3'-*O*-β-Glycosylation of nucleoside analogues using a promiscuous bacterial glycosyltransferase

Jonathan P. Dolan,[1] Tessa Keenan,[2] Caecilie M. M. Benckendorff,[1] Aisling Ní Cheallaigh,[1] Martin A. Fascione[2] and Gavin J. Miller[1]*

[1]School of Chemical & Physical Sciences and Centre for Glycoscience, Keele University, Keele, Staffordshire, ST5 5BG, UK.
[2]Department of Chemistry, University of York, York, YO10 5DD, UK.
*Author for correspondence: g.j.miller@keele.ac.uk

Abstract

Nucleoside analogue therapeutics have a proven capability within drug discovery as antimicrobial, antiviral and antineoplastic agents. However, their efficacy can be limited by poor cellular uptake, high off target toxicity and poor bioavailability. Prodrugs of such analogues contribute to an improved pharmacokinetic profile. Herein, we explore biocatalytic glycosylation of nucleoside analogues. The activity of the nucleoside-specific 3'-*O*-glycosyltransferase AvpGT from *Streptomyces sp. AVP053U2* is investigated against a panel of both natural and clinically relevant purine and pyrimidine nucleoside analogues. AvpGT demonstrates broad substrate promiscuity, with 16 of 22 nucleosides tested showing glycosylation by HILIC-MS. Of these, 13 nucleosides were successfully glycosylated on 25 μmol scale in 39-91% yields, including four nucleoside analogue therapeutics. Furthermore, a novel β-glucosidase, AvpGS, was identified from the same *Streptomyces sp.* strain, heterologously expressed, purified and shown to display high substrate promiscuity in subsequently removing glucose from the glycoconjugates.

Introduction

Nucleoside analogues, both synthetic and natural in origin, represent an essential class of small molecule pharmaceutical with broad ranging antimicrobial, antiviral and antitumour properties.[1–3] However, therapeutic intervention using nucleoside analogues can be limited by poor cellular uptake and down regulation of nucleoside transporters [e.g., human equilibrative nucleoside transporter 1 (hENT1)], low oral bioavailability, rapid degradation or clearance, development of resistance profiles and limited conversion to the active metabolite.[4,5] One strategy that has emerged to contend with some of these issues is a prodrug form of such molecules, perhaps best typified by the ProTide approach (e.g., sofosbuvir & remdesivir), which employs a 5'-phosphormamidite group, improving cell permeability and overcoming an often rate limiting initial phosphorylation step during conversion to the active nucleotide triphosphate.[6,7] Another strategy that has been explored in this prodrug context is glycosylation. Carbohydrates are abundant biomolecules and the attachment of sugars to active pharmaceuticals has been sought as a strategy to improve pharmacokinetic profiles, including for nucleoside analogues.[8,9]

Cancer cells are significantly reliant on aerobic glycolysis instead of oxidative phosphorylation.[10] As such, there is an upregulation of glycolytic enzymes, insulin-independent glucose transporters (GLUTs) and sodium-dependent glucose transporters (SGLTs) to facilitate the increased reliance on glucose as an energy source (also known as the Warburg effect).[11–13] Within solid tumours, hypoxic regions adapt by activating hypoxia-inducible factor (HIF), resulting in upregulation of a variety of genes including GLUT1.[14] Conversely, hypoxia and HIF activation has been proposed to down regulate expression of ENTs.[15,16] In humans, hENT1 is the main protein responsible for the transport of nucleosides, including many pharmaceutically relevant analogues such as gemcitabine and for which resistance profiles have rapidly developed.[17] This phenotypic change towards increased glucose uptake as a result of upregulated GLUT expression, has led to the widespread clinical use of ^{18}fluorodeoxyglucose positron-emission tomography (FDG-PET) as a method of detecting and staging

various cancers.[18,19] Glufosfamide[20] and an aroylhydrazone glycoconjugate prochelator[21] have both been demonstrated to rely on uptake by GLUT1 to facilitate cytotoxic activity.

