with the standard reference spectrum of PTX008 at the top and the STD difference spectrum at the bottom of the figure. As highlighted in red on the chemical

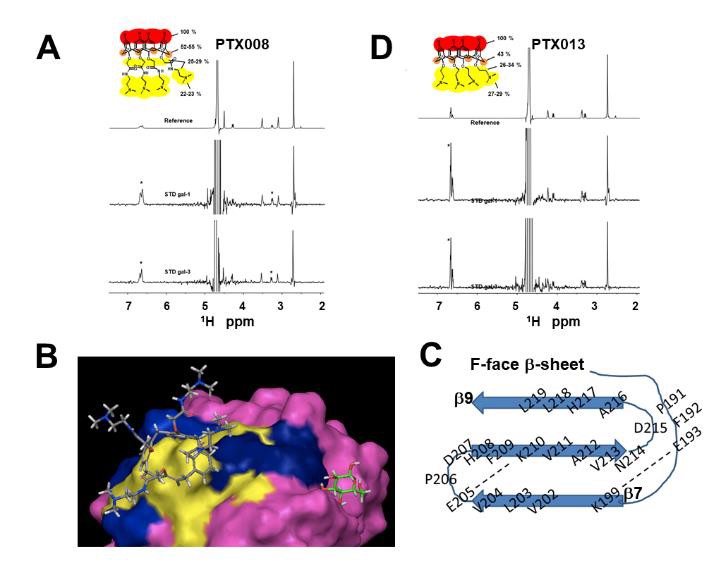
structure of the compound shown at the top of Figure 6A, the greatest effect is observed at the PTX008 phenyl groups (resonances at ~6.7 ppm). While the STD effect is 100% for these aromatic groups and ~53% for the ring-bridging methylenes, it is much less for the methylenes around the amide group (~27%) and the *N*- dimethyl groups (~22%). These data indicate, therefore, that the hydrophobic face of PTX008 is the primary site of contact with the F-face of the Gal-3 CRD. Also noteworthy is that the PTX008 binding epitope for Gal- 3 is the same for Gal-1 (Figure 6A, middle STD NMR spectal trace).

Having identified both the complementary regions on PTX008 and on the F-face of the CRD, we modeled the bound-state structure by manually docking the two and then running MD simulations to define the low- energy form of the Gal-3:PTX008 complex (Figure 6B). Most of the residues within the binding site on the Gal- 3 F-face (-sheet strands 7, 8 and 9) are hydrophobic and/or negatively charged (Figure 6C). In this orientation, these Gal-3 residues promote interactions with PTX008. Analysis of this modeled complex (Figure 6B) suggested that Gal-3 binding could be enhanced by reducing both the length of the *N*-dimethyl chains on the hydrophilic face of PTX008 and the chains’ overall polar character. We accomplished this by removing the intra-chain amide groups, resulting in PTX013, whose structure is given at the top of Figure 6D. STD NMR spectroscopy also demonstrated that PTX013 and PTX008 have the same contact profile to Gal-3 (Figure 6D, bottom spectral trace).

On the protein side, an HSQC titration with 15N-labeled Gal-3 and PTX013 (Figure 7A) demonstrates that PTX013 binds Gal-3 at the same site on its CRD F-face as does PTX008 (Figure 7B). Moreover, because PTX013-induced chemical shift changes are similar (Figure 7B,D) and resonance broadening is greater (Figure 7C,E) at the same PTX008/Gal-3 molar ratio, interactions with PTX013 appear to fall further into the intermediary exchange regime. Thus, it seems that PTX013 associates with the Gal-3 F-face more strongly than does PTX008. This difference in binding capacity is illustrated in Figure 7D and 7E that plots and

Intensity values, respectively, averaged over all Gal-3 resonances vs. the concentration of both compounds.

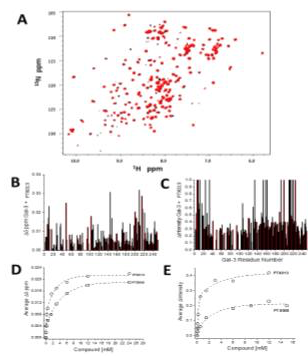
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The 50% change in Intensity with PTX013 is observed at ~150 M, about 8-fold less than with PTX008 (~ 1200

M). Although these concentrations are not KD values, the trend does reflect differences in the KD values by the synthetic adaptation.



