JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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In Silico-Aided Design of a Glycan Ligand of Sialoadhesin for in Vivo Targeting of Macrophages

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Supporting Information

ABSTRACT: Cell-specific delivery of therapeutic agents using ligand targeting is gaining interest because of its potential for increased efficacy and reduced side effects. The challenge is to develop a suitable ligand for a cell-surface receptor that is selectively expressed on the desired cell. Sialoadhesin (Sn, Siglec-1, CD169), a sialic acid-binding immunoglobulin-like lectin (Siglec) expressed on subsets of resident and inflammatory macrophages, is an attractive target for the development of a ligand-targeted delivery system. Here we report the development of a high-affinity and selective ligand for Sn that is an analogue of the natural ligand and is capable of targeting liposomal nanoparticles to Sn-expressing cells in vivo. An efficient in silico screen of a library of ∼8400 carboxylic acids was the key to identifying novel 9-*N*-acyl-substituted *N*-acetylneuramic acid (Neu5Ac) substituents as potential lead compounds. A small panel of targets were selected from the screen and synthesized to evaluate their affinities and selectivities. The most potent of these Sn ligands, 9-*N*-(4*H*-thieno[3,2-*c*]chromene-2-carbamoyl)-Neu5Acα2−3Galβ1−4GlcNAc (^{TCC}Neu5Ac), was conjugated to lipids for display on a liposomal nanoparticle for evaluation of targeted delivery to cells. The ^{TCC}Neu5Ac liposomes were found to target liposomes selectively to cells expressing either murine or human Sn in vitro, and when administered to mice, they exhibited in vivo targeting to Sn-positive macrophages.

Ligand-targeted liposomal nanoparticles offer promising applications in human medicine for selective delivery of therapeutic agents to the desired cells, resulting in increased efficacy and decreased side effects.[1−5] The challenge is to identify cell-surface receptors that are selectively expressed on the targeted cell and to develop ligands that target and bind those receptors with high selectivity. Siglecs, a family of *s*ialic acid-binding *i*mmunoglobulin-like *lec*tins with restricted expression on one or a few immune cell types, represent attractive targets for cell-directed therapies.[6,7] Among them, sialoadhesin (variously denoted as Sn, Siglec-1, or CD169) is an endocytic surface receptor that is expressed on subsets of resident and inflammatory macrophages and has a preference to bind glycan ligands with the Neu5Acα2−3Galβ1−4GlcNAc sequence (Neu5Ac = *N*-acetylneuraminic acid; Gal = galactose; GlcNAc = *N*-acetylglucosamine).[8−10] Because macrophages have both protective and pathological activities, including antitumor immune response, allergy and asthma, atherosclerosis, and wound healing,[11,12] the restricted expression and endocytic properties of Sn make it an ideal receptor for the development of a macrophage-targeted delivery system for therapeutic intervention.

In general, Siglecs bind with low intrinsic affinity (0.1−1 mM) to their natural sialoside ligands.[13] Several reports have demonstrated the importance of sialic acid substituents (e.g., at C9 of Neu5Ac) for increasing the affinity and selectivity of ligand binding to Siglecs.[8,14−17] In this regard, an exemplary ligand for CD22 (Siglec-2) on B cells, 9-*N*-BPC-Neu5Acα2−6Galβ1−4GlcNAc (6′-^{BPC}Neu5Ac; BPC = biphenylcarbamoyl) was found to support targeting of chemotherapeutic-loaded liposomes and prolong life in a murine model of human B cell lymphoma.[18] More recently, we showed that liposomes decorated with a related ligand, 9-*N*-BPC-Neu5Acα2−3Galβ1−4GlcNAc (**1**, 3′-^{BPC}Neu5Ac), are capable of targeting Sn-expressing cells in vitro. However, this ligand was not sufficiently selective for in vivo targeting.[19] Here we describe the in silico-aided design approach that we employed to develop a high-affinity ligand for Sn that can be used for in vivo targeting of macrophages.

