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Digitoxin Analogues with Improved Anticytomegalovirus Activity

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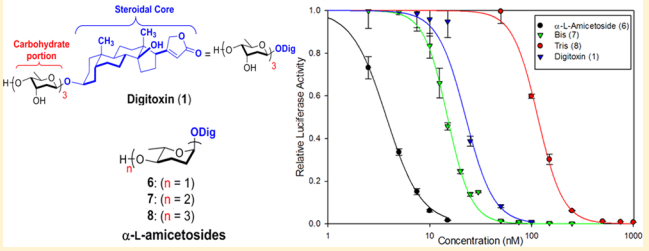
Supporting Information

ABSTRACT: Cardiac glycosides are potent inhibitors of cancer cell growth and possess antiviral activities at nanomolar concentrations. In this study we evaluated the anticytomegalovirus (CMV) activity of digitoxin and several of its analogues. We show that sugar type and sugar length attached to the steroid core structure affects its anticytomegalovirus activity. Structure–activity relationship (SAR) studies identified the L-sugar containing cardiac glycosides as having improved anti-CMV activity and may lead to better understanding of how these compounds inhibit CMV replication.



KEYWORDS: *Cardiac glycosides, digitoxin analogues, cytomegalovirus, virus inhibition*

Cardiac glycosides (CGs) have been used for centuries to treat congestive heart failure and arrhythmias, conditions

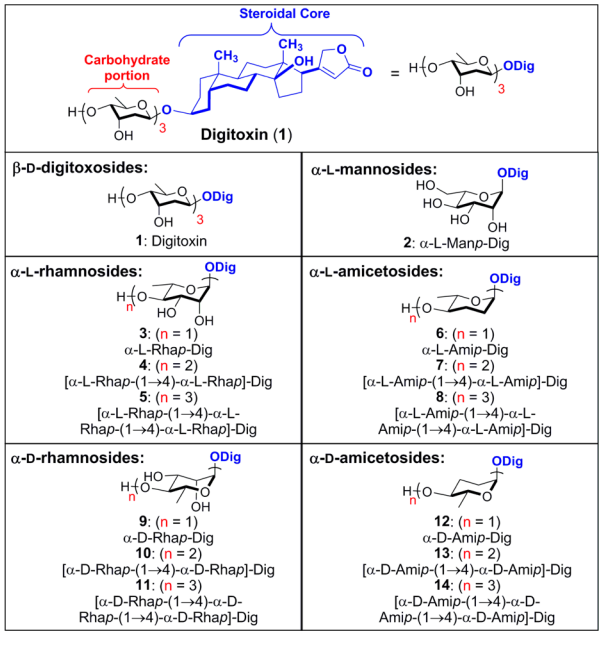


Figure 1. Structure of digitoxin analogues.

in which they bind to the Na^+/K^+ATPase and inhibit its activity.[1,2] The number of CGs identified in animals and plants is growing, and novel effects are becoming evident, including anticancer and antiviral activities.[3−6] Digoxin and ouabain were first reported to inhibit Herpes Simplex Virus 1 (HSV1) in nanomolar concentrations.[7,8] While the anticancer effects of CGs have been confirmed in multiple studies, the antiviral activities have not been well-studied. CGs were reported to inhibit human Cytomegalovirus (HCMV) replication at nanomolar concentration.[9] We reported that digoxin, ouabain, and digitoxin are potent inhibitors of the HCMV laboratory-adapted Towne strain.[10] Previous reports have shown that digitoxin had anticancer activities at concentrations commonly found in cardiac patients (20−33 nM),[11,12] which suggests that new digitoxin analogues with improved activity and selectivity may have an important role in the treatment of high-risk HCMV-infected patients.

The steroidal aglycon of the CG has long been recognized as the pharmacophore; however, more recent studies suggest the anticancer activity also depends on the sugar portion of the molecule.[13,14] These studies have shown that cancer cell cytotoxicity of CGs depends greatly upon the specific glycosidic linkage,[15,16] the length of the oligosaccharide,[16,17] the stereochemistry of the sugar,[18] the degree of substitution on the sugar,[19] and the size of the sugar's C5′-substituent.[20]

Using our de novo approach to oligosaccharides,[21] we have been able to execute medicinal chemistry studies on the carbohydrate portion of the cardiac glycosides.[22,23] These carbohydrate based structure–activity relationships (SAR) studies have led to the discovery of two new sugar motifs that significantly improved the cytotoxicity across a range of cancer cell lines (α-L-Rhap **3** and α-L-Amip **6**).[18] For instance, the α-D-sugar diastereomers (α-D-Rhap **9** and α-D-Amip **12**)