**Figure 7. HSQC spectra for Gal-3 binding to PTX013. (A)** Two 1H–15N HSQC spectra obtained in the absence (black contours) and presence (red contours) of 12 mM PTX013 are overlaid for 15N-labeled full-length Gal-3. **(B)** An HSQC chemical shift map is shown for PTX013 (1.2 mM) binding to Gal-3 (40 M). **(C)** An HSQC resonance broadening map is shown for PTX013 (10 mM) binding to Gal-3 (40 M). Intensity values are shown vs. the amino acid sequence of Gal-3. **(D,E)** Titration curves are shown for PTX013 and PTX008 binding to Gal-3 as sequence-averaged **(D)** and

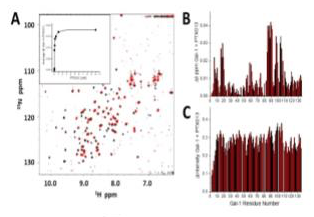
Intensity **(E)** values vs. the concentration of these compounds as labeled. The total ligand concentration is shown on the x-axis in these plots.

In Figure 5, we showed that binding of PTX008 to the CRD F-face of Gal-3 perturbed residues on the CRD S- face that resulted in attenuated lactose binding (Supplemental Figure S2A, repeat of Figure 5D). Because PTX013 binds more strongly to the Gal-3 F- face than does PTX008, we performed the same lactose titration with Gal-3 in the presence of PTX013. Supplemental Figure S2B plots values vs. lactose concentration for the same residues as shown in Figure 5D for Gal-3 plus PTX008. In the absence of either calixarene compound, the apparent KD value is ~170

M. In the presence of PTX013, the KD value is ~500 M, slightly higher than that in the presence of PTX008 (KD

~350 M). Thus the stronger binding of PTX013 to the

Gal-3 CRD F-face translates into weaker binding of lactose to the S-face.

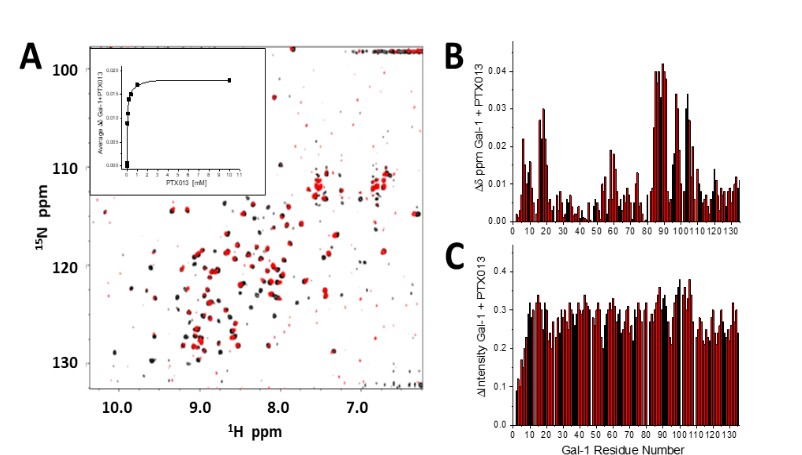
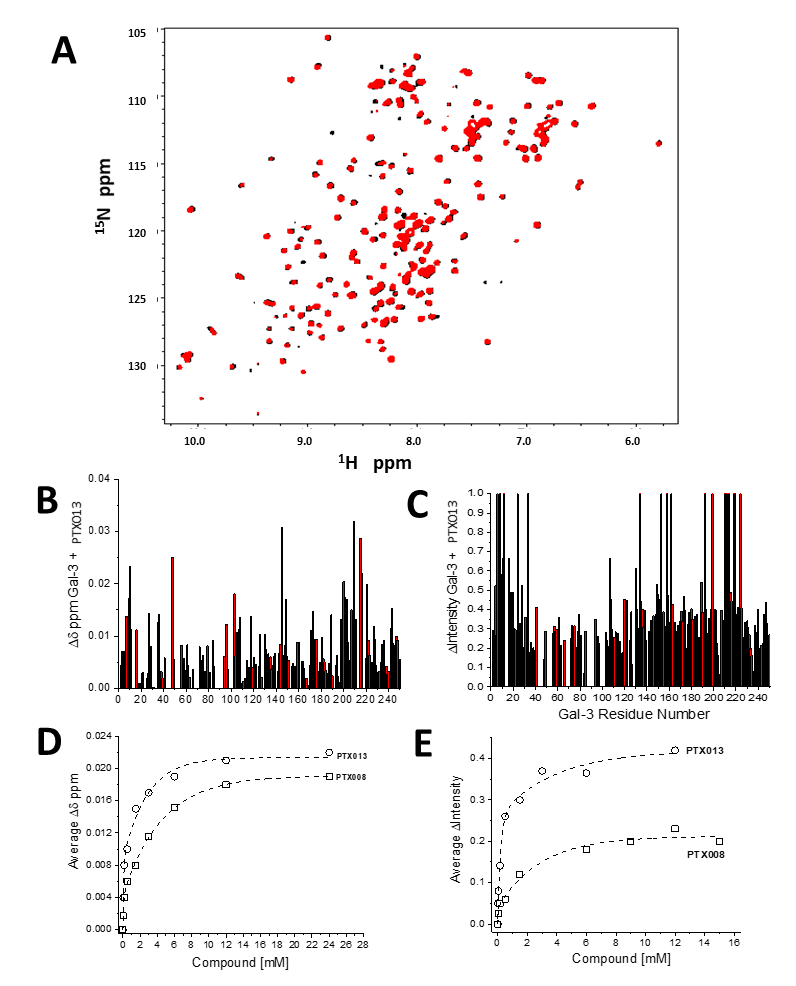


