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Supplementary Information

A practical glycosyltransferase assay for the identification of new inhibitor chemotypes

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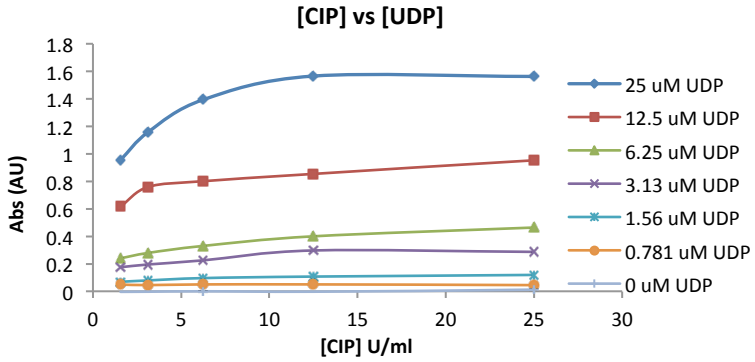
Table of contents

Preparation of malachite green reagents.

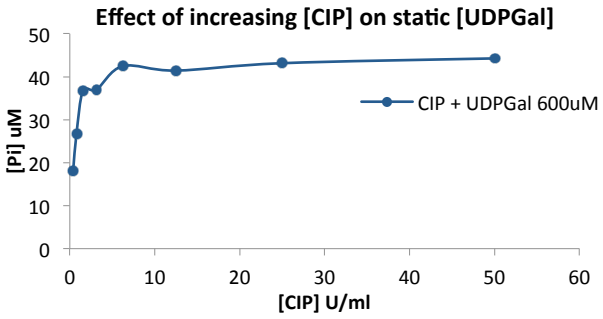
The malachite green reagents were prepared according to the method of Veldhoven et al.[1] Briefly, the Malachite A reagent is composed of 3.15 M sulfuric acid and 14.2 mM ammonium molybdate tetrahydrate. The Malachite B reagent is composed of 0.35% w/v polyvinyl alcohol (98-99% hydrolysed; low molecular weight) and 0.38 mM malachite green oxalate. Acid washed glassware was used to ensure removal of any phosphate. The A reagent was prepared by diluting the sulfuric acid into water, allowing to cool, adding the molybdate and making up to the required volume. The B reagent was prepared by adding the PVA to distilled water and microwave heating to dissolve the PVA. It was then cooled to room temperature and the malachite green oxalate added and made up to the desired volume.

Figure S1 Optimisation of CIP concentration[a]



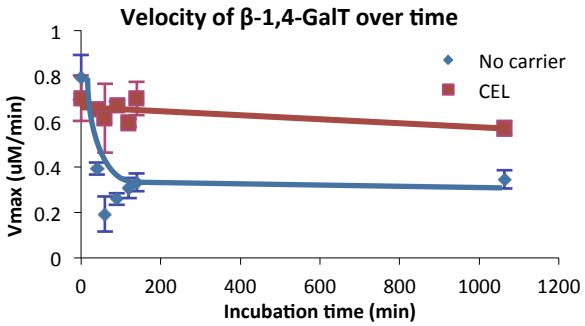
[a]*Conditions*: 13 mM HEPES, 50 mM KCl, pH 7.0, 10 mM GlcNAc, 5 mM $MnCl_2$. CIP was analysed at 1.56 - 25 U/mL. UDP was analysed at 0.781 - 25 µM. Each combination of [CIP] and [UDP] underwent a 20 minute incubation at 30 °C before development with malachite green reagents. At 25 U/mL, all UDP concentrations were stable. *NB* that this experiment was carried out without CEL and thus more CIP was used than in later experiments under optimised conditions

Figure S2 Effect of increasing [CIP] on 600 µM UDP-Gal[a]



[a]*Conditions*: 13 mM HEPES, 50 mM KCl, pH 7, 10 mM GlcNAc, 5 mM $MnCl_2$. 600 µM UDP-Gal was incubated with increasing [CIP] and incubated for 20 minutes at 30 °C before development with malachite green reagents. There is never more than 43 µM of P_i released, indicating that there is a ~7% P_i impurity (eg. 3.5% if UDP). The results also clearly show that UDP-Gal is not a substrate of CIP.

