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NMR-based exploration of the acceptor binding site of human blood group B galactosyltransferase with molecular fragments

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Abstract A substantial body of work has been devoted to the design and synthesis of glycosyltransferase inhibitors. A major obstacle has always been the demanding chemistry. Therefore, only few potent and selective inhibitors are known to date. Glycosyltransferases possess two distinct binding sites, one for the donor substrate, and one for the acceptor substrate. In many cases binding to the donor site is well defined but data for acceptor binding is sparse. In particular, acceptor binding sites are often shallow, and in many cases the dimensions of the binding pocket are not well defined. One approach to glycosyltransferase inhibitors is to chemically link donor site and acceptor site ligands to generate high affinity binders. Here, we describe a novel approach to identify acceptor site ligands from a fragment library. We have chosen human blood group B galactosyltransferase (GTB) as a biologically important model target. The approach utilizes a combination of STD NMR, spin-lock filtered NMR experiments and surface plasmon resonance measurements. Following this route we have identified molecular fragments from a fragment library that bind to the acceptor site of GTB with affinities of the order of a natural acceptor substrate. Unlike natural substrates these fragments allow for straightforward chemical modifications and, therefore will serve as scaffolds for potent GTB inhibitors. In general, the approach described is applicable to any glycosyltransferase and may assist in the development of novel glycosyltransferase inhibitors.

Keywords STD NMR · Spin-lock filter · Glycosyltransferase · Inhibitor

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This paper is dedicated to Prof. Klaus Bock on the occasion of his 65th birthday.

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Glycosyltransferases control the glycosylation of proteins, lipids, nucleic acids and natural products. It is well documented that specific glycosylation patterns on cell surfaces are essential for a variety of biological functions [1]. A number of pathological conditions are associated with the malfunction of glycosyltransferases. For instance, there is strong evidence that cancer metastasis is linked to an elevated level of expression of sialyl Lewis^x epitopes [2–4]. Clearly, inhibition of specific glycosyltransferases would have a potential for novel therapeutic approaches [5–7]. For basic research such inhibitors would be interesting tools to study the effects of glycosyltransferase inhibition in cell culture or in animals. Moreover, industrial production of glycoproteins for therapeutic purposes utilizing glycosyltransferase inhibitors to restrict the glycosylation pattern [8] is another example for the high impact such compounds will have. Therefore, the development of glycosyltransferase inhibitors is an important goal. Among various synthetic efforts the synthesis of transition state

analogues has been promising [9–11]. Yet, therapeutically suitable inhibitors are not in sight. Since glycosyltransferases are two-substrate enzymes that transfer monosaccharide moieties from donor substrates to specific acceptor substrates the development of inhibitors may be based on molecules that bind either to the donor site, or to the acceptor site, or to both sites simultaneously [6, 12, 13]. A third possibility would be to target allosteric sites to inhibit conformational transitions such as loop movements that are required for catalysis. Cross-linking any of these ligands offers the possibility to enhance affinity and specificity.

Glycosyltransferases that transfer a specific monosaccharide utilize the same donor substrate. For example, all galactosyltransferases use UDP-Gal as donor substrate. Therefore, inhibitors based on ligands that bind to the donor site are unlikely to furnish specific inhibitors. On the other hand, most glycosyltransferases are highly specific for the acceptor substrate that is processed. For those cases, where dissociation constants are available the binding affinity for acceptor substrates is much less than for the donor substrate. Therefore, potent and specific inhibitors may be derived from linking ligands that bind to the donor site to ligands that bind to the acceptor site.

Another obstacle for the development of glycosyltransferase inhibitors is the fact that their substrates are highly hydrophilic, and so are many ligands that have evolved based on the structures of donor or acceptor substrates. For any practical purpose in therapy or biotechnology it will be important to design inhibitors that are capable to penetrate cell walls and also do not suffer from poor pharmacokinetics.

Here, we propose a strategy to identify acceptor site ligands employing recently developed NMR techniques in combination with a library of molecular fragments. We have chosen human blood group B galactosyltransferase (GTB) [14] as a model target to demonstrate the feasibility of this approach. For this enzyme a number of crystal structures have been obtained, also in complex with donor and acceptor substrates [15, 16]. This offers the possibility to interpret our data on a solid structural basis. Also, the recombinant expression of the catalytic domain of this enzyme in *E. coli* has been optimized, and thus allows for follow up projects concerned *e.g.* with the synthesis and testing of GTB-inhibitors. The library of molecular fragments has been obtained from Thermo Scientific (Maybridge Ro5 fragment library) and has been especially designed for NMR screening purposes. It contains 500 fragments with a molecular weight below 500 Da and a water solubility of each component of at least 1 mM.