**Figure 8. HSQC spectra of Gal-1 with PTX013.** (**A**) HSQC spectra are overlaid for 15N- labeled Gal-1 (80 M) in the presence (black peaks) and absence (red peaks) of PTX013 (400 M). (**B**) HSQC chemical shift and resonance broadening (**C**) maps are shown for PTX013 binding to Gal-1 at a PTX013 concentrations of 1 mM. Changes are given vs. the amino acid sequence of Gal-3. The insert to **A** plots sequence-averaged values vs the concentration of PTX013.

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Having detected PTX013 and PTX008 binding for Gal-1 (Figure 6D, middle spectral trace), we performed HSQC experiments with 15N-labeled Gal-1 and PTX013 to determine the strength of the PTX013-Gal-1 interaction. Figure 8A shows an HSQC spectrum of 15N- labeled Gal-1 in the absence (black peaks) and presence (red peaks) of PTX013. Figure 8B plots chemical shift changes () at 400 M PTX013 vs. the amino acid sequence of Gal-1. The most highly shifted resonances are located within the F-face of the CRD (residues 17-21, 84-92, 95-100 and 104-108), as previously found with PTX008.[37] Other significant chemical shift changes (albeit smaller) are noted for some residues within the canonical sugar-binding S- face (residues 53-74), as well as at the N-terminus (residues 5-12). The overall greater values for F-face residues indicate that PTX013 binds to the Gal-1 F-face like PTX008. The smaller values for S-face and N- terminal residues suggest allosteric effects induced by F-face binding of PTX013, as observed with PTX008. Figure 8C shows resonance broadening caused by PTX013 binding (Intensity) vs. the amino acid sequence of Gal-1, a trend that is similar as that found previously for PTX008.[37] It appears that PTX013 binds Gal-1 more strongly than PTX008, primarily because PTX013-induced and Intensity values are greater

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than those for PTX008 at the same concentration (i.e. about twice the PTX013/Gal-1 molar ratio).[37] This is supported by a plot of sequence-averaged values vs the concentration of PTX013 (insert to Figure 8A), with the 50% change (~50 M) being somewhat lower than that for PTX008 (~100 M).[37] As noted previously, these are not KD values, yet their trends are clear.

Given the known co-expression of Gal-1 and -3 with Gal-7 in colon cancer,[47] it became pertinent to examine whether PTX013 (and PTX008) also bind to galectin-7 (Gal-7). Whereas PTX013 binds relatively strongly to both Gal-3 and Gal-1, it apparently interacts with Gal-7 very weakly, if at all. Supplemental Figure S1A shows HSQC spectra overlaid for 15N-labeled Gal-7 (80 M) in the absence (black peaks) and presence (red peaks) of 10 mM PTX013, along with values

anti-angiogenic and tumor activities.[31] In the present study, we used 15N–1H HSQC NMR spectroscopy to demonstrate that the calixarene PTX008 binds to Gal- 3, albeit more weakly, than to Gal-1 and allosterically attenuates lactose binding to the lectin. We designed PTX013 by removing the amide group within the four *N*-dimethyl alkyl chains of PTX008, thus reducing both its alkyl chain length and polar character which significantly improved affinity of Gal-3 F-face binding by PTX013 to about 8-fold stronger than that for PTX008. In addition, we found that both calixarenes only weakly (if at all) interact with Gal-7, thereby underscoring selectivity among these galectins. Recently, we reported that PTX013 induces cell cycle arrest and attenuates melanoma tumor growth in a mouse model, about 50-fold better than PTX008

[50]

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(Supplemental Figure S1B) and Intensity values

without apparent cytotoxicity

and with mice

(Supplemental Figure S1C) vs the amino acid sequence of Gal-7. Relatively weak binding is indicated by

administered either PTX008 or PTX013 all surviving,

displaying normal behavior, and gaining weight

[31,49]

minimal HSQC spectral changes and very small and

Intensity values even at a very high PTX013/Gal-7 molar ratio (125:1). In fact, for many resonances, broadening is actually attenuated (i.e. negative

Intensity values) by the presence of PTX013. This apparently weaker binding may result from PTX013 interactions with Gal-7 F-face that is also the Gal-7 dimer interface (Supplemental Figure S1D), thus the competition between PTX013 binding and Gal-7 dimerization in the monomer-dimer equilibrium may be what attenuates PTX013 binding. The decreased resonance broadening may reflect changes in that dynamic Gal-7 monomer-dimer equilibrium, shown to be operative previously.[46] Essentially the same HSQC results were observed with Gal-7 and PTX008 (data not shown). Since Gal-7 has been referred to as p53- induced gene 1 based on upregulation prior to the onset of apoptosis in human colon cancer (DLD-1) cells,[48] blocking by PTX013 or PTX008 may not be desirable with this galectin.

