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Glycomimetic Ligands for the Human Asialoglycoprotein Receptor

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Supporting Information

ABSTRACT: The asialoglycoprotein receptor (ASGPR) is a high-capacity galactose-binding receptor expressed on hepatocytes that binds its native substrates with low affinity. More potent ligands are of interest for hepatic delivery of therapeutic agents. We report several classes of galactosyl analogues with varied substitution at the anomeric, C2-, C5-, and C6-positions. Significant increases in binding affinity were noted for several trifluoromethylacetamide derivatives without covalent attachment to the protein. A variety of new ligands were obtained with affinity for ASGPR as good as or better than that of the parent *N*-acetylgalactosamine, showing that modification on either side of the key C3,C4-diol moiety is well tolerated, consistent with previous models of a shallow binding pocket. The galactosyl pyranose motif therefore offers many opportunities for the attachment of other functional units or payloads while retaining low-micromolar or better affinity for the ASGPR.

The asialoglycoprotein receptor (ASGPR), a high-capacity C-type lectin receptor expressed on mammalian hepatocytes, plays an important role in the lysosomal processing of *N*-acetylgalactosamine (GalNAc) and galactose (Gal)-containing glycopeptide substrates.[1] These motifs can therefore be employed as hepatotropic vectors for hepatocyte uptake of a variety of nanoparticles such as liposomes,[2] cyclodextrins,[3] quantum dots,[4] and polymers linked to oligonucleotides.[5] Due to the relatively low affinity of monosaccharides (K_d typically high μM to low mM),[6] multivalent GalNAc epitopes constitute the current state of the art,[7] with tetraantennary ligands exhibiting dissociation constants in the nanomolar range.[8]

Relatively little success has been reported in optimizing the affinities of small monovalent ligands as replacements for GalNAc. The X-ray crystal structure of the carbohydrate-binding H1 domain of the ASGPR protein was described in 2000 (PDB ID 1dv8).[9] Although the protein was crystallized in the presence of 20 mM lactose, the sugar does not appear in the structure. Docking calculations, as well as structures of GalNAc bound to homologous mutants of mannose-binding protein (PDB IDs 1bch, 1bcj, 1fif, 1fih),[10] suggest that the GalNAc binding region is relatively shallow but is anchored by complexation of the galactosyl C3- and C4-hydroxyls to the calcium ion characteristic of this receptor family.[11]

Early work using competition assays and rodent receptors focused on simple modification of the GalNAc amide group.[12] Ernst and co-workers recently extended the application of rational drug design principles and systematic analysis to the ASGPR ligand problem with the use of biophysical techniques and isosteric replacement of the N-acetyl group with heterocycles.[13] They identified a productive interaction with the hydrophobic π-face of Trp243 and a "dumbbell-shaped binding pocket" accommodating the rest of the motif extended at C2 by a 1,4-triazole (structure **1a**, see Figure 2).[13] Compounds **1b** and **1c** have also been reported by the same group as inhibitors of ASGPR binding with roughly the same potency as GalNAc.[14] Here we describe efforts focused on the 2- and 6-positions of the galactose motif.

A series of compounds was prepared from 2-azidogalactosyl analogues **2a** and **2b** (Figure 1A), in which the anomeric substituent was replaced with either a β-methyl or β-4-methoxyphenyl group to provide an extra measure of rigidity compared to the Ernst structures and the potential for additional linkage attachment points. The azides were elaborated into amides **3** and triazoles **4** by reduction/acylation and Cu-catalyzed azide–alkyne cycloaddition,[15] respectively. In the latter process, a variety of aromatic, heterocyclic, heterocycle-methyl, and small hydrophobic components were used in an attempt to engage the receptor in productive ancillary binding interactions along the binding pocket. A second series of structures, shown in Figure 1B, explored the consequences of placing triazole connectors on the C5/6 end of the GalNAc motif while retaining the C2-acetamide. In this case, the precursor C5-alkyne **5** and C6-azide **7** were constructed with a glycosidic α-allyloxy group to allow for subsequent connection to multivalent scaffolds. The synthetic routes are detailed in the Supporting Information (SI).