STD NMR [17] and spin-lock filtered NMR experiments [18] were employed to identify ligands with binding affinity. Both techniques are so called ligand-based techniques, *i.e.* resonance signals of the free ligand are observed in order to yield information on the bound state. Spin-lock filtering is plainly based on the fact, that transverse relaxation is enhanced in the bound state. This leads to an attenuation of resonance signals that bind to a receptor protein and can be used for the detection of ligand binding, especially in the presence of spin-labels [19]. In STD NMR experiments, saturation is transferred from a receptor protein to ligand molecules and leads to specific signal attenuation of resonance signals of ligands that bind to the receptor protein. This attenuation is made visible by difference spectroscopy and allows identification and characterization of ligands with binding affinity. Many applications of this very robust technique have been described since its introduction and are summarized in several review articles, *e.g.* [20–25].

The resulting hits were then further confirmed and refined by surface plasmon resonance (SPR) and activity assays.

Materials and methods

Abbreviations GTB: Human Blood Group B Galactosyltransferase; RU: Resonance Units; SPR: Surface Plasmon Resonance; STD NMR: Saturation Transfer Difference NMR; TSP: trimethylsilyl-2,2,3,3-tetradeuteropropionic acid; UDP: Uridine Diphosphate.

Chemicals and reagents UDP was purchased from Sigma. H-antigen disaccharide (α-L-Fuc-(1,2)-β-D-Gal-*O*-$^{13}CH_3$) was chemically synthesized (W. Hellebrandt, unpublished results). UDP-α-D-Galactose was a kind gift from Beat Ernst (University of Basel, Switzerland). All buffer exchanges of the GTB were performed using centrifugal filtering with Vivaspin 15R 10K MWCO filter devices at 3,000×*g* followed by protein concentration measurements using NanoDrop ND 1000 Spectrophotometer (PEQLAB Biotechnologie GmbH) and a colorimetric assay that is based on the Bradford Assay using Bio-Rad Protein Assay Dye Reagent Concentrate and bovine γ-globuline (Pierce) as a standard.

Expression and purification of GTB Recombinant human-10GTB (residues 63-354) [26] was overexpressed in *Escherichia coli* BL21 cells and purified by successive ion exchange chromatography with SP Sepharose FF (GE Healthcare) and affinity chromatography with UDP-Hexanolamine Sepharose as described elsewhere (manuscript in preparation). Purified GTB was dialyzed against 50 mM MOPS (pH 7.0), 100 mM NaCl, 1 mM DTT, 5 mM $MnCl_2$ and stored at 4°C.

Before use for NMR experiments the enzyme was exchanged four times with 25 mM phosphate buffer,

100 mM NaCl and 10 mM $MgCl_2$ in D_2O (99.97%) having a final concentration of 200 μM.

Handling of the Ro5 Maybridge library Compounds of the Ro5 Maybridge Library (Thermofischer Inc., 2006) were dissolved at a final concentration of 2 mM in the same buffer as the glycosyltransferase. Compounds with a lower calculated solubility as provided by Thermofischer Inc. stock solution were at 1 mM. Finally, compounds that show any kind of precipitates or aggregates readily visible were spun down for 20 min at 10,000×*g* and the supernatant was introduced into the screening process after concentration determination with NMR using 0.4 mM TSP as an internal reference. Peak picking of all reference ^1H NMR spectra of the compounds enabled distribution of the compounds from the Maybridge library into 25 bins using the peaks of the individual compounds as selection rules. The compounds were distributed so that all compounds would have at least one unique peak that did not overlap with any other peaks by 0.01 ppm in chemical shift. The number of compounds in each bin ranged from 11 to 20 with an average of 18 compounds.

NMR experiments All NMR screening data were measured at 282K using Norell ST500-7 5 mm tubes. Samples for STD [17] and $T_{1\rho}$ filtered [18] NMR screening contained 40 μM GTB, fragments at a concentration of approximately 60 μM per compound, 300 μM of UDP, and 10 μM of TSP in 25 mM phosphate buffer containing 50 mM NaCl and 5 mM $MgCl_2$ dissolved in D_2O at pH* 7.0 (99.97%). A single point titration by addition of H-antigen disaccharide was performed, giving a final concentration of 500 μM of H-antigen disaccharide in solution. Reference bins, containing only the Maybridge compounds in deuterated phosphate buffer and TSP, were also prepared. All STD and $T_{1\rho}$ filtered NMR experiments, for the screening, were carried out on a Bruker Avance III 700 MHz NMR spectrometer with a 5 mm TCI Z-gradient high resolution cryogenic probe and a Bruker Automatic Sample Changer B-ACS 60.

The experiments were recorded using the standard STD pulse sequence [17] with a 15 ms 5 kHz spin-lock pulse to reduce the background protein resonances [18] and excitation sculpting [27] with a 164 Hz square pulse to suppress residual HDO. Saturation of the protein NMR signals of the enzyme were performed using a train of 80 selective 70 Hz Gaussian pulses with duration of 50 ms, adding up to a total saturation time of 4 s. The on-resonance frequency was set to 0.4 ppm and off-resonance irradiation was applied at 80 ppm. STD NMR spectra were acquired with a total of 320 transients in addition to 16 scans to allow the sample to come to equilibrium. Spectra were performed with a sweep width of 9 kHz and 20480 data points corresponding to an acquisition time of 1.1 s and with an additional relaxation delay of 0.1 s giving a total repetition time of 5.2 s for the experiment. The same pulse sequence and water suppression were used for the $T_{1\rho}$ filtered experiments, but without saturation of the protein, with 32 transients and a spin-lock pulse of 400 ms at 6 kHz.