With a view to developing a novel prodrug strategy for glycosylated nucleoside analogues, indicatively invoking uptake through glucose transporters and release by intracellular glycosidase activity (Figure 1), we explore herein the substrate profile of a 3'-*O*-β-nucleoside specific glycosyltransferase (GT) from *Streptomyces sp. AVP053U2* (AvpGT) against natural purine and pyrimidine nucleosides alongside a series of clinically relevant nucleoside analogue drugs.

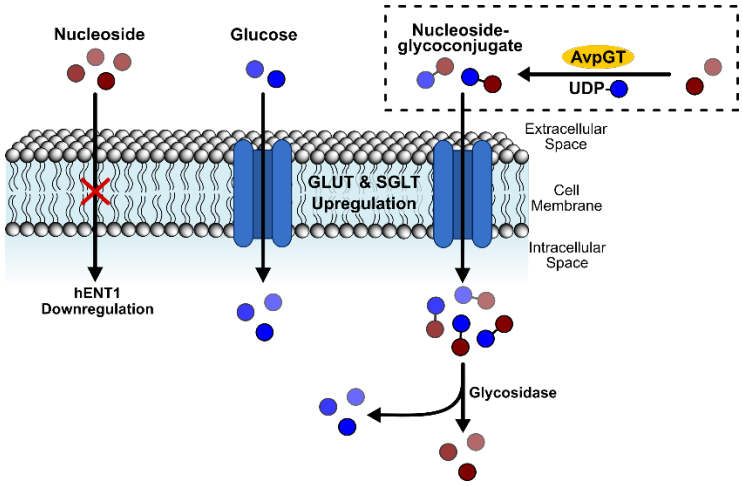


Figure 1: Targeted transport of nucleoside analogue prodrugs across a cell membrane utilising upregulated glucose-transporters followed by glycosidase induced release of nucleoside therapeutic. Boxed: Enzymatic glycosylation of nucleosides using bacterial nucleoside-specific glycosyltransferase (AvpGT).