Figure S3 Time course data for β-1,4GalT[a]

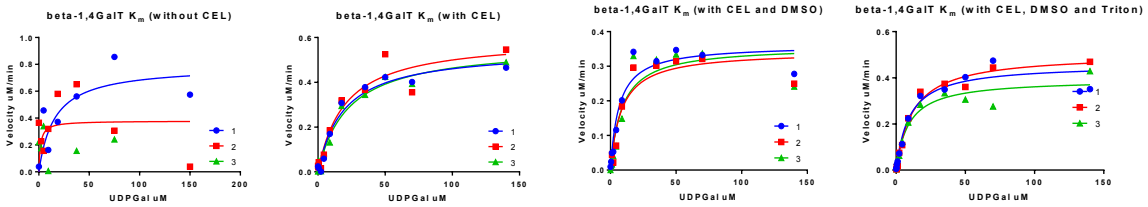


[a]*Conditions*: 13 mM HEPES, 50 mM KCl, pH 7, 5 mU/mL β-1,4-GalT, 10 mM GlcNAc, 5 mM $MnCl_2$, 25 mU/mL CIP, 150 μM UDP-Gal. Incubation at 30 °C was carried out at increasing time points and the velocity of the β-1,4-GalT determined by linear regression and dividing the recorded [UDP] concentration by time (minutes). CEL shows a large protectant effect on the β-1,4-GalT.

Figure S4 Effect of CEL, DMSO and Triton-X 100 on K_m and K_{cat}

Each graph shows one independent experiment carried out on a separate microplate. Where 3 sets of data are shown, the experiment was carried out in triplicate. Where 2 sets of data are shown, the experiment was carried out in duplicate. 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 experiments included 10 U/mL CIP, and were incubated for 20 mins at 30 °C, followed by development with the malachite green reagents. For kinetic data (K_m, k_{cat}) extracted from these experiments see Tables.

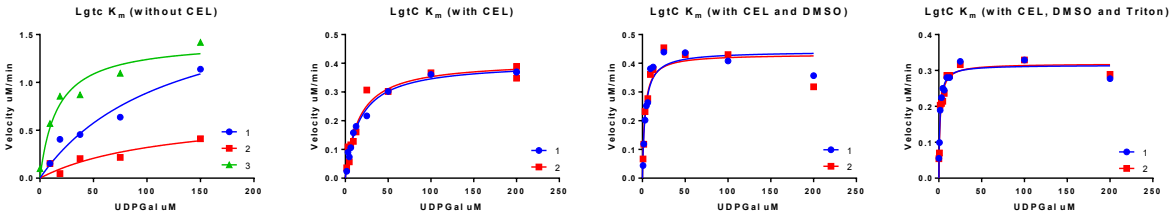
(a) β-1,4GalT



	Additive			Literature
	CEL[a]	CEL[a] + DMSO[b]	CEL[a] + DMSO[b] + Triton[c]	
K_m (μM)[d]	20.4 ± 1.8	8.8 ± 6.3	13.3 ± 2.5	25[2]
	34.4 ± 6.5	7.9 ± 1.2	16.2 ± 2.1	28[3]
	11.3 ± 2.7	15.1 ± 2.3	10.2 ± 1.6	
		1.5 ± 0.6		
		11.1 ± 3.7		
k_{cat} (s^{-1})[d]	0.595 ± 0.026	0.209 ± 0.070	0.464 ± 0.011	3.6[4]
	0.477 ± 0.030	0.369 ± 0.010	0.693 ± 0.103	0.435[4]
	0.336 ± 0.033	0.539 ± 0.093	0.470 ± 0.057	
		0.075 ± 0.008		
		0.263 ± 0.023		

[a]1 mg/mL CEL; [b]10% v/v DMSO; [c]0.01% v/v Triton-X 100; [d]Individual K_m and k_{cat} values are averages ± S.D. from n = 3 experiments. Data from experiments without added CEL are not included in the table as they were variable and did not afford values that fitted Michaelis-Menten curves.