Biacore experiments Surface plasmon resonance studies were carried out on a Biacore 3000 instrument using a standard streptavidin coated chip (SA chip, Biacore). At a flow rate of 10 μL/min 5 μL of a 1 μg/mL stock solution of biotinylated multivalent H-antigen disaccharide (Glycotech) were immobilized to yield a final surface density of 44 RU. A reference cell was established using only the biotinylated polyacrylamide backbone at the same density.

GTB stock solution was exchanged two times with MOPS buffer in the presence of 15 mM EDTA and then two times with 50 mM MOPS buffer, containing 100 mM NaCl and 5 mM $MgCl_2$. The final protein concentration was 2 mg/mL. The calculated RU_{max} value for this chip set-up was 534 RU.

Compounds from the Maybridge Ro5 Library were dissolved in the running buffer close to their calculated aqueous solubility provided by Thermofischer Inc. GTB was pre-incubated with a 7.5-fold molar excess of UDP (as in the NMR experiments). This solution was then injected at constant concentration while varying fragment concentration with five 4:1 dilutions at 10 μL/min flowrate for 1.5 min. No regeneration of the chip was necessary due to fast off-rate. Experiments were performed in duplicates and evaluated using Biacore evaluation software and scientific python for curve fitting of IC_{50} curves including an error estimate using Monte Carlo methods. The equation used to fit the data assuming zero response units at full inhibition was:

$$RU(c) = \frac{RU_{\max}}{1 + \left(\frac{IC_{50}}{c}\right)^{hillslope}}$$

with $RU(c)$ being the measured intensity at a given concentration c, RU_{max} being the maximal intensity measured in absence of inhibitor, and the *hillslope* describing the steepness of the curve.

Enzyme activity assay The influence of selected compounds from the Maybridge Ro5 Library on the activity of GTB was determined with a radiochemical assay according to a procedure described elsewhere [28]. Briefly, the reaction mixtures with final volumes of 10 μL contained 1 μM GTB, 100 μM α-L-Fuc-(1-2)-β-D-Gal-O-octyl as acceptor substrate, 500 μM UDP-Gal with 786 Bq UDP-[U-^{14}C]-Gal (lithium salt, Amersham Biosciences) as donor substrate, and 500 μM of the Maybridge library compound in 50 mM MOPS, pH 6.7, 5 mM $MgCl_2$, and

100 mM NaCl. Reference samples without compounds were also prepared. The mixtures were incubated for 30 min at 37°C and the reaction was stopped by addition of 170 μL ice-cold water. The radiolabeled trisaccharide product was separated from the donor substrate using reversed-phase Sep-Pak® Vac RC C18 Cartridges (100 mg, Waters) and radioactivity was measured in a Wallac 1409 Liquid Scintillation Counter.

Docking The crystal structure of the GTB in complex with UDP and the H-disaccharide (pdbid 2RJ8) [15], with ligands removed, was used as the receptor and the AutoDock 4.0 program [29] was used for docking. The atomic coordinates for the Maybridge compounds were optimized using the Calculator Plugins of Instant JChem, Marvin 5.2.0, ChemAxon (www.chemaxon.com). The grid dimensions were 50×50×50 points, with points separated by 0.375 Å. The grids were chosen to be centred on the acceptor site and sufficiently large to cover the whole binding site. One hundred fifty individual docking runs employing the Lamarckian genetic algorithm were performed with 5×10^6 energy evaluations, while other parameters were set to default values.

Results and discussion

GTB has a donor- and an acceptor-binding site. The donor site is well defined [15], and leaves relatively little variation for improvement. Therefore, we have focussed on the acceptor site that is much less well defined in its shape and extension. In order to study binding of compounds to this site it was necessary to block the donor site. We have shown previously [30] that uracil is the minimum fragment of UDP-Gal that binds to the donor site, and that UDP is a very good binder with binding affinity similar to that of UDP-Gal. Therefore, UDP was chosen to block the donor binding-site in our experiments.

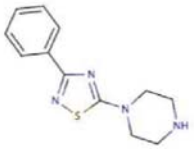
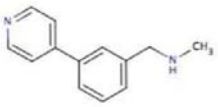
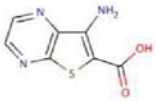
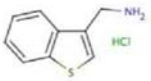
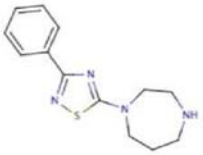
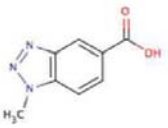
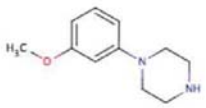
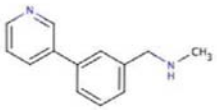
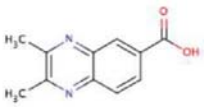
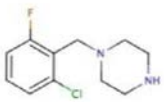
In order to identify fragments from the Maybridge library with binding affinity for the acceptor site of GTB we performed an NMR screening based on STD NMR experiments [17] and spin-lock filtered NMR spectra [18] in the presence of excess UDP to block the donor site. The compounds referred to in the following are found in Table 1.