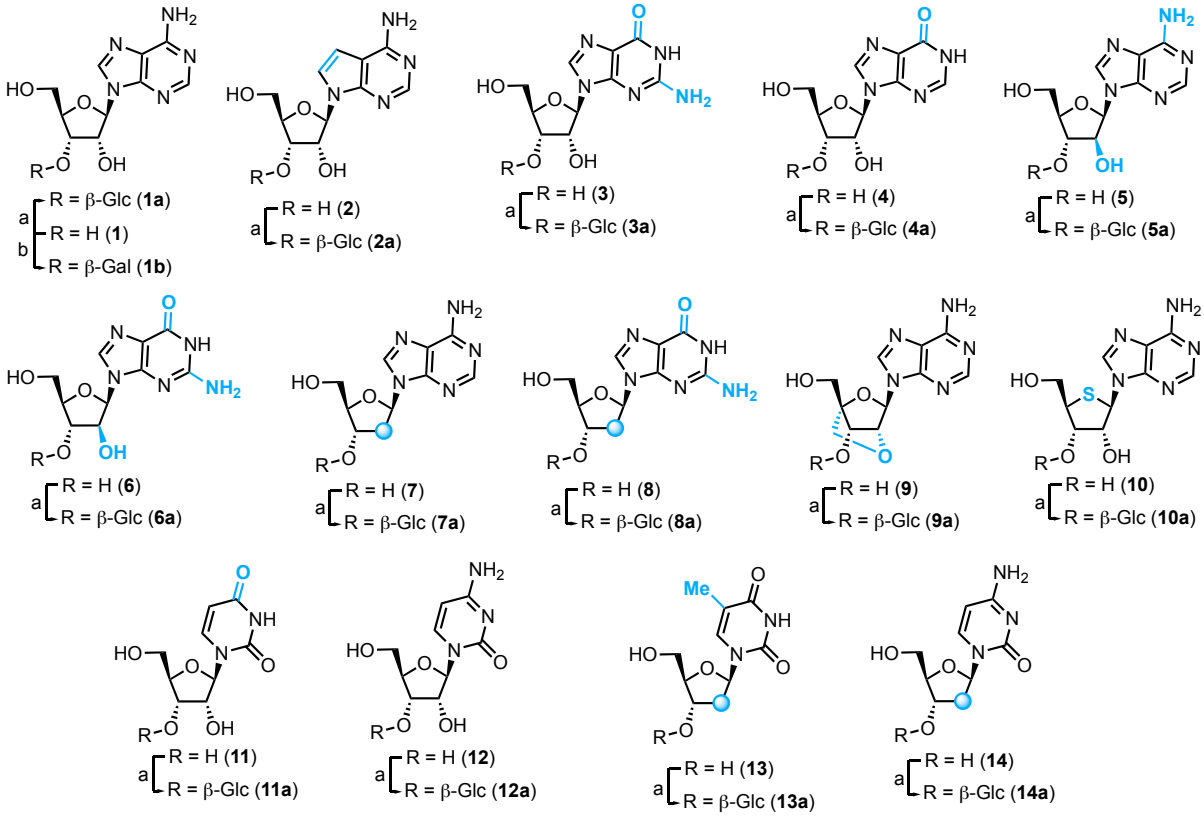
Results & Discussion

Only two nucleoside specific GTs have been identified and examined in detail: NucGT from *S. calvus,*[22,23] and AvpGT from *Streptomyces sp. AVP053U2*.[23] Both enzymes are members of the GT2 family,[24] whereby they catalyse glycosyl transfer through an inverting mechanism. Both NucGT and AvpGT share a high degree of sequence similarity, sharing 66% sequence identity.[23] AvpGT was selected, expressed and purified based on previously published protocols.[23] In the host species, AvpGT catalyses the 3'-*O*-glucosylation of tubercidin (7-deazadenosine, **2**), a potent antimycobacterial and antineoplastic agent produced by various species of *Streptomyces*.[25] Previously, work by Pasternak *et al.* showed AvpGT displayed a promiscuity towards 5'-modification and changes of purine nucleobase (adenosine & guanosine).[23] To further explore AvpGT substrate promiscuity, we sought to characterise activity against both purine and pyrimidine nucleosides in addition to a series of clinically relevant analogues, featuring modifications to both the nucleobase and ribose ring.

Exploring AvpGT Activity Profile Towards Purine & Pyrimidine Nucleosides

The specific activity of glycosyltransferase reactions was determined for a range of purine & pyrimidine nucleoside analogues (Table 1). Analysis was performed monitoring the release of uridine-diphosphate from the glucose donor, UDP-glucose, using strong anion exchange-HPLC. The formation of glycosylated nucleoside products was confirmed by HILIC-MS (see ESI, Figures S2-S18).

Table 1: Specific glycosyltransferase activity of AvpGT with purine and pyrimidine nucleosides **1–14**.



Entry	Substrate	Product	Specific Activity (mU mg^{-1})[a]	[AvpGT]	Time	% Yield[b] (isolated)
1	**1**	**1a**	940.00 ± 9.23	10 µM	16 h	85 (9.1 mg)
2	**1**	**1b**	239.30 ± 1.16	-	-	-
3	**2**	**2a**	1467.39 ± 7.36	10 µM	16 h	87 (9.3 mg)
4	**3**	**3a**	62.56 ± 0.81	10 µM	16 h	79 (8.9 mg)
5	**4**	**4a**	41.98 ± 1.22	10 µM	16 h	86 (9.3 mg)
6	**5**	**5a**	115.37 ± 0.85	10 µM	16 h	91 (9.8 mg)
7	**6**	**6a**	*n.q*[c]	-	-	-
8	**7**	**7a**	19.32 ± 0.28	-	-	-
9	**8**	**8a**	*n.q*[c]	-	-	-
10	**9**	**9a**	4.53 ± 0.19	-	-	-
11	**10**	**10a**	314.54 ± 5.67	-	-	-
12	**11**	**11a**	17.01 ± 0.18	30 µM	24 h	72 (7.3 mg)
13	**12**	**12a**	148.99 ± 1.24	10 µM	16 h	68 (6.9 mg)
14	**13**	**13a**	*n.o*[d]	-	-	-
15	**14**	**14a**	*n.o*[d]	-	-	-