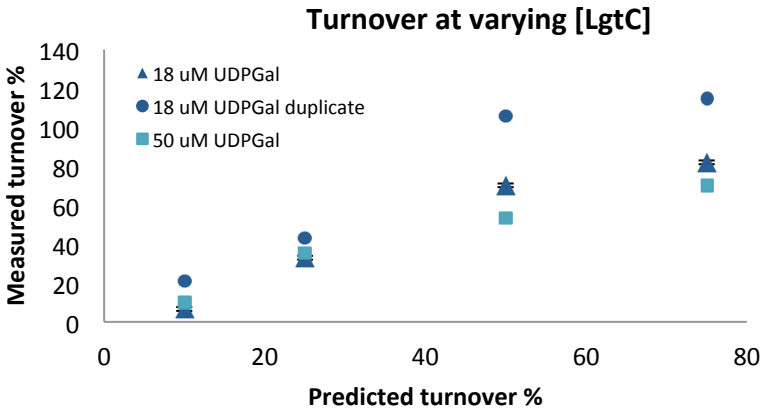
(b) LgtC



	Additive			Literature
	CEL[a]	CEL[a] + DMSO[b]	CEL[a] + DMSO[b] + Triton[c]	
K_m (μM)[d]	16.2 ± 0.8[e] 16.6 ± 2.0[e] 21.1 ± 0.3[e]	8.8 ± 6.3 7.9 ± 1.2 15.1 ± 2.3 1.5 ± 0.6 11.1 ± 3.7 3.5 ± 1.9 3.8 ± 0.3[e] 3.1 ± 0.3[e] 4.0 ± 0.4[e] 3.6 ± 0.7[e]	1.5 ± 0.2[e] 8.0 ± 5.2[e] 3.0 ± 0[e]	18[5] 20[6] 4.4[7]
k_{cat} (s^{-1})[d]	0.358 ± 0.034 0.352 ± 0.043 0.339 ± 0.010	0.030 ± 0.003 0.060 ± 0.005 0.147 ± 0.085 0.083 ± 0.014 0.041 ± 0.010 0.339 ± 0.047[e] 0.322 ± 0.061[e] 0.352 ± 0.058[e] 0.289 ± 0.005[e]	0.311 ± 0.082[e] 0.383 ± 0.108[e] 0.271 ± 0.009[e]	14.2[5] 24[6]

[a]1 mg/mL CEL; [b]10% v/v DMSO; [c]0.01% v/v Triton-X 100; [d]Individual K_m and k_{cat} values are averages ± S.D. from n = 3 experiments unless stated otherwise; [e]n = 2. Data from experiments without added CEL are not included in the table as they were variable and did not afford values that fitted Michaelis-Menten curves.

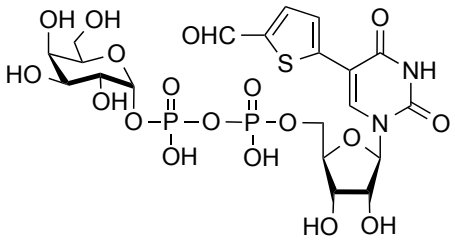
Figure S5 Predicted *vs* actual turnover rates for LgtC[a]



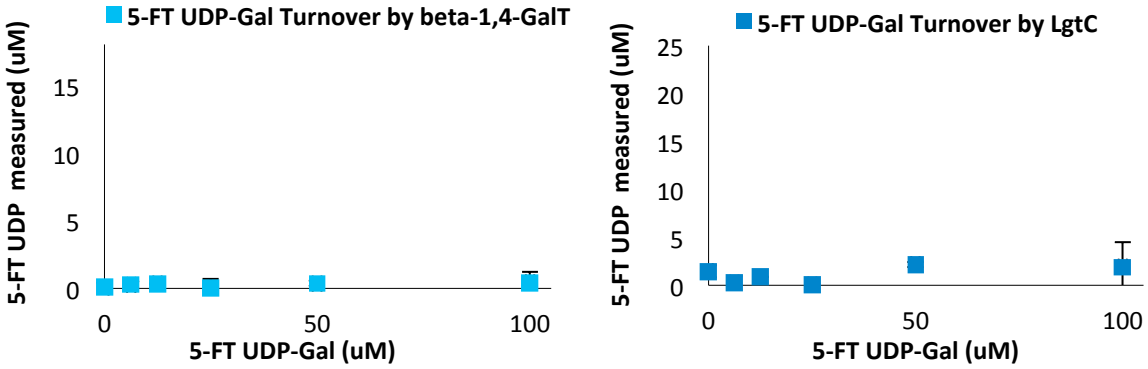
[a]*Conditions*: experiments were conducted with various concentrations of LgtC, 18 μM or 50 μM UDP-Gal as the donor, 5 mM $MnCl_2$, 2 mM lactose as the acceptor, 13 mM HEPES, pH 7.0, 10 U/mL CIP, 1 mg/mL CEL, 10% v/v DMSO and 0.01% v/v Triton-X 100. Reactions were incubated at 30 °C for 20 mins before development with malachite reagents. Turnover was predicted from previously measured enzyme activity.