##### Conclusions

Previously, we produced a library of calix[4]arene- based compounds,[31,49,50] and showed that PTX008 binds strongly to the F-face of Gal-1, resulting in potent

normally.

The difference in PTX013/008 Gal-3 binding affinity and affect on lactose binding parallels the difference in cell- based and *in vivo* functional assays in that PTX013 is more active than PTX008.[50] Binding of PTX013 to both Gal-3 and Gal-1 likely explains why PTX013 is considerably more effective biologically than PTX008.[50] Put into perspective and considering that

|  |  |  |
| --- | --- | --- |
| galectins like Gal-1 | and -3 can | cooperate |
| pathophysiologically[51] | and exhibit | functional |

antagonism,[52] pursuing a structure-based design approach will allow for screening of related compounds and selective targeting of galectins. This report thus contributes to the development of calixarene-based therapeutics and sets this class of compounds apart from other galectin antagonists that target the canonical sugar-binding S-face of galectins.

##### Experimental Section

**Calixarene Synthesis.** Calix[4]arene compounds were synthesized as previously described for PTX008[31] and PTX013.[50]

**Gal-3 Expression and Purification.** Full-length human Gal-3 (residues 1-250) and its CRD (residues 108-250)



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were recombinantly made using [15N]NH4Cl in *E. coli* BL21 DE3 cells (Promega, Mannheim, Germany) with respective cDNA inserted into a pET12a expression vector (Novagen, Darmstadt, Germany) at 30 °C and 400 µM IPTG (16 h induction). Protein was purified using affinity chromatography on lactosylated- Sepharose 4B. Lactose was removed by ultrafiltration and PC-10 gel filtration. Protein purity and activity were assessed as previously described.[53,54] Usually,

~45 milligrams of pure protein was obtained from 1 liter culture. 15N-Labeled Gal-1 and -7 were prepared, as described elsewhere.[19,46]

**Preparation of 15N-enriched NT of Gal-3.** 15N-Labeled NT of Gal-3 was prepared using 15N-enriched His- tagged NT by thrombin digestion. In brief, cDNA for His- tagged NT was inserted into a pET28a vector between the *BamHI* and *EcoRI* sites. *E. coli* BL21 (DE3) cells were transformed using the construct, and cultured at 37 oC for 12 h in minimal media with [15N]NH4Cl as the nitrogen source. Expression was induced with 0.2 mmol/L IPTG at 25 oC, cultures maintained for 16 h. Suspensions were centrifugated, cell pellets were resuspended in phosphate buffer, lysed by sonication, and then the cleared solution (by centrifugation) was batchwisely incubated with Ni-NTA agrose beads. Following extensive washing, His-tagged protein bound to the beads was treated with 2 U/mg thrombin, and His-tag-free NT was released, the solution put into dialysis.

**Peptide Synthesis.** Peptides were synthesized using tert-butyloxycarbonyl (tBoc)-based solid-phase synthesis and purified by HPLC, as previously described.[43,55] Two NT parts of 1-50 and residues 51- 107 were generated. Peptides were prepared on the

0.2 mmol scale. Following synthesis, resin-bound protected peptide was treated with 4 ml

mercaptoethanol /2 ml diisopropylethylamine in 14 ml

v-% *p*-cresol as scavenger. Peptides were precipitated in ice-cold diethylether, redissolved in 0.1 M sodium acetate buffer (pH 4) with 6 M guanidinium hydrochloride, and purified by HPLC (Vydac C-18 column, 250 x 10mm, 10µm) using a linear gradient of acetonitrile in water/0.1%TFA (flow rate of 5 ml/min; 0.2% B/min). The product of each run was characterized by ESI-MS (Applied Biosystems SCIEX API 150 EX electrospray ionization quadrupole (ESI-Q) mass spectrometer), fractions pooled and lyophilized. Masses were calculated using the Analyst 1.4.2 software (Sciex).