The compounds were tested for binding to immobilized human ASGPR by surface plasmon resonance (SPR), with results reported as dissociation constants (K_d).[16] In order to follow the binding of the small-molecule analogues, the lone free cysteine of the ASGPR H1 domain was derivatized with a biotin-maleimide linker, followed by immobilization onto the streptavidin biosensor to establish a stable ASGPR surface. GalNAc and most of the compounds tested to date exhibited fast-on/fast-off kinetics consistent with relatively weak

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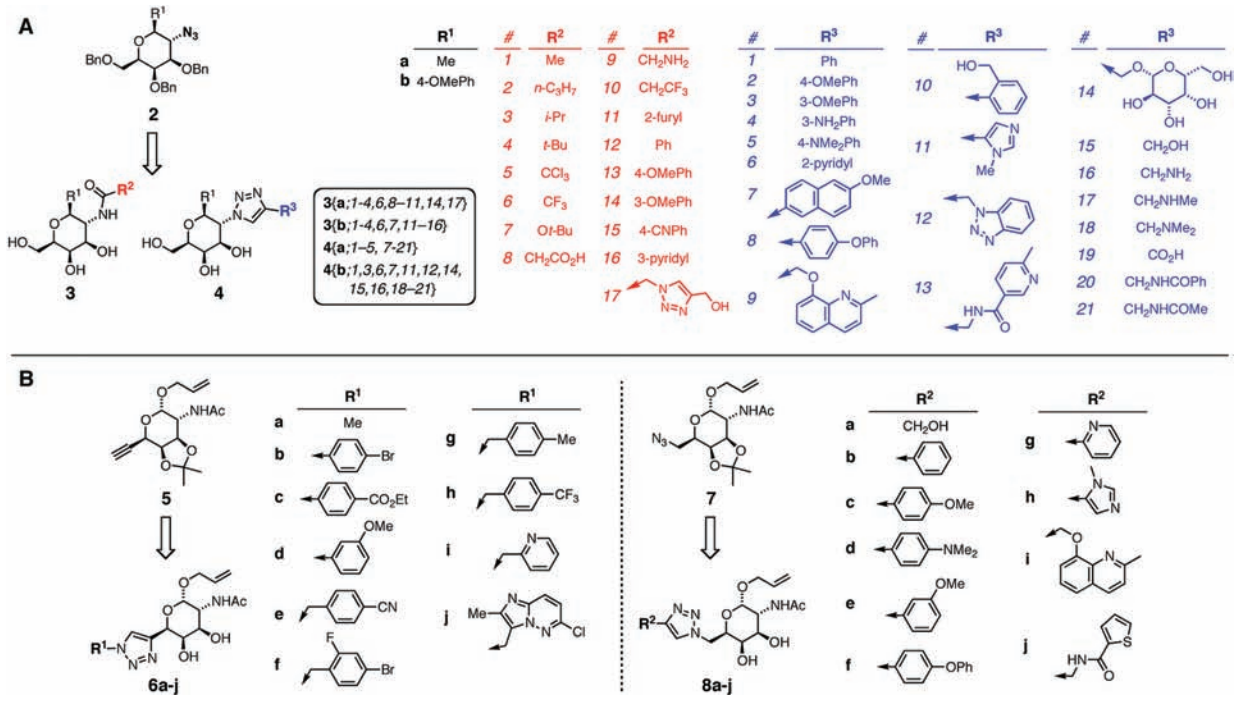

Figure 1. (A) Synthesis of β-substituted triazole and acetamide galactosyl analogues. (B) Synthesis of derivatives focused on the GalNAc C6 position.

