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A practical glycosyltransferase assay for the identification of new inhibitor chemotypes

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Abstract

We have developed an operationally simple assay protocol for the identification and evaluation of small molecular glycosyltransferase inhibitors. Wu and co-workers have recently reported that the formation of the nucleoside diphosphate during the glycosyltransferase reaction can be monitored with a phosphatase/malachite green detection system at 620 nm. Here we demonstrate, for the first time, that this assay principle can be exploited for enzyme inhibition studies, and report the optimisation of several key parameters of this assay. We have significantly improved the reproducibility of the assay by addition of chicken egg-white lysozyme as a carrier protein. We have also substantially reduced the assay running costs by using the inexpensive and widely available calf intestinal phosphatase. Critically for inhibition studies with small organic molecules, our assay protocol tolerates additives such as the solubiliser DMSO and the surfactant Triton-X 100, and we have validated its application in inhibition experiments with two known galactosyltransferase ligands. The new assay protocol is robust and inexpensive, requires only short incubation times, and can be carried out in a microplate format. These characteristics make it ideal for high-throughput screening (HTS) campaigns. As it does not require specialist equipment and can readily be performed in non-biochemistry laboratories, we anticipate that the assay will help expedite the identification of new glycosyltransferase inhibitors both in industry and academia.

Introduction

Glycosyltransferases (GTs) are a large family of enzymes that are essential in nature for the biosynthesis of oligo- and polysaccharides as well as complex glycans.[1,2] To date, more than 87,000 protein sequences with proven or putative GT activity have been reported, and it is estimated that 1% of open-reading frames (ORFs) in the human genome code for GTs.[3] GTs catalyse the transfer of a sugar from a glycosyl donor, most commonly a sugar-nucleotide, to an acceptor substrate (Fig. 1). While individual GTs often display exquisite selectivity for their respective donor and acceptor substrate, between them, GTs recognise a broad variety of acceptors, including mono- and oligosaccharides, lipids, peptides, proteins and small molecules. The resulting glycosylation products are often involved in fundamental biological processes, such as cell signalling,[4] cellular adhesion[5] and bacterial virulence.[6] Individual GTs have been identified as potential drug targets in a number of important therapeutic areas including diabetes, cancer, inflammation and infection.[7] Small molecular inhibitors of GTs are therefore sought after not only as chemical tool compounds to study these processes, but also as lead compounds for drug discovery.[7] Most existing GT inhibitors are substrate analogues with limited potential for further development, while non-substrate-based, “drug-like” inhibitors of this important enzyme class remain very rare.[8-10]

< Figure 1 here >

One potential source for the discovery of such alternative inhibitor chemotypes is the screening of drug-like compound collections. GT bioassays which allow the rapid screening of inhibitor candidates are therefore of considerable interest for drug discovery and chemical biology.[11,12] Ideally, such a screening assay will be

operationally simple, cheap, adaptable for a high-throughput format and applicable across a wide range of different GTs. While several new GT assays have been reported in the past decade,[11,12] the most popular assay formats to date are probably still a radiochemical assay[13] and an enzyme-coupled UV assay based on the NAD/NADH interconversion,[14] which were both developed two decades ago. Both assays have seen a wide uptake in the field and are used extensively for monitoring enzyme kinetics. They are, however, less suitable for the screening of inhibitor libraries, due to their relatively low-throughput format. In addition, the UV readout of the coupled assay at 340 nm is susceptible to assay artefacts resulting from interference with inhibitor candidates that absorb at or around that region.

Recently, a biochemical GT assay has been reported in which GT activity is coupled to the phosphatase-catalysed hydrolysis of the nucleoside diphosphate (e.g. UDP) that is formed as the secondary product of the GT reaction.[15] The free phosphate released by the phosphatase is quantified by a well-established phosphate detection system based on the malachite green reagent (Fig. 1). To date, this new assay has only been used for kinetic studies with different GTs.[15] In principle, the format is also very attractive for inhibitor screening. Due to the detection of the secondary reaction product, it is broadly applicable to different GTs. Importantly, the spectrophotometric readout occurs at wavelengths above 600 nm, which significantly reduces potential interference from most small molecular inhibitor candidates.

Herein, we describe the adaptation of this assay format for the identification and characterisation of small molecular GT inhibitors in a 96-well plate format. We have used two galactosyltransferases, *N. meningitidis* LgtC[16] and bovine β-1,4-GalT, as representative model enzymes. LgtC is of particular interest as a potential target

for anti-virulence drug discovery.[16,17] We have optimised assay parameters which are critical for applications in inhibitor screening, in order to make the assay operationally simple and economically viable, two important prerequisites for its application in screening campaigns. Finally, we demonstrate that the assay can be used to evaluate inhibitors of GTs, including small molecules that are structurally unrelated to either GT donor or acceptor.

