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Stereochemical survey of digitoxin monosaccharides: new anticancer analogues with enhanced apoptotic activity and growth inhibitory effect on human non-small cell lung cancer cell

Hua-Yu Leo Wang[†], **Wenjun Xin**[‡], **Maoquan Zhou**[‡], **Todd A. Stueckle**[*,§], **Yon Rojanasakul**[*,§], and **George A. O'Doherty**[*,†]

[†]Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA, 02115

[‡]Department of Chemistry, West Virginia University, Morgantown, WV, 26506

[§]Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, WV, 26506

Abstract

A stereochemically diverse array of monosaccharide analogues of the trisaccharide based cardiac glycoside natural product digitoxin has been synthesized using a *de novo* asymmetric approach. The analogues were tested for cytotoxicity against the NCI panel of 60 human cancer cell lines and in more detail against non-small cell human lung cancer cells (NCI-H460). The results were compared with digitoxin and its aglycone digitoxigenin. Three novel digitoxin monosaccharide analogues with β-D-digitoxose, α-L-rhamnose, and α-L-amicetose sugar moieties showed excellent selectivity and activity. Further investigation revealed that digitoxin α-L-rhamnose and α-L-amicetose analogues displayed similar anti-proliferation effects, but with at least 5-fold greater potency in apoptosis induction than digitoxin against NCI-H460. This study demonstrates the ability to improve the digitoxin anti-cancer activity by modification of the stereochemistry and substitution of the carbohydrate moiety of this known cardiac drug.

Keywords

Digitoxin; Digitoxin monosaccharide; Rhamnose; Amicetose; Cancer; Apoptosis; NCI-H460

Digitoxin (Figure 1, **1**), a cardiac glycoside found in *Digitalis purpurea*, has been well studied and used over centuries, and still today, for treating congestive heart failure by the inhibition of plasma membrane Na^+/K^+ ATPase.1 The accumulated intracellular Na^+ concentration from this inhibition causes an increase in Ca^{2+} concentration which enhances myocardial cell contractility. In addition, digitoxin is known to have anti-proliferative effects on cancer cells. Stenkvist found that breast cancer patients on digitalis (digitoxin **1**, digoxin **1a**) had better outcomes than untreated patients.2 They also found a 9.6 fold lower rate of recurrences for digitalis treated patients after mastectomy.3 Due to its faster elimination rate from the body, digoxin (**1a**) is more widely prescribed as a cardiac drug than digitoxin (**1**). Conversely we believe that pharmacokinetic properties may make digitoxin a better candidate for cancer therapy.

yrojan@hsc.wvu.edu, g.odoherty@neu.edu. [*]To whom correspondence should be addressed..

SUPPORTING INFORMATION AVAILABLE: Assay protocols, statistical analysis data, synthetic procedures, characterization data, and NMR spectra. This information is available free of charge via the Internet at http://pubs.acs.org.

While no detailed mechanism of tumor cell death has been demonstrated, many apoptosis signal pathways have been found to be effected by digitoxin.4 For instance, digitoxin was shown *in vitro* to induce apoptosis and inhibit cancer cell growth at sub-cardio-toxic concentration in plasma.5 It is known that the inhibition of Na^+/K^+ ATPase activates tyrosine kinase Src, phosphoinositide-3 kinase (PI3K), and phospholipase C signalosome complex, which can induce many anti-proliferative downstream effects related to cell growth and apoptosis.6,7 Because the anti-cancer effects of digitoxin occur below its cardio-toxic concentration, many Na^+/K^+ ATPase independent pathways have also been suggested.8,9,10