To develop ligands with higher selectivity and affinity for Sn, we adopted a strategy that takes advantage of the existing crystal structure to identify novel 9-*N*-acyl-Neu5Ac substituents that could favorably bind to Sn in the hydrophobic pocket occupied by the previously described biphenyl substituent. To this end, we conducted a virtual screen of a library of carboxylic acids guided by the cocrystal structure of murine Sn (mSn) and ^{BPC}Neu5Ac-OMe.[8] As an alternative to solution-based screening methods, which would require extensive synthetic effort, the in silico approach has the advantage of rapidly screening large compound libraries to identify lead structures.[20,21] Figure 1 outlines the in silico screening approach for the representative carboxylic acid shown in Figure 1A. Initially, up to 250 conformers were calculated for each of ∼8400 carboxylic acids from a commercial building-block library. The resulting conformations were treated as unique acid structures and virtually coupled to the amino group of 9-NH_2-Neu5Ac fixed within the binding pocket. An aromatic ring pharmacophore was implemented using the coordinates of the first benzene ring of the biphenyl substituent in ^{BPC}Neu5Ac-OMe. The tethered docking of the acid conformers was scored on the basis of London dispersion energy using Molecular Operating Environment (MOE). Four representative solutions from this tethered docking approach for the acid in Figure 1A are shown in Figure

Received: July 30, 2012
Published: September 11, 2012

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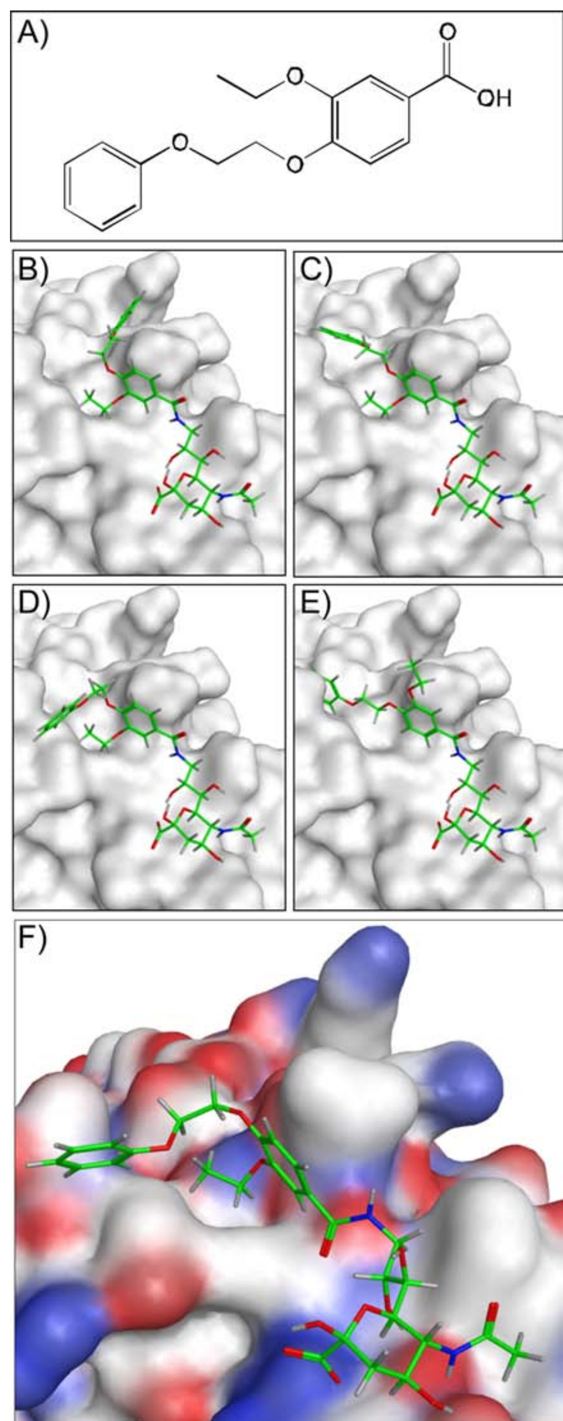
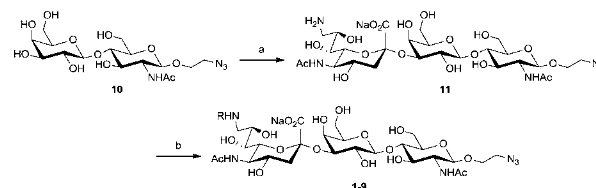