[a]Assay conditions: Substrate (1 mM), UDP-Glc or UDP-Gal (1.5 mM), AvpGT (10 µM), Tris (50 mM, pH7.4), 100 mM NaCl, 10 mM DTT, 10 mM $MgCl_2$, 30 °C, 100 rpm, 1-60 mins. [b]Isolated yield following purification by semi-prep HPLC. [c]*n.q* – Not quantifiable: UDP release observed by SAX-HPLC (after 60 mins), and product formation <5% as observed by HILIC-MS (after 22 hrs). [d]*n.o* – No product formation observed by HILIC-MS (after 22 hrs). Blue circles on structures denote structural modification is 2'-deoxy.

Based on the kinetic parameters reported previously,[23] adenosine **1** and tubercidin **2** were superior substrates for AvpGT (Table 1, entries 1 and 3), with activity towards guanosine **3** reduced by ~15-fold compared to **1** (Table 1, entry 4). Switching the sugar nucleotide donor to UDP-galactose (UDP-Gal) was possible, with ~3-fold reduction in activity in forming **1b** (Table 1, entry 2), compared

to the ~4-fold reduction in activity reported previously.[23] The enzyme demonstrated no turnover of UDP-*N*-acetyl-glucosamine (UDP-GlcNAc) or UDP-glucuronic acid (UDP-GlcA) donors (see ESI, Figures S2-5). Whilst, changing the purine 6-position substitution from -NH_2 in **1** to C=O for inosine **4** showed a reduced activity (Table 1, entry 5), comparable to that observed for **3**. Next, we explored changing ring substitution pattern at the 2'-*O*-position. Using arabinoadenosine **5** resulted in an ~8-fold reduction in activity (Table 1, entry 6) and no quantifiable activity could be detected for arabinoguanosine **6** (Table 1, entry 7). Furthermore, removal of 2'-OH (2'-deoxy analogues, blue sphere) resulted in a ~50-fold reduction compared to **1** for 2'-deoxyadenosine **7** and again no quantifiable activity was measured for a guanosine variant **8** (Table 1, entries 8 and 9).

Overall, these initial results for purine analogues hint at an importance in maintaining hydrogen bonding capability and canonical D-ribo configuration at the 2'-position. 2-Position purine base substitution appears unfavourable (substrates **3**, **6** and **8**), whilst changes at the 6-position are better tolerated. Finally, for purines, we evaluated a locked adenosine analogue **9** (Table 1, entry 10) which showed low activity, alongside 4'-thioadensone **10**, which was active (Table 1, entry 11), albeit ~3-fold reduced compared to **1**. In addition, AvpGT demonstrated acceptance of both uridine **11** and cytidine **12** as substrates, with the latter showing ~9-fold higher activity over uridine (Table 1, entries 12 and 13). This combined with higher activity observed towards tubercidin **2** and adenosine **1** compared to guanosine **3** and inosine **4** suggests the presence of a hydrogen bond donor within the nucleobase (at C6 in purines and C4 in pyrimidines) may be a key interaction for activity. Lastly, and in alignment to results observed for the purine series, 2'-deoxygneation of D-ribose was not tolerated for thymidine **13** or cytidine **14** (Table 1, entries 14 and 15). Substrates **1**-**5**, **11** & **12** were selected for scale up and purified by semi-preparative RP-HPLC, delivering multimilligram quantities of glycosylated nucleosides **1a**-**5a**, **11a** & **12a** in isolated yields of 68-91%. The regioselectivity and stereoselectivity of AvpGT-mediated glycosylation was confirmed at 3'-*O*-β for each glycoconjugate using a combination of ^{1}H, ^{1}H-decoupled HSQC and ^{1}H-^{13}C HMBC NMR, illustrated for **2a** in Figure 2.

