Biomimetics of Carbohydrate Nucleotides

DOI: 10.1002/anie.201400394

Fluorescent Mimetics of CMP-Neu5Ac Are Highly Potent, Cell-Permeable Polarization Probes of Eukaryotic and Bacterial Sialyltransferases and Inhibit Cellular Sialylation**

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Abstract: *Oligosaccharides of the glycolipids and glycoproteins at the outer membranes of human cells carry terminal neuraminic acids, which are responsible for recognition events and adhesion of cells, bacteria, and virus particles. The synthesis of neuraminic acid containing glycosides is accomplished by intracellular sialyl transferases. Therefore, the chemical manipulation of cellular sialylation could be very important to interfere with cancer development, inflammations, and infections. The development and applications of the first nanomolar fluorescent inhibitors of sialyl transferases are described herein. The obtained carbohydrate-nucleotide mimetics were found to bind all four commercially available and tested eukaryotic and bacterial sialyl transferases in a fluorescence polarization assay. Moreover, it was observed that the anionic mimetics intruded rapidly and efficiently into cells in vesicles and translocated to cellular organelles surrounding the nucleus of CHO cells. The new compounds inhibit cellular sialylation in two cell lines and open new perspectives for investigations of cellular sialylation.*

Sialic acids like 5-*N*-acetylneuraminic acid (Neu5Ac) are attached to glycoconjugates such as glycoproteins and glycolipids at the outer surface of eukaryotic cells and act as multivalent ligands in cell-adhesion events. The regulation of sialylation is of general importance for the maintenance of healthy cells and organisms.[1] Hypersialylation of cells is found in inflammation and enables immune cells to intrude into infected tissue.[2] Moreover, strongly hypersialylated cancer cells are capable of leaving their primary tissue environment, migrating, and forming metastases in remote tissues.[3] Hypersialylation is, therefore, strongly indicative of a bad prognosis of neoplasia and inhibition of this event could be an alternative therapeutic strategy against cancer.[4]

Sialylation of glycoconjugates proceeds in the Golgi apparatus by the action of at least 20 distinct sialyltransferases found in both the human and the murine genome.[5] The degree of sialylation of cells is strongly linked to the expression level of sialyltransferases, which can thus be considered to be potential targets for pharmacological interference.[6] For a more detailed understanding of the functions and the significance of protein sialylation, protein-binding probes for this enzyme class are highly desirable. In addition, specific and generic inhibitors of this enzyme class would be valuable for the validation of sialyltransferases as potential targets for the treatment of metastasizing neoplasms.[7]

The development of inhibitors for sialyltransferases is a demanding task for several reasons. First, few of the genetically encoded enzymes have been expressed and isolated as stable, soluble, and bioactive proteins for homogeneous assays so far. Secondly, the elaboration of functional assays has been difficult, as many of the enzymes require specific oligosaccharide substrates, which must be prepared or isolated. Thirdly, the monitoring of the enzymatic reaction typically requires product analysis by chromatography rendering the assay time-consuming, expensive, and low throughput,[8] though the recent progress in the area of glycan arrays might lead to an alternative to solution assays in the future.[9]

We concluded from these considerations that a generic assay for sialyltransferases can be realized only if not the enzyme-specific conversion of acceptor substrates but binding at the recognition site of the generic donor substrate of these membrane-bound enzymes, 5-*N*-acetyl-neuraminic acid cytidine monophosphate (CMP-Neu5Ac, Figure 1), was detected. In order to realize such a binding assay for sialyltransferases, generic probes for this enzyme class were required.

Already two decades ago, Reutter et al. established that the enzymes in sialic acid metabolism including the sialyltransferases are promiscuous with respect to donor substrates carrying various substitutions in the C5 position.[10] Besides the native *N*-acetyl group, larger amide residues and azides are accepted, a finding that was subsequently exploited for the on-cell derivatization of 5-azidosialic acids.[11] Potent inhibitors of sialyltransferases were identified by the group of R. R. Schmidt that contained phosphate esters of α-hydroxybenzylphosphonic acid derivatives.[12] Combination of these distinct findings prompted us to propose arylic amide **1** as

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[**] Support from the DFG (FOR 806, SFB 765, TRR 67), and the Berlin School of Integrative Oncology is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201400394.