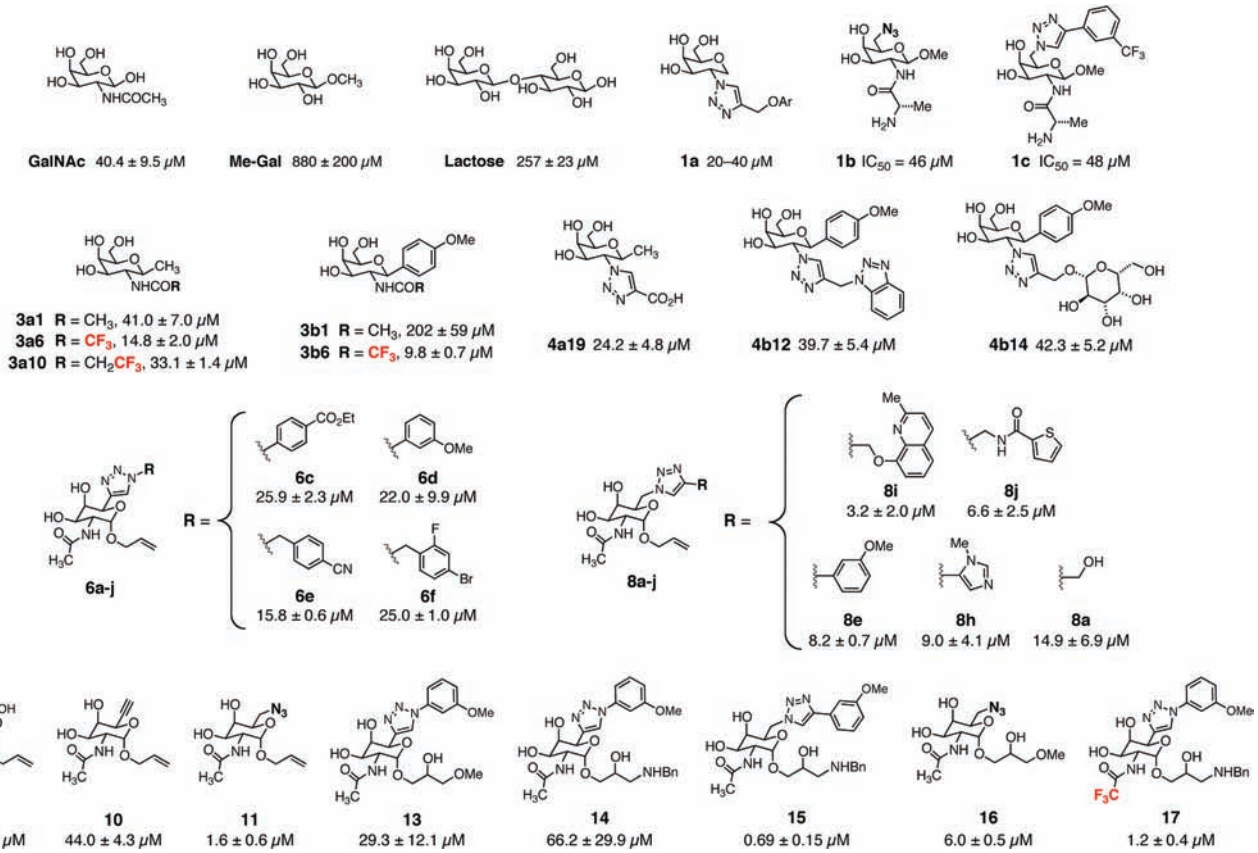

Figure 2. Compounds discussed in the text with K_d values determined by SPR (this work), or IC_{50} values from the literature.

interactions with the protein; the results for GalNAc, methyl galactoside, and lactose (Figure 2) were very similar to previously reported values.[17] Results of the SPR measurements for compounds exhibiting especially significant affinities are summarized in Figure 2, and a complete table of results (82 compounds) is given in the SI.

Replacement of the anomeric hydroxyl of D-GalNAc with β-4-methyl (**3a1**) or β-4-methoxyphenyl (**3b1**) substituents

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gave rise to a 5-fold loss of affinity for ASGPR in the latter case, but no loss of binding ability in the former (Figure 2). Replacement of the *N*-acetyl with larger amide groups on these scaffolds made for poorer binding (SI), unlike previous observations made for GalNAc derivatives.[12] Contrariwise, trifluoroacetamides **3a6** and **3b6** showed ∼3- and 20-fold better affinity for ASGPR than their respective acetamide analogues. This stands in contrast to an early publication by Lee and co-workers, which reported the trifluoroacetamide of a β-aminohexyl O-glycoside derivative to be ∼3 times *less* tightly bound by rodent ASGPR than the corresponding acetamide.[18] These differences with prior reports may be due to the difference in source species (rat vs human). The 2,2,2-trifluoroethyl amide **3a10** also had good affinity, only 2-fold weaker than the trifluoromethyl structure **3a6** (33.1 vs 14.8 μM). Early mutagenesis and binding studies involving an ASGPR-mimic form of rat serum mannose-binding protein pinpointed His256 as a critical residue for acetamide binding,[10a,11] but it is not yet clear what interaction(s) the CF_3 groups of **3a6** or **3a10** may be engaging in. When we installed carboxylate, amine, or triazole groups in place of CF_3 to try to create a new H-bond or salt bridge to His256 or other polar side chains that may exist in the vicinity, the resulting compounds (**3a8**, **3a9**, and **3a17**, SI) bound much more weakly to the protein ($K_d \approx 200$, >500, and 490 μM, respectively).

