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Glycosyldisulfides from dynamic combinatorial libraries as *O*-glycoside mimetics for plant and endogenous lectins: Their reactivities in solid-phase and cell assays and conformational analysis by molecular dynamics simulations

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Abstract—Dynamic combinatorial library design exploiting the thiol-disulfide exchange readily affords access to glycosyldisulfides. In order to reveal lectin-binding properties of this type of non-hydrolyzable sugar derivative, libraries originating from a mixture of common building blocks of natural glycans and thiocompounds were tested against three plant agglutinins with specificity to galactose, fucose or *N*-acetylgalactosamine, respectively, in a solid-phase assay. Extent of lectin binding to matrix-immobilized neoglycoprotein presenting the cognate sugar could be reduced, and evidence for dependence on type of carbohydrate was provided by dynamic deconvolution. Glycosyldisulfides also maintained activity in assays of increased physiological relevance, that is, using native tumor cells and also adding to the test panel an endogenous lectin (galectin-3) involved in tumor spread and cardiac dysfunction. *N*-Acetylgalactosamine was pinpointed as the most important building block of libraries for the human lectin and the digalactoside as most potent compound acting on the toxic mistletoe agglutinin which is closely related to the biohazard ricin. Because this glycosyldisulfide, which even surpasses lactose in inhibitory capacity, rivals thiodigalactoside as inhibitor, their degrees of intramolecular flexibility were comparatively analyzed by computational calculations. Molecular dynamics runs with explicit consideration of water molecules revealed a conspicuously high degree of potential for shape alterations by the disulfide's three-bond system at the interglycosidic linkage. The presented evidence defines glycosyldisulfides as biologically active ligands for lectins.

1. Introduction

The growing realization that the carbohydrate part of cellular glycoconjugates is a rich source of biochemical signals relevant for the cells' communication with their environment inspires innovative medical applications.[1–7] In detail, distinct carbohydrate determinants, whose synthesis and/or spatially suitable presentation mode is under exquisite control, are recognized by endogenous receptors (lectins) or cognate sites of infectious microorganisms.[8–12] This interaction can then lead to medically relevant modulation of cell proliferation or migration, tissue invasion, cell-specific glycan uptake or to adhesion as crucial step in infection or metastasis formation.[11–13] The immediate clinical relevance of these cell responses or contacts prompts to envision the synthetic design of optimized ligands. Their availability should enable to manipulate particular recognition processes by either blocking interactions or artificially triggering post-binding signaling with the perspective of therapeutic benefit.

Enormous advances in oligosaccharide synthesis have made it possible to deliver chemically pure glycans, for examble, complex-type *N*-glycans without/with common natural substitution, for biological testing.[14–20] Nonetheless, the high-coding capacity of oligosaccharides, embodied by the involvement of different hydroxyl groups and anomeric positions in code word genera-

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tion,[21,22] poses a new dimension of problems en route to chemical glycan assembly. In order to test a large variety of oligosaccharides and to add new glycomimetic or otherwise reactive compounds, which offer pharmaceutical advantages such as formation of non-hydrolyzable bonds, library approaches have been introduced to this field.[23–29] Following the establishment of library generation by parallel synthesis the alternative to prepare dynamic combinatorial libraries with rapid interconversion between their constituents and the possibility for an adaptive selection process during screening has opened a different route in the mentioned long-term quest.[28,30] Initial experiments into this direction with *N*-acetyl-D-galactosamine (GalNAc) chelated glycoclusters, disulfide-spacered *p*-aminophenyl glycosides, and carbohydrate aldehydes/amines together with hydrazides/aldehydes have revealed proper randomization to yield the desired panoramic display of constituents.[31–34] A plant lectin and lysozyme were test substances at this stage. The ease to control dynamic interconversions, which are driven by thiol–disulfide interchange, by pH shifts is directing attention to thiosaccharides as building blocks of dynamic combinatorial libraries. After all, *S*-glycosides such as thiodigalactoside or *N*,*N*′-diacetyl-4-thiochitobiose indeed appear to be accommodated by lectins binding *O*-galactosides or oligomers of *N*-acetylglucosamine.[35–39] Evidently, the non-hydrolyzable *S*-glycosides can thus maintain binding properties despite the increased length of the C–S bond (1.78 Å vs 1.41 Å) and its geometry differing from that of the C–O bond (98° for C–S–C vs 117° for C–O–C).[40] In consequence, they are becoming valuable additions to the panel of compounds tested for blocking glycosidase or lectin activities.[41,42] But it is an entirely open question whether this will also be true for glycosyldisulfides.

Glycosyldisulfides constitute a new class of carbohydrate derivatives with interesting chemical and physical properties.[43–45] Their generation from thioglycosides via thiol-disulfide exchange, also operative in formation of disulfide-linked glycopeptides,[46] has recently been reported,[47] revealing them to be suitable building blocks for dynamic combinatorial chemistry. With the principle of the set-up having been established, the next step is to test the hypothesis whether this library type can come up with potent lectin-binding compounds. This report thus addresses the issue whether libraries of glycosyldisulfides can successfully be screened against different lectins. To set an example with impact beyond a single case, four main building blocks from natural glycans (Fig. 1) and several lectins were selected. Among them, a biohazard-

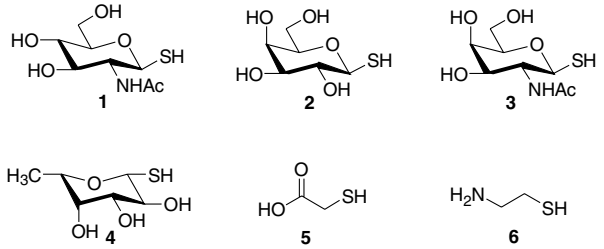


Figure 1. Thiol-containing components for generating dynamic combinatorial libraries.

Table 1. Composition of the tested dynamic combinatorial libraries and glycosyldisulfides[a]

Library	Components
L-01	Complete library
L-02	Without **4**
L-03	Without **1**
L-04	Without **2**
L-05	Without **3**
L-06	Without **5**
L-07	Without **6**
L-08	**2**–**2**
L-09	**2**, **4**
L-10	**2**, **1**
L-11	**2**, **3**
L-12	**2**, **5**
L-13	**2**, **6**

[a] For compound listing, please see Figure 1; 2–2 thus refers to the digalactosyldisulfide.

ous plant toxin and a clinically relevant member of the family of human galectins (galectin-3), which is a factor in tumor progression and cardiac dysfunction, are emblematic of the medical perspective.[48–51] Equally important, the inhibitory potency of the compound mixtures was examined: (i) in two test systems, deliberately placing special emphasis on in vitro assays with tumor cells, and (ii) after the first step of systematically decreasing the number of constituents. Table 1 gives a precise account on library composition to enable assessment of efficiency of dynamic deconvolution. In detail, the following questions will be answered by the experiments:

(a) Will glycosyldisulfides, where the interglycosidic oxygen atom is replaced by a disulfide bridge, be specific binding partners for plant lectins such as the toxin from *Viscum album* using a solid-phase system with neoglycoproteins which present the respective cognate saccharide?
(b) Will glycosyldisulfides interfere with lectin binding in an assay system of increased physiological relevance, that is, using cells in culture instead of a plastic surface coated with a neoglycoprotein and also testing a tissue lectin?
(c) Will the dynamic deconvolution strategy and disulfide synthesis come up with compounds whose activity can rival that of the common *O*-glycoside?
(d) Will a glycosyldisulfide, if shown to be a ligand as pure test substance, differ in conformational properties from the corresponding *S*-glycoside which has ligand properties?