The fragments were distributed in several bins containing eighteen individual compounds on the average. Compounds in one bin had been selected via a computer algorithm such that overlap of ^{1}H NMR resonance signals was minimized, and that there was at least one isolated resonance signal for each compound of the mixture. The saturation time in the STD experiments was chosen long enough (4 s) such that saturation transfer to each proton of the ligand is facilitated. This ensures that saturation transfer is observed even if a resonance signal has been chosen for observation that belongs to a proton that is more distant to protons in the binding pocket. For ranking STDs and signal attenuations from spin-lock filtering a reference was required. It is well known that small molecules such as water, DSS, or TSP bind non-specifically and weakly to proteins. Such weak binding can be readily observed with STD NMR experiments as well as with spin-lock filtered NMR experiments. Here, we have employed TSP as internal chemical shift reference, and at the same time as reference for classifying STD effects and signal attenuations from spin-lock filtering. Four categories, 0 to 3 were used where category 0 corresponds to no STD or no signal reduction from spin-lock filtering. Category 1 corresponds to equal or lower STD or signal reduction than observed for TSP. Ligands in category 2 show larger STDs or stronger signal attenuation than observed for TSP, and category 3 comprises fragments that display large STDs (>8%) or complete signal suppression with spin-lock filtering. Assignment of a compound to a particular category was favourable towards a higher class in the sense that if a compound had resonances showing STD effects classified as *e.g.* 2 for a resonance and another resonance as 3 this compound was assigned to category 3. The ligands were further subdivided into fragments that compete with the natural acceptor (α-L-Fuc-(1-2)-β-D-Gal-O-octyl, "H- antigen disaccharide") for the acceptor binding site, and those that do not. This division was quickly achieved by single point titrations with the H-antigen disaccharide. For compounds that bind to the acceptor site addition of H-disaccharide led to a reduced STD or to less pronounced signal attenuation in the spin-lock filtered NMR experiments. In Fig. 1 this is demonstrated for compound 382 in a mixture of 20 compounds.

The overall result of this combined STD NMR and spin-lock filter screening is summarized in Fig. 2. Those 103 fragments that compete with the H-disaccharide for the acceptor site, and that were classified as belonging to category 3 either for STD or for spin-lock filtering, or alternatively category 2 for both types of experiments, were subjected to further evaluation (brick red squares in Fig. 2a). We also considered seven fragments that did not compete for the acceptor binding site but that fulfilled the category 3 or 2 criterion (dark blue squares in Fig. 2b) and additional five fragments that did not fulfil these criteria (light blue squares in Fig. 2b). The supporting material provides an Excel spreadsheet that contains all experimental data from the NMR screening.

Surface plasmon resonance (Biacore) experiments were performed for 120 compounds of the library. Since the molecular weights of the fragments were rather low and would yield only weak SPR responses we used a "reverse" setup where streptavidin chips were coated with biotinylated multivalent H-disaccharide. GTB was pre-incubated with a

Table 1 Fragment structures of the best ten binders and binding affinity

compound		STD [0-3]	spin lock [0-3]	IC_{50} [mM]	compound		STD [0-3]	spin lock [0-3]	IC_{50} [mM]
382		3	2	0.79 ± 0.16	281		2	2	1.61 ± 0.21
435		3	2	0.97 ± 0.13	127		1	3	2.11 ± 0.35
10		3	3	1.04 ± 0.05	197		3	2	2.16 ± 0.01
4		3	3	1.28 ± 0.06	298		2	2	2.61 ± 0.63
226		3	1	1.56 ± 0.25	13		3	2	2.72 ± 0.44

The top ten hits ranked according to the IC_{50} measured with the Biacore assay. The best 5 hits all display strong STDs (category 3, see text) whereas signal reduction due to spin-lock filtering varies. It should be remembered that neither STD intensities nor signal reductions due to spin-lock filtering directly reflect the size of dissociation constants. Some common structural features are readily identified: Three of the compounds display a five-membered ring fused to an aromatic six-membered ring system, *e.g.* compound 435. Four compounds carry a piperazine, or a structurally similar homopiperazine ring that is attached to an aromatic entity via one of the nitrogen atoms in the ring