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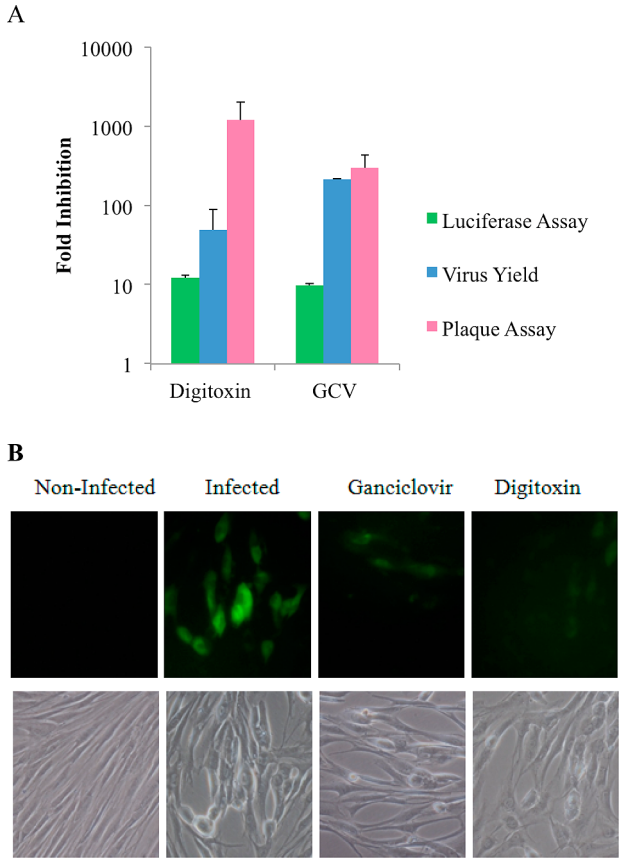

Figure 2. Inhibition of HCMV replication with digitoxin. (A) Inhibition of Towne HCMV: pp28-luciferase activity was measured in cell lysates of HCMV-infected human foreskin fibroblasts (HFFs) collected at 72 h post infection (hpi). Virus DNA yield in supernatants of HCMV-infected cells collected at 96 hpi was measured by real-time PCR. Plaque reduction assay performed at 8 days postinfection. Data represent mean values (±SD) of triplicate determinations from three independent experiments. (B) Inhibition of TB40 HCMV: HFFs were infected with HCMV-TB40 strain at MOI of 1 pfu/cell and treated with GCV or digitoxin for 3 days. Top panel is GFP signals of HCMV, bottom panel is bright field.

were less cytotoxic than the α-L-sugar diastereomers (α-L-Rha*p* **3** and α-L-Ami*p* **6**) (Figure 1). Similarly, the cytotoxicity of the L-rhamnose and L-amicetose mono-, di-, and trisaccharide CGs (**3**–**5** and **6**–**8**, respectively) reduced with increase in sugar-chain length, where the most active α-L-Rha*p* **3** and α-L-Ami*p* **6** were at least 10-fold more potent than the corresponding di- and trisaccharides (**4**–**5**, and **7**–**8**).[13,18] These anticancer structure–activity SAR studies led us to hypothesize that similar modification of the carbohydrate portion of digitoxin may lead to novel analogues with improved anti-HCMV activities.

The inhibition of HCMV replication by digitoxin was tested. At 50 nM, treatment with digitoxin resulted in complete inhibition of HCMV replication, similar to the anti-HCMV activities of ganciclovir (GCV, 5 μM or 10 μM). These results were based on the following antiviral assays: pp28 expression of HCMV Towne (by luciferase, at 72 hpi), virus DNA yield of HCMV Towne (by real-time PCR, at 96 hpi), GFP signal of HCMV-TB40 strain at 3 days, and plaque reduction of HCMV Towne at 8 days postinfection (Figure 2).