Figure 1. Representative structures from the in silico screening strategy used to identify high-affinity ligands for sialoadhesin (Sn). (A) Representative carboxylic acid from a commercial building-block library whose members were screened as potential substituents of 9-NH_2-Neu5Ac. (B–E) Four representative solutions for the acid from (A) obtained using the tethered docking approach, highlighting varying docking poses of the tethered acid substituent. Conformations of the respective substituents were calculated and conjugated via in silico amide coupling to 9-NH_2-Neu5Ac, which was fixed within the binding site. The top 3000 hits from this initial docking evaluation were further inspected using AutoDock. (F) Representative AutoDock solution for the selected carboxylic acid coupled to 9-NH_2-Neu5Ac. O, C, and N atoms are highlighted in red, white, and blue, respectively.

Scheme 1. Chemo-enzymatic Synthesis of C-9 *N*-Acyl-Modified Sialoside Analogues[a]



[a]Reagents and conditions: (a) CMP-9-NH_2-Neu5Ac, PmST1; (b) RC(O)NHS, THF, H_2O (see Table 1 for R).

Table 1. Inhibitory Potencies of Sialoside Analogues 1–9 against Murine Sn[a]

Compound	R =	IC_{50} (μM)
1		4.82 ± 0.14
2		0.38 ± 0.04
3		7.25 ± 0.61
4		21.6 ± 2.1
5		25.8 ± 1.4
6		>200
7		>400
8		26.5 ± 3.8
9		>500

[a]All of the titrations were performed in triplicate, and standard deviations (SDs) of three independent measurements are given.

1B–E. From this preliminary evaluation, the top 3000 poses were selected for further inspection using AutoDock 4.2.[22] Figure 1F depicts a tethered AutoDock solution for the representative carboxylic acid. The final nontethered docking solutions resembled the canonical sialic acid binding pose and provided a ranking of the acids based on calculated binding energies. From this ranking, a small panel of six target structures (**2**–**7**) were selected from the top-ranked 100 for their structural diversity based on the computed two-dimensional molecular fingerprints of the corresponding acids; their structures are shown in Table 1. In addition, as "nonranked" controls, we selected two additional sialosides (**8** and **9**) with *N*-acyl substituents that were eliminated from the screen at an early stage.

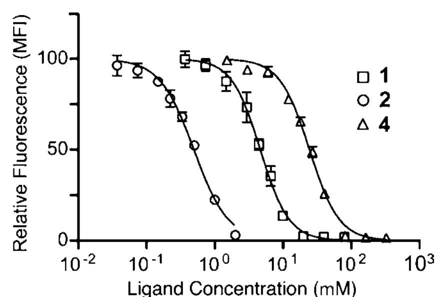


Figure 2. Inhibitory potencies of selected sialoside analogues in a mSn competitive bead-binding assay. Each compound was analyzed in triplicate for inhibition of the binding of mSn-Fc chimera to Neu5Acα2−3Galβ1−4GlcNAc-coated magnetic beads as described in the Supporting Information. See Table 1 for glycans **1**, **2**, and **4**.

