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Glycosyloxyamine Neoglycosylation: A Model Study Using Calicheamicin

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Neoglycosylation is an efficient chemoselective glycosylation method enabled by a uniquely reactive alkoxyamine ‘handle’ which, distinct from primary or secondary alkylamines,[1] induces acid-catalyzed cyclization of reducing aldoses to provide desired closed-ring neoglycosides.[2] While a variety of *O*-substituted oxyamines (methyl, ethyl, allyl, benzyl, and acetoxy)[3] have been successfully employed in the context of neoglycosylation to increase the potency and/or alter the mechanism of various natural products and small molecules, the susceptibility of glycosyloxyamines toward neoglycosylation is unknown. While rare, this unique functional group is found within the two structurally-related natural products calicheamicin (CLM, **1** and **2**, Scheme 1) and esperamicin. In the case of CLM, the novel glycosyloxyamino functionality derives from P450-catalyzed oxidation of an aminosugar nucleotide precursor followed by sequential glycosyltransferase-catalyzed sugar attachments.[4–7] The corresponding CLM glycosyloxyamino-containing aryltri- or aryltetrasaccharide is critical for both binding to its biological target (DNA)[8] as well as recognition by self-resistance proteins within the native calicheamicin producer *M. echinospora*. [9] In both cases, this molecular recognition element helps position the CLM warhead for radical-based hydrogen abstraction reactions,[10] the outcome of which in the case of DNA is remarkably cytotoxic. Efforts to target this incredible toxicity toward cancer cells led to the first approved antibody-drug conjugate (ADC), gemtuzumab ozogamicin or Mylotarg.[11] However, undesired peripheral toxicity in patients, potentially due to lability of the antibody-CLM linker, led to the recent removal of Mylotarg from the market.[12] Using CLM as the model, herein we describe the first example of glycosyloxyamine neoglycosylation, thereby unveiling a new reaction of synthetic utility for this well-studied natural product class. In addition, assessment of the biological activity of the corresponding CLM neoglycosides reveals the analogs to retain sufficient potency. Thus, this study also offers a potentially new calicheamicin bioconjugation method which could reinvigorate CLM-based ADC development.[13]

To assess the ability of the glycosylhydroxyamine of CLM to act as a reactive handle, multiple CLMs were tested as substrates for neoglycosylation. The nature of the substitution within *O*-substituted oxyamines influences the reactivity, neoglycoside product distribution, and stability of final products.[3,14] For example, while relatively-electron rich

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hydroxyamines have a tendency toward the formation of open chain nitron saccharides,[3f] in situ *O*-acetylation leads to a dramatic switch to the desired cyclic neoglycoside (wherein formation of an electron poor acetoxyminium intermediate favors cyclization). Based upon this precedent, the proximal hemiacetal of the CLM glycosyloxyamine was anticipated to exert similar influence and thereby favor formation of the desired cyclic products. Using D-ribose as the representative reducing sugar, neoglycosylation with calicheamicin α_3^1 (CLM α , **1**), or the Mylotarg 'cargo' *N*-acetyl γ_1^1 (*N*-Ac CLM γ , **2**) under mild conditions (e.g., 1.5 equiv. HOAc in MeOH at 40 °C) successfully led to neo-D-ribosides **3** and **4**, respectfully (Scheme 1). ^1H NMR analysis revealed the β -anomer as the predominate product in both **3** and **4** (**3**, β : α 3:1; **4**, β : α 2:1), as commonly observed among neo-D-ribosides.[3e,f] The reaction was concentration-dependent. Specifically, reactions performed at 90 mM of CLM led to the desired products while more dilute reactions (10–20 mM aglycon) failed to provide the desired neoglycoside. While the added steric bulk in *N*-Ac CLM γ clearly did not prohibit the overall reaction, it is interesting to note that CLM α reacted more rapidly than *N*-Ac CLM γ under identical conditions.