Figure S6 Control experiments with 5-FT UDP-Gal

(a) Structure of 5-FT UDP-Gal

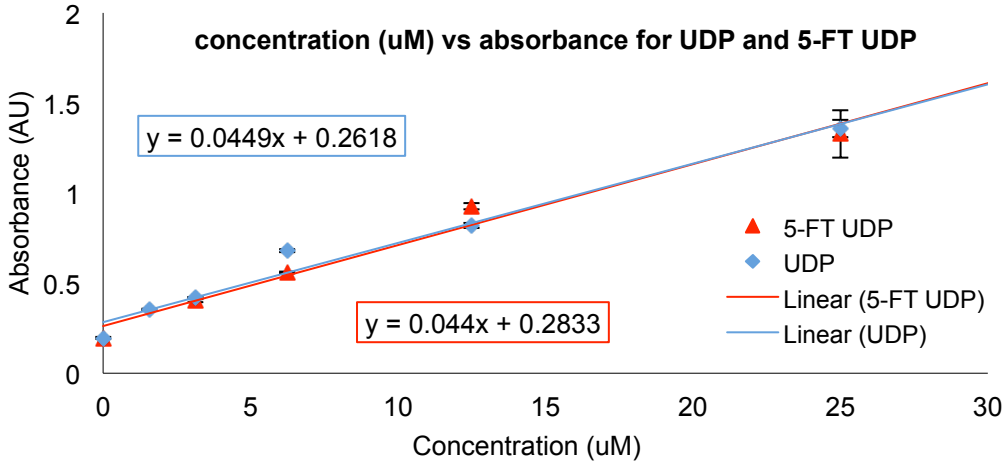


(b) 5-FT-UDP-Gal is not a substrate of β-1,4-GalT or LgtC[a]



[a]Experiments were conducted with 13 mM HEPES (pH 7.0), 5 mM $MnCl_2$, 10 U/mL CIP, 1 mg/mL CEL, 10% v/v DMSO, 0.01% Triton-X-100 and either 0.4 mU/mL β-1,4-GalT and 50 mM KCl, or 1 mU/mL LgtC. Reactions were incubated at 30 °C for 20 mins before development with malachite reagents. Data shown are averages from n = 2 experiments, with error bars representing standard deviation. Under these conditions, no significant formation of 5-FT UDP was observed.

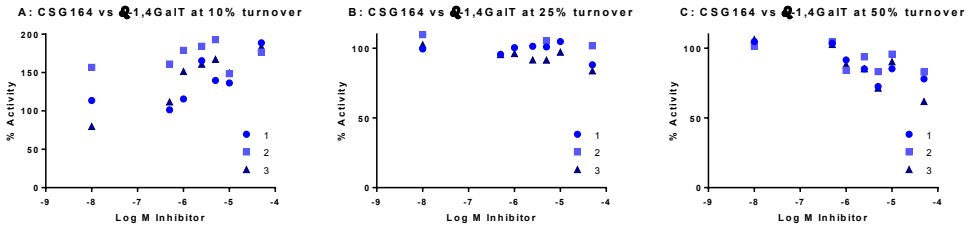
(c) 5-FT UDP is a substrate of CIP[a]



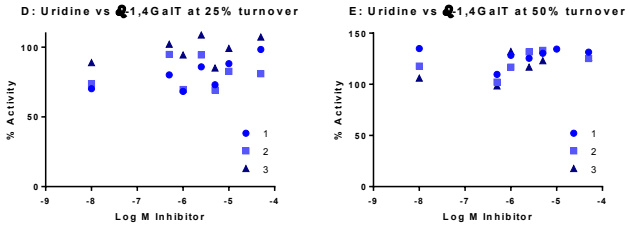
[a]Experiments were conducted with 13 mM HEPES (pH 7.0), 5 mM $MnCl_2$, 10 U/mL CIP, 1 mg/mL CEL, 10% v/v DMSO, and either UDP or 5-FT UDP (0-25 μM). Reactions were incubated at 30 °C for 20 mins before development with malachite reagents. Under these conditions, UDP and 5-FT UDP were used as a substrate by CIP with comparable efficiency.

Figure S7 β-1,4-GalT inhibition experiments with CSG164, uridine and 5-FT UDP-Gal[a]

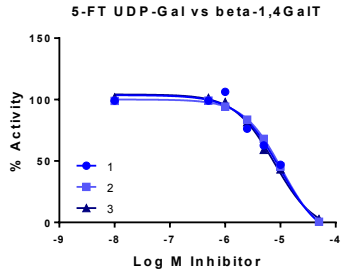
(a) CSG164[b]



(b) uridine[b]



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



[a]*Conditions*: each graph shows the results from an individual, representative experiment, carried out in triplicate (curves 1-3) on a single microplate. CSG164 was tested at concentrations from 0.01-50 μM, at three different turnover rates for β-1,4-GalT: 0.066 mU/mL (**A**), 0.166 mU/mL (**B**), and 0.332 mU/mL (**C**). Uridine was tested at concentrations from 0.01-50 μM, at two different turnover rates for β-1,4-GalT: 0.166 mU/mL (**D**) and 0.332 mU/mL (**E**). 5-FT-UDP-Gal was tested at concentrations from 0.01-50 μM, at 0.13 mU/mL β-1,4-GalT. [b]No inhibition under any of the conditions tested. [c]IC_{50}: 9.0 ± 1.4 μM (average ± S.D. of the triplicates in this experiment), donor turnover: 29%.

References

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