**NMR Spectroscopy.** Uniformly 15N-labeled recombinant galectin samples were dissolved at a concentrations of 20 M to 100 M in 20 mM potassium phosphate buffer at pH 6.9, 50 µM EDTA, made up using a 95% H2O/ 5% D2O mixture. 1H–15N HSQC and 1H TOCSY NMR experiments were used to investigate binding of PTX008 and PTX013 to 15N- labeled galectin samples (i.e. Gal-3 full-length, its truncated CRD, and the stretch of residues 1-113 of the NT, or Gal-7). 1H and 15N resonance assignments had previously been reported for Gal-1,[56] Gal-3,[57] and Gal-7.[58] NMR experiments were performed at 25 oC to 30 oC, because spectral characteristics (linewidths and S/N) were optimal vis-à-vis 15-20 oC or 40 oC. NMR experiments were carried out on Bruker 700 or 850 MHz NMR spectrometers equipped with H/C/N triple- resonance probes and *x*/*y*/*z* triple-axis pulse field gradient units. A gradient sensitivity-enhanced version of two-dimensional 1H–15N HSQC was applied with 256 (*t*1) x 2048 (*t*2) complex data points in nitrogen and proton dimensions, respectively. Raw data were converted and processed by using NMRPipe[59] and were analyzed by using NMRview.[60]

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Chemical shifts were internally referenced to 4,4- dimethyl-4-silapentane-1-sulfonic acid, and chemical

1 2

dimethlformamide (DMF) (2 × 30 minutes) to remove

shift differences (were calculated as [(H) +

15 2

1/2

the histidine Dnp protecting group; then the

(0.25 N) ]

. Intensity changes (Intensity) were

suspension was treated with trifluoroacetic acid (2 × 1 minute) to remove the N-terminal Boc group, washed in DMF, DCM and 1:1 v/v DCM/MeOH and dried. Peptides were deprotected and cleaved from the resin by treating with anhydrous HF for 1 h at 0 °C, using 4

calculated as (1 – Inti/Into), where Inti is the resonance

intensity at some new condition (addition of peptide or more Gal-3) and Into is the resonance intensity initially. A value of 0 indicates the absence of resonance broadening, and a value of 1 indicates that that resonance is no longer observable. A negative value

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indicates that resonances have increased in intensity,

i.e. become rather narrow. When conditions between samples (e.g. concentrations) differed, intensities in both spectra were first normalized to intensities of resonances from the first 10 N-terminal residues. NMR titrations were performed with the calixarene compounds in the absence and presence of lactose. Titration curves were fit to data points using a function having the general form y = 1/(1+e-x), where y is the  or Intensity value and x is the corresponding ligand concentration.

**STD NMR Spectroscopy.** Saturation Transfer Difference (STD) NMR experiments were acquired at 25 oC using a Bruker AVANCE 600 MHz spectrometer equipped with a cryoprobe. Experiments were performed in 20 mM potassium phosphate buffer, pD 6.7, 5% DMSO-d11. Samples contained 1 mM of PTX008 or PTX013, and 30 μM of Gal-1 or full-length Gal-3. The proteins were saturated on-resonance at their methyl maxima ~ 0.78 ppm, and off-resonance at 100 ppm with a train of Gaussian-shaped pulses of 50 ms each, totaling an irradiation time of 2 s. A 3 s relaxation delay was applied, and a T2 relaxation filter consisting of a 15 ms 5 kHz spin-lock was used to minimize protein background signals. STD spectra were obtained by subtracting the on-resonance spectrum from the off- resonance spectrum. STD intensities were measured, comparing each STD spectrum with the corresponding off-resonance spectrum and normalizing it to the ligand peak receiving the highest degree of saturation (i.e. aromatic protons). Data were analyzed using the Bruker TopSpin program version 3.0, and figures were drawn using the program MestReNova v.8.0.2.

**Molecular dynamics simulations.** Similar approaches and protocols were used as previously reported.[61] Briefly, the Amber 14SB force field was applied. Compounds were initially dock on the galectin structure using the program AutoDock Vina.[62] Complexes were solvated by TIP3P water models with a box size of 10 Å and were then subjected to energy minimization (5,000 steps of steepest descent followed by 5,000 steps of conjugate gradient algorithm) in order to optimize the complexes and remove close contacts. Subsequently, a position-restrained phase of

MD simulations was carried out for 500 ps by first slowly heating up the systems from 0 K to 300 K for 100 ps and then maintaining the temperature at 300 K for another 400 ps. During this phase, a soft-force constraint (10 kcal/mol•Å2) was applied to restrain the complexes. Finally, free MD simulations were performed for 200 ns.

Essential parameters (e.g. temperature (300 K), pressure (1 bar) and time steps (2 fs with SHAKE constraint)) were set at standard values as indicated during the free MD simulations. Energy minimization and MD simulations were employed by using AMBER16 program. BFE calculations were performed by extracting trajectories during the 170-200 ns period of MD simulations (100 snapshots), and the molecular mechanics/ generalized Born surface area (MM/GBSA) approach (generalized Born model 5 with standard parameters) was used for this purpose. The 3D structures of galectins were obtained from the Protein Data Bank: Gal-1 (PDB code 1W6N) and Gal-3 (PDB code 1A3K).