Replacing the acetamide group with triazole provided several compounds of moderate affinity, with the β-4-methoxyphenyl series (**4b**) generally outperforming the β-methyl series (**4a**). One notable exception to this trend was the affinity of carboxytriazole **4a19** (24.2 μM) vs **4b19** (>1 mM), suggesting perhaps an unusual binding mode for the former that must await further study. Galactosyl-triazoles such as **4b12** and **4b14** showed that large and polar groups can be accommodated as triazole substituents.

Exploration of the C5/6 derivatives (triazole adducts **6** and **8**) started with the parent allylglycoside **9**, alkyne **10** (the deprotected form of **5**, Figure 2), and azide **11** (deprotected **7**). The alkyne was equipotent compared to GalNAc and the other two compounds proved to be significantly better binders than GalNAc, an observation that seems to be novel. (Compound **9** was previously reported to be a moderate inhibitor of asialoorosomucoid binding to rat hepatocytes, showing IC_{50} = 120 μM and 200 μM for the α- and β-*O*-allyl anomers, respectively,[18] vs 90 μM for GalNAc itself.[17]) A series of aryl and benzylic triazoles derived from the C5-alkyne **5** (**6b**−**h**) bound ASGPR approximately as well as GalNAc; the most potent examples are shown in Figure 2.

C6-Triazole derivatives **8** proved to be overall the most effective family of GalNAc mimetics developed thus far, consistent with the better starting affinity of their common azide precursor, **11**. A relatively large series of compounds exhibited low-micromolar binding (3.2−19 μM) for a variety of substituents of different sizes and characteristics (Figure 2 and SI), suggesting that the substituent on the triazole ring was not very influential.

The solvent-exposed nature of the anomeric α-allyloxy group was probed in preliminary fashion by epoxidation of **6d**, **8e**, and **11**, followed by ring-opening with either methanol or benzylamine to give **13**−**16**, each as an approximately equal epimeric mixture at the new secondary alcohol. As expected, these functionalized compounds retained substantial binding ability (slightly diminished for **13** and **14** derived from **6d**, and **16** from **11**; significantly better for **15** derived from **8e**). The trifluoroacetamide analogue of **14** (**17**) was found to bind ASGPR about 55 times better than the acetamide, similar to observations made in the **3a**/**3b** series, but of greater magnitude. On- and off-rates for **17** measured by SPR ($k_{on} = 4.3 \times 10^4$ M^{-1} s^{-1}, $k_{off} = 0.05$ s^{-1}) were typical for binding to a shallow protein pocket, providing no evidence for covalent modification of the receptor.[19]

These results include some of the highest affinity monovalent ligands for the asialoglycoprotein receptor developed thus far, and suggest that modification on both sides of the C3/C4-diol "anchor" to ASGPR can be employed.[20] Significant variations at the anomeric, C2, and C5-pyranose positions were tolerated in this survey, with the triazole connector used as a convenient linkage to varied substituents.[13] In addition, the trifluoromethyl acetamide was found to be a highly effective change in the GalNAc motif. The discovery of multiple structures able to interact with the ASGPR with affinities equal to or better than the natural ligands bodes well for the development of effective carriers to bring therapeutic agents to the liver. Many other parameters must also be examined and optimized, including selectivity for desired liver cells, and the rates and destinations of cellular uptake.[21] These are the subjects of ongoing studies in our laboratories.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of binding results for all compounds; experimental details of synthetic and analytical operations; compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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