2. Results

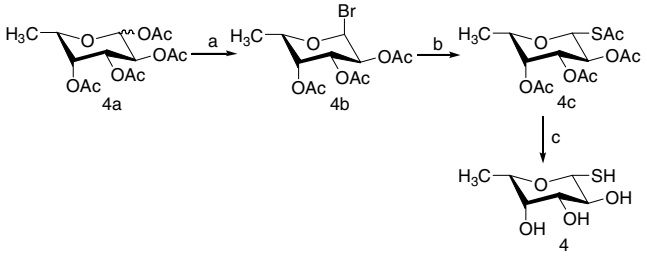
2.1. Library design

The monosaccharides D-galactose (Gal), GalNAc, and L-fucose (Fuc) are often spatially accessible headgroups of glycan antennae and branches, *N*-acetyl-D-glucosamine (GlcNAc) commonly serving as natural acceptor of β1-linked epitopes (Gal or GalNAc) of fully

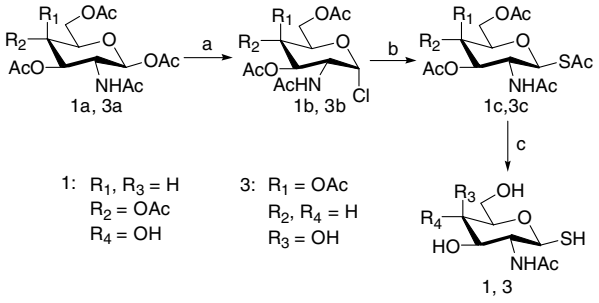
processed chains. In the branch-end position, these sugars are contact points for lectins so that their 1-thio derivatives were selected for the panel (Fig. 1). However, it should be noted that 1,1-linked disulfides will be formed instead of the β1,3(4)-linkages common to Gal-GlcNAc (LacNAc isomers)/GalNAc–GlcNAc (LacdiNAc) disaccharides or the α1,2-linkage common to the histo-blood-group H-type Gal-Fuc disaccharide.

The synthesis of the *S*-fucoside was routed via the bromoacetyl derivative (Scheme 1), whereas the two *N*-acetylated compounds were prepared from their chloroacetyl derivatives, as outlined in Scheme 2. In detail, thioacetic acid under biphasic conditions and a phase-transfer reagent were used to obtain the peracetylated 1-thioglycosides. Deprotection with lithium hydroxide solution produced the 1-thioglycosides to start generation of the dynamic combinatorial library. Of note, the β-derivative of Fuc was included, which was readily accessible.

To probe the effect of an acidic/basic center in the vicinity of the carbohydrate determinant on lectin binding, mercaptoacetic acid and mercaptoethanolamine were added to the list of building blocks (Fig. 1). As compiled in Table 1, the complete set of compounds underlied the first library, termed L-01. To be able to pinpoint the contribution of an individual component to the activity, sublibraries in the absence of one compound, hereby reducing the pool size of products in each library from 21 to 15 (L-02–L-07), were systematically prepared. Next, as a means to spot importance of a distinct extension of the 1-thiogalactoside (compound **2** in Fig. 1) the



Scheme 1. Reagents and conditions: (a) HBr, HAc, CH_2Cl_2, rt (65%); (b) HSAc, Na_2CO_3 (aq), TBAHS, CH_2Cl_2, rt (50%); (c) i—LiOH, MeOH, H_2O, rt; ii—H^+ exchange resin (98%).



Scheme 2. Reagents and conditions: (a) HCl, Ac_2O, rt (60%); (b) HSAc, Na_2CO_3 (aq), TBAHS, CH_2Cl_2, rt (50%); (c) i—LiOH, MeOH, H_2O, rt; ii—H^+ exchange resin, (98%).

full set of disulfides with this constituent (L-08–L-13) was also synthesized. Due to the possibility of anomeric inversion the compounds were subjected to spectroscopic analysis. Evidence for such a process could only be recorded for the fucose derivative at a trace level of much less than 5%. Having herewith completed the design of the test panel from the ligand side, the lectin-binding properties were subsequently characterized in order to answer the first question given at the end of the introduction.

2.2. Solid-phase assays

Two plant lectins which target primarily a monosaccharide in glycans, that is, the galactoside-specific *Viscum album* agglutinin (VAA) and the fucoside-specific *Ulex europaeus* agglutinin (UEA-I), were first tested for binding this monosaccharide in α1,2-linkage. By adsorption of neoglyoproteins bearing lactose or Fuc moieties as *p*-isothiocyanatophenyl derivatives to the surface of microtiter plate wells, a ligand-presenting matrix with characteristics of a cell surface was established. The lectins, which can conveniently be tested in solution, bound to the matrix in a carbohydrate-dependent and saturable manner. As first control of sugar specificity in this assay system, the mannose/glucose-specific plant lectin concanavalin A failed to bind to the lactose-bearing matrix. Next, only the specific haptenic monosaccharide was shown to reduce extent of binding for the two tested lectins, enabling to proceed to systematically test the libraries L-01–L-07. The final concentration of each component in the assay was 0.4 mM, distributed over 21 or 15 disulfides, respectively, in the libraries. To comparatively illustrate the potency of the corresponding haptenic monosaccharide as internal standard, Gal and Fuc were routinely used in parallel experimental series at 0.4 mM. Further assays at 5 mM (Fuc)/50 mM (Gal) ascertained that carbohydrate-dependent interaction underlied the association of the lectins to the ligand-bearing matrix. If the glycosyldisulfides harbor reactivity, lectin binding to the matrix should be reduced.

The presence of the libraries was indeed effective to inhibit lectin binding in both cases (Fig. 2). Notably, the complete compound mixture was conspicuously more effective than the monosaccharide Fuc to decrease binding of UEA-I to the matrix. Under the given conditions, the 1-thio derivatives of the monosaccharides acting as haptenic inhibitor (Gal or Fuc) maintained activity. It endowed the library, only six from 21 disulfide-linked compounds containing either Gal or Fuc, with the property to interfere with ligand binding. To exclude any non-specific effects library generation without the key 1-thioglycosides (Gal for VAA, Fuc for UEA-I) should not produce inhibitory compounds. Consequently, the omission of each of these two crucial compounds in the first step of dynamic deconvolution should readily show up in our diagram, if the reactivity is governed by carbohydrate-dependent binding. Indeed, sublibraries L-02 (without Fuc) and L-04 (without Gal) are nearly completely devoid of inhibitory capacity (Fig. 2). In contrast, reduction of constituent complexity

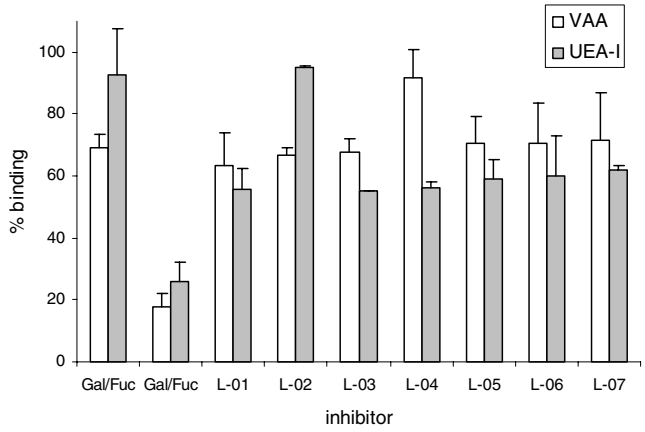


Figure 2. Illustration of inhibitory potency on extent of lectin binding (galactose-binding mistletoe (*Viscum album*) agglutinin VAA and α-L-fucoside-binding gorse (*Ulex europaeus*) agglutinin UEA-I) to surface-immobilized neoglycoproteins with lactose (VAA) or L-fucose (UEA-I) as bioactive ligands by the haptenic sugars (D-galactose and L-fucose, respectively) and libraries. Assay results with the cognate monosaccharides are given for 0.4 and 50 mM in the case of Gal as well as 0.4 and 5 mM in the case of Fuc. Compound libraries L-01–L-07 were tested at a constant concentration of 0.4 mM for each constituent to allow direct comparison.

by omission of any other compound resulted in rather small effects. These five sublibraries kept inhibitory potency now spread over 15 disulfides. There was a tendency that the absence of the two non-sugar compounds led to an extent of decrease of binding slightly lower than that for the pyranoses (Fig. 2).