Results and discussion

Assay optimisation. In order to adapt the published assay protocol for inhibition studies, we investigated a number of parameters, with a view towards maximising reproducibility and minimising cost. Our first goal was the identification of a cheap alternative to the proprietary phosphatases employed in the original report (e.g. CD39L3), which are commercially available, but expensive.[15] In order to identify a more economic replacement, we explored other phosphatases as well as the use of inorganic catalysts (e.g. Ca^{2+}). The key criterion for alternative catalysts was their capacity to selectively hydrolyse UDP in the presence of UDP-sugars. For the detection and quantification of the inorganic phosphate released from UDP we used standard malachite green reagents.[18]

The best results in these initial experiments were achieved with calf intestinal phosphatase (CIP), a cheap, commercially available phosphatase with broad substrate specificity,[19,20] which is employed routinely in molecular biology. When used in a range from 6-25 U/mL, CIP reliably and stably released inorganic phosphate from UDP (0.78-25 μM), the secondary product of the GalT reaction (ESI, Fig. S1). In further experiments at a fixed concentration of CIP (10 U/mL) and a range of UDP concentrations, absorbance at 620 nm increased in a concentration-

dependent manner, with a stable background absorbance of 0.2 (Fig. 2). Comparison with the absorbance profile of inorganic phosphate (P_i) showed that under these conditions, CIP releases both phosphate groups from UDP (Fig. 2). Importantly, the correlation between absorbance and analyte concentration was linear for UDP concentrations up to 12.5 µM (P_i: 25 µM), which allowed us to establish a viable calibration curve (Fig. 2). The loss of linearity at higher concentrations was due to the formation of a precipitate with the malachite reagents, both in the case of UDP and P_i. Critically for its application in the coupled GT assay, CIP was much less efficient in hydrolysing UDP-Gal, the GalT donor. Thus, at concentrations of 0-100 µM UDP-Gal, only very low quantities of phosphate were measured in the relevant assay window (0-12.5 µM), which could easily be accounted for through background correction (Fig. 2). Interestingly, when we investigated the release of phosphate from UDP-Gal at higher concentrations (600 µM), we detected a stable concentration of approximately 43 µM of P_i, independent from the concentration of CIP (ESI, Fig. S2). Most likely, this contaminant is UDP itself, which from our experiments appears to be present in concentrations up to 3-4%. Similar results were observed with all the different commercial sources of UDP-Gal tested in this study, while this type of contamination was much less of a problem with other UDP-sugars (UDP-Glc, UDP-GalNAc, data not shown).

< Figure 2 here >

With a suitable alternative phosphatase in hand, we next set out to establish conditions for the phosphatase-coupled GT assay that would require a minimum amount of material. For these experiments, we used bovine β-1,4-GalT and LgtC, an

α-1,4-GalT from *Neisseria meningitidis*, as model enzymes. To save on cost, we were keen to use both GTs at minimal concentrations (< 1 μg/ml). However, we found that under these conditions the reproducibility of K_m (donor) and k_{cat} in repeat experiments was very limited (Fig. 3). At a fixed concentration of enzyme and variable concentrations of donor, the measured concentrations of UDP were highly variable, especially at high UDP-Gal concentrations (Fig. 3). These considerable variations were mainly the result of an increasingly unfavourable signal-to-noise ratio at high UDP-Gal concentrations, which in turn was due to a substantial background in combination with a relatively weak signal, probably as a result of diminished enzyme activity.

< Figure 3 here >

It is known that when studying enzymes at very high dilution, transfer losses through protein adhesion to plastic surfaces (e.g. microplate wells, pipette tips etc.) can become highly significant, which in turn affects assay reproducibility. We reasoned that under the above conditions, β-1,4-GalT and LgtC may be susceptible to significant reductions in their effective concentration through such non-specific adhesion events. To circumvent such losses, we investigated the addition of a 'carrier', i.e. a soluble molecule, in high concentration (1 mg/mL), which is inactive towards the assay components, i.e. to the β-1,4-GalT assay mixture. A carrier commonly used for such applications is bovine serum albumin (BSA). However, we found that BSA significantly hindered the colour development of the malachite reagents, possibly through sequestering molybdenum. We therefore screened several amino acids, surfactants and a selection of proteins as potential alternatives to BSA, including tryptophan, phenylalanine, cysteine, leucine, casamino acids,

Triton-X 100, gelatin, α-lactalbumin and chicken-egg lysozyme (CEL). Phenylalanine and casamino acids hindered the colour development in the malachite reaction step, and only CEL and gelatin showed the desired protectant effect on β-1,4-GalT in time-course experiments. As gelatin appeared to have a phosphate background, CEL was selected as the additive of choice for subsequent experiments.

As desired, addition of CEL to the assay mixture dramatically improved the reproducibility of K_m (donor) and k_{cat} for β-1,4-GalT (Fig. 3 and Table 1). In a time-course experiment, we found that without a carrier protein, the GT activity dropped by half over 200 minutes, while the presence of CEL preserved full activity (ESI, Fig. S3). Pleasingly, the optimized assay conditions were not limited to β-1,4-GalT, but could also be applied to LgtC, where the addition of CEL also resulted in reproducible values for K_m (donor) and k_{cat} (Fig. 3). For both enzymes, the values obtained for the K_m of the UDP-Gal donor under our assay conditions were in good agreement with those reported previously in the literature (Table 1). This was also true for the k_{cat} value for β-1,4-GalT, while for LgtC we reproducibly obtained a k_{cat} which was lower than previously reported values.[2,16]

< Table 1 here >

The evaluation of small molecular inhibitors, which are often relatively non-polar, usually requires the use of solubilisers, to improve their solubility in aqueous media, and of surfactants, to minimise inhibitor aggregation. We therefore investigated the effect of DMSO and Triton-X 100 on our assay protocol. We found that the addition of 10% DMSO slightly lowered the K_m (donor) of LgtC, but not prohibitively so, while the additional inclusion of 0.01% Triton-X 100 did not affect the K_m (donor) any

further (ESI, Fig. S4). In the case of β-1,4-GalT, the effects of DMSO and Triton-X 100 led to similar, negligible variations in K_m and k_{cat} (ESI, Fig. S4). We therefore adopted the use of 10% DMSO and 0.01% Triton-X 100 for our standard protocol.

