Inhibition of the Sodium/Potassium ATPase Impairs N-Glycan Expression and Function

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Abstract

Aberrant N-linked glycans promote the malignant potential of cells by enhancing the epithelial-to-mesenchymal transition and the invasive phenotype. To identify small molecule inhibitors of N-glycan biosynthesis, we developed a chemical screen based on the ability of the tetravalent plant lectin L-phytohemagglutinin (L-PHA) to bind and crosslink surface glycoproteins with β1,6GlcNAc-branched complex type N-glycans and thereby induce agglutination and cell death. In this screen, Jurkat cells were treated with a library of off-patent chemicals (n = 1,280) to identify molecules that blocked L-PHA–induced death. The most potent hit from this screen was the cardiac glycoside (CG) dihydroouabain. In secondary assays, a panel of CGs was tested for their effects on L-PHA–induced agglutination and cell death. All of the CGs tested inhibited L-PHA–induced death in Jurkat cells, and the most potent CG tested was digoxin with an EC50 of 60 ± 20 nmol/L. Digoxin also increased the fraction of some concanavalin A–binding N-glycans. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, digoxin significantly increased GlcNAc2Man5GlcNAc2Fuc1 and GlcNAc2Man5GlcNAc2Fuc1 oligosaccharides demonstrating an impairment of the N-glycan pathway. Consistent with this effect on the N-glycan pathway, digoxin inhibited N-glycosylation–mediated processes of tumor cell migration and invasion. Furthermore, digoxin prevented distant tumor formation in two mouse models of metastatic prostate cancer. Thus, taken together, our high throughput screen identified CGs as modifiers of the N-glycan pathway. These molecules can be used as tools to better understand the role of N-glycans in normal and malignant cells. Moreover, these results may partly explain the anticancer effect of CGs in cardiovascular patients. [Cancer Res 2008;68(16):6688–97]

Introduction

Malignant cells display characteristic changes in N-glycan structures on surface glycoproteins including receptors and transporters that contribute to cancer progression and metastasis (reviewed in ref. 1). Oncogene activation stimulates increased expression of the Golgi enzymes that generate β1,6GlcNAc-branched tetraantennary N-glycans. These N-glycans are found on proteins including growth factor receptors and integrins, and have been shown to enhance growth signaling in motile tumor cells (2–4). Chemical or genetic disruption of the N-glycosylation pathway in cancer cells decreases these malignant features (5, 6). Therefore, targeting defective glycosylation pathways may be a novel approach for the treatment of malignancy and a strategy to prevent metastasis.

The N-glycosylation pathway begins in the lumen of the endoplasmic reticulum, and remodeling in the Golgi apparatus generates structural diversity. To initiate the pathway, the oligosaccharide precursor Glc3Man9GlcNAc2 is transferred en bloc from dolichol-PPi onto asparagine (Asn) residues in the sequence Asn-X-Ser/Thr (where X can be any amino acid except for proline) to form an Asn-linked glycan (N-glycan). Once attached to the protein, this precursor is modified through a well-defined pathway leading to the sequential removal of the three sugars and one mannose by the actions of rough endoplasmic reticulum glycosidases to form Man5GlcNAc2-Asn. In the Golgi, Man5GlcNAc2-Asn is further modified by the removal of mannoses via Golgi mannosidases and by the addition of GlcNAc via GlcNAc transferases, leading to the generation of hybrid and complex N-glycans. Finally, other sugars such as fucose, galactose, and sialic acid are added to the N-glycans to increase their diversity (Fig. 1; reviewed in refs. 7, 8).

To better understand the N-glycosylation pathway and identify strategies to target the aberrant N-glycosylation in neoplastic cells, we designed, automated, and conducted a chemical screen of off-patent drugs and chemicals. This high-throughput screen identified cardiac glycoside (CG) Na+/K+-ATPase inhibitors that altered N-glycosylation. Subsequent evaluation showed that inhibition of the Na+/K+-ATPase increased the fraction of concanavalin A (ConA)-binding N-glycans. Moreover, the effects of CG on N-glycosylation seemed functionally important as these compounds inhibited the glycosylation-mediated processes of cell migration and invasion as well as decreased distant tumor formation in vivo.

Materials and Methods

Reagents. The LOPAC chemical library, L-PHA, digoxin, digitoxin, dihydroouabain, and fibronectin were purchased from Sigma-Aldrich.

