Research Article
doi.org/10.1002/cbic.202200076
Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Conformationally Constrained Sialyl Analogues as New Potential Binders of h-CD22

Rosa Ester Forgione[+],[a] Ferran Fabregat Nieto[+],[a] Cristina Di Carluccio[+],[a] Francesco Milanesi,[b, c] Martina Fruscella,[b] Francesco Papi,[b] Cristina Nativi,[b] Antonio Molinaro,[a] Pasquale Palladino,[b] Simona Scarano,[b] Maria Minunni,[b] Marco Montefiori,[d] Monica Civera,[d] Sara Sattin,[d] Oscar Francesconi,[b] Roberta Marchetti,*[a] and Alba Silipo[a]

Here, two conformationally constrained sialyl analogues were synthesized and characterized in their interaction with the inhibitory Siglec, human CD22 (h-CD22). An orthogonal approach, including biophysical assays (SPR and fluorescence), ligand-based NMR techniques, and molecular modelling, was employed to disentangle the interaction mechanisms at a molecular level. The results showed that the Sialyl-TnThr antigen analogue represents a promising scaffold for the design of novel h-CD22 inhibitors. Our findings also suggest that the introduction of a biphenyl moiety at position 9 of the sialic acid hampers canonical accommodation of the ligand in the protein binding pocket, even though the affinity with respect to the natural ligand is increased. Our results address the search for novel modifications of the Neu5Ac-α(2-6)-Gal epitope, outline new insights for the design and synthesis of high-affinity h-CD22 ligands, and offer novel prospects for therapeutic intervention to prevent autoimmune diseases and B-cell malignancies.

Introduction

CD22 (Siglec-2) is a transmembrane receptor belonging to the evolutionary conserved class of Siglecs (Sialic acid binding immunoglobulin type lectins) and is selectively expressed on B-lymphocytes and, to a lesser extent, to other hematopoietic system cells.[1–3]

Upon sialoglycans engagement, CD22 negatively modulates B Cell Receptor (BCR) signaling with significant implications in maintaining tolerance to self-antigens, mandatory to prevent autoimmune diseases and B cells related malignancies.[4,5] The mechanism of BCR modulation involves the formation of CD22 homo-oligomers on resting B cells by means of *cis* interactions. Subsequently, specific antigens provoke a conformational change of BCR, leading to the activation of immune response and to the recruitment of CD22 clusters; ultimately, *trans* sialoglycan binding of autologous *self*-cells triggers BCR inhibition.[6]

The accommodation in the binding pocket of CD22 of sialic acids α-(2-6)-linked to galactose epitopes (Neu5Acα(2-6)-Gal), common terminus of mammalian surface glycoproteins, has been recently described and occurs with a $K_D = 281 \pm 10$ μM.[7] Furthermore, the conformational behavior of natural complex-type biantennary *N*-glycans interacting with CD22 was undertaken[8] thus proving that the terminal Neu5Acα(2-6)-Gal is the sole epitope recognized by and interacting with CD22, differently from other lectins-glycans interactions,[9] and also showing the key role of glycan conformation in triggering CD22 homo-oligomers formation.

Given its role in health and diseases, therapeutic tools based on CD22 as candidate target in immunomodulation and tumor therapies have been conceived, particularly focusing on the development of antibody-based approaches.[10–12] Nonetheless, alternative lines are also under evaluation, including glycan-based therapies that rely on the design of high affinity ligands with the capability to take over endogenous glycans.

In this context, low-molecular-weight Siglec ligands and their conjugates, especially those based on synthetic sialic acid derivatives, showed promising results in clinical trials as therapeutics for the nerve regeneration, oncology, and immunology.[13] The majority of sialic acid analogues are

[a] *R. E. Forgione,[+] F. F. Nieto,[+] C. Di Carluccio,[+] A. Molinaro, R. Marchetti, A. Silipo*
Department of Chemical Sciences
University of Naples Federico II,
Via Cintia 4, 80126, Napoli (Italy)
E-mail: roberta.marchetti@unina.it
[b] *F. Milanesi, M. Fruscella, F. Papi, C. Nativi, P. Palladino, S. Scarano, M. Minunni, O. Francesconi*
Department of Chemistry "Ugo Schiff"
University of Florence,
Polo Scientifico e Tecnologico
50019, Sesto Fiorentino, Firenze (Italy)
[c] *F. Milanesi*
Centro Risonanze Magnetiche,
CERM
Via L. Sacconi 6, 50019 Sesto Fiorentino, Firenze (Italy)
[d] *M. Montefiori, M. Civera, S. Sattin*
Dipartimento di Chimica,
Università degli Studi di Milano
via C. Golgi, 19, 20133, Milano (Italy)
[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200076