Inhibition studies. With a reproducible biochemical assay protocol in hand for both β-1,4-GalT and LgtC, we investigated the suitability of this assay for inhibitor studies. For these validation experiments, we used three different ligands: 5-FT UDP-Gal (5-(5-formylthien-2-yl) UDP-Gal), uridine and CSG164. 5-FT UDP-Gal is a known, broad-spectrum GalT inhibitor, which is structurally derived from the natural donor UDP-Gal.[26] 5-FT UDP-Gal has activity against both β-1,4-GalT and LgtC and served as a positive control in our experiments. As a negative control, we selected the nucleoside uridine, which is a known binder but only very weak inhibitor of GalTs, due to the absence of the galactose and pyrophosphate moieties.[27] CSG164 is a new, heterocyclic LgtC inhibitor, which was recently discovered in our laboratory.[28] CSG164 is structurally unrelated to UDP-Gal and was included in our experiments to assess if the assay protocol was suitable for the identification of new GT inhibitor chemotypes.

In principle, our assay set-up can be used to assess the potency of inhibitors by determining their K_i values, e.g. from Dixon plots. This approach requires limiting the turnover of donor substrate to <10% (with “turnover” defined as the percentage of UDP-Gal that is consumed during the GT reaction, relative to the total amount of UDP-Gal in solution). We found that in practice this can be difficult, mainly for two reasons. First, it can be practically difficult to exactly control turnover rates with the required accuracy. In principle, turnover rates can be controlled through enzyme concentration, and early control experiments indicated that increasing the

concentration of enzyme did indeed increase the turnover of donor substrate. However, this correlation was often neither completely linear, nor predictable. For example, the LgtC concentration predicted to result in around 10% turnover of donor substrate (1.8 μM in the presence of 18 μM UDP-Gal) afforded turnovers between 7 and 17%, whilst the predicted concentration for 25% turnover gave 32-45% (ESI, Fig. S5). This poor correlation between predicted and measured turnover was exacerbated at higher enzyme concentrations. Second, at very low turnover rates (~2-5%), it was difficult to distinguish the assay signal from the background, resulting in a relatively narrow assay window (Fig. 4). As the donor substrate contributes to the background of the assay, via enzymatic and chemical hydrolysis to terminal phosphates, this problem cannot be overcome by simply increasing the concentration of UDP-Gal. In fact, we found that using UDP-Gal at a concentration of 3x K_m gave the best practical results in inhibition studies. This gives a low enough background to be usable and a practically useful equation if K_i values are to be derived from a Dixon plot (i.e. $K_i = -y_{int}/4$).

< Figure 4 here >

While in principle our protocol can be used to obtain K_i values for synthetic inhibitors,[28] we found that in individual experiments, donor turnover was frequently too high or too low to generate reliable Dixon plots. This lack of predictability resulted in a waste of time and valuable reagents. In order to develop a screening protocol that is less susceptible to the practical problems arising from the narrow assay window at 10% turnover, we decided to explore inhibition at higher turnover rates. In principle, such an approach will allow the generation of relative IC_{50} values at a

defined turnover rate, if not of K_i values. We reasoned that the determination of IC_{50} values would be entirely appropriate for our primary purpose of assessing the potency of different inhibitor candidates, while imposing fewer restrictions with regard to enzyme turnover. The absolute levels of maximal and minimal enzyme activity in the presence of inhibitor varied between experiments (see e.g. Fig. 5a). Following the guidelines developed by Sebaugh for accurate EC_{50}/IC_{50} estimation,[29] we therefore determined relative, rather than absolute, IC_{50} values throughout this study. A relative IC_{50} value is defined as the inhibitor concentration that corresponds to the response midway between the lower and the upper plateau of the dose-response curve.[29] The use of relative IC_{50} values has been recommended specifically for scenarios, as in our case, in which the maximal (minimal) inhibitor activity is not identical to the 100% (0%) plate control.[29]

In order to identify a suitable turnover "window" for the reproducible determination of IC_{50} values, we first investigated the inhibitory activity of our new LgtC inhibitor CSG164 at three different turnover rates from 20-90% (Fig. 5a). In order to allow the construction of full IC_{50} curves, for these experiments, CSG164 was used in concentrations from 10 nM-50 μM at turnover rates of 20%, 50% and 90% respectively. At 20% turnover, two sets of experiments, each performed in triplicate (n = 3), gave relative IC_{50} values between 7-17μM (Fig. 5a). Interestingly, at 50% turnover, very similar IC_{50} values between 4-10 μM were obtained, but with a narrower spread of values between individual experiments. Not unexpectedly, maximal inhibition was slightly lower at 50% than at 20% turnover. Finally, the data obtained at a turnover rate of 90% did not allow the construction of meaningful sigmoidal curves for the calculation of IC_{50} values. Taken together, these results demonstrate that within a window of 20-50% turnover, reproducible and robust IC_{50}

