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Multivalent S-sialoside protein conjugates block influenza hemagglutinin and neuraminidase

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ABSTRACT

A new class of S-sialoside Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA) conjugates were prepared to enhance the binding affinity to hemagglutinin (HA) and neuraminidase (NA). The valency of glycoconjugates was controlled by the reaction ratio of the S-sialoside monomer and protein. Hemagglutination inhibition assay showed that these synthetic glycoproteins have higher affinity to HA than the small clusters of sialosides with lower valency, due to multivalent effect and optimized three dimensional presentation of sialosides on the protein platform. The results of fluorescent NA inhibition assay showed that some of the conjugates have moderate NA inhibitory activity, in comparison to the monomer and low valent conjugates with weak or none inhibitory activity. These synthetic sialylated proteins were not cytotoxic with concentrations up to 100 μM, since the sialylation did not change the secondary structure of protein. This new kind of conjugates can be used as lead compounds for antiviral drug design and the construction of pseudo sialoside-protein conjugates library to investigate the carbohydrate-HA/NA recognition process and a platform for the influenza virus capturing.

1. Introduction

Influenza is an acute and fast spreading respiratory disease, which remains a great threat to global health and burden to the economy [1]. Infection of influenza viruses causes 13 deaths and 76, 498 of hospitalizations in March 2016 alone in China [2]. Recently, highly pathogenic avian flu outbreaks, such as H5N1 [3] and H7N9 [4] with a mortality rate higher than 30% have raised the concerns all over the world. To better fight the battle against influenza, the World Health Organization (WHO) has created FluID [5], a global influenza epidemiological data sharing platform to facilitate the tracking of global trends, spread, intensity, and impact of influenza.

Hemagglutinin (HA), Neuraminidases (NA) and ion-channel protein M2, which present on the surface of viral particles, are main targets for small molecule anti-influenza drug design [6,7], as well as vaccine development. Two M2 channel blockers: Amantadine and Rimantadine, and two NA inhibitors: Zanamivir (**ZA**) and Oseletamivir (**OS**), which are both sialic acid (**SA**) analogs (Fig. 1.), have been approved by FDA for treating influenza. However, due to the central nervous system (CNS) side-effects of the M2 blockers [8], antigenic drift [9] and the emerging drug-resistant strains [10], there is still an urgent need for development of new antiviral drugs.

The majority of viral envelope proteins are HA (80%, ~300 copies of trimer) and NA (17%, ~50 copies of tetramer), with an approximately 4:1 ratio [11]. These two glycoproteins play key roles in the

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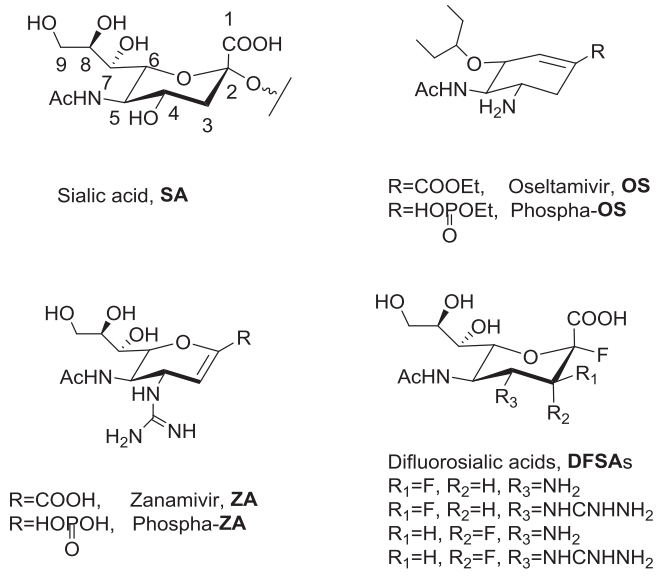

Fig. 1. Structure of sialic acid and NA inhibitors.

initial and final steps of the replication cycles of influenza viruses. Both HA and NA recognize and bind to terminal **SA** residues of glycoproteins and glycolipids on the membrane of host cells [12]. HA binds to **SA**-containing receptors and promotes clathrin-mediated endocytosis, which results in the internalization of viruses in the endosome, while NA cleaves terminal **SA** from cell surface to release the newly assembled viruses from old host cells and facilitate the spread of the infection. Due to their abundant and clustered distribution, these two viral proteins are ideal targets for anti-viral drug design with several ongoing developments [13,14].