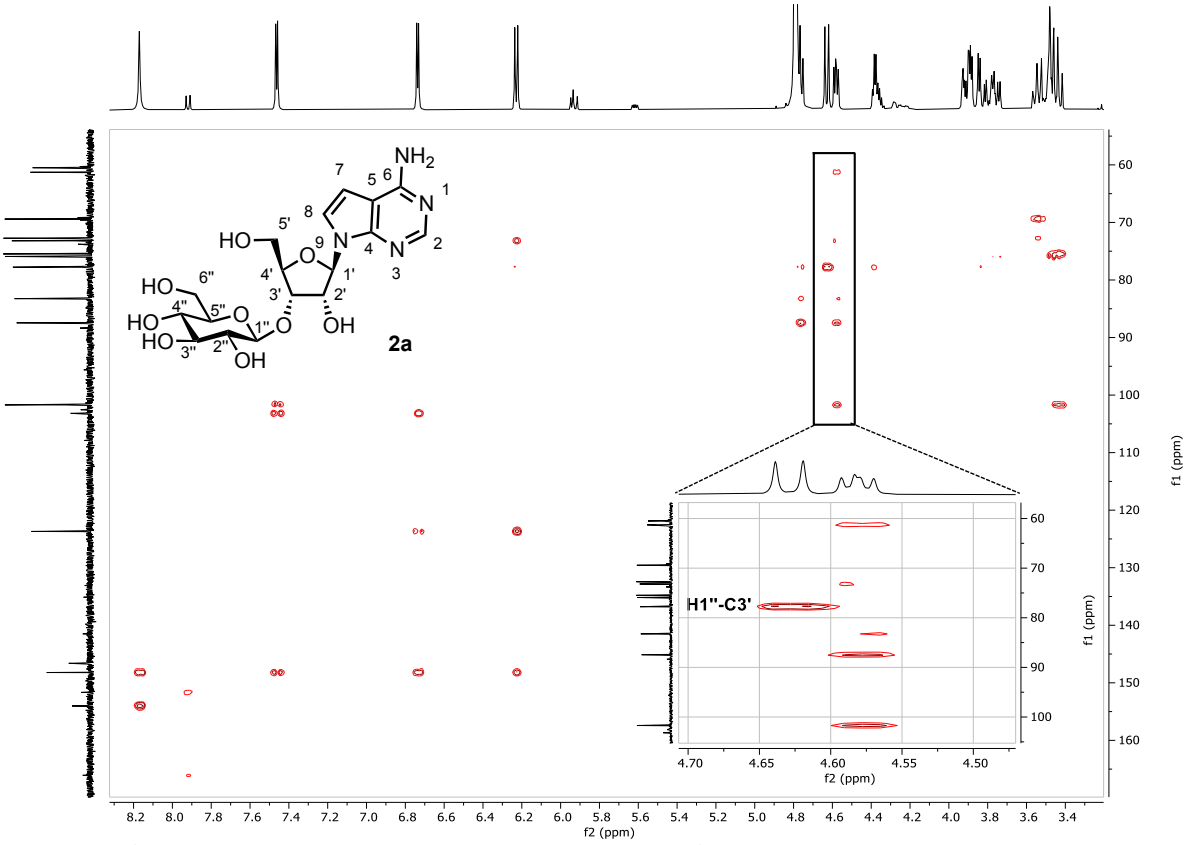
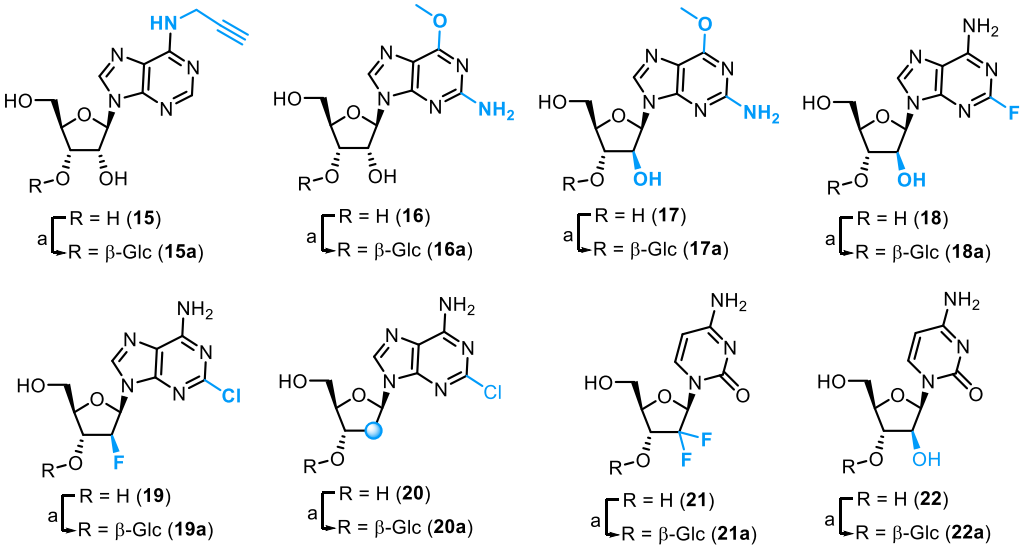