values can be obtained with this assay set up. As positive and negative controls, we carried out similar experiments with, respectively, 5-FT UDP-Gal and uridine instead of CSG164. As expected, the weak GalT binder uridine showed no inhibitory activity, irrespective of the UDP-Gal turnover rate (Fig. 5b), while the known GalT inhibitor 5-FT UDP-Gal inhibited LgtC with an IC_{50} value of 0.9 ± 0.03 μM (Fig. 5c). Interestingly, despite its structural similarity to the natural donor UDP-Gal, 5-FT UDP-Gal was not recognized as a substrate by LgtC (ESI, Fig. S6). Thus, no significant formation of 5-FT UDP was observed when 5-FT UDP-Gal was used as a "donor", instead of UDP-Gal, under the conditions of the inhibition assay. Importantly, the lack of signal in this experiment is not due to the phosphatase step, as additional control experiments showed that CIP recognises both UDP and 5-FT UDP as substrates with similar efficiency. Taken together, these results demonstrate that our assay set up is suitable to discriminate between strong and weak inhibitors from different structural classes, including both substrate-based and non-substrate-based inhibitors, and can therefore be used to identify new LgtC inhibitor chemotypes.

< Figure 5 here >

Finally, we repeated the same set of inhibition experiments with β-1,4-GalT. We have recently discovered that CSG164, while a potent inhibitor of the bacterial α-1,4-GalT LgtC, does not inhibit bovine β-1,4-GalT.[28] In the present study, CSG164 showed no inhibitory activity against β-1,4-GalT at turnover rates of 10, 25 and 50%, in keeping with these previous findings (ESI, Fig. S7). Importantly, this result not only confirms the selectivity of CSG164 for LgtC over β-1,4-GalT, it also rules out the

possibility that CSG164 may be merely inhibiting the CIP used in our assay, as inhibition of the phosphatase would have resulted in "activity" against both GTs. As expected, in additional control experiments with uridine and 5-FT UDP-Gal, uridine demonstrated no inhibitory activity against β-1,4-GalT, while 5-FT UDP-Gal inhibited β-1,4-GalT with an IC_{50} value of 9.0 ± 1.4 μM (ESI, Fig. S7). Taken together with the results from the LgtC inhibition experiments, these results show that our assay protocol is suitable to establish not only the inhibitory potency of a given GalT inhibitor, but also its selectivity profile against different enzymes.

Summary and conclusion

Recently, Wu and co-workers have reported a phosphatase-coupled GT assay for determining enzyme kinetics.[15] In this study, we have developed this new assay protocol into a format that can be used to identify and evaluate small molecular GT inhibitors. Using two galactosyltransferases as model enzymes, we have optimised key parameters such as assay reproducibility and cost. We have identified CIP as a reliable and inexpensive alternative to the proprietary phosphatase employed in the original assay protocol. We also demonstrate that the addition of chicken egg-white lysozyme as a carrier protein significantly increases the reproducibility of the assay. These modifications, together with the "in-house" preparation of the malachite green reagents, have dramatically reduced the material cost of the assay. Completing a single microplate, with a volume of 150 μL per well, costs around £4 (Table 2). This compares very favourably with the commercial assay, despite several additional components in our protocol (e.g. CEL, DMSO, Triton-X 100). These savings will be particularly attractive to academic laboratories, which often operate on a tight budget.

< Table 2 here >

Importantly, we also show that our assay protocol is compatible with the use of DMSO and Triton-X 100. This is a crucial prerequisite for the screening of small molecule inhibitors, which are often poorly soluble in aqueous media, or behave as aggregators. We have validated the application of our assay conditions in inhibition experiments with three ligands, including both substrate-based and non-substrate-based inhibitors. For these experiments, we have established conditions that allow the reliable calculation of relative IC_{50} values, within a large assay window. In view of its low cost, operational simplicity and potential broad applicability to other GTs, we anticipate that the new assay protocol will find wide application in the search for new GT inhibitors, both in industry and academia.

Acknowledgement

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Figure 1. The glycosyltransferase reaction, exemplified by the retaining α-1,4-galactosyltransferase LgtC and the inverting β-1,4-GalT. The assay used in this study is based on the release of inorganic phosphate from the secondary reaction product UDP and detection of the resulting inorganic phosphate (P_i) with malachite green.