7.5 molar excess of UDP to block the donor site and with increasing concentrations of inhibitor. The solution of GTB complexed with UDP (GTB/UDP) was then passed over the chip with immobilized H-disaccharide on the surface to yield inhibition curves and corresponding IC_{50} values (Fig. 3). As a reference we also pre-incubated GTB/UDP with increasing concentrations of H-disaccharide, and we performed one experiment where we pre-incubated GTB with increasing concentrations of UDP. The inhibition curves yielded an IC_{50} value of 180 μM for the H-disaccharide. Interestingly, binding of GTB to immobilized H-disaccharide on the chip was enhanced by increased concentrations of UDP, and an EC_{50} value of 8 μM resulted for UDP. This corresponds very well with affinity data from mass spectrometry that have been published recently [31]. In general, the compounds investigated were of limited solubility such that data points at higher concentrations could not be obtained. In some cases artifacts negative RU values were observed at higher concentrations of test compounds. We considered this as an artifact resulting from beginning precipitation that is not yet detectable by eye inspection. The sensorgrams in Fig. 3a and the corresponding inhibition curve in Fig. 3b document this with compound 382 as an example. Although this led to larger experimental errors we considered the data to be good enough for ranking affinities.

In order to relate the relative binding affinities to the modulation of enzymatic activity we performed activity assays with selected fragments. For this purpose, the fragments were ranked according to their IC_{50} values (Table 1, Excel spreadsheet of the supporting material), and the best ten hits were subjected to activity assays. It was found that all fragments that exhibited binding to the acceptor site also reduced enzymatic activity. The correlation between IC_{50} values and enzyme inhibition (Fig. 3d) is

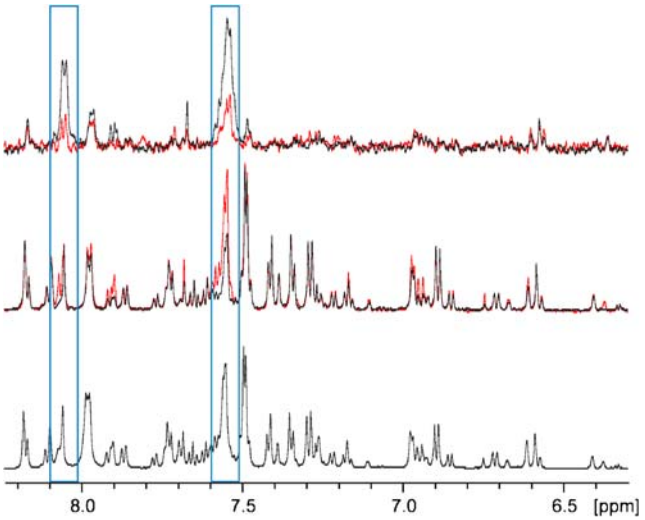


Fig. 1 NMR spectra of a bin of 20 compounds (60 μM each) in the presence of GTB (40 μM) and UDP (300 μM) recorded at a proton frequency of 700 MHz. The bottom spectrum shows the ^{1}H NMR reference spectrum of the compound mixture, the middle spectrum is the overlay of the spin-lock filtered (400 ms spin-lock filter) spectrum in the presence (*red*) and absence (*black*) of an 8-fold excess of H-disaccharide, and the top spectrum depicts the STD NMR spectrum (4 s saturation time) in the presence (*red*) and absence (*black*) of an 8-fold excess of H-disaccharide. The spectra allow identification of those fragments from the library that bind to GTB and compete with H-disaccharide for the acceptor binding pocket. Blue boxes highlight resonances originating from compound 382

very satisfying and supports the findings from the NMR screening. The correlation also shows that only the best binders from NMR and Biacore screening showed a notable reduction of enzyme activity (Fig. 3d). From the results presented it is estimated that only ca. 1% of the fragments would have been identified as binders if solely the enzymatic assay had been used for screening.

Inspecting the fragments identified to bind to the acceptor binding site one recognizes some structural similarities. Three of the ten compounds presented have a five-membered ring fused to an aromatic six-membered ring, both rings containing a varying number of nitrogen and sulfur atoms. Three of the compounds in Table 1 display a piperazine ring attached to aromatic systems via one of the ring nitrogen atoms. The fragments presented in Table 1 are a basis for the synthesis of potent GTB inhibitors. For instance, they can be covalently linked to donor site ligands or derivatives thereof in order to generate *e.g.* bidentate inhibitors that simultaneously bind to the acceptor and to the donor site.

In order to evaluate the efficiency of the screening process we correlated the NMR "ligand-category", *e.g.* 3/2 for strong STD and moderate signal reduction upon spin-lock filtering (*vide infra*) with the IC_{50} values from the Biacore assay (Fig. 4). Only IC_{50} values below a value of 10 mM were considered to be binders of interest. With this threshold none of the compounds with neither STDs nor spin lock effects (category 0/0) are classified as binders according to the Biacore assay. Also, a fraction of compounds that were identified as binders by NMR did not show any binding activity in the Biacore assay. For some of the ligands further scrutiny showed that the classification according to the strength of the STD or the spin-lock effects was too conservative in order not to overlook potential ligands but more general, this observation of "false positives" is not surprising, as the size of STD signals, or the occurrence of signal reduction upon application of a spin-lock filter is not directly linked to the size of the dissociation constant K_D or to the IC_{50} value used here. For example, significant STD signals can be observed even for very low affinity binding ligands with K_D values in the high mM range. Therefore, it is necessary to either perform a STD titration, or to independently determine K_D, *e.g.* with Biacore experiments. It should be finally noted that the temperatures at which the NMR screening (282K) and the Biacore assays (298K) were performed were not identical for practical reasons. This could also account for some of the deviations observed.

