Stereochemical Survey of Digitoxin Monosaccharides

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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 nonsmall 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 antiproliferation 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 anticancer 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
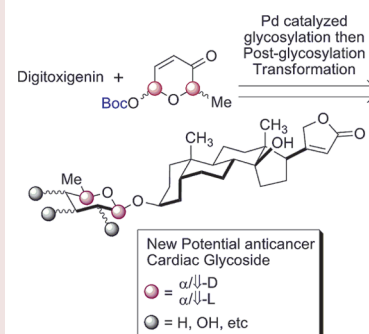
Digitoxin (Figure 1, **1**), a cardiac glycoside found in *Digitalis purpurea*, has been well studied and used over centuries and is still used today for treating congestive heart failure by the inhibition of plasma membrane Na^+/K^+ ATPase.[1] The accumulated intracellular Na^+ concentration from this inhibition causes an increase in Ca^{2+} concentration, which enhances myocardial cell contractility. In addition, digitoxin is known to have antiproliferative 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] Because of 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.

While no detailed mechanism of tumor cell death has been demonstrated, many apoptosis signal pathways have been found to be affected by digitoxin.[4] For instance, digitoxin was shown in vitro to induce apoptosis and inhibit cancer cell growth at a subcardiotoxic 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 antiproliferative downstream effects related to cell growth and apoptosis.[6,7] Because the anticancer effects of digitoxin occur below its cardiotoxic concentration,

many Na^+/K^+ ATPase independent pathways have also been suggested.[8−10]

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.[12−14] To date, the most significant SAR study of carbohydrate portion of digitoxin on cancer cells is that of Thorson.[15] Thorson demonstrated the potentiality to enhance the anticancer activity of digitoxin by screening a library of *MeON*-neoglycoside link monosaccharides to digitoxigenin.[16] 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
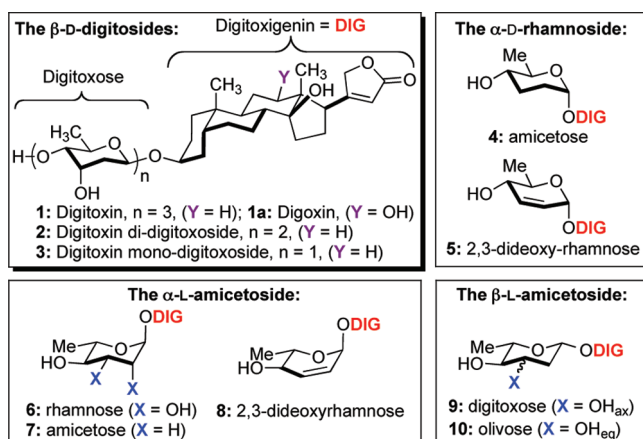
**Figure 1.** Digitoxin **1** and related carbohydrate analogues.

with C2′-deoxy D-sugar as a 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 disaccharide **2** and trisaccharide **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 anticancer effect. In this present study, we synthesized a series of digitoxin monosaccharide analogues (Figure 1, **4−10**), as both α- and β-anomer, via a highly stereo/chemoselective 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.[19−21] 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

Scheme 1. De Novo Synthesis of Digitoxin Pyranone Monosaccharides **16a**, **16b**, **17a**, and **17b**[a]
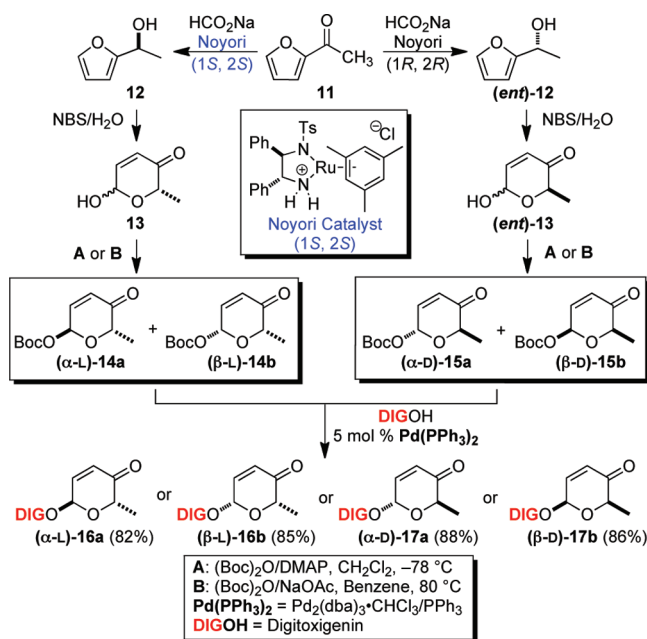

