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Design and synthesis of glycoprotein-based multivalent glyco-ligands for influenza hemagglutinin and human galectin-3

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ABSTRACT

We report a facile synthesis of glycoprotein-based glyco-ligands and their binding with influenza hemagglutinin and human galectin-3. Human serum albumin (HSA) was used as the scaffold and an Asn-linked complex type N-glycan prepared from chicken eggs was used as the glycan building block. It was found that Cu(I)-catalyzed alkyne–azide cycloaddition reaction (click chemistry) between the alkyne-labeled glycan and the azide-tagged HSA led to an efficient formation of the glycoconjugates. The density of glycan ligands on the protein scaffold was readily varied by changing the molar ratios of the two reactants. Binding studies indicated that the sialylated and desialylated multivalent glycoligands could selectively bind to influenza hemagglutinin and human galectin-3, respectively, with high affinity. In the two glycan–lectin interactions, a clear multivalent effect was observed. Moreover, a cell-based assay showed that the synthetic multivalent glyco-ligands could efficiently inhibit the attachment of galectin-3 to human prostate cancer and lung cancer cell lines. This study suggests that the synthetic glycoprotein-based glyco-ligands can be useful for different applications, including blocking the function of galectin-3 in cancer metastasis.

1. Introduction

Protein–carbohydrate recognition plays an important role in many physiological and disease processes, such as viral infection, cancer metastasis, and immune recognition.[1–6] In biological systems, protein–carbohydrate recognition is often characterized by multivalent interactions between glycan clusters in glycoconjugates (e.g., glycoproteins) and glycan-binding proteins (e.g., lectins) with multiple carbohydrate recognition domains.[6–13] Thus, multivalent interactions involving simultaneous binding of multiple sites represent a common strategy that has arisen in evolution for enhancing the strength and specificity between the two recognition parties.[7] This shared process also provides important clues for guiding the design of efficient multivalent glyco-ligands as inhibitors for interventions of key host–pathogen interactions.[13,14]

Influenza hemagglutinin (HA) is a glycoprotein found on the viral surface that is responsible for virus entry by binding to the sialic acid (SA) ligands (e.g., sialoglycoproteins) on the host cell membrane.[7,15,16] On the other hand, galectin-3 (Gal3), a lectin that recognizes β-galactoside-terminating glycoconjugates such as the Thomsen-Friedenreich (T_F) antigen (Galβ1,3GalNAc-Thr/Ser) plays an active role in cancer progression and metastasis. Gal3 may exert its effects via different mechanisms, including mediation of cell adhesion between endothelial and cancer cells, mediation of tumor cell homotypic aggregation, regulation of intracellular antiapoptotic function, and modulation of cell migration.[10,17–22] It has been demonstrated that Gal3 plays an important role in the preferential adhesion of the prostate cancer cell to bone marrow endothelial cells,[23] and over-expression of Gal3 occurs in many aggressive cancers, strongly suggesting a key participation of Gal3 in neoplastic progression and metastasis.[24,25] These discoveries have stimulated extensive interest in recently years aiming at the design of effective inhibitors targeting the protein–carbohydrate interactions mediated by influenza HA and cancer-associated Gal3. For example, multivalent sialylated glycoconjugates, built on various templates including small-molecule scaffolds, polypeptides, polysaccharides, polyacrylamides, and nanoparticles, have been previously reported as inhibitors for competing the binding between SA and HA.[7,26–30] Different Gal3 inhibitors that mimic the natural carbohydrate ligands have also been prepared and tested for targeting cancer metastasis.[20,31–34] Despite these progresses, there is still an urgent need for improved design to address issues of the relatively low activity observed in some classes of the multivalent ligands, the cytotoxicity of polyacrylamide-based inhibitors, and the unwanted immunogenicity of protein-based ligands. We report herein the design and synthesis of biocompatible

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glycoprotein-based glyco-ligands by employing natural N-glycans as the carbohydrate ligands and endogenous human serum albumin (HSA) as the carrier protein (Fig. 1), which should be well tolerated in biological systems and are no or less immunogenic than foreign-protein based multivalent ligands carrying synthetic oligosaccharides. The glycoconjugates were assembled using the copper-catalyzed alkyne–azide cycloaddition reaction between an alkyne-labeled glycan and an azide-modified HSA. Binding studies show that the synthetic sialylated glycoproteins are high-affinity ligands for the influenza HA while the asialoglycoproteins are high-affinity ligand for Gal3. A preliminary cell-based assay indicates that the asialoglycoproteins act as potent inhibitors capable of efficiently blocking the attachment of Gal3 to cancer cells including the PC3 and A549 cancer cell lines.

2. Results and discussion

2.1. Design of the glycoprotein-based glyco-ligands

Our design strives to use biocompatible natural carrier protein and natural N-glycan ligands to construct multivalent glyco-ligands as antiviral or anticancer agents that are expected to be of low cellular toxicity and be of no or low immunogenicity. For this reason, we chose to make a glycoconjugate using an endogenous human protein, human serum albumin (HSA) as the scaffold and a natural bi-antennary complex-type N-glycan as the carbohydrate ligand (Fig. 1). For the conjugation, we decided to apply the bio-orthogonal Cu(I)-catalyzed azide–alkyne cycloaddition reaction as the key step, which has been shown to be highly selective and efficient for biomolecule conjugation in water.[35–38]

For this purpose, an asparagine-linked sialylated complex-type bi-antennary glycan (SCT-Asn), prepared from the sialoglycopeptide (SGP) isolated from chicken egg yolk,[39,40] was used as starting material and was labeled with an alkyne group at the Asn moiety, while the HSA was tagged with azide functionality to provide the two partners for conjugation. One advantage for this reaction design is that the density of glycan ligands loaded can be readily controlled by changing the ratio of ligands to the carrier in the reaction to provide glycoproteins with varied multivalency. The sialylated glycoconjugates are expected to act as inhibitors against SA/HA interactions in influenza infection. On the other hand, the terminal sialic acids (SA) can be easily removed by neuraminidase treatment to expose the galactose moieties in the non-reducing end, yielding a terminal galactosyl-HSA glycoconjugate for inhibition of Gal3 (Fig. 1). This design presents an efficient approach to attain multivalent glycoproteins with desired terminal carbohydrate moieties for different targets.

