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Modifying the glycosidic linkage in digitoxin analogs provides selective cytotoxins

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Abstract—A chemoselective reaction between oxyamines and unprotected, unactivated reducing sugars was used to construct for the first time a panel of linkage-diversified neoglycosides. This panel of digitoxin analogs included potent and selective tumor cytotoxins; cytotoxicity was dependent on the structure of the glycosidic linkage. These results validate linkage diversification through neoglycosylation as a unique and simple strategy to powerfully complement existing methods for the optimization of glycoconjugates.

Glycoconjugates are vital probes for the exploration of glycobiology[1] and have long been used as powerful therapeutics.[2] Optimization of these molecules has typically involved modification of aglycons and attached sugars since both entities influence target recognition, selectivity, and pharmacology. Tailoring the atomic structure of the glycosidic linkage *between* aglycons and attached sugars represents a third distinct optimization strategy that has received little attention. We hypothesized that such structural variants would also provide compounds with enhanced activities, and employed the well-known model glycoside digitoxin (**1**) as a test platform. Here we validate our hypothesis by developing chemoselective glycosylation chemistry that readily supplies different glycosidic linkages and showing that some of the resulting digitoxin analogs are more selective tumor cytotoxins than the parent natural product.

Digitoxin (**1**) is a cardiac glycoside drug used to treat congestive heart failure and supraventricular arrhythmias through inhibition of plasma membrane Na^+/K^+-ATPase.[3] In addition to this well-known activity, several studies have suggested an enhancement in the survival rate of cancer patients taking cardiac glycosides.[4] Digitoxin has also been shown to display anticancer properties in vitro,[5] including the inhibition of cancer cell growth[6] and proliferation,[7] and the inducement of apoptosis.[7a] The sugar and aglycon region that encompasses the glycosidic linkage of cardiac glycosides such as **1** appears to strongly influence the rich spectrum of biological properties exhibited by these molecules.[3,8] Thus, digitoxin is an excellent system to test the influence of the intervening glycosidic linkage. However, creating structural variation at this position requires non-traditional glycosylation chemistry.

While most glycoconjugates are generated by labor intensive chemoenzymatic or synthetic methods,[9] many chemoselective ligation strategies have been employed to form glycosides quickly and conveniently.[10] One of the most successful methods is oxyamine neoglycosylation, the chemoselective reaction between secondary oxyamines and unprotected, unactivated reducing sugars to form stable 'neoglycosides' (Scheme 1).[11] This method has provided glycopeptide mimics,[12] oligosaccharide mimics,[13] and large libraries of natural product *MeON*-neoglycosides[14] including potent digitoxin-based cytotoxins (e.g., **3a**, IC_{50} = 18–59 nM).[14a] Since digitoxin neoglycosides containing a non-natural *MeON*-linkage displayed strong cytotoxicity, we suspected tuning the structure of such glycosidic linkages would modulate glycoconjugate activities. Testing this hypothesis required the development of oxyamine neoglycosylation to provide other *RON*-linkages.

Literature precedents suggest a variety of secondary oxyamines can be chemoselectively glycosylated to provide

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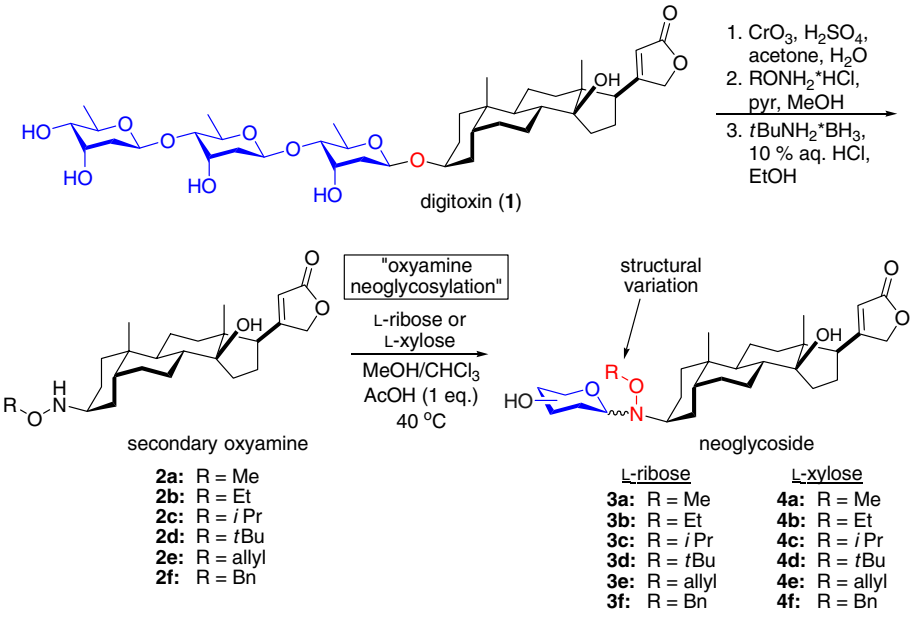