Figure 2: ^{1}H-^{13}C HMBC of 3'-O-β-glucosyl-tubercidin (**2a**) showing $^{3}J_{H1''\text{-}C3'}$ correlation between the 1'' proton on glucose and 3' carbon on ribose.

AvpGT Glycosylates Nucleoside Analogue Therapeutics

We sought next to prepare a range of 3'-*O*-glycosylated analogues of known nucleoside therapeutics. Analogues such as nelarabine **17**,[26] fludarabine **18**,[27] clofarabine **19**,[28,29] and cladribine **20**[30] act as antimetabolites and are approved treatments for lymphoblastic leukaemia, acute myeloid leukaemia, lymphocytic leukaemia and hairy cell leukaemia. In the case of nelarabine **17**, the compound is demethylated to the active compound Ara-G **6** by adenosine deaminase (ADA).[26] Gemcitabine **21**, an analogue of cytidine bearing a C2'-deoxy-2'-gemdifluoro modification, is currently the second most used anticancer fluorinated nucleoside analogue towards metastatic pancreatic, bladder, epithelial ovarian and breast cancers.[31] These nucleoside analogue therapeutics were examined using AvpGT and the previously established HILIC-MS and strong anion exchange-HPLC methods to detect and quantify product formation were employed (Table 2).

Table 2: Specific glycosyltransferase activity of AvpGT with clinically relevant nucleosides **15-23**.



Entry	Substrate	Product	Specific Activity (mU mg^{-1})[a]	[AvpGT]	Time	Isolated Yield[b] (%)
1	**15**	**15a**	141.03 ± 8.71	10 µM	16 h	88 (41.2 mg)
2	**16**	**16a**	331.46 ± 1.42	10 µM	16 h	88 (10.2 mg)
3	**17**	**17a**	42.51 ± 0.25	10 µM	16 h	88 (10.1 mg)
4	**18**	**18a**	12.71 ± 0.12	30 µM	24 h	64 (7.2 mg)
5	**19**	**19a**	2.85 ± 0.29	30 µM	24 h	39 (4.5 mg)
6	**20**	**20a**	*n.o*[c]	-	-	-
7	**21**	**21a**	1.76 ± 0.23	30 µM	24 h	91 (10.2 mg)
8	**22**	**22a**	*n.o*[c]	-	-	-

[a]Assay conditions: Substrate (1 mM), UDP-Glc (1.5 mM), AvpGT (10 µM), Tris (50 mM, pH7.4), 100 mM NaCl, 10 mM DTT, 10 mM $MgCl_2$, 30 °C, 100 rpm, 1-60 mins. [b]Isolated yield following reaction for 16 hrs and purification by semi-prep HPLC. [c]*n.o* – No product formation observed by HILIC-MS (after 22 hrs).