Structurally, digitoxin (**1**) consists of digitoxigenin (aglycone/pharmacore) and the trisaccharide moiety, which is known to play a crucial role in cell growth inhibition and tumor cell death.11 Several research groups have studied the structure-activity relationship (SAR) of the carbohydrate moiety of digitoxin, although no systematic study of stereochemistry (α/β/D/L) has been conducted.12,13,14 To date the most significant SAR study of carbohydrate portion of digitoxin on cancer cells is that Thorson.15 Thorson demonstrated the potentiality to enhance the anti-cancer activity of digitoxin by screening a library of *MeON*-neoglycoside link monosaccharides to digitoxigenin.16 While a limited SAR-conclusion could be made to conclude that a C2'-axial alcohol in the D-sugar was essential for cytotoxicity in many human cancer cell lines. Importantly, they also noted that these substrates were inactive toward Na^+/K^+ ATPase inhibition in human embryonic kidney (HEK-293) and mammalian (CHO-K) cell lines. In a direct comparison of digitoxin *MeON*-neoglycoside to natural *O*-glycoside against a range of cancer cell lines, our recent study has found digitoxin *O*-glycoside to be more potent than *MeON*-neoglycoside in both apoptosis and caspases activity.17 This study showed the viability of digitoxin with C2'-deoxy D-sugar as cytotoxic agent. In addition, we demonstrated that the cytotoxicity was dependent upon the sugar chain length with digitoxin monosaccharide **3** showing at least 10-fold stronger potency than di- **2** and tri-saccharide **1** (digitoxin) against a range of cancer cell lines.

Although Thorson's neoglycorandomization study was groundbreaking for digitoxin analogue drug discovery, it was limited in its search of stereochemistry (e.g. β-anomer predominately without C2'-deoxy sugar).16 Thus, we decided to test additional digitoxin monosaccharide analogues to further elucidate the role that carbohydrate chemistry plays in the anti-cancer effect. In this present study, we synthesized a series of digitoxin monosaccharide analogues (Figure 1, **4** to **10**), as both α- and β-anomer, via a highly stereo/chemo-selective palladium-catalyzed glycosylation.18 This *de novo* sugar replacement strategy can be essentially used to systematically install a broad range of carbohydrates on natural products in a SAR-amenable fashion. Herein, we report the synthesis and the study of a range of digitoxin monosaccharides by the use of our *de novo* glycosylation strategy in a stereochemistry divergent manner. The analogues were screened against the National Cancer Institute's (NCI) panel of 60 human cell lines with further study of the apoptotic effects of the most potent analogue.

Recently, we have had success using our *de novo* asymmetric approach for the introduction of rare/unnatural sugars in biologically important natural products.19,20,21 This approach has great potential for the systematic synthesis of stereochemically diverse sets of carbohydrates (α/β-D/L). The route begins with the use of a palladium-catalyzed glycosylation to install any of the four corresponding digitoxigenin-pyranones (e.g. α-L-**14a** to α-L-**16a**, β-L-**14b** to β-L-**16b**, α-D-**15a** to α-D-**17a**, and β-D-**15b** to β-D-**17b**) from a given aglycone (Scheme 1).

The desired glycosyl donor pyranones of any given stereochemistry were easily prepared by a three-step sequence from acylfuran **11** (Scheme 1). The absolute stereocenters were securely installed via a highly enantioselective Noyori asymmetric reduction to obtain the corresponding optically pure furanyl alcohols **12** and (*ent*)-**12** with high enantioselectivity (*ee* > 98%).22,23 An Achmatowicz oxidative rearrangement and diastereoselective Boc-protection gives the resulting α-/β-diastereomeric mixtures, which were separated by flash chromatography to give a moderate yield of the diastereomeric pure α-/β-D- and α-/β-L-Boc-pyranones (**15a/b** and **14a/b**).24,25 With a complete set of Boc-pyranones in hand, we employed our Pd-catalyzed glycosylation (2.5 mol% $Pd_2(dba)_3CHCl_3$, 10 mol% PPh_3) to convert each sugar building block **15a/b** and **14a/b** to the desired digitoxin monosaccharide precursors **16a/b** and **17a/b** with excellent yield and stereo-retention at the anomeric center. The resulting enone functionality was then set for further post-glycosylation transformation into various stereochemistries and functionalities without the reliance on protecting groups.