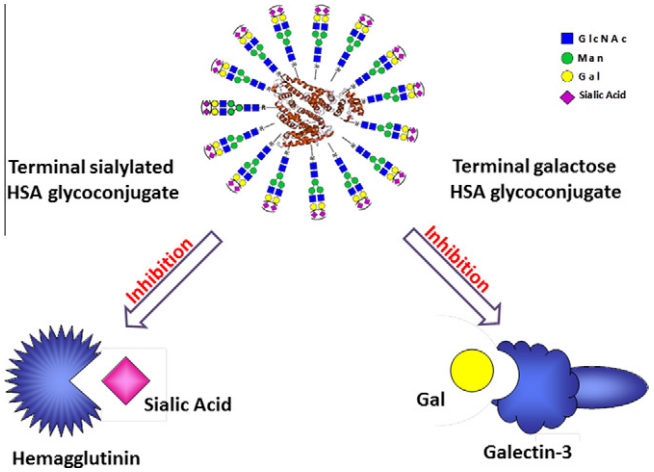

Figure 1. Design of multivalent HSA-based neoglycoproteins.

2.2. Synthesis of alkyne-labeled complex type N-glycan

A sialoglycopeptide (SGP), Lys-Val-Ala-Asn($Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$)-Lys-Thr, was purified from chicken egg yolks and used as the starting material for the preparation of the alkyne-labeled glycan ligand. The isolation of the SGP (Scheme 1) glycopeptide followed the previously described procedures[39,40] with some modifications. Egg yolks were separated from egg white and treated with aqueous phenol. After removal of pellet by centrifugation, the supernatant was washed with chloroform to remove the phenol and lipids. The residue was subject to size-exclusion chromatography and reverse-phase HPLC to give the sialoglycopeptide (**1**). The product was characterized by HPLC, HPAEC-PAD, ESI-MS, and NMR, and showed properties identical to the authentic SGP.

To synthesize the alkyne-labeled N-glycan, SGP was thoroughly digested with pronase to remove the peptide portion, leaving the asparagine (Asn)-linked N-glycan (SCT-Asn). The process was monitored by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (Dionex). The retention time (t_R) of SCT-Asn was 24.8 min under the HPAEC conditions (see Section 4), which was well separated from the SGP peak (t_R = 19.8 min) under the same chromatographic condition. After the digestion was complete, the residue was subject to size-exclusion (Sephadex G-25) chromatography to give the SCT-Asn. The identity of SCT-Asn was confirmed by ESI-MS analysis (calcd for SCT-Asn, M = 2338.10 Da; found (m/z): 1169.48 $[M+2H]^{2+}$ and 780.43 $[M+3H]^{3+}$). An alkyne group was then introduced into SCT-Asn by selective reaction of the free Asn amino group with an activated ester, *N*-(4-pentynoyloxy)succinimide in an aqueous solution. The resulting SCT-Asn-alkyne was purified by reverse-phase HPLC in 71% yield, and its identity was confirmed by ESI-MS (calcd for SCT-Asn-alkyne, M = 2418.18 Da; found (m/z), 1209.90 $[M+2H]^{2+}$ and 807.21 $[M+3H]^{3+}$).

2.3. Synthesis of HSA-based glycoproteins

For conjugation with the alkyne-labeled glycan via click chemistry, we sought to incorporate azide groups on human serum albumin (HSA) taking advantage of free lysine residues on the protein surface (Scheme 2). The mature HSA protein consists of 585 amino acids with 60 lysine residues. Treatment of HSA with an excess of the activated azide derivative, NHS-PEG4-azide (**3**), in an aqueous $NaHCO_3$ solution resulted in the introduction of multiple azide groups in the protein. The modified protein (HSA-azide) was purified by gel filtration. Comparison of the MALDI-TOF MS spectra of HSA and HSA-azide (Fig. 2, panels A and B) indicated that there was a gain of ca. 17 kDa for the azide-tagged HSA, suggesting an incorporation of about 60 azide moieties (with the linker) into HSA. This data implicates that almost all free amino groups in the HSA were reacted to the azide-containing reagent.

The conjugation of SCT-Asn-alkyne and HSA-azide was achieved via the Cu(I)-catalyzed alkyne–azide cycloaddition reaction. A solution of the alkyne and azide in a phosphate buffer (pH 7.5) was incubated together with $CuSO_4$, L-ascorbic acid, and a newly reported copper ligand, BTTP.[41] To achieve varied degree of ligand loading, different molar ratios of SCT-Asn-alkyne to the HSA-azide were applied. After reaction, the resultant HSA-SA glycoconjugates were purified by size-exclusion chromatography. Treatment of the HSA-SA glycoconjugates with neuraminidase gave the corresponding asialoglycoproteins, HSA-Gal (Scheme 2). An initial attempt to