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##### Conflict of Interest

None of the authors have a conflict of interest.

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[1] V. I. Teichberg, I. Silman, D. D. Beitsch, G. Resheff,

*Proc. Natl. Acad. Sci. USA* **1975,** *72*, 1383-

1387.



.

# 

[2] J. Hirabayashi, J. (ed) *Trends Glycosci. Glycotechnol.*

**1997,** *9*, 1-180.

[3] J. Hirabayashi, J. (ed) *Trends Glycosci. Glycotechnol.*

**2018,** *30*, SE1-SE223.

[4] C. G. H. M. de Jong, H.-J. Gabius, W. Baron, *Cell.*

*Mol. Life Sci.* **2020,** *77*, 1289-1317.

[5] G. García Caballero, H. Kaltner, T. J. Kutzner, A.-K. Ludwig, J. C. Manning, S. Schmidt, F.

Sinowatz, H.-J. Gabius, *Histol. Histopathol.* **2020,** *35*, 509-539.

[6] S. Nakahara, N. Oka, A. Raz, *Apoptosis* **2005,** *10*, 267-275.

[7] M.-H. Hong, I.-C. Weng, F.-T. Liu, *Trends Glycosci.*

*Glycotechnol.* **2018,** *30*, SE179-SE184.

[8] V. Eckardt, M. C. Miller, X. Blanchet, R. Duan, J. Leberzammer, J. Duchene, O. Soehnlein, R. T. Megens, A.-K. Ludwig, A. Dregni, A. Faussner, K. Wichapong, H. Ippel, I. Dijkgraaf, H. Kaltner, Y. Doring, K. Bidzhekov, T. M. Hackeng, C. Weber, H.-

J. Gabius, P. von Hundelshausen, K. H. Mayo,

*EMBO Rep.* **2020,** *21*, e47852.

[9] S. Kamitori, S. *Trends. Glycosci. Glycotechnol.* **2018,** *30*, SE41-SE50. [10] L. Ingrassia, I. Camby, F. Lefranc, V. Mathieu, P. Nshimyumukiza, F. Darro, R. Kiss, *Curr. Med. Chem.* **2006,** *13*, 3513-3527.

[11] R. P. M. Dings, K. H. Mayo, *Acc. Chem. Res.* **2007a,**

*40*, 1057-1065.

[12] A. Girard, J. Magnani, *Trends Glycosci. Glyco- technol.* **2018,** *30*, SE211-SE220.

[13] A. Romero, H.-J. Gabius, *Expert Opin. Ther. Targets*

**2019,** *23*, 819-828.

[14] J. Tejler, H. Leffler, U. J. Nilsson, *Bioorg. Med. Chem.*

*Lett.* **2005,** *15*, 2343-2345.

[15] I. Cumpstey, S. Carlsson, H. Leffler, U. J. Nilsson,

*Org. Biomol. Chem.* **2005a,** *3*, 1922-1932.

[16] D. Giguère, S. Sato, C. St-Pierre, S. Sirois, R. Roy,

*Bioorg. Med. Chem. Lett.* **2006,** *16*, 1668-1672. [17] S. Sirois, D. Giguere, R. Roy, *Med. Chem.* **2006,** *2*,

481-489.

[18] D. Giguère, S. André, M. A. Bonin, M. A. Bellefleur,

A. Provencal, P. Cloutier, B. Pucci, R. Roy, H.-J. Gabius, *Bioorg. Med. Chem.* **2011,** *19*, 3280-7.

[19] S. R. Rauthu, T. C. Shiao, S. André, M. C. Miller, E.

Madej, K. H. Mayo, H.-J. Gabius, R. Roy,

*ChemBioChem.* **2015,** *16*, 126-39

[20] H. Kaltner, T. Szabo, K. Fehér, S. André, S. Balla, J.

C. Manning, L. Szilágyi, H.-J. Gabius, *Bioorg. Med. Chem.* **2017,** *25*, 3158-3170.

[21] B. A. Salameh, H. Leffler, U. J. Nilsson, *Bioorg. Med.*

*Chem. Lett.* **2005,** *15*, 3344-3346.

[22] B. A. Salameh, A. Sundin, H. Leffler, U. J. Nilsson,

*Bioorg. Med. Chem.* **2006,** *14*, 1215-1220.

[23] S. André, P. J. Cejas Ortega, M. Alamino Perez, R. Roy, H.-J. Gabius, *Glycobiol.* **1999,** *9*, 1253-1261.

[24] I. Cumpstey, A. Sundin, H. Leffler, U. J. Nilsson,

*Angew. Chem.* **2005b,** *44*, 5110-5112.