Cell culture. Jurkat human leukemia, WRO human thyroid carcinoma, PPC-1 human prostate cancer, and Colo320 colorectal adenocarcinoma cells were maintained in RPMI 1640. HT1080 human fibrosarcoma, 5637 human bladder carcinoma, and HeLa human cervical cancer cells were maintained...
Cardiac Glycosides Impair the N-Glycosylation Pathway

Figure 1. Scheme of the N-glycan biosynthesis pathway. The N-glycan biosynthesis pathway is shown. The biosynthesis of N-glycans involves the following steps: (a) sequential conversion of the high mannose N-glycan M9Gn2 to M8Gn2, M7Gn2, M6Gn2, and M5Gn2 (oligosaccharides before α-mannosidase II in Table 1); (b) addition of α2-linked GlcNAc to the terminal α3-linked Man of M5Gn2 by GlcNAcTI to form Gn1M3Gn2 (oligosaccharides before α-mannosidase II in Table 1); (c) removal of two Man residues from Gn1M3Gn2 by α-mannosidase II to form Gn1M3Gn2 (oligosaccharides after α-mannosidase II-Complex structures in Table 1); (d) addition of α-L-Fucose (F) and Galactose (G) residues. Swainsonine (SW) is a known inhibitor of the α-mannosidase II enzyme. OT, oligosaccharide/transferase; GI and GIi, the α-glucosidases; TI, TIi, and TV, α-N-acetylgalactosaminyltransferases; MI, the α-1,2mannosidases; MIi and MIii, α-1,3/6mannosidases; Gal-T, α-sialyltransferases; Sa T, α-sialyltransferases; UDP-GlcNAc, UDP-N-acetylgalactosamine; UDP-Gal, UDP-galactose; CMP-SA, CMP-sialic acid. The Golgi apparatus subdomains (Cis, Medial, and Trans) are shown by separate boxes. Gray highlights, the N-glycan structures that bind ConA and L-PHA.

in DMEM. All cells were supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics. WRO cells were also supplemented with 1 mmol/L sodium pyruvate. All cell lines were cultured in a standard humidified incubator at 37°C in a 5% CO2 atmosphere.

High-throughput screen for inhibitors of L-phytohemagglutinin-induced cell death. Liquid handling was performed by a Biomek FX Laboratory Automated Workstation (Beckman Coulter). Jurkat cells (5,000 cells per well) were seeded in 96-well plates followed by the addition of aliquots from the LOPAC library of 1,280 off-patent drugs and chemicals with a final DMSO concentration of 0.05%. Jurkat cells were selected for this assay as their growth in suspension conditions facilitated the automated nature of this screen. Twenty-four hours after addition of the compound library, L-phytohemagglutinin (L-PHA) was added at a final concentration of 20 μg/mL (9). Forty eight hours after the addition of L-PHA, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay according to the manufacturer’s protocols (Promega) and as previously described (10). Cell viability was calculated relative to vehicle-treated (0.1% DMSO) control cells on each plate. To identify statistically significant hits, we calculated the Z score for each hit relative to the negative control. These Z scores were transformed into p values based on the standard-normal distribution. To control for multiple testing, we used a false discovery rate (FDR) correction to generate a q value for each compound (11). Statistically significant hits were selected as those with a q value of <5%, at which level four compounds were identified.

Measurement of ConA-binding glycopeptides. Quantification of ConA-binding glycans after swainsonine and digoxin treatment was performed as follows. Briefly, Jurkat cells (1 × 10⁶) were treated with 2 μmol/L swainsonine, 100 mmol/L digoxin, or buffer control for 24 h. After treatment, cells were harvested, washed, and sonicated. The homogenate was centrifuged and the pellet was solubilized in 6 mol/L guanidine-HCl in 0.1 mol/L Tris buffer (pH 8.0) containing 20 mmol/L DTT to reduce disulfide bonds. Solid iodoacetamide was then added to a final concentration of 60 mmol/L, and the solution was incubated for 1 h to block free thiol groups. The solution was then centrifuged to remove any insoluble residue. The solubilized, reduced, and alkylated proteins were precipitated with 10 volumes of cold absolute ethanol-glacial acetic acid.

The pellet was collected, dried, and suspended in 50 mmol/L NH₄HCO₃. Trypsin (Promega) at 1:100 (w:w), relative to total protein, was added (half at the beginning of the digestion and the other half 3 h later), and after an overnight digestion, the enzyme was denatured by boiling. After proteolysis and centrifugation, the supernatant was dried and redissolved in PBS. The peptide mixture was loaded onto a ConA-Sepharose column equilibrated with PBS buffer. After washing the column using PBS, the ConA-bound glycopeptides were eluted with 15% methyl-α-D-mannoside in PBS. A Sep Pak-C18 cartridge was then used to remove the methyl-α-D-mannoside. The glycopeptides were eluted from Sep Pak-C18 with 50% acetonitrile containing 0.1% trifluoroacetyl, and the solution was lyophilized. Equal amounts of material were dissolved in H2O, and the amount of hexose in the sample was determined by the phenol-sulfuric acid method (12).