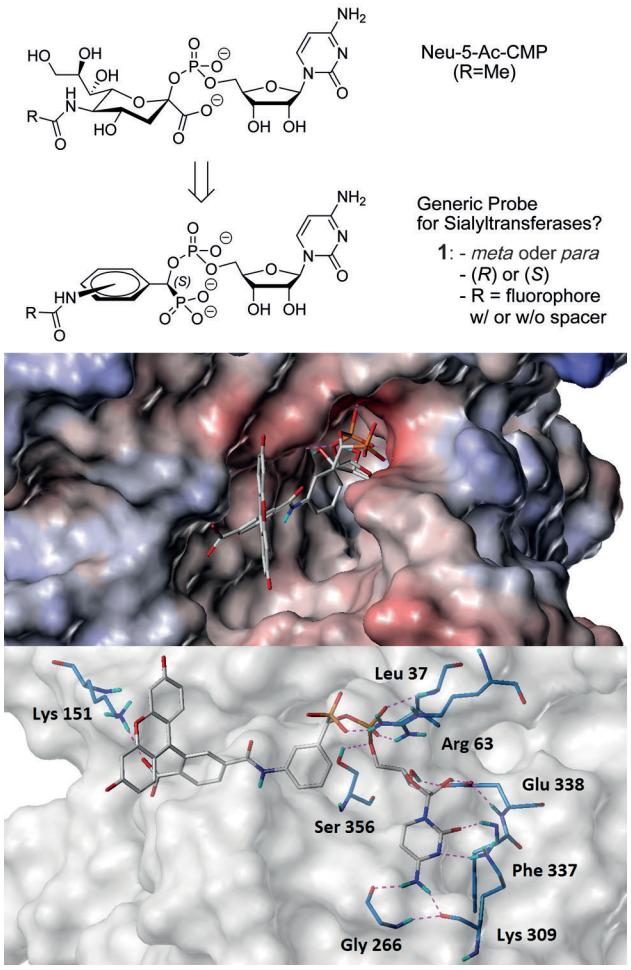


Figure 1. Design of potential probe molecules for sialyltransferases: The native substrate of sialyltransferases, CMP-Neu5Ac (top), can be varied considerably at the 5-position of neuraminic acid. Docking studies of the derived biomimetic (*R*)-**1-m** with the bacterial sialyltransferase PmST1 (PDB: 2IHZ)[13] and later (see the Supporting Information) with the mammalian sialyltransferase pST3Gal I (PDB: 2WNB[14]).

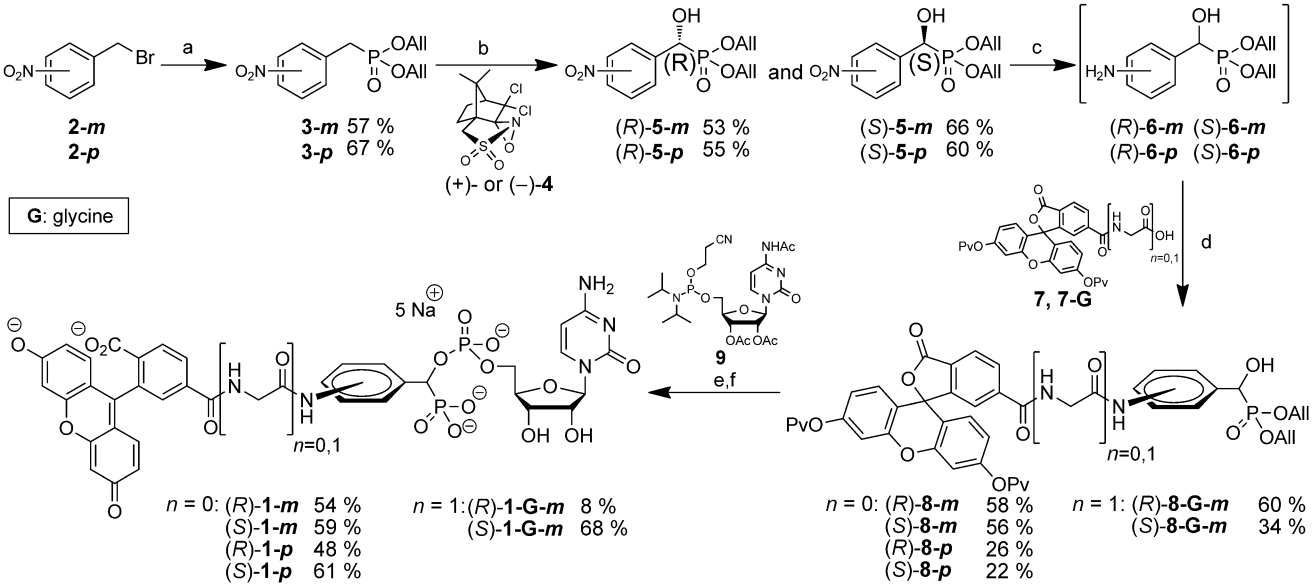
a potential, generic structure for sialyltransferase probes (Figure 1): While **1** was calculated to retain the binding mode of the substrate, the substrate tolerance in the C5 position of CMP-Neu5Ac should translate into variable substituents in the *meta* and *para* positions of the inhibitors, which possibly would enable the integration even of larger groups such as fluorophores, functionalities for pull-down experiments, and photoactive cross-linking groups. We decided to focus on the development of fluorescent sialyltransferase probes first as these molecules could be directly employed in a binding assay exploiting shifts in fluorescence polarization observed during the competitive replacement of such molecules.