N-Propargylation at the 6-position of adenosine **15** was tolerated, but resulted in a moderate loss in activity (~7-fold, Table 2, entry 1). However, this result does support a prospect to utilise **15a** as a glycosylated nucleoside analogue probe for nucleic acid synthesis, as has been accomplished for **15**.[32] C6-*O*-methylation of guanosine **16** interestingly resulted in an ~5-fold restoration of activity, compared to guanosine (**3**, Table 2, entry 2). Inverting the 2-OH stereochemistry in substrate **16** to give nelarabine **17** showed an ~8-fold reduction in activity (Table 2, entry 3). Retaining this D-arabino configuration but switching to a C2-halogenated adenosine derivative **18** saw a further ~4-fold reduction in activity (Table 2, entry 4). A C2 arabinofluoro analogue with purine C2 halogenation **19** was the lowest performing analogue tested (Table 2, entry 5). Finally, in this series, 2'-deoxyribo cladribine **20** was not active with AvpGT (Table 2, entry 6). Taken together, these purine substrates

indicate an exciting and previously unestablished activity profile for AvpGT towards glycosylation of nucleoside analogues with C2 and C6 nucleobase modifications beyond those observed in canonical systems, alongside accepting a C2'-arabino configuration, but noting that C2'-fluorination or deoxygenation is not well tolerated. Switching to pyrimidine bases, gemcitabine (C2'-*gem*-difluoro) was active (Table 2, entry 7), albeit with ~75-fold lower activity that cytidine **12**. A 2'-arabino configured system, cytarabine **22** was not active (Table 2, entry 8). Comparatively, the results for pyrimidine systems, whilst not as widely explored, are particularly encouraging from the prospect of a novel C3'-glucosylated gemcitabine conjugate. With the exception of inactive analogues **20** and **22**, all substrates were scaled up and purified by semi-preparative RP-HPLC providing a library of glycosylated nucleoside therapeutics on 4.5-10.2 mg scales.

Exploring Glycosyl Hydrolase-Mediated Cleavage of Nucleoside Analogue Conjugates

As a preliminary study to explore the capability of our nucleoside analogue glycoconjugates, we examined their activity in combination with a commercially available cytosolic glucosidase. In humans the cytosolic glucosidase GBA3 (EC 3.2.1.21) is thought to hydrolyse xenobiotic glycosides in particular demonstrating activity against a variety of substrates with a β-glucose, β-galactose, β-xylose, or α-arabinose moiety linked to a hydrophobic aglycone.[33,34] However no hydrolysis was detected for any of the glycosylated nucleosides synthesised following incubation with GBA3 overnight (see ESI Figures S36 & 37). However, analysis of the genome of *Streptomyces sp. AVP053U2* revealed a putative glucosidase (*avpGS*) with homology to the GH1 family of glycosyl hydrolases.[24,35] Moreover it shared a 92% sequence identity (95% sequence similarity) to a recently disclosed glucosidase involved in the biosynthesis of nucleocidin, *nucGS*,[22] highlighting its potential activity on glucosylated nucleosides. Therefore the gene for *avpGS* was inserted into a pET28a plasmid and overexpressed in *E.coli* Rosetta2 cells. After purification by nickel column, soluble protein was obtained which migrated in line with the predicted molecular weight (54.4 kDa) upon analysis by SDS-PAGE (see ESI, Figure S27). The substrate promiscuity of AvpGS was then explored using HILIC-MS for substrates **1a**, **3a**, **12a** (Figure 3), **15a**, **17-19a** & **21a**, with quantitative hydrolysis observed for both natural and therapeutic nucleosides following overnight incubation at a concentration of 1 mol% (see ESI, Figures S28-S35).