Together with the observation of STD effects and the reduction of signal intensities upon spin-lock filtering we also followed qualitatively line broadening effects. For

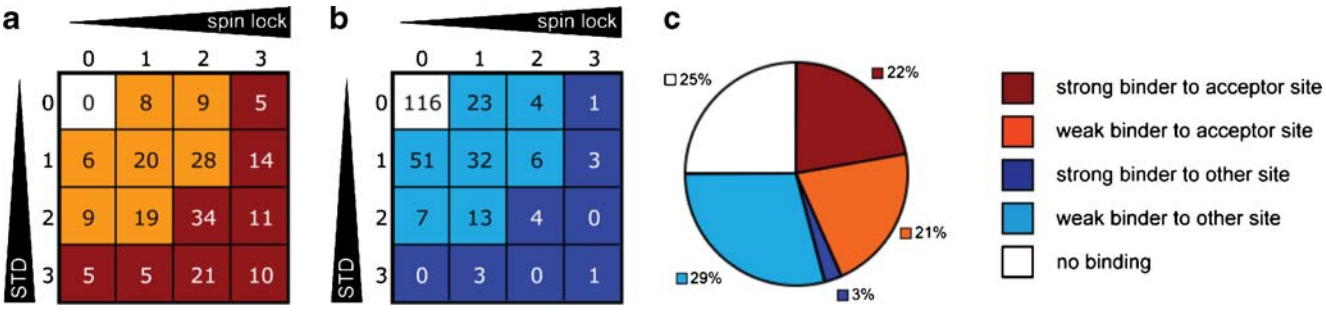


Fig. 2 Results from NMR screening. Rows reflect the category (see text) according to the signal attenuation from spin-lock filtering. Columns report the category according to the intensity of the STD experiment. **a** Fragments showing competition with H-disaccharide for the acceptor site of GTB. **b** Fragments binding to another binding site. **c** Distribution of binding compounds according to STD and spin-lock filter experiments