Docking simulations of the designed molecules in the crystal structure of sialyltransferase PmST1 (PDB: 2IHZ[13]) and later the nonhomologous, mammalian enzyme pST3Gal I (PDB: 2WNB[14]) encouraged us to challenge this hypothesis (Figure 1 and Figure S2). Several combinations of a spacer and fluorescein as the fluorophore fitted well into the protein and did not interfere with the recognition of the presumed CMP-Neu5Ac mimetic. Accordingly, a set of potential fluorescence probes **1** for sialyltransferases was designed (Figure 1, Scheme 1) containing variations with respect to the substitution at the phenyl ring of the inhibitor (*meta* or *para*), the introduction of a glycine linker between fluorescein and the inhibitor, and the configuration at the α-hydroxymethylphosphonate (*R* or *S*).

The designed probe molecules were synthesized starting from 3- and 4-nitrobenzyl bromides **2-*m*** and **2-*p***. Alkylation of triallyl phosphite with these electrophiles furnished the phosphonates **3-*m*** and **3-*p***, which were deprotonated with sodium hexamethyldisilazane (NaHMDS). The obtained intermediary phosphoryl-stabilized benzyl anions were oxidized stereoselectively employing either (−)- or (+)-[(8,8-dichlorocamphoryl)sulfonyl]oxaziridine **4** as the oxidant[15] and provided the corresponding (*R*)- and (*S*)-α-hydroxymethylphosphonates (*R*)-**5-*m*** or **-*p*** and (*S*)-**5-*m*** or **-*p***, respectively. The enantiomeric excesses (*ee*) were determined by the preparation and NMR analysis of the derived Mosher's esters and were found to be between 83–87% (Mosher's esters not shown; for details see the Supporting Information).

The four nitro-substituted (*R*)- and (*S*)-α-hydroxymethylphosphonates of structure **5** were reduced to the corresponding four aromatic amines (*R*)-**6-*m*** or (*R*)-**6-*p*** and (*S*)-**6-*m*** or (*S*)-**6-*p*** using tin(II) chloride as the reducing agent. The anilines **6** were not stable when isolated or stored for a longer time and thus after aqueous workup they were immediately coupled with pivaloyl-protected, 6-carboxyfluorescein[16] **7** or with the glycine spacer derivative **7-G**. Six stable secondary benzylic alcohols of the general structure **8** were isolated, (*R*)-**8-*m*** and (*S*)-**8-*m***, (*R*)-**8-*p*** and (*S*)-**8-*p***, and (*R*)-**8-G-*m*** and (*S*)-**8-G-*m***, the latter two containing the glycine spacer. All six alcohols **8** were coupled with the protected cytidine phosphoramidite **9**,[17] oxidized with *tert*-butyl hydroperoxide, and then deprotected with $[Pd(PPh_3)_4]$ and K_2CO_3 in MeOH, a procedure removing all allyl, acetyl, pivaloyl, and cyanoethyl protecting groups in one step.[18] The six obtained fluorescein-labeled inhibitors (*R*)- and (*S*)-**1-*m***, (*R*)- and (*S*)-**1-*p***, and (*R*)- and (*S*)-**1-G-*m*** were purified by HPLC and transformed into their sodium salts employing a cation-exchange resin. The final diastereomeric excesses (*de*) of the six potential FP probes **1** were determined by integration of the signals of the cytidine H-5 or H-1′ protons in the ^{1}H NMR spectra and were between 71 and 84%. This indicated that no significant epimerization had occurred throughout the multistep protocol from **6** to **1**.