Scheme 1. Synthesis of oxyamines followed by oxyamine neoglycosylation.

hydrolytically stable glycosides without sugar activation or protection,[11,12] but in most published examples few oxyamines other than *methoxy*amines such as **2a** have been utilized, leading only to *MeON*-glycosidic linkages (e.g., **3a**).[13–15] To determine if neoglycosides with alternative linkages can be generated easily, we synthesized aglycons **2** from digitoxin in three simple steps[16] and incubated them in parallel with L-ribose and L-xylose, sugars that previously produced cytotoxic digitoxin neoglycosides.[14a] We were gratified to learn that under the optimized reaction conditions indicated in Scheme 1, all digitoxin aglycons containing non-methyl *O*-alkyl groups except for **2d** (R = *tert*-Bu) were compatible with oxyamine neoglycosylation, leading to a panel of 10 digitoxin analogs (**3a**–**c,e,f** and **4a**–**c,e,f**) containing different *RON*-glycosidic linkages.[17] The panel was purified in parallel via solid phase extraction on silica gel cartridges to remove unreacted aglycon and sugar, and LC–MS was used to confirm product identity and assess product purity. Similar to previous results with *MeON*-glycosides,[14] the average purity of the panel of neoglycosides as estimated by LC–MS was 87%, and all but one compound (**3b**) contained greater than 90% of a single product isomer. The isolated yields for these compounds ranged from 7% to 61% (average 30%).[18] To our knowledge, this represents the first panel of natural products that contain diversified neoglycosidic linkages.

The anticancer activities of the panel and digitoxin (**1**) were assessed using a high-throughput cytotoxicity assay on four human cancer cell lines, representing lung, colorectal, and ovarian carcinomas, as well as NCI/ADR-RES, a drug-resistant ovarian carcinoma line (Fig. 1).[19] Our data clearly show that the structure of the glycosidic linkage affects both the potency and selectivity of neoglycosides. Digitoxin displayed strong, non-specific cytotoxicity toward three of the four cell lines tested, and *MeON*-glycosides **3a** and **4a** displayed a non-specific

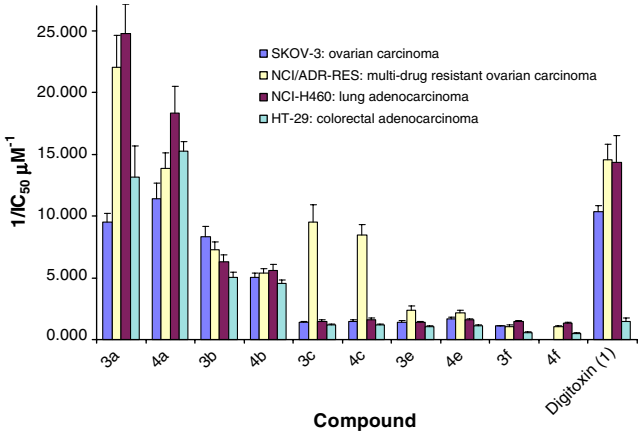

Figure 1. Summary of IC_{50} data from cytotoxicity assays. Reciprocal IC_{50} values are displayed for clarity; standard errors are depicted with error bars. In the assay, live cells are quantified by measuring the luminescent signal resulting from the reaction between cellular ATP and luciferin to form oxyluciferin. The IC_{50} value for each compound represents at least four replicates of dose–response experiments conducted over six concentrations at twofold dilutions. The IC_{50} of **4f** against HT-29 cells was >1 μM.

cytotoxicity profile similar to digitoxin, in accord with previous results.[14a] However, *EtON*-, *AllylON*-, and *BnON*-glycosides (**3b**/**4b**, **3e**/**4e**, and **3f**/**4f**, respectively) were markedly less potent cytotoxins than digitoxin or *MeON*-glycosides **3a** and **4a**; like digitoxin, **3a**, and **4a**, they were also non-specific.