ConA binding to cell surface glycans. Cells (500 cells per well) were seeded in 96-well plates. After adhesion overnight, cells were treated with increasing concentrations of digoxin for 48 h. After treatment, cells were fixed with 3.7% formaldehyde and washed. Surface N-glycans were stained.


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with ConA (20 µg/mL) conjugated to tetramethylrhodamine B isothiocyanate (TRITC; EY Laboratories) and nuclei were stained with Hoechst 33342. The total intensity of ConA staining on each cell was quantified using Cellomics ArrayScan II (Cellomics; ref. 2).

**siRNA transfections.** Cells (1 × 10⁶) were seeded in 6-well plates and transfected the next day using Lipofectamine 2000 (Invitrogen) and double-stranded siRNAs targeting either the human Na⁺/K⁺-ATPase, or Non-Targeting siRNA (siControl; Smartpool, Dharmacon). Cells were harvested 72 h posttransfection and then assayed for ConA binding to cell surface glycans.

**Reverse-transcriptase real-time PCR.** First-strand cDNA was synthesized from 1 µg of DNase-treated total cellular RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Real-time PCR assays were performed in triplicate with 5 ng of RNA equivalent cDNA, SYBR Green PCR Master mix (Applied Biosystems), and 400 nmol/L of gene-specific primers. Reactions were processed and analyzed on an ABI 7900 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: murine Na⁺/K⁺-ATPase, Forward 5'-TGT GAT TCT GGC TGA TGA AA GCT GCG-3' and Reverse 5'-TCT AGA TGA CCA AGT CG-3'; 18S, Forward 5'-AGG AAT TGA CCG AAC ACG AC-3' and Reverse 5'-GGA CAT CTA AGG GCA TCA CA-3'. Relative mRNA expression was determined using the ΔΔCT method as described (13).

**DNA constructs and generation of stable cell lines.** Stable cell lines expressing the α subunit of the murine Na⁺/K⁺-ATPase were engineered by transfecting PPC1 human prostate cancer cells with cDNA corresponding to the murine Na⁺/K⁺-ATPase in pCDNA3 vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells stably expressing murine Na⁺/K⁺-ATPase were selected with 800 µg/mL G418 (Invitrogen).

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to semiquantify N-glycan expression.** Jurkat cells (1 × 10⁶) were treated with 100 µmol/L digoxin, 2 µmol/L Swainsonine, or buffer control. After incubation, cells were lysed in 35 mmol/L Tris, 8 mol/L urea, 4% CHAPS, and 65 mmol/L DTT (pH 8.0) followed by sonication and freezing. Equal amounts of protein were dialyzed with cassettes (Slide-A-Lyzer; molecular weight cutoff of 7,000; Pierce) in 10 mmol/L NH₄HCO₃/0.02% SDS. After dialysis, samples were concentrated via vacuum centrifugation. Samples were deglycosylated at 37°C for 48 h with PNGase F (New England Biolabs). Oligosaccharides were purified via C18 and porous graphitized carbon solid phase extraction. The composition of N-glycans was semiquantified using mass spectrometry via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), using DHB as matrix, on a MALDI-CFR System (Applied Biosystems). Forward/reverse PCR primer pairs for human Na⁺/K⁺-ATPase were designed using the online tool (14). The intensities of the peaks were arbitrarily normalized to the level of (Hexose)2(2HexNAc)2(-Deoxyhexose).1

**Scratch wound–healing assay.** Scratch wound–healing assay was performed as previously described (15, 16). Briefly, HT1080 (1 × 10⁶) cells were treated with digoxin or buffer control. After 24 h, cells were harvested and seeded (1 × 10⁶) on fibronectin-coated 4-well chamber slides. After adhering overnight, the monolayer was scratched with a plastic pipette tip. Migration of the cells over 6 h was captured with a digital camera (Nikon) mounted on an inverted microscope (Nikon). Cellular migration was measured in relative units (pixels).