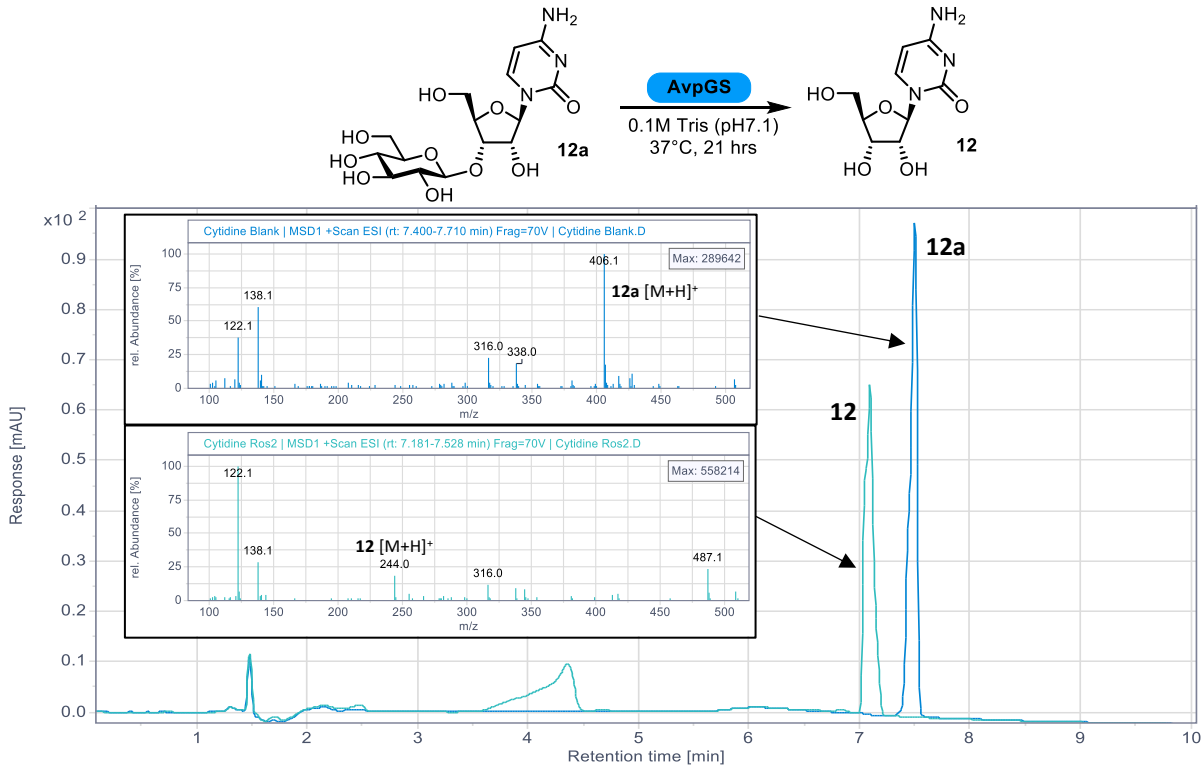


Figure 3: Hydrolysis of glucosylated cytidine (**12a**) to cytidine (**12**) following overnight incubation with AvpGS (1 mol%, teal trace); negative control reaction without AvpGS added (blue trace). Reaction followed by HILIC-DAD-MS and shows two A260 traces overlaid.

Conclusion

We have established capability for a bacterial glycosyltransferase to glycosylate a range of nucleosides, including both natural systems and analogues that are currently used clinically. We exemplify the utility of this enzymatic approach through scalable milligram access to thirteen glucosylated conjugates and further disclose preliminary studies regarding removal of the glucose moiety using a related bacterial glycosyl hydrolase. That none of the nucleoside glucoside conjugates tested were active towards a human glycosidase encourages further exploration of these bioconjugates in the context of developing prodrug approaches that target systems upregulated in the cancer microenvironment, such as GLUTs. Furthermore, an orthogonal activity of the bacterial hydrolase AvpGS to GBA3 posits an opportunity to explore target specific drug delivery systems, similar to those reported for lectin- and antibody-directed prodrug therapies.[36]

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Associated Content

The Supporting Information is available free of charge at:

Full experimental procedures for the expression of AvpGT & AvpGS, enzymatic glycosylation of nucleoside analogues; Analytical HPLC traces for enzymatic glycosylation of nucleoside analogues and glucosidase; Spectral NMR data (^{1}H, ^{13}C and ^{19}F NMR) & HRMS data available for all compounds.

Author Contributions

CRediT: **Jonathan P. Dolan** conceptualization, methodology, investigation, formal analysis, data curation, visualisation, writing-original draft, writing-review & editing;
Tessa Keenan methodology, investigation, formal analysis, data curation;
Caecilie M. M. Benckendorf methodology, investigation, formal analysis, data curation;
Aisling Ní Cheallaigh methodology, resources, supervision;
Martin A. Fascione conceptualization, methodology, writing-review & editing, supervision, project administration, funding acquisition;
Gavin J. Miller conceptualization, methodology, writing-original draft, writing-review & editing, visualisation, supervision, project administration, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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