To collect further evidence for lectin-binding activity of glycosyldisulfides, a third protein with different binding property, that is, the GalNAc-specific soybean agglutinin, was tested under the same conditions. Again, potent activity of the library was seen (57% inhibition by L-01 vs 22% inhibition by 0.4 mM GalNAc), and this activity was lost only in sublibrary L-05 lacking GalNAc. Evidently, glycosyldisulfides with a haptenic sugar retain lectin reactivity to impair carbohydrate-dependent binding to a matrix presenting a homogeneous ligand structure. These results encourage refinement of the assay system toward medical applicability. Because this matrix differs from cell surfaces—among other characteristics—by restricting the natural complexity of glycan display to a single type of ligand and excluding lateral glycoconjugate mobility, two salient factors to regulate lectin affinity, cells in culture were subsequently used as test objects. Explicitly, rational manipulation of the interaction between lectins and cells is what will matter for a medical perspective.

2.3. Cell-binding assays

The principle of the assay is illustrated in Figure 3. The binding properties of VAA and UEA-I were initially analyzed. Signal intensity in this assay system should depend on lectin concentration and on presence of haptenic monosaccharide. The validity of these two prerequisites is exemplarily documented in the first row of Figure 4, using cells of a B-lymphoblastoid line.

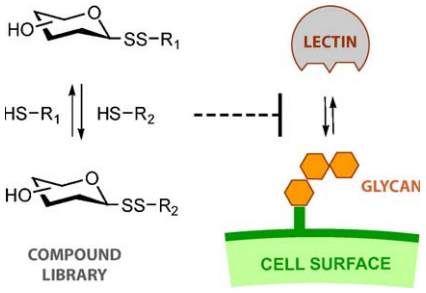


Figure 3. Schematic representation of the principle of the cell-binding assay (right part), in which components of a glycosyldisulfide-based dynamic combinatorial library (left part) are tested for interference of lectin binding to cell surface glycans. Quantitative aspects of cell binding (percentage of positive cells, median fluorescence intensity) are determined by FACScan analysis.

Presence of Gal (but not any other monosaccharide) reduced both percentage of positive cells and staining of VAA intensity. Library L-01 caused a reduction especially of the first parameter, and the assumed pivotal role of Gal was underscored by showing that its absence in library L-04 abolished inhibitory capacity (Fig. 4). These results can readily be reconciled with the data from the solid-phase measurements. To ensure that reaching this conclusion is not confined to the special case of VAA and the tested B-lymphoblastoid line, the same set of experiments on cells originating from a solid tumor, that is, a colon adenocarcinoma line, was carried out with VAA. Moreover, the libraries' impact on UEA-I binding was also quantitated. Because malignancy can be associated with increased expression of a glycosyltransferase and then increased presence of certain glycan epitopes, a new model system to determine the activity of the libraries on UEA-I binding was established. In detail, cell transformation with an expression vector carrying cDNA for α-fucosyltransferase I and selection of stable transfectants were performed to generate a carcinoma cell clone with especially strong lectin binding. The results obtained in the four systems are compiled in Figure 5, in which the first column of each panel represents the data shown as scans in Figure 4.

As a first lesson emerging from Figure 5 (left part), binding parameters were sensitive to presence of the haptenic sugar. When libraries were tested at the same concentration of 4 mM set for each individual component, extents of inhibition rivaled that of the haptenic sugar or even exceeded them in the case of the pancreatic carcinoma line Capan-1 and UEA-I (Fig. 5). Again, absence of the 1-thioglycoside of Gal or Fuc, respectively, in a sublibrary (L-04 or L-02) made its mark on the activity of the mixture. Another remarkable result concerns the only minor extent of cell-type-dependent differences in changes of staining parameters. Overall, the glycosyldisulfide mixture reached at least the potency of the haptenic inhibitor in the cell-binding studies, answering a part of the second question of the introduction. Results after the first step of dynamic deconvolution pointed to potential for improvements when the 15 disulfides lacked GlcNAc (please compare results of L-01 vs L-03), hereby answering the third question of the introduction.