**Migration and invasion assays.** Inversion and migration assays were performed as previously described (15). Briefly WRG cells (2 × 10⁶) were treated with digoxin or buffer control for 24 h. After treatment, cells were harvested and seeded in uncoated invasion chambers for migration assay, or BioCoat Matrigel Invasion Chambers (BD Biosciences) in serum-free RPMI 1640 containing 0.2% bovine serum albumin were used for invasion assays. Growth medium containing 5% FBS was used as a chemoattractant in the bottom well. After 24 h of incubation, cells that had migrated or invaded the lower surface of the membrane were stained with Diff-Quik Stain (BD Biosciences). The number of migrating or invading cells were imaged and counted using the Aperio ScanScope CS whole slide scanner (Aperio Technologies) and Image-Pro Plus Software (version 4.5; Media Cybernetics, Inc.).

**Results**

**Inhibitors of L-PHA–induced cell death identified via a high-throughput screen.** Phytothemagglutinin is a tetravalent plant lectin that binds to crosslinks complex N-glycoproteins on cell surfaces and induces agglutination and cell death (18). To identify compounds that alter N-glycan remodeling in malignant cells, we developed, automated, and conducted a chemical screen for inhibitors of L-PHA–induced cell death in Jurkat cells. In the optimized assay, Jurkat cells were seeded in 96-well plates treated with aliquots of the LOPAC (n = 1,280) library of off-patent drugs and chemicals (final concentration, ~5 µmol/L and 0.05% DMSO). Twenty-four hours after incubation, cells were treated with L-PHA (final concentration, 20 µg/mL), and 48 hours later, cell viability was measured by the MTS assay (Fig. 2A). As controls, cells received L-PHA or buffer alone. To identify molecules that inhibited L-PHA–induced cell death, we used a statistically robust methodology. For each compound in the LOPAC library, we calculated a Z score using the extensively replicated L-PHA control wells. These Z scores were converted into P values using the standard normal distribution. As we screened 1,280 distinct compounds, we then applied a FDR correction for multiple hypothesis testing to the vector of P values (11). Statistically significant hits were defined as compounds with a q value (adjusted P value) of <0.05, indicating a false-positive rate of at most 5%. Four statistically significant hits were identified in this manner. Of the four statistically significant hits, secondary screening validated one compound: the Na⁺/K⁺-ATPase inhibitor dihydroouabain. This compound restored viability of L-PHA–treated cells to 70% or over of untreated cells.

**Other cardiac glycosides inhibit L-PHA–induced cell death.** Dihydroouabain belongs to the CG family of Na⁺/K⁺-ATPase inhibitors, which are used clinically to treat patients with heart...
failure and atrial arrhythmias (19). Moreover, CGs have potential antitumor properties in patients (20, 21), but the mechanism by which they exert this effect is unknown. To determine whether the effects on N-glycan remodeling were specific to dihydroouabain or a class-effect of CGs, we evaluated a panel of CGs for inhibition of L-PHA-induced cell death. Jurkat cells were treated with L-PHA along with increasing concentrations of digoxin, digitoxigenin, digitoxin, and dihydroouabain, and cell viability

![Chemical structures of cardiac glycosides](image)