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

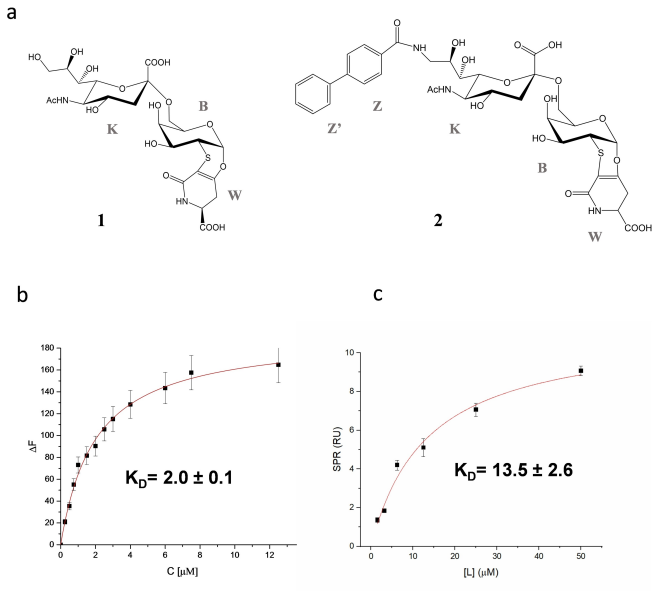
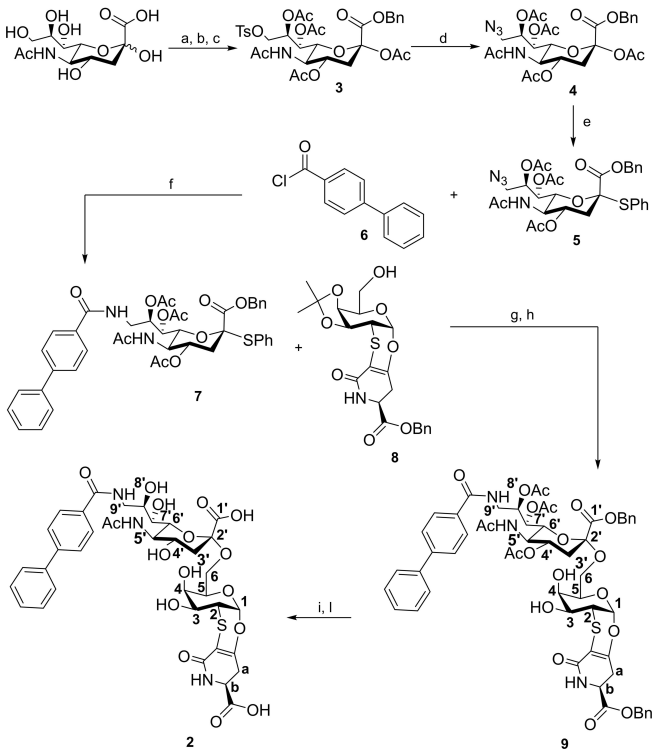


Figure 1. Binding affinity of h-CD22 and sialic acid analogues. a) Structures of the sialic acid analogues used in this study. b) Fluorescence titration of h-CD22 upon the addition of analogue **1**. Each emission spectrum was recorded at the excitation wavelength of 285 nm and a temperature of 10 °C. The relative binding isotherm and the value of the dissociation constant (K_D) is reported. For each data point, 10% Y error bars are shown. c) SPR binding curve for analogue **2** vs h-CD22 on Protein A. Each point is representative of 3 replicates ($RU_{mean} \pm SD$).



Scheme 1. Synthesis of sialo derivative **2** with atom labelling. Reagents and conditions: (a) BnBr, DBU, DMF, room temperature, 18 h; (b) TsCl, Py, room temperature, 18 h; (c) Ac_2O, DMAP, Py, room temperature, 5 h; (d) NaN_3, dry DMF, 70 °C, 5 h; (e) PhSH, $BF_3{\cdot}Et_2O$, dry DCM, room temperature, 18 h; (f) PPh_3, CH_2Cl_2, room temperature, 48 h; (g) NIS, TfOH, dry CH_3CN/CH_2Cl_2 (10:1), −40 °C, 4 h; (h) AcOH 80%, 40 °C, 18 h; (i) Pd/C, H_2, MeOH, room temperature, 72 h; (l) NH_3 4 M in MeOH, room temperature, 120 h.

characterized by the modifications of functional groups, as at carbons 2, 4, 5, and 9. In particular, substitutions at the hydroxyl/*N*-acetyl groups at position 9 and 5 respectively have shown to significantly enhance the binding affinity to Siglecs.[13–16] In parallel, the design of polyvalent constructs that include Siglecs specific- high affinity sialylated probes, to be conjugated to multivalent carriers, is a further step for biomedical applications.[13–17]

In a recent paper,[18] the synthesis and biological activity of a Neu5Ac-α(2-6)-Gal derivative, developed as an analogue of the Sialyl TnThr tumor antigen, were reported. From the structural viewpoint, the synthetic compound is a constrained tricyclic glycoside, that retains the α-*O*-glycosidic linkage at the Gal moiety and in which the terminal lactam ring mimics the Thr residue of the Tn/STn natural antigens. The Sialyl TnThr analogue belongs to a class of compounds exhibiting interesting inhibitory activity also toward other biological targets.[19–20]

With the believe that the rigid galactoside moiety would affect the binding specificity of CD22, here we report the description of the molecular recognition by human CD22 of the Sialyl-TnThr analogue and of a newly synthesized derivative, with the aim to identify novel ligands with therapeutic and diagnostic potential. The binding mode of such sialylated analogues was depicted by using NMR, fluorescence, Surface Plasmon Resonance (SPR), and molecular modelling techniques. The obtained results might contribute to rational design of CD22 potent inhibitors and/or regulators.