These efforts began with the synthesis of α-D-/L-rhamnose and α-D-/L-amicetose digitoxin monosaccharide analogues. The desired C4'-sugar stereochemistry was diastereoselectively installed via Luche reduction ($NaBH_4/CeCl_3$, −78 °C) to give α-D-/L-allylic alcohol **5** and **8** as a single isomer (Scheme 2). The resulting C2'-C3' olefin was readily hydrolyzed to give exclusively rhamnose **18** and **6** through Upjohn dihydroxylation (OsO_4/NMO) in excellent yield.26 In addition, this functional enemoiety was reduced under a facile diimide condition to generate α-D-/L-amicetose **4** and **7**. The addition of NMM as solvent seemed to improve the overall yield of this reaction.27,28

Our effort toward the β-D/L-sugars began from our previously reported synthesis of **3**.29,30 Briefly, a reduction of digitoxin pyranones **17b** and **16b** under Luche reduction, followed by Myers reductive rearrangement (NBSH, PPh_3/DIAD, NMM, −30 °C to rt) and Upjohn dihydroxylation gave exclusively diol **3** and **9** with excellent yield in 3 steps (Scheme 3).31 The resulting axial C3'-alcohol **9** was inverted via Mitsunobu reaction (DIAD/PPh_3, *p*-NO_2PhCO_2H) and subsequently hydrolyzed with K_2CO_3 to generate β-L-olivose analogue **10**. We then submitted our eight novel digitoxin monosaccharides (**3** to **10**), along with digitoxin di- **2** and tri-saccharide **1** to NCI for the evaluation of growth inhibitory effect toward panel of 60 human cancer cell lines.

Previously we have shown that **1**, **2** and **3** possess potent anti-tumor activity against 60 members of NCI cancer cell lines.17 Expectedly, we found that nearly all the cell lines significantly reduced growth ability when treated with digitoxin monosaccharide analogues (**3** to **10**, GI_{50} ~ 1 to 10 nM), which showed at least 5-fold greater potency than digitoxin **1** and digitoxin disaccharide **2** (GI_{50} ~ 50 to 100 nM, Figure 3). In particular, digitoxin monosaccharide **3**, and α-L-amicetose **7** showed excellent selectivity and growth inhibition against several cancer cell lines, including leukemia (MOLT-4), human lung carcinoma (A459/ATCC, NCI-H460), human colon carcinoma (HCT-116), human ovary adenocarcinoma (OVACR-3/8), human prostate carcinoma (DU-145), and all six human renal adenocarcinoma cell lines. Interestingly, α-L-amicetose **7** (GI_{50} ~ 12 nM across the 60 cell lines) appeared to be a better inhibitor of tumor cell growth by factor of 10 than its D-sugar diastereomer α-D-amicetose **4** (GI_{50} ~ 113 nM across the 60 cell lines). Similarly, we also found β-D-digitoxose **3** (GI_{50} ~ 25 nM across the 60 cell lines) showing greater potency than its L-sugar diastereomer β-L-digitoxose **9** (GI_{50} > 50 nM across the 60 cell lines). Due to the instability of the glycosidic bond upon long term storage, allylic alcohol **5** was not tested. We were, however, able to test the activity of its L-sugar diastereomer **8**, although its decreased cytotoxicity could also be due to the same issue of instability. The GI_{50} value for α-L-rhamnose **6** was below the minimum concentration that could be accurately determined. On the basis of these findings, we decided to further test the most active digitoxin analogues β-D-digitoxose **3**, α-L-amicetose **7**, and α-L-rhamnose **6** against the non-small cell human lung

cancer cell (NCI-H460) to investigate its mode of action. The choice of NCI-H460 was based on its intermediary activity (i.e. not the most or least sensitive cell line) to be the representative *in vitro* model for our SAR study.

We have previously shown that β-D-digitoxose **3** induces apoptosis in SK-OV-3, NCI-H460, NCI/ADR-RES, and HT-29 cell lines, which is consistent with other findings for digitoxin.[5,11,17] Digitoxin initiates apoptosis via both intrinsic and extrinsic pathways recognized to associate with different downstream effectors. To evaluate the pro-apoptotic properties of our novel digitoxin monosaccharide analogues **3**, α-L-rhamnose **6** and α-L-amicetose **7**, we conducted a cytotoxicity assay with both digitoxin **1** and the aglycone as a control. This assay was performed with a 12 hour exposure of drugs in increasing concentration (10 nM to 10 μM). Apoptosis activity was differentiated by Hoechst 33342 nuclear stain and propidium iodide to score the cells having intensely condensed chromatin and/or fragmented nuclei from the cells that undergo necrosis.