[a] Reagents: NBS, *N*-bromosuccinimide; DMAP, 4-dimethylaminopyridine.

rearrangement and diastereoselective Boc protection give 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 % Pd₂(dba)₃CHCl₃, 10 mol % PPh₃] 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 stereoretention at the anomeric center. The resulting enone functionality was then set for further postglycosylation 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 (NaBH₄/CeCl₃, 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 (OsO₄/NMO) in excellent yield.[26] In addition, this functional ene moiety was reduced under a facile diimide condition to generate α-D-/L-amicetose **4** and **7**. The addition of NMM as a 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, PPh₃/DIAD, NMM, −30 °C to room temperature) and Upjohn dihydroxylation gave exclusively diol **3** and **9** with excellent yield in three steps (Scheme 3).[31] The resulting axial

Scheme 2. Synthesis of α-D-/L-Rhamnose **18** and **6** and α-D-/L-Amicetose **4** and **7** Digitoxin Analogues[a]
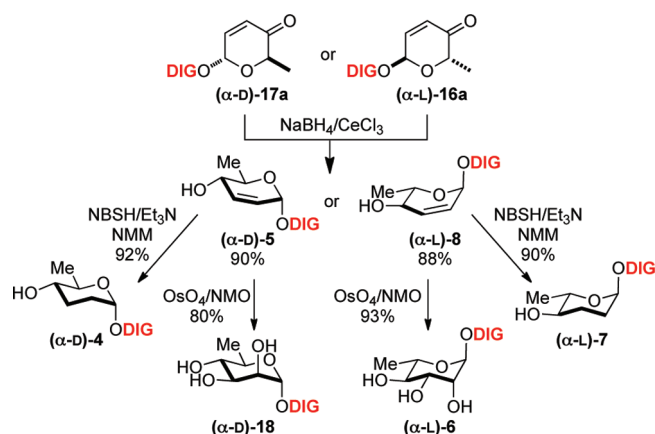
[a] Reagents: NMO, *N*-methylmorpholine-*N*-oxide; NBSH, *o*-nitrobenzenesulfonylhydrazide; and NMM, *N*-methylmorpholine.