Most significantly, *i-PrON*-glycosides **3c** and **4c** displayed enhanced selectivity relative to digitoxin and *MeON*-glycosides **3a** and **4a**. Specifically, while **3c** and **4c** were only modestly cytotoxic toward lung, colorectal, and ovarian carcinomas, these molecules were 5–6 times more potent cytotoxins against NCI/ADR-RES cells (IC_{50} = 110 ± 20 nM and 120 ± 10 nM, respectively) than any other cell line tested. To the best of our knowl-

edge, this is the first observation of cell line selectivity resulting from a simple alteration of glycosidic linkage. This outcome is particularly important since NCI/ADR-RES is a multi-drug resistant line that has high levels of P-glycoprotein expression.[19] While cardiac glycosides are generally substrates for P-glycoprotein,[21] such tumor specificity suggests that **3c** and **3d** may no longer serve as P-glycoprotein substrates. Alternatively, the *i-PrON*-glycosides may be interacting with a unique cellular target. Supporting this notion, neoglycosides have previously been shown to be significantly less potent Na^+/K^+-ATPase inhibitors in HEK-239 human embryonic kidney cells than digitoxin.[14a] Also, digitoxin itself can target cellular components in addition to Na^+/K^+-ATPase[21] such as the TNF-α/NF-κB signaling pathway, a regulator of inflammation responses relevant to cancer therapy.[22] Neoglycosides **3a**–**c** and **4a**–**c** were more potent cytotoxins than the corresponding aglycons (**2a**–**c**), confirming the importance of sugar attachments to the cytotoxicity of cardiac neoglycosides (data not shown).

In summary, we harnessed a mild, chemoselective reaction between oxyamines and unprotected, unactivated reducing sugars to construct for the first time a panel of linkage-diversified neoglycosides. This modestly-sized panel of digitoxin analogs included selective tumor cytotoxins, validating linkage diversification through neoglycosylation as a unique and simple strategy to powerfully complement existing methods for the optimization of glycoconjugates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.058.