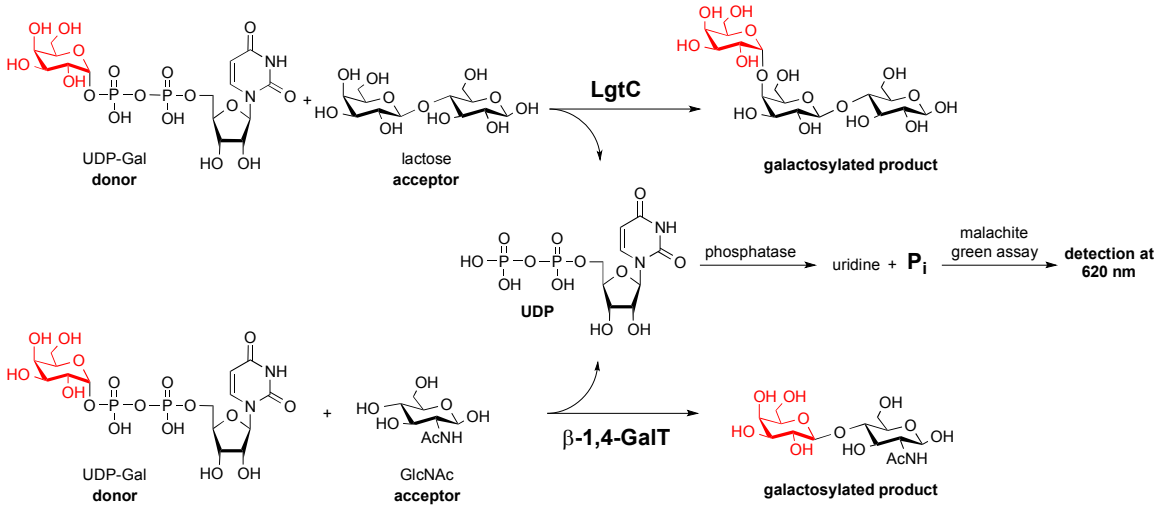
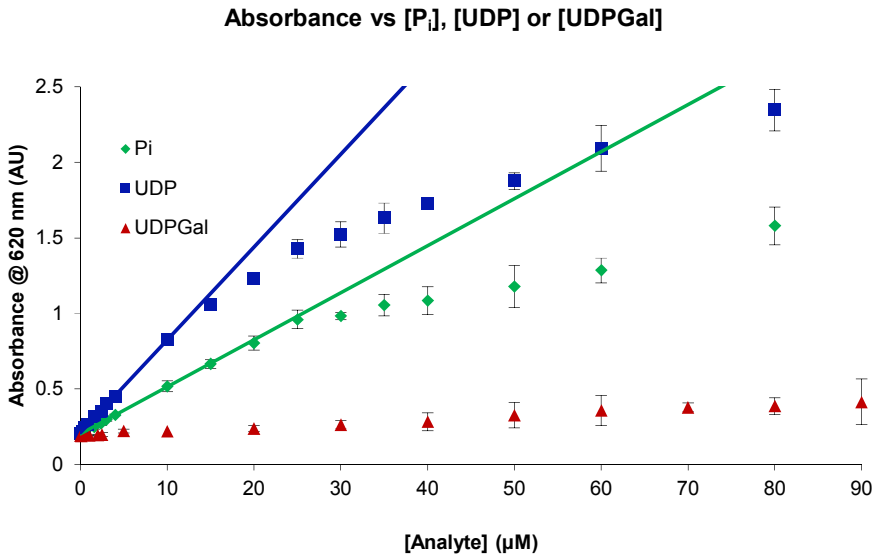


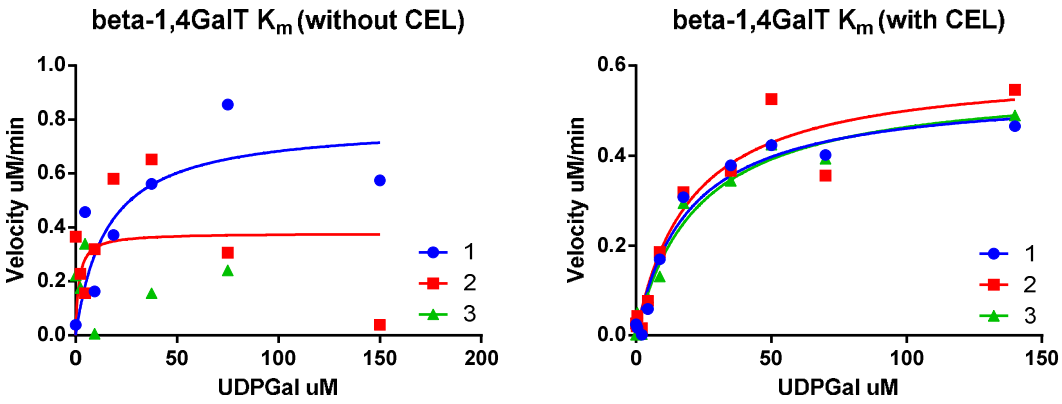
Figure 2. Colorimetric detection of inorganic phosphate (P_i) released by CIP from UDP (purple) or UDP-Gal (red) with the malachite green reagent.[a]



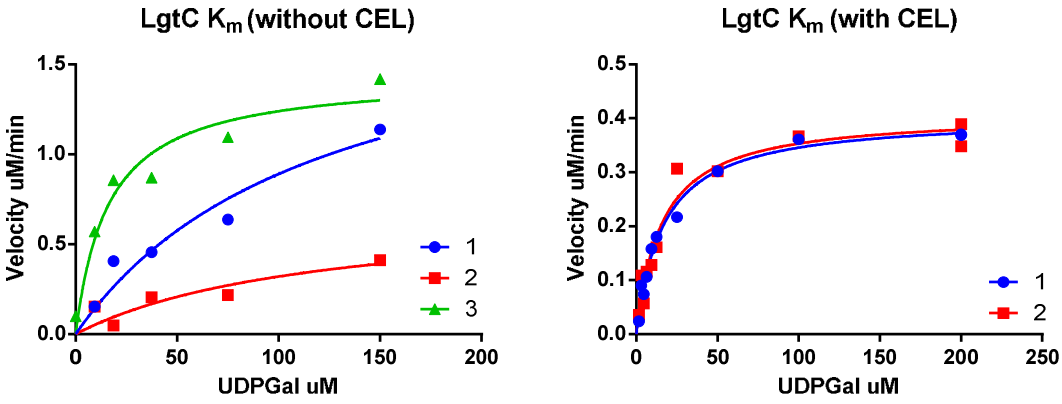
[a]*Reagents and conditions*: all experiments were conducted with 1 mg/mL CEL, 5 mM $MnCl_2$, 10 U/mL CIP, 10% DMSO, 13 mM HEPES (pH 7.0) and either inorganic phosphate, UDP or UDP-Gal. A background absorbance of 0.2 was measured in the absence of any added phosphates. CIP releases two equivalents of phosphate from UDP. All experiments were incubated for 20 min at 30 °C, followed by addition of the malachite green reagents.