Scheme 3. Synthesis of β-D-/L-Digitoxose **3** and **9** and β-L-Olivose **10** Digitoxin Analogues
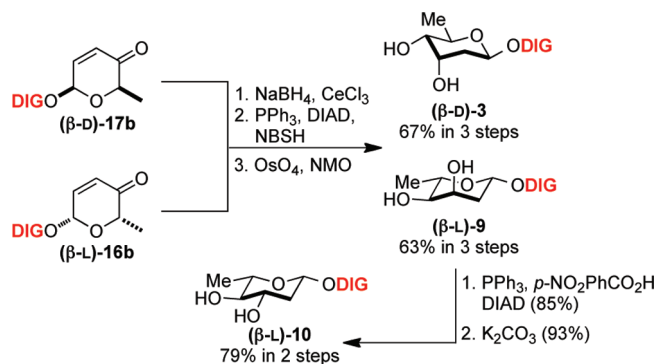
C3′-alcohol **9** was inverted via Mitsunobu reaction (DIAD/PPh$_3$, *p*-NO$_2$PhCO$_2$H) and subsequently hydrolyzed with K$_2$CO$_3$ to generate β-L-olivose analogue **10**. We then submitted our eight novel digitoxin monosaccharides (**3−10**), along with digitoxin disaccharide **2** and trisaccharide **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 antitumor activity against 60 members of NCI cancer cell lines.[17] Expectedly, we found that nearly all of the cell lines significantly reduced growth ability when treated with digitoxin monosaccharide analogues (**3−10**, GI$_{50}$ ∼ 1−10 nM), which showed at least 5-fold greater potency than digitoxin **1** and digitoxin disaccharide **2** (GI$_{50}$ ∼ 50−100 nM, Figure 2). 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 and 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 a 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). Because of 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 nonsmall 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 h exposure of drugs in increasing concentration (10 nM to 10 μM). The 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 10−500 nM (*P* < 0.001) in a dose-dependent manner (Figure 3A). A nonlinear 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 50−250 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 3C). It is worth noting that the ratio of apoptosis and necrosis becomes difficult to estimate at a high dose concentration (> 500 nM) due to the high cell
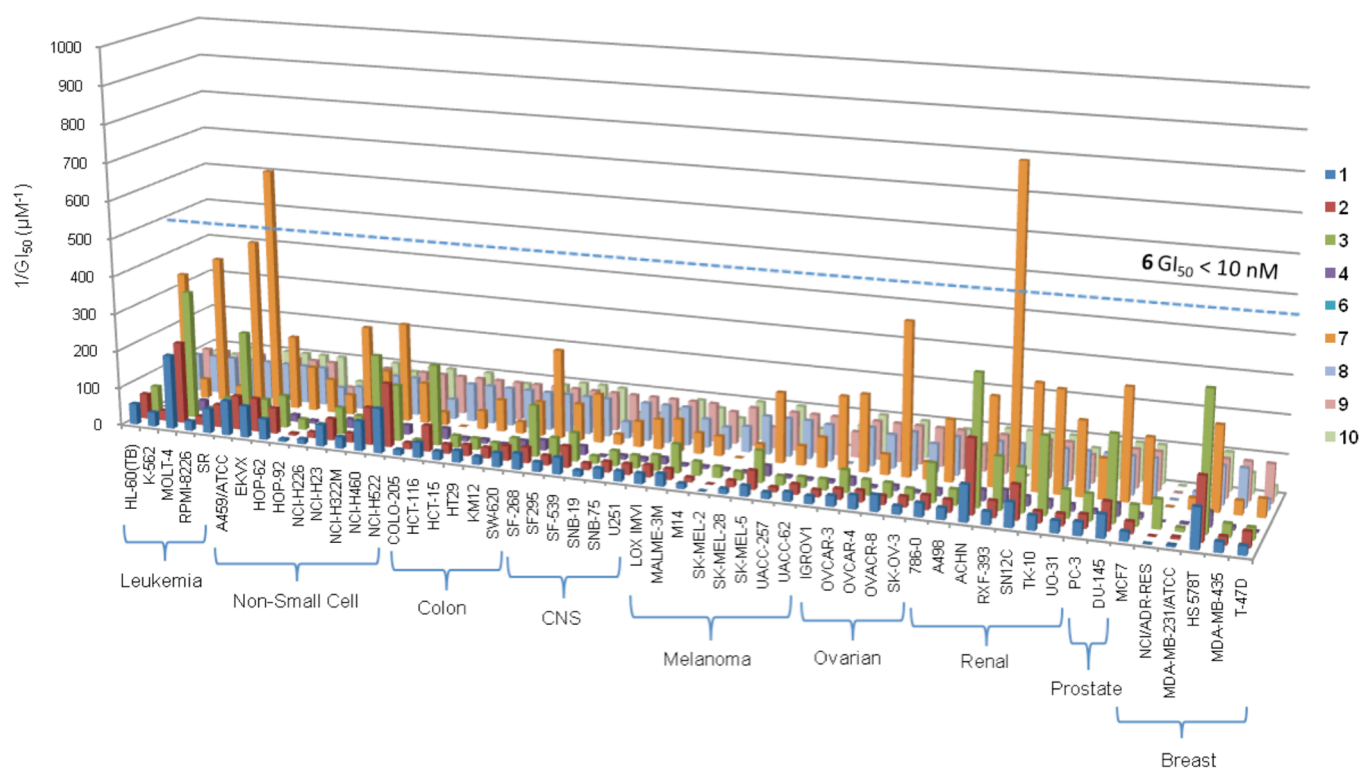
Figure 2. Carbohydrate survey of digitoxin monosaccharide analogues (**1** to **5** and **7** to **10**) against NCI panel cell lines, where the values for **6** are represented by the dotted line. Reciprocal GI_{50} value is displayed for clarity.
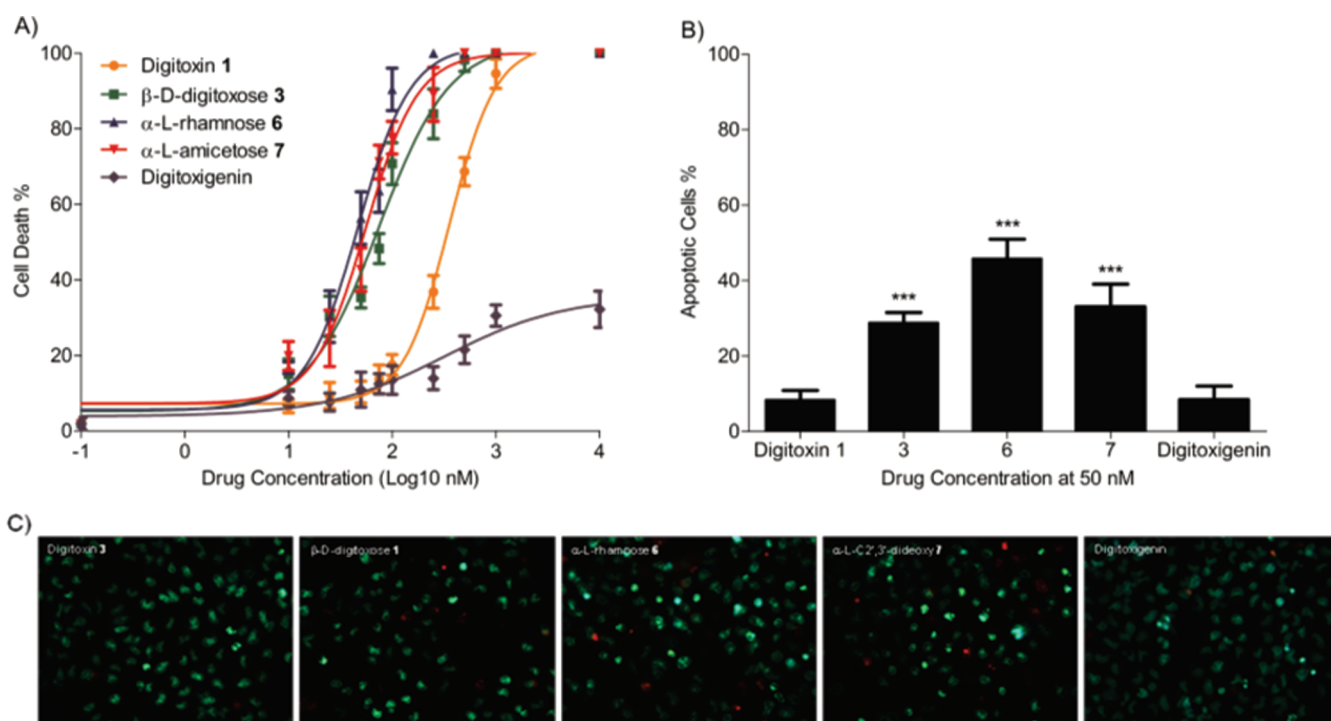