Accepted Manuscript

[25] S. André, C. Grandjean, F. M. Gautier, S. Bernardi,

F. Sansone, H.-J. Gabius, R. Ungaro, *Chem. Commun. (Camb)* **2011,** *47*, 6126-6128.

[26] K. H. Mayo, R. P. M. Dings, C. Flader, I. Nesmelova,

B. Hargittai, D. W. van der Schaft, L. I. van Eijk, D. Walek, J. Haseman, T. R. Hoye, A. W. Griffioen, *J. Biol. Chem.* **2003,** *278*, 45746-45752.

[27] R. P. M. Dings, D. W. van der Schaft, B. Hargittai, J. Haseman, A. W. Griffioen, K. H. Mayo, *Cancer Lett.* **2003a,** *194*, 55-66.

[28] R. P. M. Dings, B. W. Williams, C. W. Song, A. W. Griffioen, K. H. Mayo, R. J. Griffin, *Int. J. Cancer* **2005,** *115*, 312-319.

[29] R. P. M. Dings, E. S. van Laar, J. Webber, Y. Zhang,

R. J. Griffin, S. J. Waters, J. R. MacDonald, K. H. Mayo, *Cancer Lett.* **2008**, *265*, 270-280.

[30] V. L. Thijssen, R. Postel, R. J. Brandwijk, R. P. M. Dings, I. V. Nesmelova, S. Satijn, N. Verhofstad, Y. Nakabeppu, L. G. Baum, J. Bakkers, K. H. Mayo, F. Poirier, A. W. Griffioen, *Proc. Natl. Acad. Sci. USA* **2006,** *103*, 15975-15980.

[31] R. P. M. Dings, X. Chen, D. M. Hellebrekers, L. I. van Eijk, Y. Zhang, T. R. Hoye, A. W. Griffioen, K. H. Mayo, *J. Natl. Cancer Inst.* **2006,** *98*, 932-936.

[32] R. P. M. Dings, K. B. Vang, K. Castermans, F. Popescu, Y. Zhang, M. G. Oude Egbrink, M. F. Mescher, M. A. Farrar, A. W. Griffioen, K. H. Mayo, *Clin. Cancer Res.* **2011,** *17*, 3134-3145.

[33] D. W. van der Schaft, R. P. M. Dings, Q. G. de Lussanet, L. I. van Eijk, A. W. Nap, R. G. Beets-Tan,

J. C. Bouma-Ter Steege, J. Wagstaff, K. H. Mayo, A. W. Griffioen, *Faseb J.* **2002,** *16*, 1991-1993.

[34] R. P. M. Dings, Y. Yokoyama, S. Ramakrishnan, A.

W. Griffioen, K. H. Mayo, *Cancer Res.* **2003b,** *63*, 382-385.

[35] R. P. M. Dings, M. Loren, H. Heun, E. McNiel, A. W.

#### .



# 

Griffioen, K. H. Mayo, R. J. Griffin, *Clin. Cancer Res.*

**2007b,** *13*, 3395-3402.

[36] R. Lotan, P. N. Belloni, R. J. Tressler, D. Lotan, X.-C. Xu, G. L. Nicolson, *Glycoconjugate J.* **1994,** *11*, 462-

468.

[37] R. P. M. Dings, M. C. Miller, I. Nesmelova, L. Astorgues-Xerri, N. Kumar, M. Serova, X. Chen, E. Raymond, T. R. Hoye, K. H. Mayo, *J. Med. Chem.* **2012**, *55*, 5121-5129.

[38] N. Nagy, H. Legendre, O. Engels, S. André, H. Kaltner, K. Wasano, Y. Zick, J.-C. Pector, C. Decaestecker, H.-J. Gabius, I. Salmon, R. Kiss, *Cancer* **2003,** *97*, 1849-1858.

[39] H. Dawson, S. André, E. Karamitopoulou, I. Zlobec, H.-J. Gabius, *Anticancer Res.* **2013,** 33, 3053-9.

[40] E.-M. Katzenmaier, S. André, J. Kopitz, H.-J. Gabius,

*Anticancer Res.* **2014,** *34*, 5429-38.

[41] T. Funasaka, A. Raz, P. Nangia-Makker, *Glycobiol.*

**2014,** *24*, 886-91.

[42] K. Nakajima, D. H. Kho, T. Yanagawa, M. Zimel, E. Heath, V. Hogan, A. Raz, *Cancer Metastasis Rev.* **2016,** *35*, 333-46.

[43] H. Ippel, M. C. Miller, S. Vértesy, Y. Zheng, D. Suylen, K. Umemoto, C. Romanò, T. M. Hackeng, G. Tai, H. Leffler, J. Kopitz, S. André, D. Kübler, J. Jiménez-Barbero, S. Oscarson, H.-J. Gabius, K. H. Mayo, *Glycobiol.* **2016**, *26*, 888-903.

[44] M. P. Williamson, *Prog. Nucl. Magn. Reson.* **2013**,

*73*, 1-16.