Our result clearly showed that digitoxin monosaccharide analogues **3**, **6** and **7** have significantly stronger potency than digitoxin **1** within a range of 10 to 500 nM ($P < 0.001$) in a dose dependent manner (Figure 4A). A non-linear regression analysis showed that α-L-rhamnose **6** was at least 7-fold more active (IC_{50} ~ 46.7 nM) than digitoxin **1** (IC_{50} ~ 357 nM). Interestingly, α-L-rhamnose **6** also showed better cytotoxicity than α-L-amicetose **7** (IC_{50} ~ 55.7 nM), and β-D-digitoxose **3** (IC_{50} ~ 74.8 nM) in a range of 50 to 250 nM ($P < 0.001$). In addition, α-L-amicetose **7** is comparatively more potent than β-D-digitoxose **3** at 50 nM ($P < 0.01$). The aglycone digitoxigenin was nearly ineffective ($IC_{50} > 323$ nM) against NCI-H460 cells *in vitro*, which was consistent to the results reported by Lopez-Lazaro.[11]

To further differentiate the cytotoxicity between apoptosis and necrosis, the NCI-H460 cells were treated with Hoechst and propidium iodide. Cellular degradation with condensed and fragmented nuclei as shown by Hoechst stain in blue is indicative of apoptosis, while necrotic cells appear completely ruptured in red when stained with propidium iodide. We found for the most potent digitoxin analogue **6** that the ratio of apoptotic and necrotic cell death was 80:20 at 50 nM concentration (Figure 4C). It is worth noting that the ratio of apoptosis and necrosis become difficult to estimate at high dose concentration (> 500 nM) due to the high cell mortality rate. However, in the comparison of digitoxin and its analogues in apoptosis activity at 50 nM, we found that α-L-rhamnose **6** (45.8%) showed the highest apoptosis activity than the others ($P < 0.001$, Figure 4B). The β-D-digitoxose **3** (28.8%) and α-L-amicetose **7** (33%) displayed less activity, but both showed better apoptosis induction than digitoxin **1** (8.4%) and digitoxigenin (8.5%).

We next quantified NCI-H460 cell viability by measuring mitochondrial activity via MTT colorimetric assay to compare cell growth inhibition of our digitoxin analogues. Consistent with our apoptosis results, digitoxin monosaccharide analogues **3**, **6** and **7** all showed significantly stronger cytotoxicity than digitoxin **1** in both time-and dose-dependent manner ($P < 0.001$, Figure 5A and B). In particular, we found that digitoxin monosaccharide analogues **3**, **6** and **7** showed better growth inhibition than digitoxin **1** in a low concentration range (10 to 25 nM) over 48 hour treatment ($P < 0.001$, Figure 5B). Specifically, both α-L-rhamnose **6** (GI_{50} ~ 2 nM) and α-L-amicetose **7** (GI_{50} ~ 2.2 nM) showed a greater growth inhibitory effect than β-D-digitoxose **3** (GI_{50} ~ 3.8 nM), as well as digitoxin **1** (GI_{50} ~ 10.7 nM). These results also agree to the GI_{50} value from our NCI data, where α-L-rhamnose **6** (GI_{50} is undetermined), α-L-amicetose **7** (GI_{50} ~ 3.8 nM), and β-D-digitoxose **3** (GI_{50} ~ 4.5 nM) displayed at least 3-fold greater potency than digitoxin **1** (GI_{50} ~ 12.8 nM) in NCI-H460 cells.