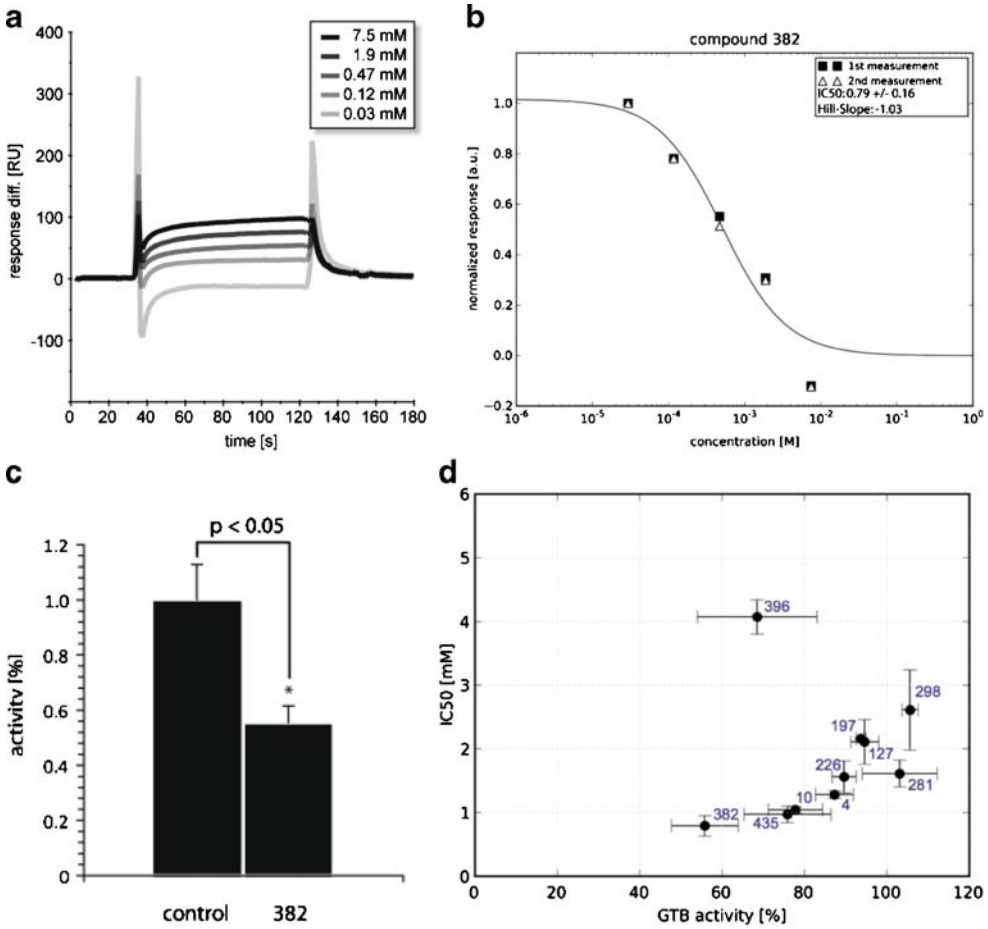


Fig. 3 Correlation of surface plasmon resonance data (**a**, **b**) and inhibition data (**c**) for compound 382. **a** Sensorgrams of the competition assay measured for compound 382 at the indicated concentrations. **b** Normalized RU values as a function of the concentration of fragment 382. Data were from two independent measurements. The negative data point at the highest concentration of 382 is an artifact. **c** Inhibition of enzymatic activity of GTB in the presence of 382. The activity was measured in triplicate as described in Materials and Methods. The graph shows the *p*-value from a two sided *t*-test. **d** Correlation of the IC_{50} values and inhibition of enzymatic activity of GTB. The activity assay was performed in triplicates and then averaged. Except for one outlier (compound 396) there is a clear correlation between binding affinity as reflected by the IC_{50} and inhibition of enzyme activity. For the formulas of the compounds *cf.* Table 1

instance, fragments 127 and 202 (*cf.* Table 1 and for the structure of 202 see the supporting material) showed significant line broadening in the presence of GTB. Nevertheless, the observation of line broadening effects turned out to be critical and could lead to false answers. For instance, compound 278 shows line broadening that is removed by addition of H-disaccharide but no effects were observed in the STD as well as in the spin-lock filtered experiments, and no inhibition was measured in the Biacore assay. Also, no competition was found in the Biacore assay. Although the reason for this remains unclear, it appears that line broadening effects should be treated with care to infer binding activity in the case of compound mixtures.

Since the fragments from the library had been used in small excess only, changes of chemical shifts were observed for some compounds in the presence of GTB. For instance, the second best binder, compound 435 showed large chemical shift changes in the ^{1}H NMR spectrum in the presence of GTB that were reversible upon addition of H-disaccharide. Such effects may be interesting in future investigations but are not further evaluated for the purpose to identify fragments that bind to the acceptor binding site of GTB.

The best binder from our study (compound 382) has been subjected to docking with Autodock utilizing the coordinates from a recent crystal structure analysis [15]. The binding site was restricted to the acceptor-binding pocket. Figure 5 shows the best binding mode for compound 382. Docking suggests that compound 382 only partly fills the binding pocket, which would allow for chemical modifications to improve binding affinity. Certainly, this binding mode has to be substantiated by experimental data.

The NMR experimental setup was chosen to minimize the amount of material and to allow automated screening. A rather low ligand to protein (GTB) ratio of 1.2:1 was chosen in order to allow for the observation of STD effects and signal reduction upon spin-lock filtering with the same sample. Clearly, these conditions are suboptimal for STD experiments, and in order to compensate for this we applied long saturation times of 4 s. Under these conditions epitope mapping is not possible but otherwise twice the amount of protein as applied here would have been required (36 mg instead of 18 mg). The total NMR measurement time was 60 h, and Biacore experiments required 100 h on the equipment.

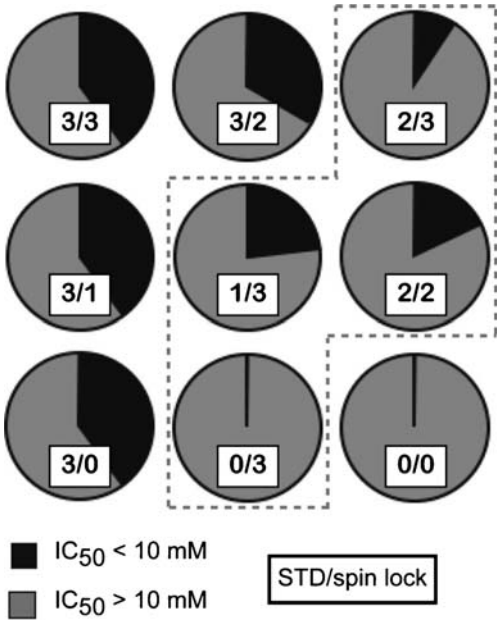


Fig. 4 The graph shows the fraction of binders with IC_{50} values lower than 10 mM for each of the categories that classify the strength of the STD effect and the signal reduction upon spin-lock filtering. Diagrams enclosed by dotted lines highlight compounds with strong spin-lock filter effects while STD intensities are weak. The graph suggests that a large STD effect has a better predictive value for a good binder than a large spin lock filter effect