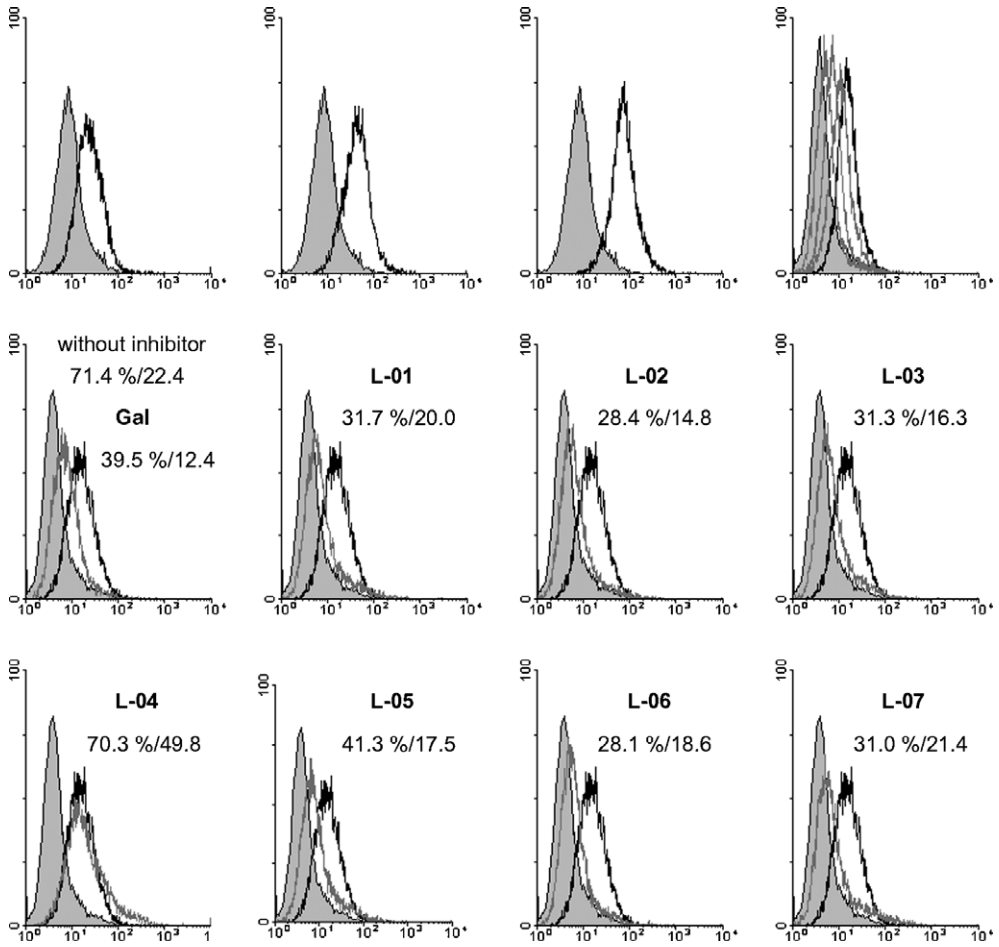


Figure 4. Semilogarithmic representation of fluorescent surface staining of cells of the human B-lymphoblastoid line Croco II in the absence of incubation with biotinylated mistletoe lectin (negative control; shaded) and after incubation with increasing concentrations of lectin (0.05, 0.1, and 0.2 μg/ml; first three scans in first panel) and with increasing concentrations of haptenic inhibitor (0.5, 8, and 32 mM Gal) at a lectin concentration of 0.05 μg/ml (fourth diagram in first panel). Scans in the second and third panels document effect of haptenic sugar and libraries tested at a concentration of 4 mM for each constituent (black line: positive control without inhibitor; gray line: measurement in the presence of test compound). Quantitative data on percentage of positive cells (%) and mean channel fluorescence are given in each case.

Having so far worked with plant lectins as model, the next step toward medical applicability was to confront the libraries with an endogenous lectin. For this purpose, a human lectin, that is, galectin-3 which had been mentioned in the introduction due to its involvement in tumor spread and cardiac dysfunction, was introduced to this assay system. Binding parameters fulfilled the same prerequisite of concentration dependence as ascertained for plant lectins (not shown), and the ensuing tests with T-lymphoblastoid cells revealed that fluorescent staining was decreased by presence of Gal/lactose, as illustrated in Figure 6. The complete library at compound concentration of 4 mM was more effective than 32 mM Gal and subject to activity decreases by withdrawing a sugar from the mixture (Fig. 6). Of note, absence of Gal, generally known as haptenic inhibitor for galectins, was rather tolerable (L-04), whereas absence of GalNAc, precluding, for example, formation of GalNAc-GlcNAc disulfides, impaired the activity more strongly (L-05) (Fig. 6). The omission of the two aliphatic thiocompounds led to mixtures which were as effective as library L-01, pointing to a lack of effect of their presence (not shown). When next working with a line from a solid tumor, similar data were obtained in the case of the ovarian adenocarcinoma cells NIH-OVCAR3. Binding of galectin-3 at 10 μg/ml reached levels of 61% positive cells and a median fluorescence of 104.2, which was lowered by Gal (4 mM) to 52%/62.6 and by L-01 to 43.8%/48.6. This level of activity was attained by 10–12 mM Gal in direct comparison to library L-01. Libraries L-04 (40.2%/46.4) and L-05 (50.0%/56.8) showed effects comparable to those of the lymphoma cell system but L-06 failed to reach the activity of library L-01 on the ovarian cancer cells (52.0%/63.1). Thus, as measured for plant lectins which are popular models, the libraries could also exert a negative impact on cell binding of an endogenous lectin. This result answers the remaining part of the second question and provides an argument for a medical perspective.

An important factor for the comparison of the relative inhibitory potencies of haptenic sugar and libraries is the inherent averaging due to the presence of 15 or 21 compounds in libraries L-02–L-07 or starting library L-01, respectively. In order to facilitate a direct comparison between a haptenic sugar and a glycosyldisulfide,

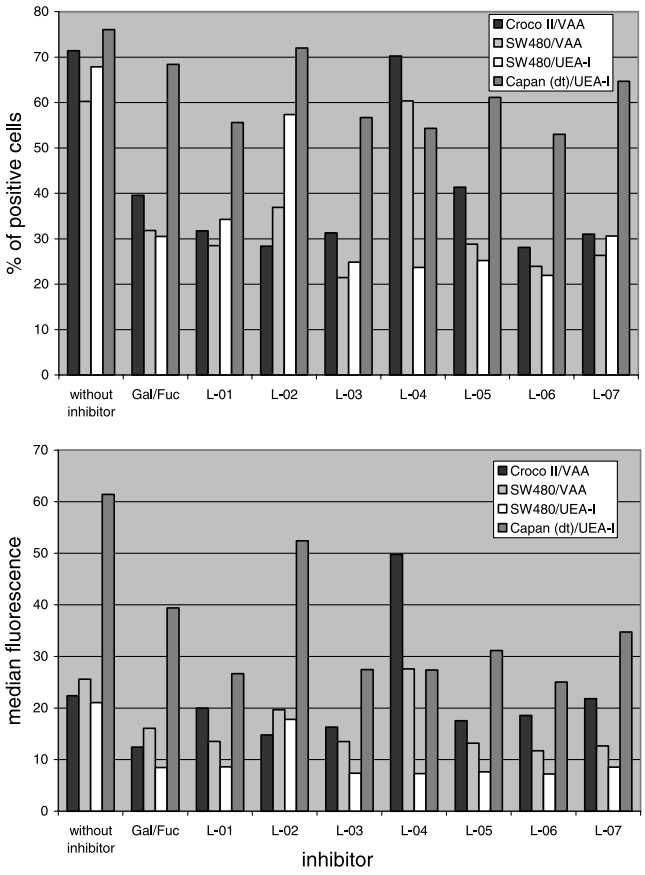


Figure 5. Comparison of the effect of the two haptenic monosaccharides (Gal or Fuc) at 4 mM and the libraries (each constituent at 4 mM) on staining parameters (upper panel: percentage of positive cells; bottom panel: median fluorescence) in flow cytofluorimetric analysis (for information on cell lines and lectin, please see inset; for illustration of scans on Croco II/VAA, please see Fig. 4).

only two compounds to initiate dynamic glycosyldisulfide formation, as listed in Table 1, were mixed, and each solution was tested at a product concentration of 4 mM. Using VAA as marker, the colon cancer line as target and the results presented in Figure 5 to give reasons for picking these two compounds, 1-thiogalactoside was selected as constant constituent. Each of the six thiocompounds was added in separate batches. The question to be answered was whether and to what extent the six different thiocompounds caused enhancement in inhibitory capacity of the galactoside-containing disulfide. Figure 7 shows that this was indeed the case. In fact, the prepared digalactoside even surpassed lactose in inhibitory capacity, whereas presence of Fuc, GalNAc or GlcNAc in the disaccharide had a comparatively small effect. The type of substitution of the Gal headgroup in the disulfide could evidently exert an influence on lectin binding, constituting a positive answer to the third question. The Gal-containing disulfides can thus act as binding partners for this lectin. Among them, the digalactoside was clearly superior to the other disulfides evoking to draw a parallel to thiodigalactoside. Structurally, our disulfide differs from this known ligand sharing the 1,1-linkage by turning the *S*-glycosidic bond into a disulfide linkage. Because a ligand's shape and conformational dynamics are key factors for their affinity,[52,53] the results obtained so far consequently prompted us to employ computational methods to comparatively look at the molecular dynamics behavior of this disulfide and thiodigalactoside.