The effects of digitoxin and its analogues (**1**–**14**) on HCMV replication were tested. Human Foreskin Fibroblasts (HFFs)

Table 1. EC_{50}, CC_{50}, and Selectivity Index (SI) of Digitoxin Analogues

ID	EC_{50} (nM)	CC_{50} (μM) MTT	SI	CC_{50} (μM) trypan blue
1 (digitoxin)	23.3 ± 0.7	2.8 ± 0.7	120	6 ± 2
2	7.3 ± 0.1	0.9 ± 0.4	127	1.9 ± 0.5
α-L-rhamno				
3	4.8 ± 0.2	0.7 ± 0.2	139	2 ± 7
4	36.7 ± 0.9	1.5 ± 0.6	41	6 ± 1
5	232 ± 4	7.0 ± 0.9	30	11 ± 2
α-L-amiceto				
6	3.8 ± 0.1	0.7 ± 0.2	174	4 ± 1
7	14.8 ± 0.4	1.1 ± 0.1	76	7 ± 1
8	113 ± 3	2.4 ± 0.3	21	9 ± 2
α-L-rhamno				
9	26.6 ± 0.9	2.3 ± 0.6	86	3.7 ± 0.9
10	209 ± 6	6.7 ± 0.7	32	10 ± 2
11	320 ± 12	10 ± 1	31	16 ± 2
α-L-amiceto				
12	37.5 ± 0.5	1.1 ± 0.3	29	4 ± 1
13	790 ± 40	12.1 ± 0.7	15	1 ± 1
14	2080 ± 90	15 ± 1	7	19 ± 1

were infected with pp28-luciferase HCMV and treated with **1**–**14** at a range of concentrations (Table 1 and Figure 3A). Dose–response curves were measured for HCMV-infected cells and noninfected HFFs, as a control. For each compound, the anti-HCMV activity in infected HFFs was expressed as EC_{50}, whereas cytotoxicity in noninfected HFFs was expressed as CC_{50}. Selectivity index (SI), defined as CC_{50}/EC_{50}, was calculated for each analogue, using the CC_{50}; virus yield was determined for selected compounds (Figure 3B).

The data showed that the L-sugar diastereomers had improved anti-HCMV activity compared to the D-isomers. This modification provided an almost 10-fold increase in anti-HCMV activity along with an improved SI (Table 1). For each set of sugar diastereomer (i.e., both α-L-/α-D-Rha*p* and -Ami*p*), there was an inverse correlation between the sugar length and anti-HCMV activity; the longer the oligosaccharide chain, the less effective the compound was against HCMV replication. There was also decreased cytotoxicity in HFFs as the sugar length increased; however, the anti-HCMV activity did not directly correlate with cell cytotoxicity as reflected by the SI. The compounds with the best SI against HCMV replication were α-L-Rha*p* **3**, α-L-Ami*p* **6**, and α-L-Man*p* **2**.

The SI shown in Table 1 is the more conservative estimate of selectivity as it uses the CC_{50} measured by MTT. Evaluation of cell toxicity in uninfected HFFs by MTT assay revealed that cell proliferation decreased to approximately 50% at a dose of approximately 0.1 μM and remained at that level even on further increasing the dose of digitoxin and α-L-amicetoside (Figure 4A). This unusual toxicity pattern of CGs in HFFs, in which cell viability was decreased to ∼50% and resistant to high drug concentrations suggested that the MTT assay may overestimate the cellular toxicity for digitoxin analogues in HFFs. The limitation of the MTT assay is that it measures cellular metabolic activity, which can vary throughout the lifecycle of cells. Thus, it does not actually measure the number of viable cells.[24] Because of this pattern of cell viability as well as the effects of CGs on enzymes that depend on ATP, a trypan blue assay was also performed for the digitoxin analogues **1**–**14** (Supporting Information, Table S2). This assay showed