For evaluating the binding of the probes to the CMP-Neu5Ac pocket, two mammalian sialyltransferases were selected, rat rST3Gal II,[19] for sialylation of galactose in position 3, and human hST6Gal I,[20] for sialylation of galactose in position 6; both of these enzymes were commercially available in soluble form and recombinantly expressed in insect cells. In addition, the two prokaryotic sialyltransferases PmST1 from *Pasteurella multocida*[21] and Pd2,6ST(N) from *Photobacterium damsela*[22] for sialylation of galactose in position 3 and position 6, respectively, were employed. Potential enzyme probes **1** were dissolved (10 nM) in buffer with serial dilutions of the four sialyltransferases. The dissociation constant K_D of the ligand–protein complex was determined by



Scheme 1. Reaction conditions for the synthesis of probes 1: a) $P(OAll)_3$, toluene, microwaves, 105 °C, 15 h; b) 1. NaHMDS, THF, −95→−45 °C, 1–3 h, 2. (−)- or (+)-**4**, THF, −95 °C→−78 °C, 3.5 h; *ee* 84–87 %; c) $SnCl_2$, EtOH, 20–40 °C, 1–3 d; d) **7** or **7-G**, EDC, DCM, 2–18 h; e) 1. **9**, 1*H*-tetrazole, DCM, 2.5–18 h, 2. *t*BuOOH, 1–21 h; f) 1. $[Pd(PPh_3)_4]$, K_2CO_3, MeOH, 4–24 h, 2. HPLC, 3. Na^+, *de* 71–84 %).

recording the anisotropy changes in fluorescence polarization as a function of protein concentration. All probes proved to be ligands of the four STs investigated. Binding affinities to the mammalian STs were very high—in the two-digit nanomolar range—with surprisingly low affinity differences between the different probes. Best binding affinities with respect to the bacterial STs were also in the lower nanomolar range with more variations between the probes.

The configuration of the α-hydroxymethylphosphonate had only little influence on the binding properties of the probes toward the mammalian STs while the *R*-configurated probes bound much more strongly to the bacterial STs than the *S*-configurated. The position of the aromatic substituent had only little influence on the affinities of the probes to the mammalian STs but the *para*-substituted probes were much stronger binders of the bacterial STs than the *meta*-substituted. Both the configuration of the benzylic stereocenter and the aromatic substitution influenced the anisotropic properties of the probes regarding the maximal anisotropy and the quantum yield of fluorescence. The glycine linker seemed not to be crucial for the binding of the probes to the STs; however, it increased significantly the dynamic range of anisotropy for rST3Gal II. Differences in the dynamic range between the probes might arise from flexibility of the fluorophore tag, different rotational relaxation times of the probe–protein complex, and the influence of the protein environment on the fluorescence lifetime.[23] The specificity of the ST probes was scrutinized for one selected example. (*S*)-**1-G-*m*** was incubated with increasing concentrations of the commercially available β-1,4-galactosyltransferase from cow milk and fluorescence polarization was measured. Even at the maximal protein concentration of 250 μM only partial retention of the FP was recorded indicating $K_D > 250$ for the probe and a selectivity for the two eukaryotic sialyltransferases of more than 10000.

Next, an FP-based displacement assay was to be established for each of the sialyltransferases (Figure 2). Thus, for each ST a probe was selected which provided an optimal combination of high affinity, large dynamic anisotropy window, and constant fluorescence quantum yield. This was (*S*)-**1-G-*m*** for the rat enzyme rST3Gal II, (*S*)-**1-*p*** for the human protein hST6Gal I, (*R*)-**1-*p*** for Pd2,6ST(N), and (*S*)-**1-*p*** for PmST1. The initial anisotropy was optimized for each binding assay with respect to the sensitivity by adjusting the protein concentration.[24]

Subsequently, the bound probes were replaced competitively by addition of cytidine diphosphate (CDP) and the simplified, nonfluorescent CMP-Neu5Ac biomimetic **1-Ac** (Figure 1, R = Me). The experiments clearly confirmed the reversible binding mode of all selected probes. The inhibition constants K_i were calculated by the method of Nikolovska-Coleska et al.[25] and indicated inhibition of the mammalian STs by CDP in the μM range, whereas **1-Ac** was a sub-μM inhibitor (see Table 1). Remarkably, the inhibition potencies of CDP are similar to or even better than those of the nonfluorescent derivative **1-Ac** with both bacterial STs and were in the low nM range. The displacement assays proved to be robust against 1 % DMSO, an important feature for the application of this assay in the screening of chemical libraries.