A number of protocols have been published that have applied NMR experiments to screen fragment libraries [19, 22, 23, 32–39]. To our knowledge, the combination of STD NMR and spin-lock filtered NMR experiments as a "read-out system" for screening has not been reported before. This combination allows an efficient pre-screening to identify fragments with binding affinity whereas Biacore experiments allow a ranking of the affinities. A reverse Biacore setup where the protein is immobilized on the chip would normally be an alternative for the NMR screening and would require even less protein. In the specific example described here saturation of the donor binding site was required in order to identify fragments that bind to the acceptor site. This is readily achieved in a competitive assay where the acceptor substrate is immobilized to the chip as it is described above but would have been difficult to monitor if GTB had been immobilized. The main reasons are as follows. First, the expected resonance signal for any of the low molecular weight fragments from the library would be only a few resonance units, and, second, the signal would have been detected on top of the signal of UDP binding to the chip. In turn a double difference signal would have been necessary to be detected, which would have introduced large errors. Therefore, the Biacore experiments required a larger amount of GTB. Since only about one third of the library fragments had been subjected to the Biacore experiments the amount of GTB for the competitive Biacore screening was significantly reduced. Another aspect is that binding affinities may differ once a protein target is immobilized, or in the worst case, binding affinity is lost completely upon immobilization. Thus, the "reverse" setup ensures that this does not happen.

In summary, the combined NMR/Biacore setup suggested here for identification of ligand binding to receptor

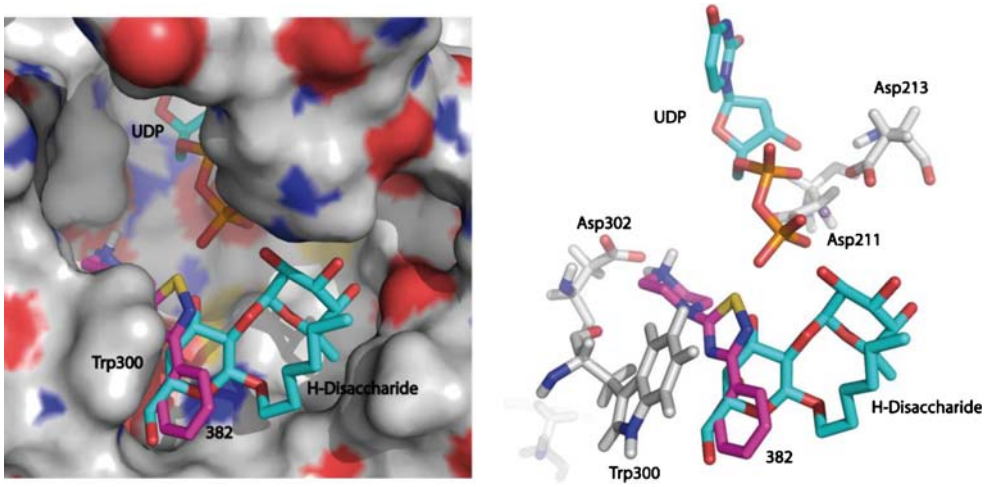


Fig. 5 Proposed binding mode of the best binder, compound 382, overlaid with UDP and H-disaccharide in the binding pocket of GTB. The pose shown represents the convergence of over 90% of 150 docking runs. *Left:* Surface representation of the binding pocket of GTB. In the back, UDP occupies the donor site, and in the front compound 382 competes with the H-disaccharide for the acceptor site. A stacking interaction of 382 with Trp300 and coordination of piperazine N-4 of 382 with Asp302 stabilizes this orientation. *Right:* Only part of the binding pocket is shown as capped sticks. Asp211 and Asp213 that complex Mn^{2+} or Mg^{2+} are also shown. This view shows that according to the docking compound 382 only partially overlaps with the H-disaccharide. The image was produced with the program PyMOL (http://www.delanoscientific.com)

proteins requires less than a week if no further complications are encountered.

Conclusions

The combination of STD/spin-lock filter based NMR screening with a suitable Biacore assay represents a robust and fast method to identify fragments with binding affinity from a fragment library. Here, the ranking of ligands with binding affinity has been achieved via surface plasmon resonance. It should be noted that STD NMR titrations would have offered an alternative way to perform the ranking of ligands, although this would have required more enzyme. Nevertheless, this approach may be necessary for ligands or proteins were immobilization of one or both of the partners loose their function upon immobilization.

This study shows that the NMR experiments identify a larger subset of fragments as binding ligands than the Biacore assay. Using a reasonable threshold for the IC_{50} the Biacore assay allows to narrow down on the number of binding ligands and at the same time delivers a ranking of the affinities. Obviously, there is redundancy in the experimental setup chosen but it helps to identify "false positives" as well as "false negatives".

For a further development of GTB inhibitors the binding ligands from Table 1 form a basis set of scaffolds that can be further functionalized to deliver potent and selective inhibitors. The optimization of such ligands will require the application of STD NMR experiments under conditions that allow an epitope mapping of the ligands, and the identification of the precise binding site using protein based NMR experiments. Such experiments are under progress in our laboratory.

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Supporting information available

Structures of the Maybridge Ro5 library and all experimental data from the screening are available.