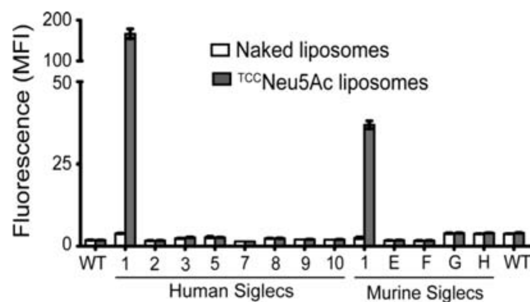


Figure 3. Fluorescence-assisted cell sorting (FACS) analysis of in vitro binding of targeted ^{TCC}Neu5Ac or nontargeted "naked" liposomes to cells expressing murine and human Siglecs. Binding is shown as mean fluorescence intensity (MFI) ± SD (n = 3).

All of the targets were synthesized chemo-enzymatically (Scheme 1). Briefly, Galβ1−4GlcNAc-ethyl azide (**10**) was reacted with CMP-9-NH_2-Neu5Ac (CMP = cytidine monophosphate) using *Pasteurella multocida* α2−3-sialyltransferase 1 (PmST1)[23] to afford the trisaccharide scaffold **11**. Divergent reaction of **11** with the panel of NHS-activated carboxylic acids afforded the final targets **2**−**9**. The reference ligand **1** substituted with BPC was also prepared.

The inhibitory potencies (IC_{50}) of the glycan derivatives **1**−**9** were evaluated in a flow cytometry assay based on the competitive binding of mSn-Fc chimera to beads decorated with the natural ligand Neu5Acα2−3Galβ1−4GlcNAc. The IC_{50} values required to displace the bound mSn-Fc were determined with serial dilutions of the competitors (Table 1 and Figure 2).

A wide range of binding affinities was observed for sialosides **2**−**7** containing the top-ranked substituents. Relative to the reference compound **1** (IC_{50} = 4.8 μM), two were weak inhibitors (**6** and **7**; IC_{50} > 200 μM), three were of comparable affinity (**3**−**5**; IC_{50} of 7.3−26 μM), and one had ~13-fold higher affinity (**2**; IC_{50} = 0.38 μM). This result is particularly notable in view of the affinity of the natural nonsubstituted ligand, Neu5Acα2−3Galβ1−4GlcNAc, which had an IC_{50} of 1300 μM in a comparable assay.[13] Analysis of **2** in complex with Sn suggested that the bioisostere replacement of the first benzyl group in **1** provides improved shape complementarity and additional contacts to the protein (data not shown). Of the two unranked substituents, one was weakly active (**9**; IC_{50} > 500 μM) and the other showed relatively high affinity (**8**; IC_{50} = 26.5 μM). Thus, while our approach of using the in silico screen to identify target substituents requires further investigation to validate its potential and general utility, it provided a significant lead with minimal investment in synthetic resources. Accordingly, we proceeded to evaluate further the high-affinity lead structure, 9-*N*-(4*H*-thieno[3,2-*c*]chromene-2-carbamoyl)-Neu5Acα 2−3Galβ1−4GlcNAc (**2**, ^{TCC}Neu5Ac).