References and notes

1. (a) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357; (b) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873; (c) Sawa, M.; Hsu, T.-L.; Itoh, T.; Sugiyama, M.; Hanson, S. R.; Vogt, P. K.; Wong, C.-H. *Proc. Natl. Acad. Sci.* **2006**, *103*, 12371; (d) van Kasteren, S. I.; Kramer, H. B.; Jensen, H. H.; Campbell, S. J.; Kirkpatrick, J.; Oldham, N. J.; Anthony, D. C.; Davis, B. G. *Nature* **2007**, *446*, 1105.
2. (a) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461; (b) Clardy, J.; Walsh, C. *Nature* **2004**, *432*, 829; (c) Thorson, J. S.; Hosted, T. J., Jr.; Jiang, J.; Biggins, J. B.; Ahlert, J. *Curr. Org. Chem.* **2001**, *5*, 139.
3. Paula, S.; Tabet, M. R.; Ball, W. J., Jr. *Biochemistry* **2005**, *44*, 498.
4. (a) Stenkvist, B. *Anti-Cancer Drugs* **2001**, *12*, 635; (b) Haux, J.; Klepp, O.; Spigset, O.; Tretli, S. *BMC Cancer* **2001**, *1*, 11.
5. Johansson, S.; Lindholm, P.; Gullbo, J.; Larsson, R.; Bohlin, L.; Claeson, P. *Anticancer Drugs* **2001**, *12*, 475.
6. López-Lázaro, M.; Pastor, N.; Azrak, N.; Ayuso, M. J.; Austin, C. A.; Cortés, F. *J. Nat. Prod.* **2005**, *68*, 1642.
7. (a) Haux, J. *Medical Hypotheses* **2002**, *59*, 781; (b) Yeh, J. Y.; Huang, W. J.; Kan, S. F.; Wang, P. S. *J. Urol.* **2001**, *166*, 1937.
8. For a flexible synthetic approach that provides ready access to mono- and disaccharide analogs see: (a) Zhou, M.; O'Doherty, G. A. *J. Org. Chem.* **2007**, *72*, 2485; (b) Zhou, M.; O'Doherty, G. A. *Org. Lett.* **2006**, *8*, 4339; (c) Zhou, M.; O'Doherty, G. A. *Org. Lett.* **2006**, *8*, 5677.
9. (a) Thibodeaux, C. J.; Melancon, C. E.; Liu, H.-W. *Nature* **2007**, *446*, 1008; (b) Blanchard, S.; Thorson, J. S. *Curr. Opin. Chem. Biol.* **2006**, *10*, 263; (c) Langenhan, J. M.; Griffith, B. R.; Thorson, J. S. *J. Nat. Prod.* **2005**, *68*, 1696; (d) Griffith, B. R.; Langenhan, J. M.; Thorson, J. S. *Curr. Opin. Biotechnol.* **2005**, *16*, 622.
10. Langenhan, J. M.; Thorson, J. S. *Curr. Org. Synth.* **2005**, *2*, 59, and references cited therein.
11. Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269.
12. (a) Carrasco, M. R.; Brown, R. T.; Doan, V. H.; Kandel, S. M.; Lee, F. C. *Biopolymers* **2006**, *84*, 414; (b) Carrasco, M. R.; Brown, R. T. *J. Org. Chem.* **2003**, *68*, 8853; (c) Carrasco, M. R.; Brown, R. T.; Serafimova, I. M.; Silva, O. *J. Org. Chem.* **2003**, *68*, 195; (d) Carrasco, M. R.; Nguyen, M. J.; Burnell, D. R.; MacLaren, M. D.; Hengel, S. M. *Tetrahedron Lett.* **2002**, *43*, 5727.
13. (a) Peri, F.; Nicotra, F. *Chem. Comm.* **2004**, 623; (b) Peri, F.; Jimenez-Barbero, J.; Garcia-Aparicio, V.; Tvaroska, I.; Nicotra, F. *Chem. Eur. J.* **2004**, *10*, 1433.
14. (a) Langenhan, J. M.; Peters, N. R.; Guzei, I. A.; Hoffman, F. M.; Thorson, J. S. *Proc. Natl. Acad. Sci.* **2005**, *102*, 12305; (b) Ahmed, A.; Peters, N. R.; Fitzgerald, M. K.; Watson, J. A., Jr.; Hoffmann, F. M.; Thorson, J. S. *J. Am. Chem. Soc.* **2006**, *128*, 14224; (c) Griffith, B. R.; Krepel, C.; Fu, X.; Blanchard, S.; Ahmed, A.; Edmiston, C. E.; Thorson, J. S. *J. Am. Chem. Soc.* **2007**, *129*, 8150.
15. The distribution of pyranose, and sometimes furanose, anomers in these glycosides is dependent on sugar identity, but usually a single isomer predominates (see Ref. 14a).
16. Example procedure: Digitoxigenone (see Ref. 14a) (1.82 g, 4.89 mmol) was dissolved in methanol (11 mL, 2.2 mL/mmol) and pyridine (0.87 mL, 10.8 mmol). Methoxyamine hydrochloride (0.653 g, 7.82 mmol) was added, and the solution was stirred for 2.5 h and then concentrated. The resulting residue was dissolved in CH_2Cl_2 and washed with 1 M HCl, brine, dried over $MgSO_4$, filtered, and then concentrated. The mixture of oxime diastereomers was suspended in ethanol (7 mL, 0.7 mL/mmol) and cooled to 0 °C. Borane *tert*-butylamine complex (1.40 g, 16.2 mmol) was added, followed by the dropwise addition of 10% aq HCl (13.2 mL, 2.7 mL/mmol). The reaction mixture was stirred at 0 °C for 2.5 h. After this time, Na_2CO_3 was added until gas evolution ceased, and the mixture was partitioned between water and $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The resulting diastereomeric mixture was purified by SiO_2 column chromatography eluting with 3:2 EtOAc/hexane to elute **2a** (TLC $R_f = 0.33$ in 3:2 EtOAc/hexane). ^1H NMR ($CDCl_3$, 400 MHz) δ 5.88 (s, 1H), 4.99