2.4. Molecular dynamics calculations

The type of the interglycosidic linkage will account for the relative positioning of the hexopyranose rings of both Gal units. Thiodigalactoside and the corresponding disulfide differ with respect to the number of bonds

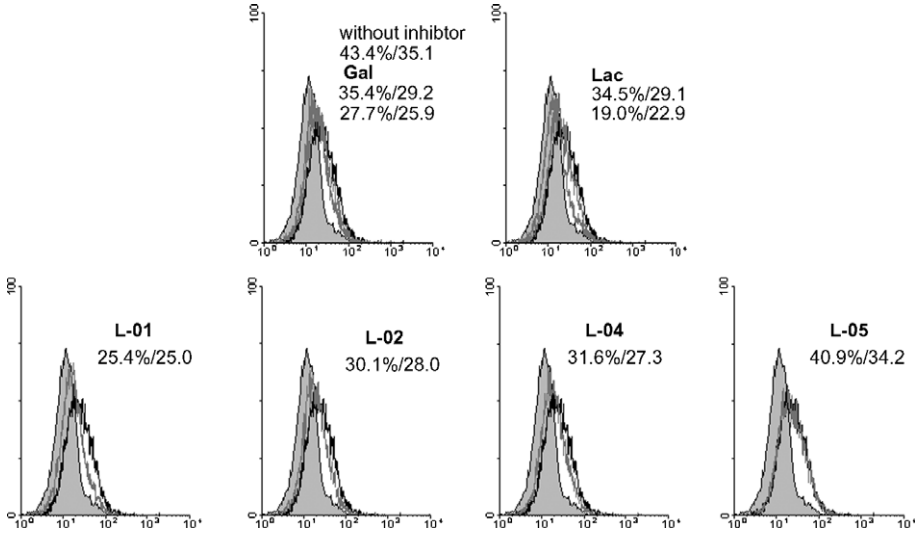


Figure 6. Semilogarithmic representation of fluorescent surface staining of cells of the human T-lymphoblastoid cell line CCRF-CEM in the absence of labeled lectin (negative control, shaded) and in the presence of 10 μg human galectin-3/ml (solid line), next using 4 and 32 mM Gal or lactose (gray lines) as haptenic inhibitors with increasing efficiency (top panel) as well as four libraries with the constituent concentration set to 4 mM. Quantitative data on control value (top panel, left side), effects of haptenic inhibitors and libraries (center and bottom panels) are given in the illustrations (for library assignment, please see Table 1).

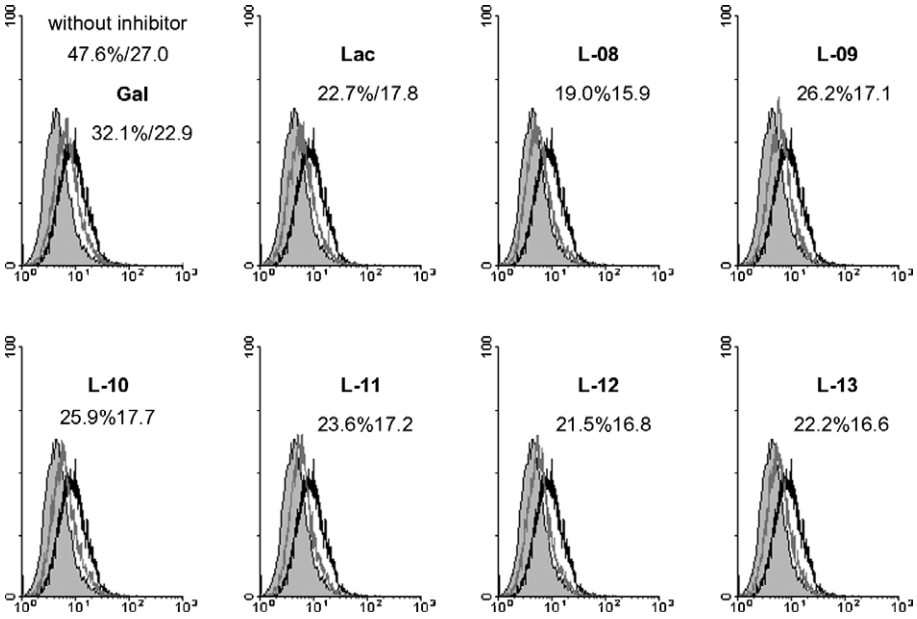


Figure 7. Semilogarithmic representation of fluorescent surface staining of cells of the human colon adenocarcinoma line SW480 in the absence of labeled lectin (negative control, shaded) and in presence of 2 μg VAA/ml (solid line), next using 4 mM Gal or lactose as haptenic inhibitors and defined 1-thiogalactoside-containing disulfides (for compound listing, please see Table 1) at the same concentration (gray line). Quantitative data on control value and effects of inhibitors are given in the illustrations.

(Fig. 8). The conformational space of the two disaccharides is established by rotations around the two torsion angles Φ and Ψ for thiodigalactoside, whereas the disulfide has three degrees of rotational freedom with Φ, Ψ, and Ω angles (for definitions, please see Fig. 8). To depict the individual features how the two disaccharides populate the conformational space, molecular dynamics calculations were performed for total simulation periods of 10 ns at 300 K, starting with fixed values of the dielectric constant at $\varepsilon = 4$ and $\varepsilon = 80$, the latter one to account for properties in water. Because the Ω-angle of the disulfide appeared to be arrested around values of about 180° due to an interresidual hydrogen bond between Gal-OH_2 and Gal'-OH_6 groups, the relevance of this result was put to the test by running the calculations with explicit consideration of water molecules. The resulting trajectories revealed an increased level of intramolecular flexibility without the noted restraint so that we continued to routinely perform the calculations of the comparative analysis under this condition. The obtained set of trajectories for each angle reveals that the Φ, Ψ-angles of thiodigalactoside rapidly fluctuate between two main areas without accessing other portions of the conformational space (Fig. 8, left part). In contrast, the Φ, Ω-angles of the disulfide allow a comparatively higher degree of flexibility, the Ψ-angle being limited to fluctuations around −80° to −120° (Fig. 8, right part). Snapshots from the populated area sections, presented in the bottom part of Figure 8, give an idea on the way the different angle combinations translate into shape. For the convenience of the reader, one galactose moiety was always kept in a constant position to enable comparison by visual inspection readily. Regarding intramolecular distance parameters of the flexible compounds the two protons at the anomeric centers are separated by 2.13–4.14 Å in thiodigalactoside and 2.75–5.18 Å in the disulfide. In summary, the three-bond interglycosidic linkage engenders a comparatively high level of flexibility, what answers the last question posed in the introduction. This variability accounts for rather broad positional screening during contact with a lectin to reach an optimal fit of binding without necessity of distortions from nearly rigid low-energy positions. Examples from the rather large conformer population of the disulfide are depicted in Figure 8 (bottom part). The noted potential to eventually reach an optimal fit necessarily comes at the expense of an entropic penalty, if the ligand is arrested in one of the many low-energy conformers.