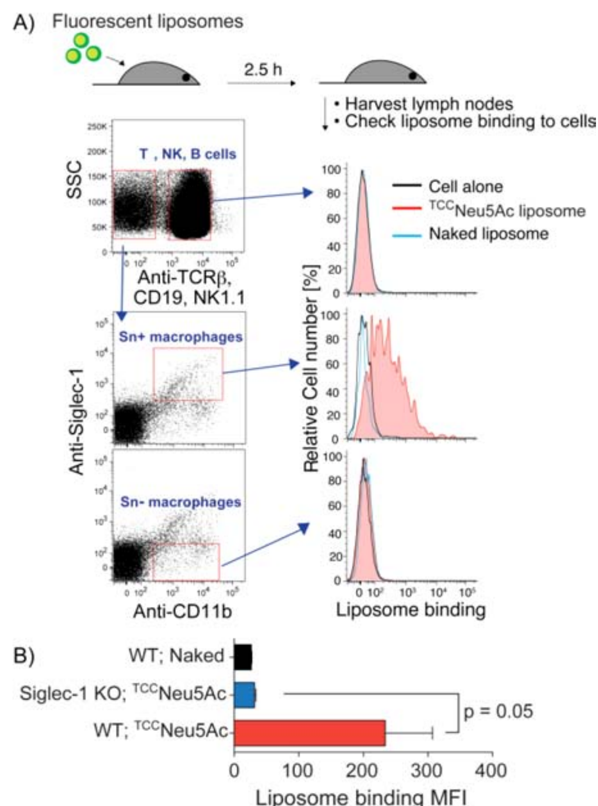


Figure 4. ^{TCC}Neu5Ac liposomes target Sn-positive macrophages in vivo. (A) Wild-type mice were injected subcutaneously with fluorescently labeled naked or ^{TCC}Neu5Ac liposomes, and the neighboring lymph nodes were harvested after 2.5 h. Cells were stained with various antibodies as described in the Supporting Information and analyzed by flow cytometry. (B) Wild-type and Sn knockout mice (n = 3 in each group) were injected with the liposomes. The binding of liposomes to macrophages ($TCRb^-NK1.1^-CD19^-CD11b^+$) was analyzed by flow cytometry. The plots show the geometric mean of fluorescent intensity (MFI).

We next tested the selectivity of ^{TCC}Neu5Ac (**2**) for Sn when incorporated into targeted liposomes. To accomplish this, fluorescent targeted liposomes were formulated to include high-affinity glycan ligand **2** coupled to PEGylated lipid (see the Supporting Information), and these liposomes were then tested for binding to a panel of cell lines expressing human and murine Siglecs.[18,19] Cells expressing Siglecs were incubated with fluorescently labeled targeted liposomes, washed, and analyzed by flow cytometry. The results revealed that the ^{TCC}Neu5Ac ligand is highly selective for Sn (Figure 3). Only cells expressing murine or human Sn bound the targeted liposomes, while nontargeted "naked" liposomes did not bind any of the cells.

To evaluate the specificity of the ^{TCC}Neu5Ac ligand in an in vivo setting, we sought to determine whether ^{TCC}Neu5Ac-decorated liposomes could target Sn-expressing macrophages in peripheral lymph nodes. Accordingly, wild-type (WT) or Sn knockout (KO) mice were subcutaneously injected in the flank with Alexa Fluor 647-labeled ^{TCC}Neu5Ac liposomes. After 2.5 h, immune cells from neighboring lymph nodes on the same

side were harvested and analyzed by flow cytometry. Populations of T, B, and NK lymphocytes and myeloid cells were identified using specific antibodies (Figure 4). Furthermore, Sn-positive myeloid cells (macrophages) were identified using an anti-Sn antibody. The gated cell populations revealed that the ^{TCC}Neu5Ac liposomes effectively targeted Sn-positive myeloid cells, which was observed as a right shift in the histogram (Figure 4A). There was no binding of targeted liposomes to T, B, NK, or Sn-negative macrophages. Naked liposomes did not bind to any cells. Furthermore, no binding of targeted liposomes to macrophages from Sn KO mice was detected, indicating that uptake of the liposomes was Sn-dependent (Figure 4B).

In summary, we have described the successful development of a high-affinity ligand for Sn suitable for use in targeting liposomal nanoparticles to Sn-expressing cells in vivo. An efficient in silico screen of a commercial building-block library was performed to identify novel substituents of potential lead compounds. From this screen, a small panel of selected target structures were translated into sialoside derivatives with limited synthetic effort as a result of the design strategy. Consequently, a novel high-affinity ligand of Sn was identified. Targeted liposomal nanoparticles displaying the ligand showed high selectivity for human and murine Sn-expressing cells in vitro. Further evaluation of the targeted liposomes in mice showed effective in vivo targeting of Sn-positive macrophages. We anticipate that delivery systems incorporating this novel ligand for Sn will provide a powerful means of active in vivo delivery of antigens or therapeutic agents to macrophages.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary synthetic scheme, experimental protocols, synthetic methods, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

C.R. thanks EMBO for a long-term fellowship. This work was supported by the National Institutes of Health (P01HL107151 and R01AI05143).