Figure 3. Effect of the addition of the carrier protein chicken egg-white lysozyme (CEL) on assay reproducibility. The graphs show replicates of Michaelis-Menten plots for β-1,4-GalT and LgtC experiments at different UDP-Gal concentrations. *Left*: experiment conducted without CEL. *Right*: identical experiment conducted in the presence of 1 mg/mL CEL.[a]

(a) β-1,4-GalT

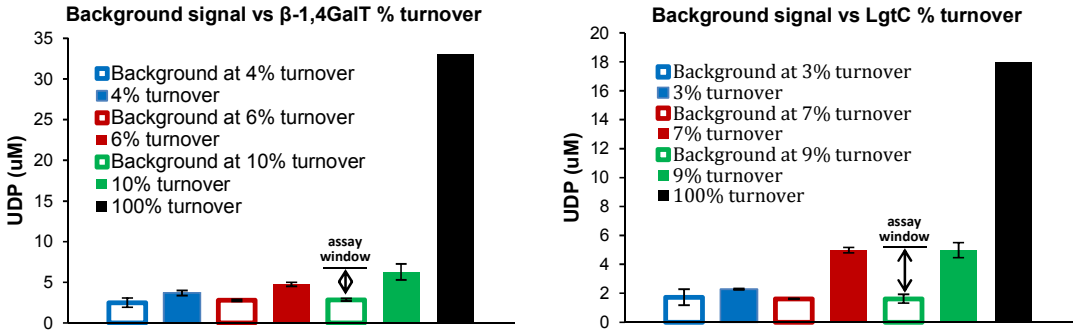


(b) LgtC



[a]Each graph shows the results from an individual, representative experiment, carried out in triplicate (curves 1-3) or duplicate (curves 1 & 2) on a single microplate. β-1,4-GalT experiments were conducted with 3 μg/mL β-1,4-GalT, 33 μM UDP-Gal, 10 mM GlcNAc as the acceptor, 13 mM HEPES (pH 7.0), 50 mM KCl and 5 mM $MnCl_2$. LgtC experiments were conducted with 14 μg/mL LgtC, 18 μM UDP-Gal as the donor, 2 mM lactose as the acceptor, 13 mM HEPES (pH 7.0) and 5 mM $MnCl_2$. All reactions included 10 U/mL CIP and were incubated for 20 mins at 30 °C, followed by development with the malachite green reagents. Experiments in the presence of CEL were repeated three times in total (Exp. 1-3, Table 1). For kinetic data (K_m, k_{cat}) extracted from these three experiments see Table 1.

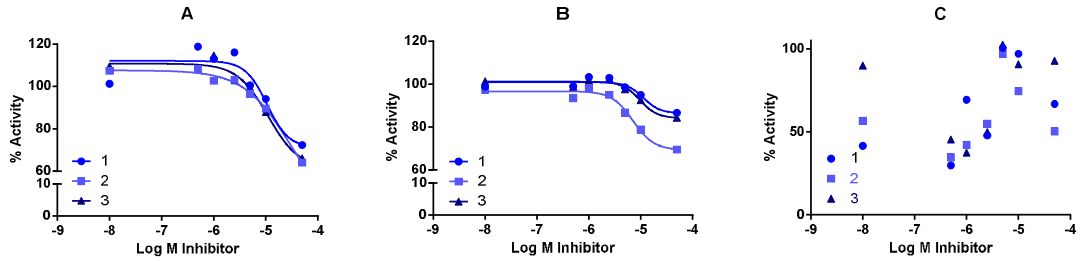
Figure 4. Assay window at low GalT turnover rates (4-10%) for β -1,4-GalT (left) and LgtC (right).[a]



[a]*Conditions*: β-1,4-GalT experiments were conducted with 3 µg/mL β-1,4-GalT, 33 µM UDP-Gal as the donor, 10 mM GlcNAc as the acceptor, 13 mM HEPES (pH 7.0), 50 mM KCl and 5 mM $MnCl_2$. LgtC experiments were conducted with 14 µg/mL LgtC, 18 µM UDP-Gal as the donor, 2 mM lactose as the acceptor, 13 mM HEPES (pH 7.0) and 5 mM $MnCl_2$. All experiments included 10 U/mL CIP, 1 mg/mL CEL, 10% v/v DMSO, 0.01% v/v Triton-X 100, and were incubated for 20 mins at 30 °C, followed by the addition of the malachite green reagents. Data shown is an average of three separate experiments (each with n = 3), error bars represent the standard deviation between the three experiments.