In summary, we investigated our newly synthesized digitoxin monosaccharide analogues, in which we note a distinct structural activity shift between the D- and L-sugars in their NCI growth inhibition data. For instance, this data showed that α-L-amicetose **7** and β-D-digitoxose **3** with greater potency than their sugar enantiomers (i.e., diastereomer), αDamicetose **4** and β-L-digitoxose **9**. We also found that the β-D-digitoxose **3**, α-L-rhamnose **6** and α-L-amicetose **7** sugar moieties showed the most promising anti-neoplastic activity. Both of our apoptosis and cell viability assays showed enhanced activity for the digitoxin monosaccharide analogues **3**, **6** and **7** as compared to natural digitoxin **1**. Our results suggested an overall activity trend with α-L-rhamnose **6** ≥ α-L-amicetose **7** > β-D-digitoxose **3** > digitoxin **1** > digitoxigenin in both apoptosis induction and anti-proliferation effects in NCI-H460 cells. It is interesting to note that the cancer cell cytotoxicity for structures **6**, **7**, and **3** is not inconsistent with the SAR conclusion by Fullerton for the cardioglycoside's cardiotonic activity.32

This study highlights our *de novo* asymmetric method for the unique introduction of different α/β-D/L stereoisomers, which can be easily established via palladium-catalyzed glycosylation coupled with a series of post-glycosylation transformations. This approach not only shows an efficient preparation of novel digitoxin analogues, but also demonstrates its use for systematic SAR studies. Moreover, this comparison of digitoxin monosaccharide stereoisomers provides better insight into the effect of the carbohydrate moiety toward anti-cancer activity for designing potential cardiac glycoside anti-cancer agents. Further investigations along this line are ongoing and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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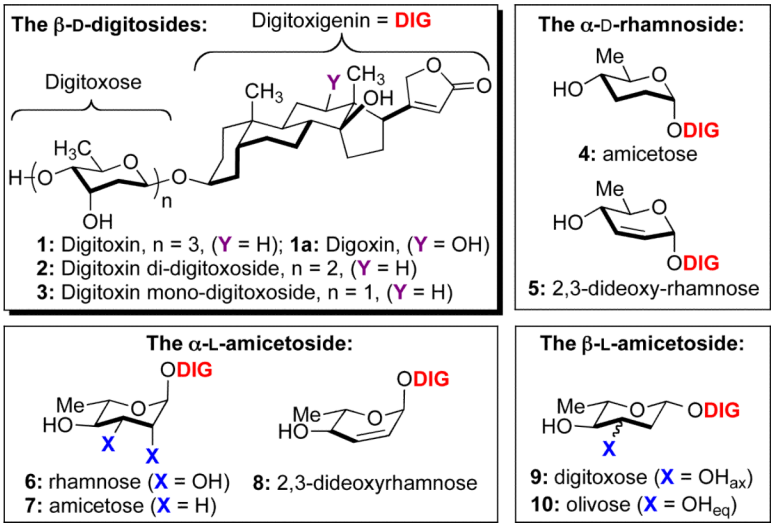
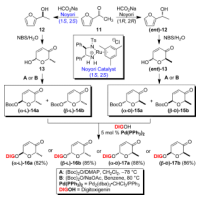


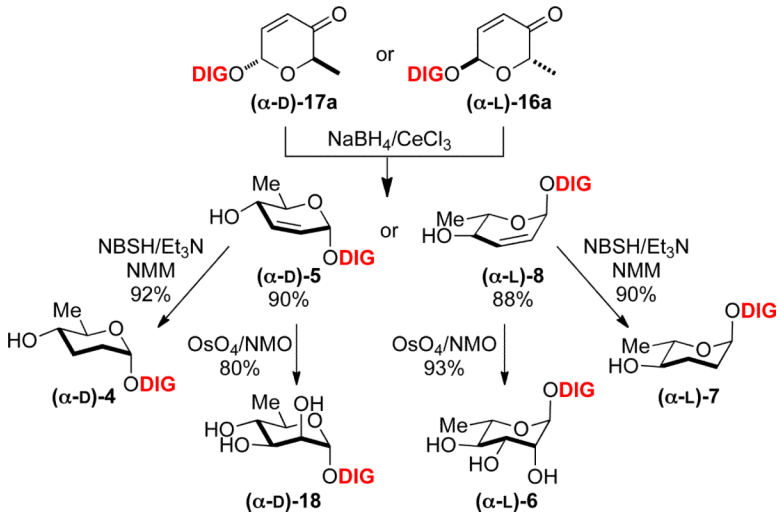
Figure 1.
Digitoxin **1** and related carbohydrate analogues



Scheme 1.