3. Discussion

The reversible thiol-disulfide conversion affords access to dynamic combinatorial carbohydrate libraries using 1-thioglycosides as building blocks. After having documented the chemical feasibility of this approach,[43–45] it is next essential to prove that products originating from this type of library design have a perspective as binding partners for lectins. Toward this end, three plant lectins with different monosaccharide specificity were deliberately selected as study objects. First, a library comprising four main constituents of natural glycans was used in a solid-phase assay as inhibitor of lectin binding. In contrast to chip technology both lectin and the test compound are in solution. Any potentially troublesome surface effects are excluded. Inhibitory potency was indeed recorded, and it was at least similar to the haptenic monosaccharide in each case. When starting library formation with the β-derivative of fucose, the interaction of UEA-I, which binds α1,2-linked fucosides in natural glycans,[54,55] with the α-fucoside-bearing neoglycoprotein

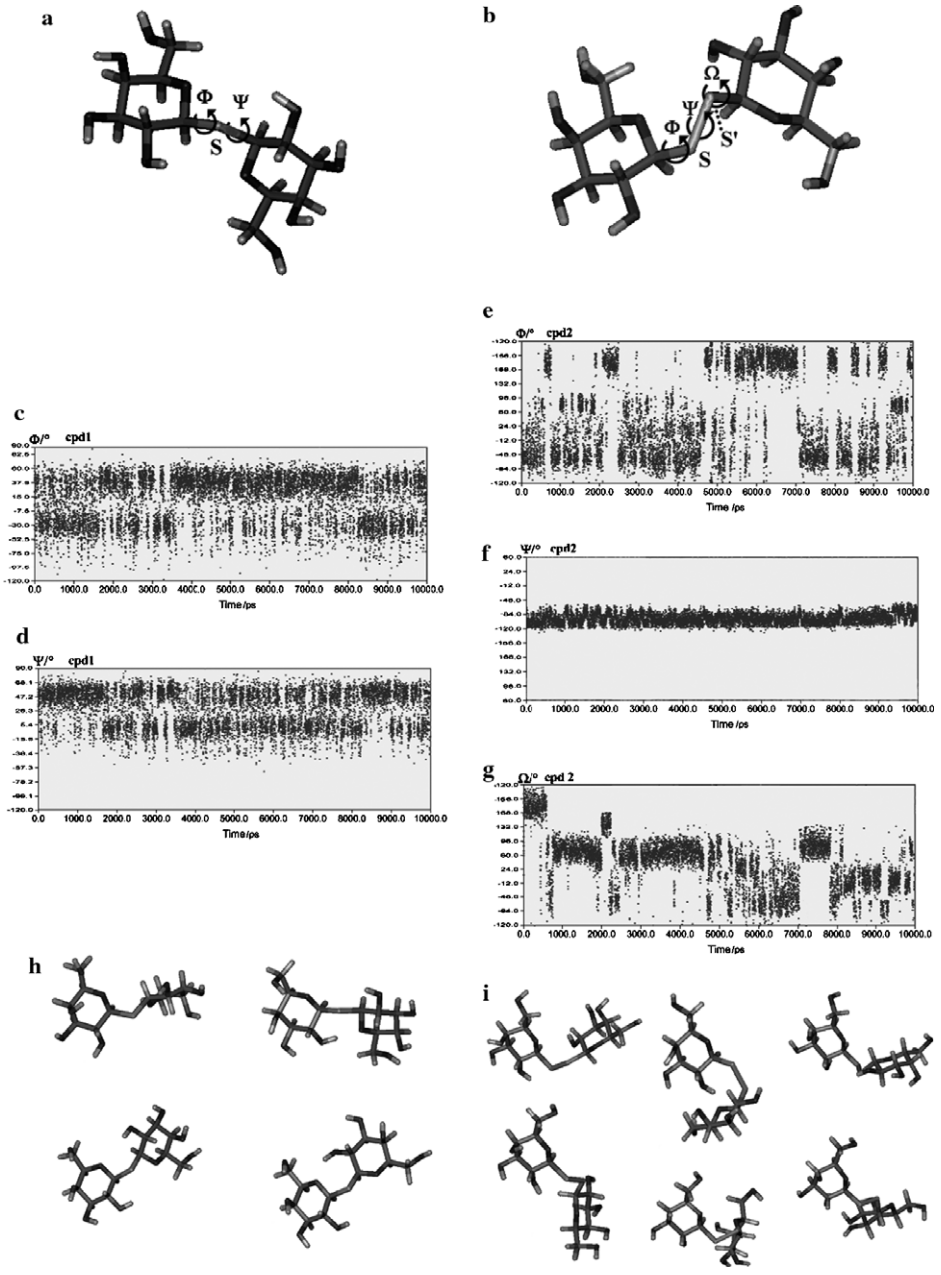


Figure 8. Illustration of conformational aspects of thiodigalactoside (left, compound **1**) and β1,1-thio-based digalactosyldisulfide (right, compound **2**). The angles of the *S*-glycosidic linkages are defined as H1–C1–S–C1 (*Φ*) and C1–S–C1–H1 (*Ψ*) for the symmetrical thiodigalactoside as well as H1–C1–S–S′ (*Φ*), C1–S–S′–C1 (*Ψ*), and S–S′–C1–H1 (*Ω*) for the digalactosyldisulfide (top panel). Trajectories from MD runs (300 K, 10 ns) with explicit consideration of water molecules are shown in the center part for the *Φ*, *Ψ*-angles of compound **1** (left) and the *Φ*, *Ψ*, *Ω*-angles of compound **2** (right). They describe the conformational space accessible for the two compounds, as further illustrated in the bottom part by drawing representative conformers for which the angle combinations given as follows occur during the simulations: *Φ*/*Ψ* 39°/51°, −22°/53°, 38°/15°, and 43°/−42° (from top left to bottom right) and *Φ*/*Ψ*/*Ω* 104°/−91°/−70°, −65°/−99°/−167°, 157°/−93°/78°, −9°/−88°/48°, 37°/−88°/140° and, 11°/−103°/−65° (from top left to bottom right). The position of one galactose moiety is deliberately kept constant throughout the given snapshots to let positional changes become easily apparent.

was also affected. This reactivity intimates inversion to the α-anomer in the course of disulfide formation.[56] Its occurrence was yet restricted to much less than 5% for 1-thio-β-L-fucopyranoside and could not at all be detected for any other thiosaccharide. Another possibility concerns tolerance of the β-anomer in the disulfide for binding. Such a case of tolerance for anomeric positioning in fucose derivatives presented as ligand part of neoglyoproteins had been reported for the rat Kupffer cell receptor.[57] In our assays, binding specificity was invariably underscored by the impact of omission of the key 1-thioglycoside from the starting set to generate a library in each tested case. Because the three plant lectins home in on a monosaccharide for primary contact,[54,55,58–60] it is not surprising to see no drastic enhancement in binding activity by adding a second sugar unit. However, additions of β1,2(3)-linked Gal units to a Gal moiety to form *O*-glycosidic digalactosides had produced potent blocking reagents of VAA binding.[61–63] In full accord with these data our respective tests with disaccharides revealed a remarkable activity of the disulfide-linked digalactoside standing out from the tested

panel. These results were obtained in cell-binding assays. They simulate the clinical situation in which an inhibitor should preclude association of the toxic lectin to cells. Also in view of the clinical situation, a cell clone was engineered to assess efficiency of impairing lectin binding to tumor cells with increased expression of α-fucosyltransferase I. This overexpression appears to contribute to benign and malignant prostate growth.[64] In this cell system, already the complete library L-01 proved to be more effective than the free monosaccharide.