[45] I. V. Nesmelova, E. Ermakova, V. A. Daragan, M. Pang, M. Menendez, L. Lagartera, D. Solis, L. G. Baum, K. H. Mayo, *J. Mol. Biol.* **2010**, *397*, 1209- 1230.

[46] E. Ermakova, M. C. Miller, I. V. Nesmelova, L. Lopez-Merino, M. A. Berbís, Y. Nesmelov, L. Lagartera, V. A. Daragan, S. André, F. J. Cañada, J. Jiménez-Barbero, D. Solis, H.-J. Gabius, K. H. Mayo, *Glycobiol.* **2013**, *23*, 508-523.

[47] S. Ueda, I. Kuwabara, F.-T. Liu, *Cancer Res.* **2004**,

Astorgues-Xerri, J. MacDonald, T. R. Hoye, E. Raymond, K. H. Mayo, *Investigat. New Drugs* **2013**, *31*, 1142-1150.

[51] D. Weinmann, M. Kenn, S. Schmidt, K. Schmidt, S.

M. Walzer, B. Kubista, R. Windhager, W. Schreiner,

S. Toegel, H.-J. Gabius, *Cell. Mol. Life Sci.* **2018,** *75*, 4187-4205.

[52] H. Kaltner, J. Abad-Rodríguez, A. P. Corfield, J. Kopitz, H.-J. Gabius, *Biochem. J.* **2019,** *476*, 2623-

2655.

Accepted Manuscript

[53] D. Kübler, C.-W. Hung, T. K. Dam, J. Kopitz, S. André, H. Kaltner, M. Lohr, J. C. Manning, L. He, H. Wang, A. Middelberg, C. F. Brewer, J. Reed, W. D. Lehmann, H.-J. Gabius, *Biochim. Biophys. Acta*, **2008**, *1780*, 716-722.

[54] H. Sanchez-Ruderisch, C. Fischer, K. M. Detjen, M. Welzel, A. Wimmel, J. C. Manning, S. André, H.-J. Gabius, *FEBS J.* **2010**, *277*, 3552-3563.

[55] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S.

B. H. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180- 193.

[56] I. V. Nesmelova, M. Pang, L. G. Baum, K. H. Mayo,

*Biomol. NMR Assign.* **2008,** *2*, 203-205.

[57] H. Ippel, M. C. Miller, M. A. Berbís, D. Suylen, S. André, T. M. Hackeng, C. Weber, H.-J. Gabius, J. Jiménez-Barbero, K. H. Mayo, *Biomol. NMR Assign.* **2015**, *9*, 59-63.

[58] I. V. Nesmelova, M. A. Berbis, M. C. Miller, F. J. Cañada, S. André, J. Jiménez-Barbero, H.-J. Gabius,

K. H. Mayo, *Biomol. NMR Assign.* **2012,** *6*, 127-129. [59] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J.

Pfeifer, A. Bax, *J. Biomol. NMR* **1995,** *6*, 277-293.

[60] B. A. Johnson, *Methods Mol. Biol.* **2004,** *278*, 313-

352.

[61] K. [Wichapong, J. E.](https://www.ncbi.nlm.nih.gov/pubmed/?term=Wichapong%20K%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) [Alard, A. Ortega-Gomez,](https://www.ncbi.nlm.nih.gov/pubmed/?term=Alard%20JE%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) [C.](https://www.ncbi.nlm.nih.gov/pubmed/?term=Alard%20JE%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) [Weber, T. M.](https://www.ncbi.nlm.nih.gov/pubmed/?term=Weber%20C%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) [Hackeng,](https://www.ncbi.nlm.nih.gov/pubmed/?term=Hackeng%20TM%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) O. [Soehnlein,](https://www.ncbi.nlm.nih.gov/pubmed/?term=Soehnlein%20O%255BAuthor%255D&amp;cauthor=true&amp;cauthor_uid=26871718) G. A. [*J. Med.*](https://www.ncbi.nlm.nih.gov/pubmed/?term=PMID%3A%2B26871718)[*Chem.*](https://www.ncbi.nlm.nih.gov/pubmed/?term=PMID%3A%2B26871718)**2016**, *59,* 4289-4301.

[62] O. Trott, A. J. Olson, *J. Comput. Chem*. **2010**, *31*, 455-461.

*64*, 5672–5676.

[48] K. Polyak, Y. Xia, J. L. Zweier, K. M. Kinzler, B. Vogelstein, *Nature* **1997,** *389*, 300-305.

[1]

…

[49] X. Chen, R. P. M. Dings, J. R. Haseman, J. Maxwell,



T. R. Hoye, K. H. Mayo, *J. Med. Chem.* **2006**, *49*, 7754-7765.

[50] R. P. M. Dings, J. I. Levine, S. G. Brown, L.

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