Models of **3**-DNA and **4**-DNA complexes (Figure 1; Figure S1, Supporting Information), derived from previously-obtained structural data,[8e,15] suggest the neoribosyl moiety to project out from the minor groove and thereby not significantly impact the requisite CLM-DNA interaction. Based upon this model, the corresponding CLM neoglycosides were thereby anticipated to retain sufficient DNA cleavage activity and cancer cell cytotoxicity. To assess the impact of neoglycosylation upon the putative DNA interaction, neoglycoside **4** was first tested in the 'molecular break light' (MBL) assay, a high throughput fluorescent-based continuous assay for DNA cleavage.[16] Specifically, neoglycoside **4** was incubated in the presence of DTT and a MBL hairpin oligonucleotide previously demonstrated to be efficiently cleaved by CLM γ and the corresponding activity compared to parent aglycon **2** under identical conditions. Using this assay, 40 μM *N*-Ac CLM γ (**2**) efficiently cleaved DNA at a rate equivalent to ~5U of Dnase while the corresponding initial rate of DNA cleavage by neoglycoside **4** was ~7-fold lower than the parent **2** (roughly equivalent to the rate of DNA cleavage by aglycon **1** in this assay) (see Figure S2, Supporting Information).

Calicheamicins **1–4** were also assayed for their ability to inhibit cell growth using a range of cancer cell lines including lung (A549), prostate (Du145), breast (MCF7), ovarian (SKOV3) and colorectal (HT29). All CLMs displayed notable potency with values for parent aglycons (GI_{50} values ranging from 200 pM to 12 nM) outperforming the corresponding neoglycosides (GI_{50} values ranging from 4.7 nM to 190 nM) (Table 1). Of the two parent aglycons, the activity of the Mylotarg cargo (*N*-Ac CLM γ , **4**) was notably less influenced by neoglycosylation (with potency reductions ranging from 5 to 11-fold) consistent with a similarly reduced DNA cleavage ability for this agent based upon the MBL assay. In contrast to the MBL assay, CLM α was more cytotoxic than the *N*-Ac CLM γ counterparts, suggesting that the MBL hairpin utilized may not be optimized for CLM α recognition or other factors (e.g., cellular uptake and/or efflux) may contribute to the observed cytotoxicity differences among parents **1** and **2**. Yet, neoglycosylation abrogated these CLM α -specific contributors to enhanced cytotoxicity as *N*-Ac CLM γ neoglycoside **4** outperformed corresponding CLM α variant **3** in all but one cell line (MCF7, where the α and γ neoglycosides were essentially equipotent).

In summary, this study reveals, for the first time, the naturally-occurring glycosyloxyamino bond of CLM to be a substrate for neoglycosylation and to provide novel CLM neoglycosides which still display notable cytotoxicity. In the context of the neoglycosylation reaction, this study extends the scope of accessible functionality beyond alkoxyamine handles and opens the door for potential new applications in the synthesis of branched oligosaccharides. Moreover, given the corresponding neoglycosides generated in this study

display potencies consistent with that required for the cargo of antibody drug conjugates, [11b,13] this study exposes a previously unrecognized method for efficient CLM bioconjugation. Within this context, tether-modified reducing sugars are anticipated to enable conjugation to a wide range of cancer-selective targeting modalities including peptides, proteins, antibodies, lipids or transport-mediating metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Model of **4** bound to DNA. DNA depicted in gray, *N*-Ac CLM γ carbon backbone in aqua and appended D-ribose in yellow.

Scheme 1.
CLM α_3^I (1) and N-Acetyl CLM γ_1^I (2) neoglycosylation.

Table 1GI₅₀ values (nM) of CLM aglycons and CLM neoglycosides.

cell line	1	3 (neo-1)	2	4 (neo-2)
A549	0.29±0.03	51±4	0.88±0.09	11±1
Du145	0.20±0.01	24±1	0.43±0.04	4.7±0.4
MCF7	12±1	32±2	3.9±0.1	44±2
SKOV3	0.20±0.01	53±3	2.9±0.1	13±1
HT29	0.88±0.04	190±10	5.6±0.2	63±3