Finally, the selected sialyltransferase probe (*S*)-**1-G-*m*** was investigated in living Chinese Hamster Ovary (CHO) cells. Commonly, the outer cell membrane is considered to be impermeable toward polyanionic molecules such as compounds **1**, a general problem for the cellular availability of organic phosphates, phosphate mimetics, nucleic acids, etc. Therefore, we expected the need for using transfection agents in order to translocate the probe (*S*)-**1-G-*m*** into cells. To our great surprise, however, treatment of CHO cells with the fluorescent probe (*S*)-**1-G-*m*** led to the rapid and prolonged intracellular staining of the cells (Figure 3). The intensity and

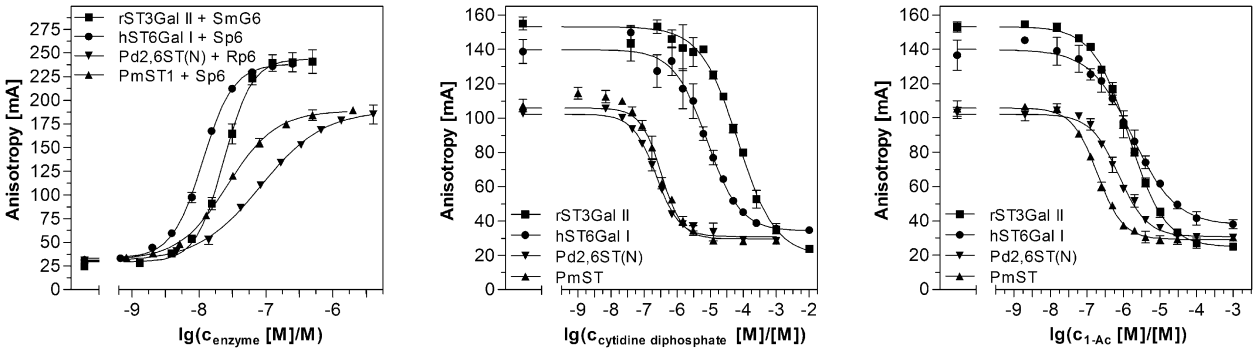


Figure 2. Fluorescence polarization binding curves of the most potent probes (left) and displacement by cytidine diphosphate (CDP) (middle) and **1-Ac** (right) (see the Supporting Information for binding plots of all probes and DMSO test for **1-Ac**).

Table 1: Binding affinities (K_D values) and anisotropy changes (ΔA) of fluorescent NeuAc5-CMP mimetics **1** as well as displacement properties (K_i values) and assay window (ΔA) of inhibitors cytidine diphosphate (CDP) and **1-Ac** with respect to four eukaryotic and bacterial sialyltransferases.

Probe	rST3Gal II		hST6Gal I		Pd2,6ST(N)		PmST1	
	K_D [nM][a]	ΔA [mA][a]	K_D/K_i [nM][a]	ΔA [mA][a]	K_D/K_i [nM][a]	ΔA [mA][a]	K_D/K_i [nM][a]	ΔA [mA][a]
(*R*)-1-*m*	41.0 ± 1.9	181.5 ± 6.1	22.2 ± 0.4	213.2 ± 2.5	924.6 ± 157.5	193.3 ± 15.5	33.6 ± 1.8	160.7 ± 6.2
(*S*)-1-*m*	44.8 ± 0.8	144.1 ± 1.6	9.0 ± 0.3	184.8 ± 2.8	> 1000	180.1 ± 28.5	183.8 ± 7.9	140.3 ± 2.7
(*R*)-1-*p*	24.5 ± 0.7	131.5 ± 2.7	21.5 ± 0.6	152.2 ± 2.7	**83.9 ± 5.1**	**159.2 ± 4.5**	11.5 ± 0.6	133.7 ± 4.2
(*S*)-1-*p*	78.9 ± 4.5	180.3 ± 6.7	**11.3 ± 0.2**	**205.0 ± 2.7**	590.7 ± 48.5	163.2 ± 4.7	**26.5 ± 1.0**	**157.7 ± 2.8**
(*R*)-1-G-*m*	23.9 ± 0.6	194.3 ± 2.9	17.9 ± 0.3	205.0 ± 2.0	53.2 ± 13.9	75.1 ± 11.6	12.7 ± 1.0	139.0 ± 4.1
(*S*)-1-G-*m*	**23.5 ± 0.9**	**214.5 ± 5.4**	12.8 ± 0.2	208.9 ± 2.3	> 1000	182.1 ± 9.4	184.6 ± 21.0	104.3 ± 5.2