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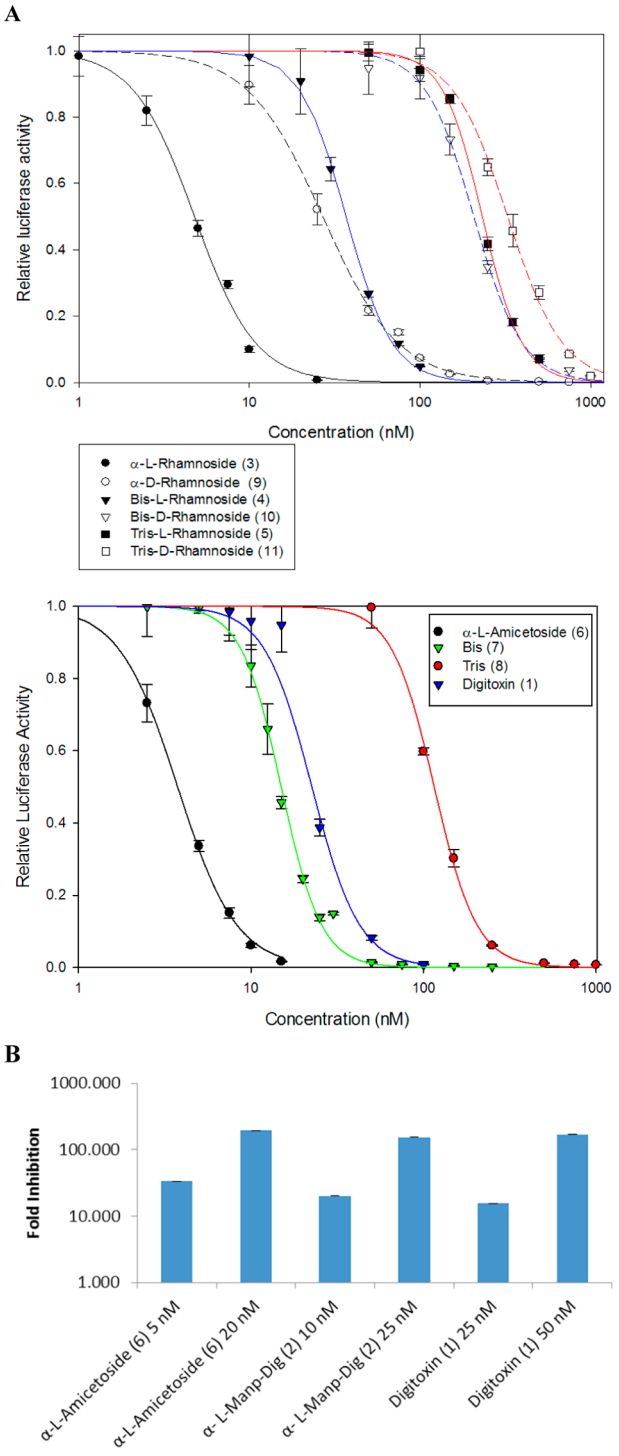

Figure 3. HCMV inhibition by digitoxin analogues. (A) Dose response curve of rhamnose (upper) and amicetose (middle) analogues: HFFs were infected with pp28-luc HCMV at MOI of 1 pfu/cell and treated with digitoxin analogues at indicated drug concentrations. Luciferase activity was measured in cell lysates collected at 72 hpi. Data represent mean values (±SD) of triplicate determinations from three independent experiments. (B) Virus DNA yield (real-time PCR) of HCMV-infected cells treated with digitoxin analogues: HFFs were infected with HCMV Towne at MOI of 1 pfu/cell. Infected cells were treated with α-L-Amip **6**, α-L-Manp **2**, and digitoxin **1** for 4 days. Supernatants were collected and virus DNA yield was quantified by real-time PCR. Data represent mean values (±SD) of triplicate determinations from three independent experiments.