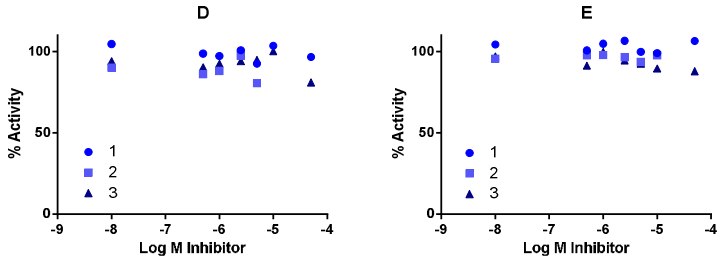
Figure 5. Determination of IC_{50} values for CSG164, uridine and 5-FT UDP-Gal against LgtC.[a]

(a) CSG164

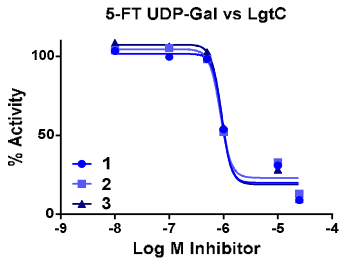


		donor turnover		
		20%	50%	90%
IC_{50} (μM)	Exp 1	16.7 ± 10.2 (**A**)	9.3 ± 2.1 (**B**)	n.m. (**C**)
	Exp 2	6.8 ± 1.6	9.6 ± 2.1	n.m.
	Exp 3		4.1 ± 1.2	n.m.

(b) uridine



(c) 5-FT UDP-Gal[b]



[a]*Conditions*: CSG164 was tested at concentrations from 0.01-50 μM, at three different turnover rates for LgtC: 0.033 mU/mL, 0.083 mU/mL, and 0.166 mU/mL. For each turnover rate, the graph for a single, representative experiment, carried out on a single microplate, is shown (**A**-**C**). In a single experiment, each inhibitor concentration was included in triplicate (curves 1-3). The entire experiment at each

turnover rate was repeated either two or three times in total (Exp. 1-3). IC_{50} values are relative IC_{50} values, as defined by Sebaugh,[29] and are averages ± S.D. of the triplicates in each independent experiment (n.m. = not measurable). Uridine was tested in triplicate (curves 1-3) at concentrations from 0.01-50 μM, at two different turnover rates for LgtC: 0.033 mU/mL (**D**) and 0.083 mU/mL (**E**). 5-FT-UDP-Gal was tested in triplicate (curves 1-3) at concentrations from 0.01-25 μM, at 0.083 mU/mL LgtC. [b]IC_{50}: 0.9 ± 0.03 μM (average ± S.D. of the triplicates in a single experiment), donor turnover: 42%.

Table 1. Kinetic data extracted from the Michaelis-Menten plots in Figure 3 Literature values are shown for direct comparison.[a]

		β-1,4-GalT		LgtC	
		this study (1 mg/mL CEL)	literature	this study (1 mg/mL CEL)	literature
K_m (μM)	Exp 1	20.4 ± 1.8[b]	25[21], 28[22]	16.2 ± 0.8[c]	18[16], 20[23], 4.4[24]
	Exp 2	34.4 ± 6.5[b]		16.6 ± 2.0[c]	
	Exp 3	11.3 ± 2.7[b]		21.1 ± 0.3[c]	
k_{cat} (s^{-1})	Exp 1	0.595 ± 0.026[b]	3.6[25], 0.435[25]	0.358 ± 0.034[c]	14.2[16], 24[23]
	Exp 2	0.477 ± 0.030[b]		0.352 ± 0.043[c]	
	Exp 3	0.336 ± 0.033[b]		0.339 ± 0.010[c]	

[a]Data from experiments without CEL are not included, as these results were highly variable and did not afford data that fitted Michaelis-Menten curves. K_m and k_{cat} values are averages ± S.D. of the replicates in each of three independent experiments (Exp. 1-3); [b]n=3; [c]n=2

Table 2. Cost comparison of the assay protocol described herein with a commercial GT assay kit.

component	cost per microplate (£)[a]	
	protocol in this study	commercial assay[b]
PVA	< 0.01	n.a.[c]
malachite green	< 0.01	n.a.[c]
ammonium molybdate	< 0.01	n.a.[c]
sulfuric acid	0.20	n.a.[c]
CIP	3.63	n.a.[c]
CEL	0.14	n.a.[c]
DMSO	0.12	n.a.[c]
Triton-X 100	< 0.01	n.a.[c]
Malachite Detection Kit	n.a.[c]	17.40
CD39L3	n.a.[c]	259.20
Total	**ca 4.10**	**276.60**

[a]96 wells, 150 μL/well; [b]from reference 15; [c]not applicable;

Table 3 Enzyme activities, and UDP-Gal and inhibitor concentrations used in individual experiments

Experiment	GalT	UDP-Gal	Inhibitor
LgtC kinetics	0.1 mU/mL	0-200 μM[a]	none
β1,4GalT kinetics	0.2 mU/mL	0-140 μM[a]	none
LgtC inhibition (20% turnover)	0.033 mU/mL	18 μM	0-50 μM[b]
LgtC inhibition at (50% turnover)	0.083 mU/mL	18 μM	0-50 μM [b]
LgtC inhibition at (90% turnover)	0.166 mU/mL	18 μM	0-50 μM[b]
β1,4GalT inhibition (10% turnover)	0.066 mU/mL	33 μM	0-50 μM[b]
β1,4GalT inhibition (25% turnover)	0.166 mU/mL	33 μM	0-50 μM[b]
β1,4GalT inhibition (50% turnover)	0.332 mU/mL	33 μM	0-50 μM[b]