With the long-term aim of medical application in mind, we not only worked with human tumor cells but also moved from assays with plant lectins to experiments with a human effector. Obviously, the libraries contained compounds which reduced carbohydrate-dependent cell binding of the tested human lectin from the family of adhesion/growth-regulating galectins. Our observation that assays with galectin-3 tolerated omission of Gal but were very sensitive to maintaining the presence of GalNAc was at first sight puzzling in view of the assumed main affinity of galectins to Gal.[35–37] However, galectin-3 has the particular ability to accommodate the GalNAcβ1,4GlcNAc (LacdiNAc) epitope, which is abundantly found in invertebrates.[65] In fact, galectin-3 is a major macrophage receptor for this epitope mediating host-parasite interactions, for instance, in the course of schistosomiasis.[65] Overall, these results teach the important lesson that this library design can come up with active compounds interfering with ligand binding of plant and human lectins. Owing to the step taken from solid-phase to cell-binding assays the physiological relevance of the data has increased. Both lectin and inhibitor could be kept in solution, and the lectin is confronted with full array of cellular glycoconjugates without any restrictions to their lateral mobility. Importantly, cellular responses to a galectin critically depend on the lectins' quaternary structure and capacity to induce ligand clustering which cannot be mimicked in a solid-phase assay.[66–68] Thus, the first three questions of the introduction could be answered positively, documenting the potential of this library design for defining a new class of binding partners of lectins. As noted above, the disulfide-linked digalactoside is such a case which passed our tests. Evidently, binding activity is not lost when moving from the *S*-glycoside thiodigalactoside, a known lectin ligand,[35–38] to the corresponding disulfide.

This result has prompted to examine the conformational properties of this disulfide more closely. The presence of a three-bond linkage system has led us to expect an analogy to the case of the two α2,3/6-linked sialogalactosides. Here, the α2,6-linkage confers a high level of flexibility to the respective disaccharide.[69] In this respect, *S*-glycosides are known to be endowed with increased flexibility compared to their *O*-glycoside, enabling them to access *anti*-conformations more easily, and particular stereoelectronic properties, facilitating interactions with receptor proteins distinct from those of the *O*-glycoside.[40,70–73] An interesting example concerns the mentioned thiodigalactoside, which binds to the galectin from *Buffo arenarum* in a manner different from *N*-acetyllactosamine (LacNAc). In detail, while the Gal-dependent contacts are conserved, the second moiety employs either the C-3 or C-2 oxygen atom for hydrogen bond formation, resulting in the same number and quality of hydrogen bonds (PDB1A78), as predicted before based on structural analogy considerations.[74,75] Notably, the measured Φ, Ψ angle pairs of 59°/13°[75] and 57°/19° for bound thiodigalactoside in the crystal correspond to a populated area in the conformational space of the free *S*-glycoside (Fig. 8, center part, left). The snapshot of the conformer with Φ, Ψ angles of 38°/15° taken during the molecular dynamics simulation illustrates its shape (Fig. 8, bottom part, left). Looking at thermodynamic binding data for VAA and galectins with lactose and thiodigalactoside more closely, there is a general trend for an increased entropic penalty which yet is more than compensated by the enthalpic gain to eventually yield an elevated affinity for the *S*-glycoside.[38,74,76,77] The lack of precise knowledge on the contributions of the sulfur atom(s) and/or the role of water restructuring notwithstanding, increased flexibility may thus not necessarily be a disadvantage. This issue is similarly discussed for the two/three-bond systems of α2,3/6-linked sialylgalactose and the four-bond system of two α2,8-linked sialic acid moieties.[69,78]

Looking at flexibility, it is intriguing that different strategies toward high affinity can be followed by a lectin. The case study on VAA delineated that it either can allow binding partners to maintain flexibility at the subterminal position or freeze this degree of conformational freedom depending of the nature on the disaccharide.[79–81] It is an open question, which option is realized in our case, giving future research a clear direction. With respect to the results of our computations it is noteworthy that experimental evidence on a fully protected β,β-1,1′-dithiodisaccharide with glucose and mannose as constituents supports the given conclusion. In detail, signals of nuclear overhauser enhancements between H2 of mannose and H2, H4, and H6a of glucose are indicative of a Ψ-angle of about −80° to −90°.[44] This result is in full accordance with the trajectory reflecting dynamic aspects of the Ψ-angle given in Figure 8 (right side). The way such a flexible ligand is accommodated in lectin domains and the actual contact sites between lectin and ligand, valuable input for further synthetic refinements, will have to be determined by a strategy combining different spectroscopic techniques recently tested for a complex glycan and a galectin in solution.[82] The demonstration of affinity of a 1,1-linked disaccharide for a member of another mammalian lectin family opens the perspective for testing disulfides also against selectins, mediators of inflammation and carcinoma adhesion.[9,12,83] In principle, the inferred high degree of flexibility makes the study of glycosyldisulfide binding to lectins an attractive model to analyze the relationship between intramolecular flexibility and free enthalpy.

Having shown the suitability of this library design and the resulting disulfides for the purpose to open a new approach toward hitherto unsuspected lectin ligands,

the following route will be taken to pursue this line of research. In detail, the recently reported access to thioglycosides other than the 1-thioglycosides tested herein, that is, 3′- and 4′-thioglycosides,[84] will team up with this experimental set-up to monitor impact of extensions especially at the 2′- and 3′-sites, following the example of the potent ABH histo-blood-group epitopes.[36,77,85–88] Besides glycosides either a sulfate group, as recognized in sulfatides with long-chain 2′-hydroxylated fatty acids by galectin-4,[89] or non-natural compounds promising improved binding properties can likewise be instrumental, for instance by 3′-derivatization,[90] in the quest to define high-affinity and selective compounds. The example of selectin blockers with a branched alkyl group as additive, an anchor-like homing device to hydrophobic lectin sections in the vicinity of the carbohydrate recognition domain[91,92] and the evidence for presence of a farnesyl-binding pocket of galectin-1, a factor in colon cancer progression and glioblastoma invasion,[93–97] makes it plausible to consider incorporation of such a functionality into library design. Obviously, our demonstration of glycosyldisulfide activity puts the underlying approach firmly on track toward a systematic variation at different positions with different compounds. Ensuing combination of headgroup optimization with an appropriate multivalent presentation, which leads to contribute to increases in affinity and selectivity for galectins,[98–102] can be expected to improve the efficiency of test compounds. In this sense, the given experimental evidence for glycosyldisulfides to serve as inhibitors in solid-phase and cell assays with plant and human lectins establishes this compound class as new substance platform for lectin-directed drug design.

4. Experimental

4.1. Materials and analytical procedures

All reagents for synthesis and compound **2** (from Senn Chemicals) as well as compounds **5** and **6** (from Aldrich) listed in Figure 1 were obtained from commercial sources. Compounds **1**, **3**, and **4b** were synthesized according to Schemes 1 and 2, and corresponded to literature data.[103–109] Neoglycoproteins used as matrix in solid-phase assays were prepared from *p*-aminophenyl glycosides and carbohydrate-free bovine serum albumin as carrier as described.[110] The lectin from *Canavalia ensiformis* seeds, the lectin from dried leaves of mistletoe, and human galectin-3, whose source was recombinant production, were purified from extracts by affinity chromatography on lactosylated or mannosylated Sepharose 4B, obtained after divinyl sulfone activation, checked for purity, quaternary structure, and activity by one- and two-dimensional gel electrophoresis, gel filtration, ultracentrifugation, and hemagglutination, and biotinylated under activity-preserving conditions with biotinyl-*N*-hydroxysuccinimide ester (Sigma, Munich, Germany) using a proteomics protocol for quantitation of biotin incorporation.[111–115] Biotinylated SBA and UEA-I were obtained from Vector Labs (distributed by Alexis Germany, Grünberg, Germany) and checked for carbohydrate-dependent activity by solid-phase assays and histochemical application.[116] Chemical reactions were monitored with thin-layer chromatography using precoated silica gel 60 (0.25 mm thickness) plates (Macherey-Nagel). Flash chromatography was performed on silica gel 60 (SDS 0.040–0.063 mm). Optical rotations were measured with a Perkin-Elmer 343 polarimeter at the sodium D line and ambient temperature. ^{1}H- and ^{13}C-spectra were recorded with Bruker Avance 400 or a Bruker DMX 500 instruments at 298 K in $CDCl_3$ or D_2O, using the residual signals from $CHCl_3$ (^{1}H: $\delta = 7.25$ ppm; ^{13}C: $\delta = 77.0$ ppm) and from H_2O (^{1}H: $\delta = 4.70$ ppm) as internal standard. Elemental analysis was performed by Analytische Laboratorien, Lindlar (Germany). HRMS was carried out by Instrumentstationen, Kemicentrum, Lund University (Sweden). ^{1}H-Assignments were made by first-order analysis of the spectra as well as ^{1}H–^{1}H correlation maps (COSY). ^{13}C-Assignments were based on ^{1}H-^{13}C correlation maps (HMQC).