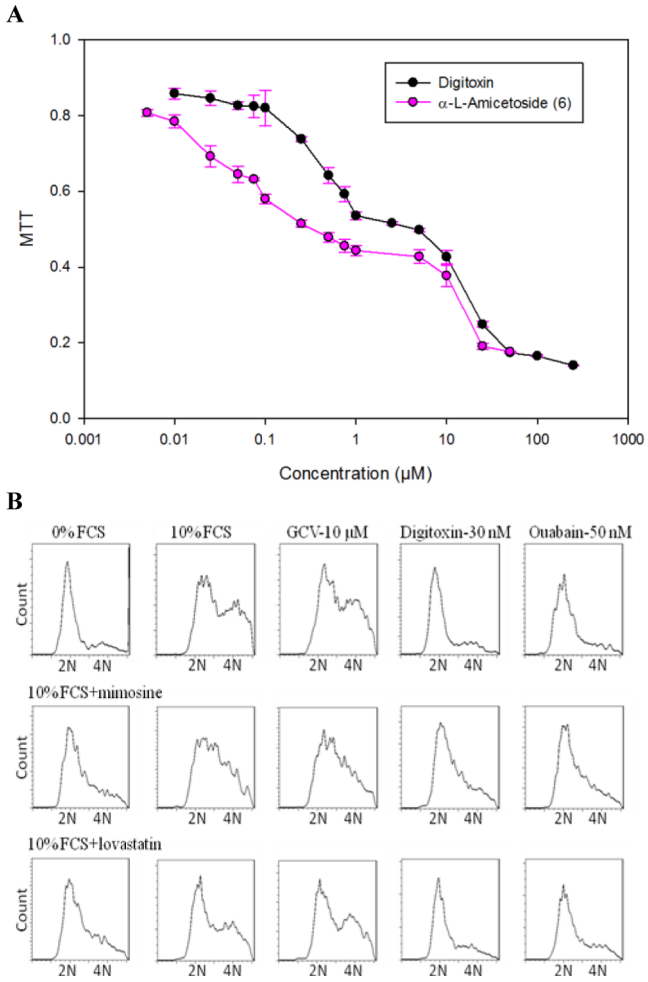

Figure 4. Cell viability and cell cycle progression of representative CGs. (A) MTT assay: HFFs were treated with indicated concentrations of digitoxin and α-L-amicetoside in 96-well plates and incubated at 37 °C for 3 days. After the addition of 20 μL/well of MTT (5 mg/mL in PBS), plates were incubated at 37 °C for 3 h. Conversion of yellow solution into dark blue formazan by mitochondrial dehydrogenases of living cells was quantified by measuring absorbance at 560 nM with DMSO. Data represent mean values (±SD) of triplicate determinations from three independent experiments. (B) Cell cycle by flow cytometry: HFFs were serum starved for 48 h followed by HCMV infection and treatment with compounds for 24 h. At that time cells were trypsinized and permeabilized in −20 °C for 12 h, followed by propidium iodide staining and FACS analysis. Shown in the middle and lower panels are experiments performed with 48 h mimosine or lovastatin treatment followed by release and treatment with the indicated compounds.

consistently higher CC_{50} values. Thus, the differences between the MTT and trypan blue assay may complicate the calculation of antiviral selectivity index (SI).

To further understand the effects of digitoxin analogues on cell proliferation/viability, a cell cycle analysis was performed (Figure 4B). HFFs were serum starved for 2 days, cells were then released from serum starvation, infected with Towne HCMV and treated with digitoxin or the cardiac glycoside ouabain for 24 h.

A propidium iodide stain and cell cycle analysis were performed by flow cytometry. Cells treated with media including serum or GCV were used as control. Both digitoxin and ouabain arrested HFFs in G0/1, while cells treated with

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GCV or maintained in serum without compound showed normal cell cycle progression. To further delineate the timing of cell-cycle arrest, HFFs were treated with mimosine (which arrests cells at mid-G1) for 48 h, then washed and treated for one day with digitoxin, ouabain, or GCV (Figure 4B). Digitoxin (**1**) or ouabain could not relieve HFFs from cell cycle arrest induced by mimosine, while GCV treatment allowed the cells to enter cell cycle. Forty-eight hours of pretreatment with lovastatin (an early G1 inhibitor) followed by release and 24 h therapy with the compounds showed a similar pattern as pretreatment with mimosine (Figure 4B). Taken together, the tested CGs arrest HFFs early in G1.

In summary, we show that improved anti-HCMV activity and selectivity can be achieved by changing the D-sugar portion of digitoxin to an L-sugar and by reducing the length of the oligosaccharide chain. It has been suggested that the differential activity of the specific cardiac glycoside analogue correlates with changes in expression/activity levels of specific α-isoforms of the Na^+/K^+-ATPase.[25] Digitoxin consists of digitoxigenin (pharmacophore) and the trisaccharide moiety, which is critical for its cardio-toxic and anticancer activity.[11] Earlier studies suggested that an α2-selective CG could result from sugar modification because the structural differences in these isoforms are primarily in the extracellular carbohydrate binding loops. Thus, sugar modification may open the potential for discovery of new and safer digitoxin analogues (i.e., more potent yet improve α-isoform selectivity). Modification of the carbohydrate section of digitoxin has been shown to significantly improve its anticancer activity. Interestingly, a very similar increased activity pattern is now seen in inhibition of HCMV replication. Thus, sugar modification may hold the potential for the discovery of new and safer digitoxin alternative for antiviral therapeutics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details for synthetic procedure and compound characterization, virus infection, and antiviral and cytotoxicity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written with contributions from all authors. HC. and R.V. were responsible for all antiviral and cytotoxicity assays. M.F. performed the real-time PCR studies. De-Xue Fu performed the flow cytometry studies. H.-Y.L.W., S.O.B., and H.L. were responsible for compounds preparation.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HCMV, human cytomegalovirus; CG, cardiac glycoside

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