(A of ABX, 1H, $J = 18.0$, 1.6), 4.81 (B of ABX, 1H, $J = 18.0$, 1.5), 3.55 (s, 3H), 3.26 (br s, 1H), 2.79 (m, 1H), 2.15 (m, 2H), 1.85 (m, 3H), 1.74–1.22 (m, 17H), 0.94 (s, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.7, 174.6, 117.8, 85.7, 73.6, 62.6, 55.1, 51.1, 49.7, 42.0, 40.1, 36.7, 35.8, 35.7, 33.3, 30.5, 28.8, 27.0, 26.7, 23.9, 22.9, 21.3, 21.2, 15.9; Electrospray ionization–MS *m*/*z* (M+H) calculated for $C_{24}H_{37}NO_4$ 404.3, observed 404.4. The undesired α isomer can be obtained by further elution with 100% EtOAc.

17. Example procedure: Aglycon **2a** (8.1 mg, 20 μmol) and L-xylose (6.6 mg, 33 μmol) were added to a glass vial equipped with a stirring flea and then were dissolved in 9:1 MeOH/$CHCl_3$ (200 μL). AcOH was added (1.1 μL, 33 μmol) and the reaction mixture was stirred at 40 °C for 4 days. The crude reaction mixture was concentrated, then suspended in 2% MeOH in $CHCl_3$. The crude suspension was purified on a disposable SiO_2 solid-phase extraction column. Three treatments with 2 mL of 3% MeOH/CH_2Cl_2 eluted unreacted aglycon and five treatments with 2 mL of 5% MeOH/CH_2Cl_2 eluted product **4b** (100% pyranose form, β-anomer). (TLC $R_f = 0.40$ in 10% MeOH/CH_2Cl_2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.91 (s, 1H), 4.97–4.90 (m, 4H), 4.52 (d, 1H, $J = 5.2$), 4.08 (s, 1H), 3.88 (d, 1H, $J = 8.7$), 3.68 (dd, 1H, $J = 10.9$, 5.1), 3.51 (s, 3H), 3.40–3.08 (m, 4H), 2.93 (dd, 1H, $J = 10.6$, 10.6), 2.73 (m, 1H), 2.05 (m, 2H), 1.93 (m, 1H), 1.87–1.05 (m, 18H), 0.89 (s, 3H), 0.77 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 176.4, 173.9, 116.2, 90.3, 83.8, 78.5, 73.1, 70.0, 69.6, 67.3, 62.9, 56.2, 50.2, 49.5, 41.1, 36.3, 35.4, 35.3, 32.3, 30.2, 28.9, 26.6, 26.3, 23.7, 23.0, 21.1, 20.8, 15.7; Electrospray ionization–MS *m*/*z* (M+H) calculated for $C_{30}H_{47}NO_8$ 550.3, obsd 550.5.
18. The isomeric mixtures were not resolved during product purification since equilibration between product isomers occurs (see Ref. 14a).
19. For years, NCI/ADR-RES cells have been misidentified as drug-resistant MCF-7 breast adenocarcinoma cells, but recently this line has been shown definitively to be a drug-resistant line derived from OVCAR-8 ovarian adenocarcinoma cells. Liscovitch, M.; Ravid, D. *Cancer Lett.* **2007**, *245*, 350.
20. Tanigawara, Y.; Okumura, N.; Hirai, M.; Yasuhara, M.; Ueda, K.; Kioka, N.; Komano, T.; Hori, R. *J. Pharmacol. Exp. Ther.* **1992**, *263*, 840.
21. (a) Johnson, P. H.; Walker, R. P.; Jones, S. W.; Stephens, K.; Meurer, J.; Zajchowski, D. A.; Luke, M. M.; Eeckman, F.; Tan, Y.; Wong, L.; Parry, G.; Morgan, T. K., Jr.; McCarrick, M. A.; Monforte, J. *Mol. Cancer Ther.* **2002**, *1*, 1293; (b) Komentiani, P.; Liu, L.; Askari, A. *Mol. Pharmacol.* **2005**, *67*, 929.
22. Yang, Q.; Huang, W.; Jozwik, C.; Lin, Y.; Glasman, M.; Caohuy, H.; Srivastava, M.; Esposito, D.; Gillette, W.; Hartley, J.; Pollard, H. B. *Proc. Natl. Acad. Sci.* **2005**, *102*, 9631.