SA is the natural ligand for both HA and NA, but attempts directly using **SA** as the inhibitor of this two proteins were not successful, primarily due to the weak binding affinity of HA and NA with **SA** monomer, with K_d in the millimolar range [15]. Several approaches have been adopted to increase the binding affinity. One strategy is to introduce chemical modifications on the structure of **SA**. This approach has led to the discovery of **ZA** and **OS** [16], which are both strong NA inhibitors. More recently, a new class of compounds including 2, 3-fluorosialosyl fluorides (**DFSA**s) [17], Phospha-**ZA** [18] and Phospha-**OS** [19,20] that exhibit superior activity against drug-resistant virus strains have also been developed. Another strategy to enhance the binding affinity between **SA** and HA/NA is inspired by the natural principle called “cluster effect” [21,22]. Synthetic **SA** [23–27] or **ZA** [14]-containing macromolecules showed broad-spectrum antiviral activities against a range of influenza viruses in *vivo* and *vitro*. However, the natural *O*-glycosides containing terminal **SA** are readily cleaved by NA [28], which may lead to the digestion of the glycopolymer. These cleavage can be prevented by replacing the *O*-sialosides with pseudo (C-, S- or triazole) sialoside, which makes the ligand more stable as we and others have demonstrated [29,30]. Synthetic small molecular clusters [31–33], polymers [34], dendrimers [35], liposome [36] and even metal nanoparticle [37,38] that coated with multiple copies of pseudo-**SA** or **SA** analog motifs have been prepared to greatly enhance the binding with HA or NA.

On the other hand, one limitation of most of the widely used macromolecule backbones such as polymers, dendrimers and nanoparticles is the lack of control over **SA** presentation. This caused the difficulties of the characterization of the surface density and spatial distribution of the **SA** after the conjugation. Moreover, numerous reports have also indicated that uncontrolled complement activation mediated by nanoparticles could be a life threatening [39–41].

To address these challenges, we chose natural proteins present in plasma (Human Serum Albumin, HSA and Bovine Serum Albumin, BSA) as the scaffolds to construct our glycomacromolecules because of their availability, biodegradability, nontoxicity and non-immunogenicity [42,43]. We attached multiple NA-resistant S-sialoside to the protein backbone, which mimics the natural receptor of the HA and NA to enhance the binding affinity. Furthermore, the density of the S-sialoside on the protein surface can be easily controlled by the reaction ratio and characterized by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). This paper reports our initial results toward the development of multivalent pseudo sialoside protein conjugates library as dual HA and NA inhibitors.

2. Results and discussion

2.1. Chemistry

At first, fully protected α-thioglycoside monomer **2** as the key intermediate was synthesized in good yield starting from known thioacetate **1** [44], which then reacted with 1-bromo-3-chloropropane in the presence of diethylamine in DMF (Scheme 1). Conversion of the chloride to the azide was achieved by the reaction of **2** with NaN_3 at high temperature to give a quantitative yield of **3**. Then Click Chemistry was used to attach the monomer to various alkynyl scaffolds as we previously reported [32,45]. Generally, **3** and alkyne were dissolved in a mixture of H_2O and tetrahydrofuran (THF), followed by addition of Cu(II) salts and sodium ascorbate. The mixture was stirred at room temperature (rt) overnight, followed by purification by column chromatography yielded fully **protected Dis**, **Trs** and **TeS**. After deacylation with $NaOCH_3$ in CH_3OH, demethylation using 0.3 M KOH, neutralization with H^+ resin and purification with Sephadex® LH-20, di- **DiS**, tri- **TrS** and tetra- **TeS** valent S-sialosides were synthesized, respectively. All of the final products were characterized by NMR and MALDI-TOF-MS.

The linker we chose for the conjugation of S-sialoside with protein is squaric acid diester [46]. This strategy involves a two-step, pH-dependent conjugation of two amines. First the amine of **5**, which was reduced with $Pd(OH)_2/C$ under hydrogen atmosphere from **4,** was reacted with one of the esters of squaric acid diethyl esters in potassium phosphate buffer (pH 7.0) to give **6**. After purification with column chromatography, the remaining ester group of **6** was conjugated with the amines on the lysine residue side chains of BSA and HSA in borate buffer (pH 9.0) to obtain the glycoconjugates. The valency of the S-sialoside on the protein was controlled by the reaction ratios of **6** and BSA or HSA (Table 1).

The resulting glycoconjugates were characterized by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), which showed shifted bands compared to unconjugated proteins. The number of sialoside **6** loaded to protein backbone was determined by MALDI-TOF-MS (see Supporting Information, SI). Mass analysis of glycoconjugates revealed that 4 to 11 S-sialosides were loaded onto one molecule of BSA or HSA (Table 1). Due to the conformation-hindrance effect, attempt to attach more than 11 S-sialosides on the protein surface failed, even when increasing the starting reaction molar ratio of **6** and protein to 200:1.

2.2. Biological assays

2.2.1. Hemagglutination inhibition assay

Hemagglutination Inhibition (HAI) assay [47] was used to evaluate the ability of glycoconjugates that bind to the HA to