Inhibitor	rST3Gal II		hST6Gal I		Pd2,6ST(N)		PmST1	
	K_i [μM][a]	ΔA [mA][a]	K_i [μM][a]	ΔA [mA][a]	K_i [μM][a]	ΔA [mA][a]	K_i [μM][a]	ΔA [mA][a]
CDP	32.83 ± 4.31	129.5 ± 4.1	3.50 ± 0.41	104.6 ± 8.0	0.09 ± 0.00	74.2 ± 2.5	0.15 ± 0.01	77.4 ± 5.6
1-Ac 0% DMSO	0.66 ± 0.03	128.4 ± 4.0	0.78 ± 0.09	101.9 ± 9.5	0.36 ± 0.02	72.5 ± 2.5	0.08 ± 0.01	75.8 ± 5.6
1-Ac 1% DMSO	0.69 ± 0.03	115.5 ± 0.7	0.88 ± 0.13	96.8 ± 11.2	0.39 ± 0.01	70.0 ± 4.8	0.09 ± 0.01	80.0 ± 7.0

[a] ± Standard error; FP probes in boldface were used for displacement studies.

homogeneity of cellular uptake was investigated by flow cytometry (Figure 3E,F). After 24 h incubation with (*S*)-**1-G-*m*** all CHO cells showed strong fluorescence (dark gray), whereas control cells treated with 6-carboxyfluorescein at the very same concentration showed less than 5% fluorescence (light gray).

Staining of cells with (*S*)-**1-G-*m*** was observed in subcellular compartments only, while the cytoplasm was not affected at all, indicating a vesicular uptake mechanism. Fluorescing vesicles translocated rapidly to the proximity of the nucleus, the nucleus itself was not stained. The dye Bodipy tr C_5 ceramide[26] was used in order to define the subcellular localization of (*S*)-**1-G-*m*** more precisely. Bodipy tr C_5 ceramide was reported to specifically stain extranuclear compartments of cells, especially the endoplasmic reticulum (ER) and the Golgi apparatus. Co-staining of CHO cells with (*S*)-**1-G-*m*** (green), the nuclear dye Hoechst 33342 (blue), and the ER/Golgi tracker (red) confirmed that the Neu5Ac biomimetic was translocated to compartments surrounding the cell nucleus. The compartments directly attached to the nucleus, the ER and the closer cis-Golgi network, which were most intensively stained with the tracker, did not contain the sialyltransferase probe, whereas compartments directly adjacent to the strongest red-stained organelles contained the highest concentration of the fluorescein probe. These compartments might be part of the trans-Golgi network, which is the reported site for sialyltransferases. Therefore, the functional effect of the probe on sialylation in living cells was investigated. Two cell lines, CHO and B35 cells, were incubated with a single dose of probe (*S*)-**1-G-*m*** (50 μM, 24 h). Cells were harvested and lyzed, and equal amounts of cell lysate were analyzed by SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and sialylated glycoproteins were detected by incubation with biotinylated sialolectin limax flavus agglutinin (LFA) followed by treatment with streptavidine labeled with horseradish peroxidase (Figure 4). In both cell lines a reduction in the chemiluminescence intensity of the LFA staining after incubation with the probe (*S*)-**1-G-*m*** was observed, indicating a reduced sialylation on the separated glycoproteins.