Results and Discussion

Based on the structure of the previously synthesized sialo-derivative **1** (Figure 1a),[18–20] the synthesis of **2** has been conceived. The sialo-derivative **2** (Figure 1a), having a similar scaffold than **1** but bearing a biphenyl substituent at position 9, was synthetized based on the observation that a similar sialo-analogue increased the bound potency and selectivity for CD22.[21]

In detail, analogue **2** was prepared (see Scheme 1) starting from *N*-acetylneuraminic acid. Esterification of the carboxylic group using benzyl bromide was followed by tosylation of the hydroxylic group in position 9 and acetylation of the remaining positions to give the protected sialic derivative **3** with a 40% yield on three steps. Tosylate group of **3** was then substituted with NaN_3 to give the azido derivative **4** with a 50% yield. Reaction of **4** with thiophenol in presence of $BF_3{\cdot}Et_2O$ gives the *S*-phenyl sialoside **5** with a 92% yield. Intermolecular Staudinger ligation between **5** and [1,1'-biphenyl]-4-carbonyl chloride **6**[22] in presence of PPh_3 gave the biphenyl substituted sialoside **7** with a 30% yield. Glycosylation reaction between the glycosidic donor **7** and the acceptor **8**, prepared according to known literature,[23] was carried out activating the *S*-phenyl group of **7** with *N*-iodosuccinimide (NIS) and catalyzing the reaction with **8**

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH

Figure 2. STD NMR analysis of analogue **1** in the interaction with h-CD22. Superimposition of the STD NMR spectrum (a) and the ^{1}H NMR spectrum (b) of h-CD22/analogue **1** mixture with a molecular ratio of 1:100, at 298 K. The interacting epitope map of analogue **1** as derived by STD-NMR data is also reported. c) 3D representation of the analogue **1** in the bioactive conformation obtained by tr-NOESY with molecular surface colored according to STD enhancements.

Figure 3. Interaction between h-CD22 and analogue **1** by molecular modelling. a) 3D model derived by docking and MD simulations for the analogue **1** bound to h-CD22 (PDB ID: 5VKM). The representative frame of the most populated MD cluster, obtained by Kmeans algorithm, was considered to depict the complex. b) Superimposition of the previously obtained X-ray complex of h-CD22/6'sialyllactose (6'SL) and the analogue **1** bound model. c) Two-dimensional plots representing the interactions between the analogue **1** and the binding site residues of h-CD22.

using TfOH. After removal of the isopropylidene group with AcOH 80%, the sialo derivative **9** was obtained with a 40% yield in two steps. Removal of the benzyl ester by hydrogenation and of the acetylic groups with an ammonia solution in methanol, gave the sialo derivative **2** with a yield of 30% on two steps.

Molecular recognition of a sialic acid analogue 1 by CD22

The binding features of the sialo-derivative **1** (Figure 1a) in the interplay with h-CD22 were described by means of fluorescence studies, ligand-based NMR techniques and molecular modelling.

Intrinsic fluorescence studies of analogue **1** in the interaction with h-CD22 showed that the tryptophane residues of the receptor were quenched by the ligand addition, thus proving the complex formation. The interpolation of the fluorescence data provided the corresponding dissociation constant $K_D = 2.0 \pm 0.1$ μM (Figure 1b and Figure S13).

Furthermore, STD NMR[24–25] and tr-NOESY[26] have been employed to dissect at molecular level the interaction between analogue **1** and h-CD22 and to map the ligand's interacting epitope and bioactive conformation (Figure 2). The STD NMR spectrum highlighted that the sialyl derivative was recognized by h-CD22 (Figure 2) as shown by the relative enhancements in the STD spectrum: a strong contribution from the neuraminic acid (Neu5Ac) moiety was detected, with the highest STD effect observed for its acetyl group. Furthermore, the involvement of the Neu5Ac lateral chain was deduced from the strong enhancements (between 50% and 70%) observed for K5, K6, K7, K8 and $K9_{R/S}$ protons. Significant STD effects were observed also for the galactose (Gal) residue, in particular for protons B3, B4 and B5. Interestingly, also the diastereoisotopic protons of the lactam moiety Wa/a' showed low STD signals, thus suggesting that also the aglycon moiety was involved in the binding.

With the aim to explore the dynamic behavior of the ligand into the h-CD22 binding site, NOE-based experiments were combined with molecular dynamics (MD) studies (Figure S14). The analogue **1** exhibited a similar conformational behavior in free and bound states, as evident by comparing the NOE and tr-NOE contacts. In detail, ϕ torsion angle around the glycosidic linkage between Neu5Ac-α-(2-6)-Gal was located around −60°

© 2022 The Authors. ChemBioChem published by Wiley-VCH GmbH