Figure 3. (A) Concentration−response curve of the apoptosis-mediated total cell death by digitoxin analogues in 12 h treatment. (B) Apoptotic cell death percentage was compared for each compound at 50 nM concentration (one-way ANOVA; $***$, $P < 0.001$). (C) Hoechst-stained apoptotic cells appear in blue, and propidium iodide-stained necrotic cells appear in red at 50 nM for compounds **1**, **3**, **6**, **7**, DIGOH.
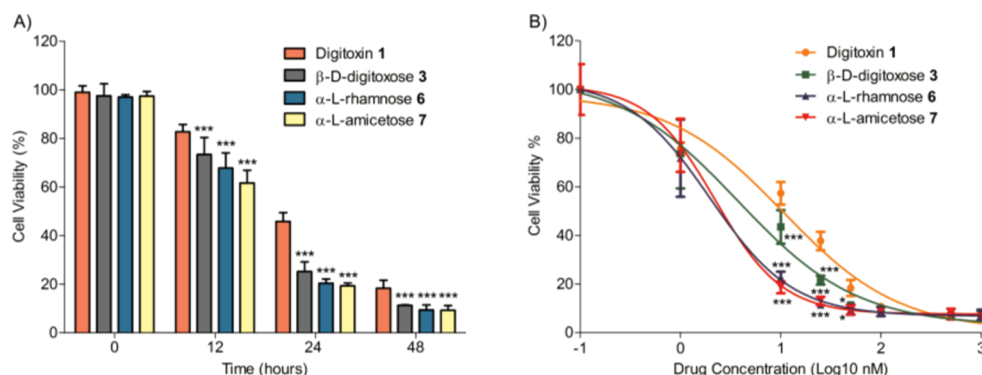
Figure 4. (A) Time-dependent experiment (50 nM). (B) Dose-dependent experiment (48 h). Both time- and dose-dependent data were analyzed by two-way ANOVA ($N = 9$, *, $P < 0.05$; ***, $P < 0.001$).

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 3B). 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 4A,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 (10−25 nM) over 48 h of treatment ($P < 0.001$, Figure 4B). Specifically, both α-L-rhamnose **6** ($GI_{50} \sim 2$ nM) and α-L-amicetose **7** ($GI_{50} \sim 2.2$ nM) showed a greater growth inhibitory effect than β-D-digitoxose **3** ($GI_{50} \sim 3.8$ nM) as well as digitoxin **1** ($GI_{50} \sim 10.7$ 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** ($GI_{50} \sim 3.8$ nM), and β-D-digitoxose **3** ($GI_{50} \sim 4.5$ nM) displayed at least 3-fold greater potency than digitoxin **1** ($GI_{50} \sim 12.8$ 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 the 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), α-D-amicetose **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 antineoplastic 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 antiproliferation 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 postglycosylation 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 anticancer activity for designing potential cardiac glycoside anticancer agents. Further investigations along this line are ongoing and will be reported in due course.

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