4.2. Typical procedure of library preparation

All libraries were prepared by mixing the chosen components (5 mM each) in neutral phosphate buffer (300 mM for libraries L-01–L-07 and 100 mM for libraries L-08–L-13) and adding hydrogen peroxide (0.75 equiv) in aliquots over a period of 24 h.

4.3. 2,3,4,-Tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-L-fucopyranose (4c)

Compound **4b** (0.23 g, 0.65 mmol) was dissolved in dry dichloromethane (10 ml), and thioacetic acid (0.099 g, 1.3 mmol) and TBAHS (0.44 g, 1.3 mmol) in 1 M Na_2CO_3 (10 ml) were added. The reaction mixture was stirred vigorously for 1 h at rt and subsequently extracted with dichloromethane. The combined organic phase was washed with saturated $NaHCO_3$, water and brine, dried, and filtered. The crude product was purified by flash chromatography on silica gel (eluent: hexane/EtOAc 6:4) to give product **4c** (yield 51%). ^{1}H NMR ($CDCl_3$, 400 MHz) 5.25–5.32 (m, 2H, H-2, H-4), 5.22 (d, 1H, $J_{1,2} = 10.4$ Hz, H-1), 5.09 (dd, 1H, $J_{3,2} = 9.8$ Hz, $J_{3,4} = 3.5$ Hz, H-3), 3.95 (bq, 1H, $J_{5,CH_3} = 6.4$ Hz, H-5), 2.37 (s, 3H, SAc), 2.16, 2.01, 1.97 (9H, 3× OAc), 1.18 (d, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) $\delta = 192.5$ (SAc), 170.6, 170.0, 169.6 (3× OAc), 80.3 (C-1), 73.8, 72.4, 70.4, 66.5 (C-2, C-3, C-4, C-5), 31.0 (SAc), 20.8, 20.7, 20.6 (3× OAc), 16.3 CH_3; $[\alpha]_D^{22}$ −18.2 (*c* 0.5 in $CHCl_3$); HRMS: calcd for $C_{14}H_{20}O_8S$ [M+Na$^+$]: 371.0778; found: 371.0778.

4.4. 1-Thio-β-L-fucopyranose (4)

Compound **4c** (0.174 g, 0.50 mmol) was dissolved in methanol (3 ml) at 0 °C, and lithium hydroxide (0.024 g, 1.0 mmol) in water (3 ml) was added dropwise at room temperature under nitrogen protection. The resulting mixture was neutralized by acidic exchange resin, concentrated, and dried to give product **4** (Yield, 98%). ^{1}H NMR (D_2O, 400 MHz) $\delta = 4.36$ (d, 1H,

$J_{1,2}$ = 9.6 Hz, H-1), 3.65–3.74 (m, 2H, H_4, H_5), 3.50 (dd, 1H, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 3.4 Hz, H_3), 3.32 (t, 1H, H_2), 1.12 (d, 3H, J_{5,CH_3} = 6.6 Hz, CH_3); $[\alpha]_D^{22}$ + 38.8 (*c* 0.5 in MeOH); HRMS: calcd for its dimer $C_{12}H_{22}O_8S_2$ [M+Na^+]: 381.0654; found: 381.0651.

4.5. Solid-phase assay

Matrix establishment was carried out by adsorption of neoglycoproteins bearing the *p*-isothiocyanato derivatives of lactose, α-Fuc, and α-GalNAc as ligand part to the surface of plastic microtiter plate wells from solutions with a concentration of 0.5 μg/ml and routinely checked for quality, concentrations of labeled lectins at 2 μg VAA/ml, 0.5 μg/ml UEA-I, and concanavalin A as well as 1.2 μg SBA/ml were routinely used in the assays using 20 mM phosphate-buffered saline (pH 7.2), and the extent of specific binding was quantitated using streptavidin–peroxidase conjugate as sensor and *o*-phenylenediamine/H_2O_2 as chromogenic substrates (β-galactosidase and chlorophenolred-β-D-galactopyranoside in the case of concanavalin A due to its reactivity with the high-mannose-type *N*-glycan of the horseradish peroxidase) as described.[117,118] Inhibitors were added in solution, and influence of buffer was excluded by mock controls without thiocompounds.

4.6. Cell-binding assay

Cell culture of the human B-lymphoblastoid line Croco II, the T-lymphoblastoid line CCRF-CEM, the ovarian carcinoma line NIH-OVCAR3, and the colon adenocarcinoma line SW480 followed routine conditions recommended by the supplier (American Type Culture Collection, Rockville, MD) or the literature.[119] The human pancreatic carcinoma line Capan-1 with reconstituted expression of the tumor suppressor p16 was kindly provided by Dr. K. M. Detjen (Berlin, Germany). Using the cDNA for human α1,2-fucosyltransferase I, kindly provided by Dr. J. B. Lowe (Ann Arbor, MI), stable transfectants with increased cell surface α1,2-fucosylation were obtained after transfection with the pcDNA3.1 vector conferring hygromycin resistance (Invitrogen/Life Technologies, Karlsruhe, Germany) and selection by cell staining using labeled UEA-I as described.[120] Cell binding was performed with biotinylated lectins in Dulbecco's phosphate-buffered saline for 30 min at 4 °C, thorough washing preceded quantitative fluorescent detection of cell-associated markers in a FACScan instrument (Becton–Dickinson, Heidelberg, Germany) using streptavidin/*R*-phycoerythrin (1:40; Sigma, Munich, Germany) as indicator as described.[121,122] A series of probe concentrations was systematically tested first to define optimal conditions for comparative analysis and carbohydrate-dependent binding was ascertained thereafter. The binding parameters of percentage of positive cells and median fluorescence were computed by the instrument's software.

4.7. Molecular dynamics calculations

Computational simulations with implicit consideration of water were carried out for a period of 10 ns at 300 K with two values of the dielectric constant at ε = 4 and 80. The calculations were run following the web-based protocol accessible at http://www.md-simulations.de with a Linux cluster of 18 processors, the parametrization of MM3 force field, and the TINKER program package. The explicit consideration of water molecules was independently included for molecular dynamics runs of 10 ns at 300 K using the AMBER (assisted model building with energy refinement) 1.6 force field as implemented in the program DISCOVER 2.98 (Accelrys Inc., San Diego, CA) and atomic charge assignment of INSIGHT II. Lack of major influence of the type of force field on the conclusions has been verified in a related system previously.[123] Graphical illustrations of the shape of thiodigalactoside and the related disulfide were generated using the VMD (visual molecular dynamics) protocol.[124]

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