[a]12 concentrations in total; [b]8 concentrations in total

Experimental section

All reagents were obtained commercially and used as received, unless otherwise stated. 5-FT UDP-Gal was prepared as previously reported.[26] The synthesis of CSG164 will be reported separately.[28] LgtC from *Neisseria meningitidis* and bovine β-1,4-GalT were expressed and purified as previously reported.[16,29,30] The plasmid for LgtC was a generous gift from Warren Wakarchuk (Toronto). The construct for bovine β-1,4-GalT (pET29b_b4GalT1Δ129 C342T) was kindly provided by Christelle Breton (Grenoble). The protocol for renaturation of inclusion bodies and refolding of β-1,4-GalT was adapted from Ramakrishnan *et al.*.[30] The malachite green reagents were prepared according to the method of Veldhoven *et al.,*[18] as described in the Supplementary Information. Absorbance measurements were carried out on a BMG Labtech POLARstar Optima multiplate reader.

General assay protocol. The colorimetric glycosyltransferase assay (see Scheme 1) was adapted from Wu et al.[15] All assays were carried out in Nunc clear, flat-bottom 96-well plates under the following, optimised conditions: the total volume per well was 150 µL and comprised of buffer (β-1,4-GalT: 13 mM HEPES, 50 mM KCl, pH 7.0; LgtC: 13 mM HEPES, pH 7.0), $MnCl_2$ (5 mM), chicken egg-white lysozyme (1 mg/ml), calf-intestinal phosphatase (10 U/mL), acceptor (LgtC: lactose at 2 mM; β-1,4-GalT: GlcNAc at 10 mM), glycosyltransferase, UDP-Gal and either inhibitor in DMSO (10% v/v) or DMSO only (10% v/v). The concentrations of glycosyltransferase, donor and inhibitor were varied depending on the experiment (Table 3). CSG164 and uridine were added in 100% DMSO to afford a final DMSO concentration of 10% v/v DMSO. 5-FT UDP-Gal is not soluble in DMSO and was

added in the appropriate buffer. In the case of 5-FT UDP-Gal, 10% v/v DMSO was therefore incorporated into the buffer master mix.

First, $MnCl_2$, lysozyme, CIP, glycosyltransferase, acceptor (replaced with buffer in all background wells) and buffer (for kinetic experiments) or inhibitor (for inhibition experiments) were added in 15 µL aliquots of the respective stock solution (10x assay concentration). The solution was made up to 135 µL with the appropriate buffer. To start the reaction, UDP-Gal was added in 15 µL aliquots of the stock solution (10x assay concentration). On each plate, a UDP calibration curve (0-12.5 µM) was included. Wells for the calibration curve included all components of the standard reaction except for the acceptor, and UDP instead of UDP-Gal. To start the reaction, UDP was added in 15 µL aliquots of the stock solution (10x assay concentration). The reaction was incubated for 20 min at 30 ºC, and stopped by the addition of 30 µL malachite reagent A. After thorough shaking, 30 µL of malachite reagent B was added and the colour allowed to develop over 20 min at 30 ºC. The absorbance at 620 nm was recorded and the data used to calculate initial reaction velocities for each well.

< Table 3 here >

Data processing. ***Standard procedure for the collection and analysis of kinetic data***: Absorbance at 620 nm (AU) was converted to [UDP] (µM) using linear regression from a calibration curve (0-12.5 µM) constructed for each experiment. The background value for each UDP-Gal concentration (no acceptor, but otherwise identical components) was subtracted from each data point. After background correction, the calculated concentration of UDP was divided by 20 to give the

velocity of the reaction at each concentration of UDP-Gal. This data was fitted to a Michealis-Menten curve using GraphPadPrism 6 software. Averages and standard deviations were calculated in Microsoft Excel.

Standard procedure for the collection and analysis of inhibition data: Absorbance at 620 nm (AU) was converted to [UDP] (μM) using linear regression from a calibration curve (0-12.5 μM) constructed for every experiment. A negative control (0 μM inhibitor) and a blank (0 μM inhibitor, 0 μM acceptor) were included on each plate in triplicate. After linear regression, subtracting the blank from the negative control afforded the assay window. The background value for each inhibitor concentration (no acceptor, but otherwise identical components) was subtracted from each inhibitor concentration data point. Once corrected for the background, the absorbance in the presence of each inhibitor concentration was divided by the assay window and represented as a percentage. This percentage was plotted against log [inhibitor] and analysed using GraphPadPrism 6 software to afford relative IC_{50} values,[29] if the data represented a sigmoidal curve. Averages and standard deviations were calculated in Microsoft Excel.

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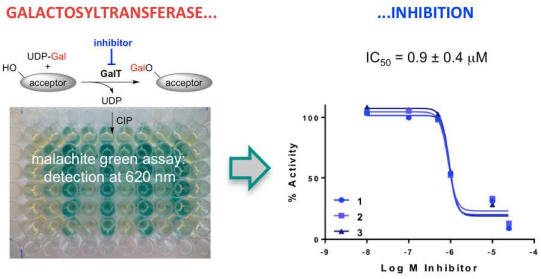

An operationally simple, colorimetric assay protocol for the identification and evaluation of galactosyltransferase inhibitors is described.