In summary, we have established compounds of general structure **1** as generic and potent fluorescent molecular probes for sialyltransferases. The developed probes were highly active toward the four tested recombinant STs, which were the four commercially available enzymes at the time of this study both of mammalian and of bacterial origin. The binding of the probes to STs was determined by fluorescence polarization experiments. Anisotropy was employed to establish a robust, competitive binding assay for each of the STs used in this study. The selected probe (*S*)-**1-G-*m*** was highly

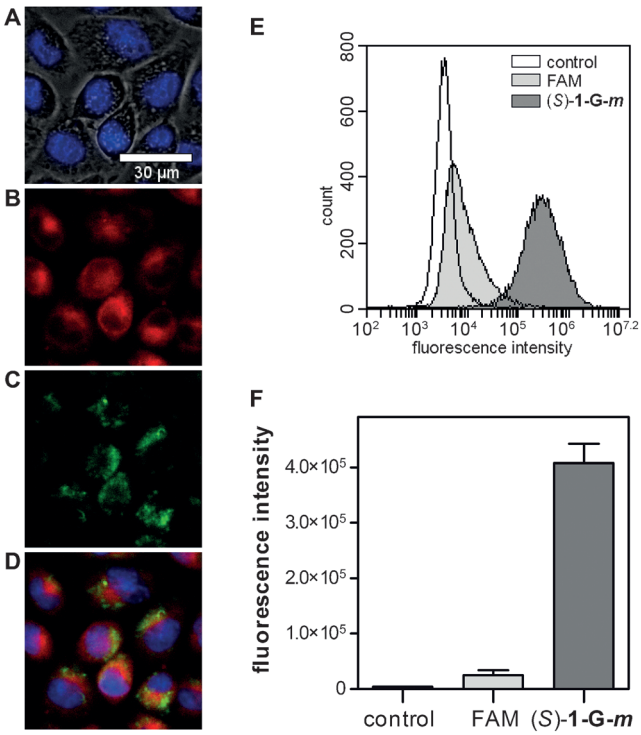


Figure 3. Cellular uptake of (*S*)-**1-G-*m*** by CHO cells: A) staining with Hoechst 33342 for nuclei and overlay with phase-contrast image; B) staining with BODIPY TR C_5-ceramide (5 μM) for labeling of the endoplasmatic reticulum and the Golgi; C) staining showing vesicular allocation of the probe; D) co-staining with three dyes (for pictures of control and treatment with carboxyfluorescein see the Supporting Information). Flow cytometry analysis of (*S*)-**1-G-*m*** (50 μM) uptake by CHO cells compared to carboxyfluorescein (FAM) and control after 24 h of incubation. E) Logarithmic plot indicates significant fluorescence in all cells incubated with the probe. F) Bar graph of absolute values (mean ± SEM, $n = 2$).

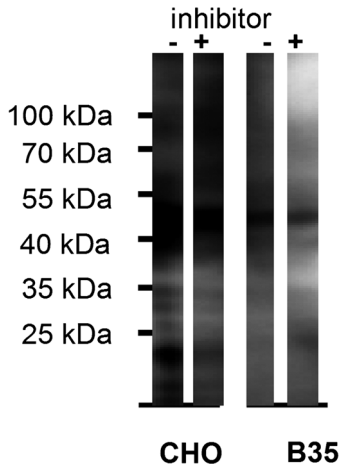


Figure 4. Lectin blot. CHO or B35 cells were grown in the absence (−) or presence (+) of **1-G-*m*** (50 μM) for 24 h. Cells were harvested and homogenized in RIPA buffer. Equal amounts (50 μg) of cell lysate in each lane were subjected to SDS-PAGE. Proteins were blotted and sialylated proteins were detected using biotinylated limax flavus agglutinin (LFA) (1:5000) and streptavidin labeled with horseradish peroxidase (1:20000).

cell-permeable and located to compartments surrounding the cell nucleus, possibly to the trans-Golgi network. (*S*)-**1-G-*m*** was active in inhibiting protein sialylation in two cell lines. These findings generate novel scientific opportunities and research lines and pose additional questions. First of all the described cell-permeable probes will be investigated further in functional biological studies in order to scrutinize their potential as inhibitors of membrane sialylation in living cells and to study the effects of altered cellular decoration with sialic acid residues. Secondly, it will be rewarding to identify the putative membrane receptor responsible for the vesicular uptake of the probes. Finally, the probes are currently used for the screening of chemical libraries in search for small-molecule inhibitors of sialyltransferases, which could be tested in the future as an innovative therapeutic approach against cancer cells.[27]

Received: January 14, 2014
Published online: April 15, 2014

Keywords: fluorescence polarization · metastasis · neuraminic acid · protein-binding probes · sialyltransferases

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