De novo synthesis of digitoxin pyranone monosaccharides **16a**, **16b**, **17a** and **17b**[a]

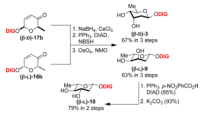
[a] Reagents: NBS, *N*-bromosuccinimide; DMAP, 4-Dimethylaminopyridine.



Scheme 2.

Synthesis of α-D-/L-rhamnose **18** and **6**, and α-D-/L-amicetose **4** and **7** digitoxin analogues

[a] Reagents: NMO, *N*-methylmorpholine-*N*-oxide; NBSH, *o*-nitrobenzenesulfonylhydrazide; NMM, *N*-methylmorpholine.



Scheme 3.
Synthesis of β-D-/L-digitoxose **3** and **9**, and β-L-olivose **10** digitoxin analogues

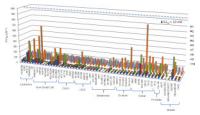


Figure 3.
Carbohydrate survey of digitoxin monosaccharide analogues (**1** to **10**) against NCI panel cell lines. Reciprocal GI_{50} value is displayed for clarity.

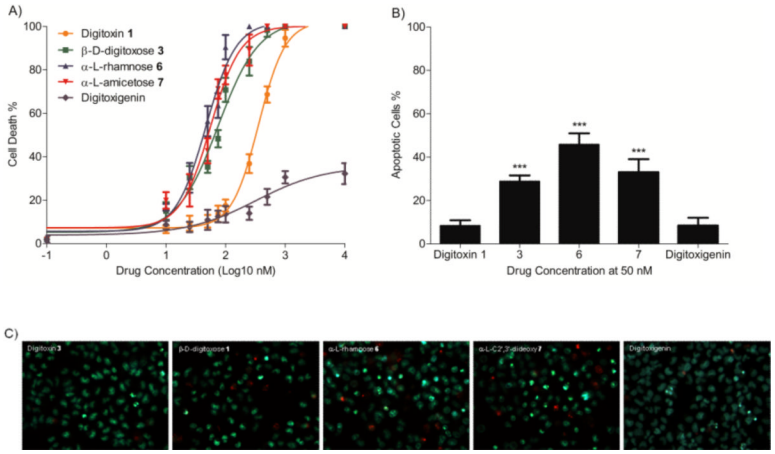


Figure 4.

A) The concentration-response curve of the apoptosis mediated total cell death by digitoxin analogues in 12 h treatment. B) Apoptotic cell death percentage was compared for each compound at 50 nM concentration (One-way ANOVA; ***, $P < 0.001$). C) Hoechst stained apoptotic cell appear in blue and propidium iodide stained necrotic cell in red at 50 nM for each compound.

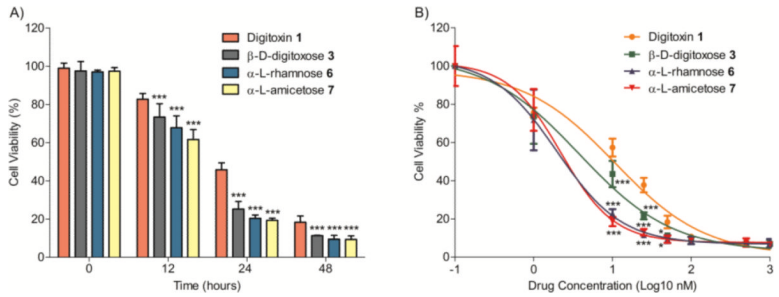


Figure 5.

A) Time-dependent experiment (50 nM). B) Dose-dependent experiment (48 hour). Both time- and dose-dependent data were analyzed by Two-way ANOVA ($N = 9$, *, $P < 0.05$; ***, $P < 0.001$).