■ REFERENCES

(1) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145.
(2) Trapani, G.; Denora, N.; Trapani, A.; Laquintana, V. *J. Drug Targeting* **2012**, *20*, 1.
(3) Lepenies, B.; Yin, J. A.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2010**, *14*, 404.
(4) Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N. K. *Biomaterials* **2012**, *33*, 4166.
(5) Xie, R.; Hong, S. L.; Feng, L. S.; Rong, J.; Chen, X. *J. Am. Chem. Soc.* **2012**, *134*, 9914.
(6) Crocker, P. R.; Paulson, J. C.; Varki, A. *Nat. Rev. Immunol.* **2007**, *7*, 255.
(7) O'Reilly, M. K.; Paulson, J. C. *Trends Pharmacol. Sci.* **2009**, *30*, 240.
(8) Zaccai, N. R.; Maenaka, K.; Maenaka, T.; Crocker, P. R.; Brossmer, R.; Kelm, S.; Jones, E. Y. *Structure* **2003**, *11*, 557.
(9) Delputte, P. L.; Van Gorp, H.; Favoreel, H. W.; Hoebeke, I.; Delrue, I.; Dewerchin, H.; Verdonck, F.; Verhasselt, B.; Cox, E.; Nauwynck, H. J. *PLoS One* **2011**, *6*, No. e16827.
(10) Hartnell, A.; Steel, J.; Turley, H.; Jones, M.; Jackson, D. G.; Crocker, P. R. *Blood* **2001**, *97*, 288.
(11) Murray, P. J.; Wynn, T. A. *Nat. Rev. Immunol.* **2011**, *11*, 723.
(12) Kelly, C.; Jefferies, C.; Cryan, S. A. *J. Drug Delivery* **2011**, No. 727241.
(13) Blixt, O.; Collins, B. E.; van den Nieuwenhof, I. M.; Crocker, P. R.; Paulson, J. C. *J. Biol. Chem.* **2003**, *278*, 31007.
(14) Blixt, O.; Han, S. F.; Liao, L.; Zeng, Y.; Hoffmann, J.; Futakawa, S.; Paulson, J. C. *J. Am. Chem. Soc.* **2008**, *130*, 6680.
(15) Mesch, S.; Moser, D.; Strasser, D. S.; Kelm, A.; Cutting, B.; Rossato, G.; Vedani, A.; Koliwer-Brandl, H.; Wittwer, M.; Rabbani, S.; Schwardt, O.; Kelm, S.; Ernst, B. *J. Med. Chem.* **2010**, *53*, 1597.
(16) Mesch, S.; Lemme, K.; Wittwer, M.; Koliwer-Brandl, H.; Schwardt, O.; Kelm, S.; Ernst, B. *ChemMedChem* **2012**, *7*, 134.
(17) Magesh, S.; Ando, H.; Tsubata, T.; Ishida, H.; Kiso, M. *Curr. Med. Chem.* **2011**, *18*, 3537.
(18) Chen, W. C.; Completo, G. C.; Sigal, D. S.; Crocker, P. R.; Saven, A.; Paulson, J. C. *Blood* **2010**, *115*, 4778.
(19) Chen, W. C.; Kawasaki, N.; Nycholat, C. M.; Han, S.; Pilotte, J.; Crocker, P. R.; Paulson, J. C. *PLoS One* **2012**, *7*, No. e39039.
(20) Fadda, E.; Woods, R. J. *Drug Discovery Today* **2010**, *15*, 596.
(21) Ernst, B.; Magnani, J. L. *Nat. Rev. Drug Discovery* **2009**, *8*, 661.
(22) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785.
(23) Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B. Y.; Zhang, J. B.; Zhang, Y. X.; Jia, Q.; Chen, X. *J. Am. Chem. Soc.* **2005**, *127*, 17618.