**Figure 2.** A chemical screen identifies cardiac glycosides as inhibitors of L-PHA–induced cell death. A, Jurkat cells (5,000) were seeded in 96-well plates and treated with aliquots of the LOPAC chemical library (final concentration, ~5 μmol/L). Twenty four hours after treatment, L-PHA (final concentration, 20 μg/mL) was added. Forty eight hours after L-PHA addition, cell viability was measured by an MTS assay. Viability was expressed as a percentage of buffer treated control cells. Statistically significant hits were selected as those with a q value of <5%. Four statistically significant hits were identified in this manner, which restored viability of L-PHA–treated cells to 70% or over of untreated cells (dashed line). B, Jurkat cells (5,000) were seeded in 96-well plates and treated with increasing concentrations of dihydroouabain, digitoxigenin, digitoxin, and digoxin. Twenty four hours after treatment, L-PHA (final concentration, 20 μg/mL) was added. Forty eight hours after L-PHA addition, cell viability was measured by an MTS assay. Viability was expressed as a percentage of buffer treated control cells. Points, mean percent viable cells; bars, SD.
Figure 3. Digoxin increases levels of ConA-binding glycoproteins through inhibition of the Na⁺/K⁺-ATPase. A, Jurkat cells (1 × 10⁷) were treated with 100 nmol/L digoxin, 2 μmol/L Swainsonine, or buffer control for 24 h. After treatment, cells were harvested and the levels of ConA-binds glycopeptides were measured by the colorimetric technique as described in the Materials and Methods. Columns, mean concentration of ConA-binding glycopeptides; bars, SD. B, Colo320, 5637, PPC-1, HeLa, WRO, and HT1080 cells (1 × 10⁴ cells/mL) were seeded in 96-well plates and treated with increasing concentrations of digoxin for 48 h. After incubation, cells were stained with TRITC-labeled ConA and Hoechst 33342. The intensity of ConA staining was measured using the automated Arrayscan microscope as described in the Materials and Methods. Points, mean fluorescent intensity; bars, SD. C, PPC-1 cells stably transfected with cDNA corresponding to the α₁ subunit of murine Na⁺/K⁺-ATPase or vector control were treated with digoxin (50 nmol/L) for 48 h. After incubation, cells were stained with TRITC-labeled ConA and Hoechst 33342 and the intensity of ConA staining was measured using the automated Arrayscan microscope as described in the Materials and Methods. Columns, mean fluorescent intensity; bars, SD. D, PPC-1 cells were transfected with siRNA corresponding to the human Na⁺/K⁺-ATPase or a control siRNA. Three days after transfection, cells were harvested and stained with TRITC-labeled ConA and Hoechst 33342 and the intensity of ConA staining was measured using the automated Arrayscan microscope as described in the Materials and Methods. Columns, mean fluorescent intensity; bars, SD.
was measured by the MTS assay (Fig. 2B). All CGs tested blocked L-PHA–induced cell death. The most potent CG tested was digoxin that inhibited L-PHA–induced cell death with an EC50 of 60 ± 20 nmol/L. This result is in keeping with digoxin being a more potent inhibitor of the Na+/K+-ATPase than dihydrouoabain and digitoxigenin (22). In addition to inhibition of L-PHA–induced cell death, CGs also blocked L-PHA–mediated agglutination of the Jurkat cells (data not shown). Of note, at higher concentrations of the CGs or longer periods of incubation, the CGs directly induced cell death, consistent with previous reports (23, 24). Compared with the human isoform, CGs bind the α1 subunit of murine Na+/K+-ATPase with less affinity, thereby rendering murine cells resistant to any effects of CGs that are mediated through this ATPase (25, 26). Consistent with this prediction, the tested CGs did not block L-PHA–induced cell death nor agglutination of MDAY-D2 murine leukemia cells (data not shown), suggesting that CGs inhibition of L-PHA toxicity to Jurkat cells requires its known function as inhibitors of human Na+/K+-ATPase.

Digoxin increases the levels of total and cell surface ConA-binding glycoproteins. To explore the effect of inhibiting the Na+/K+-ATPase on the N-glycan profile of cancer cells, we measured the effects of digoxin on levels of ConA-binding high mannos and hybrid N-glycans. Jurkat cells were treated with digoxin (100 nmol/L), the known Golgi α-mannosidase II inhibitor, swainsonine (2 μmol/L; refs. 18, 27), or buffer control for 24 hours. After treatment, the abundance of ConA-binding glycopeptides was measured by the phenol-sulfuric acid method as described in the Materials and Methods section. Digoxin and swainsonine increased the abundance of ConA-binding glycopeptides 2.3- and 1.8-fold, respectively, compared with controls (Fig. 3A).

To further explore the effects of Na+/K+-ATPase inhibition on ConA-binding glycans and to evaluate the generalizability of this finding, we measured the binding of ConA to the surface of a panel of solid tumor cell lines. Colo320, 5637, HeLa, WRO, PPC-1, and HT1080 cells were treated with increasing concentrations of digoxin for 48 hours. After treatment, cells were fixed and stained with TRITC-labeled ConA. The binding of fluorescently labeled ConA was quantified by the Cellomics Array scan (Fig. 3B). Consistent with modification of the N-glycan pathway, digoxin increased the binding of ConA to the surfaces of all the cell lines studied.

To determine whether the effects of digoxin on the surface expression of ConA-binding proteins were due to inhibition of its known target Na+/K+-ATPase, we overexpressed in PPC1 human Table 1. Digoxin and swainsonine alter the expression of oligosaccharides

<table>
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NOTE: Jurkat cells (1 × 10⁷) were treated with 100 nmol/L digoxin, 2 μmol/L Swainsonine, or buffer control for 24 h. After incubation, oligosaccharides were isolated and analyzed by MALDI-TOF mass spectrometry. The ability of glycans to bind to ConA is based on previous studies using standard N-glycans and ConA-Sepharose columns (38, 39). The data represent the normalized intensity of each oligosaccharide relative to (Hexose)²(HexNAc)²(Deoxyhexose)¹. Previous work from several groups (e.g., ref. 40) with mass spectrometry of N-glycans has shown that the m/z values in this table correspond to structures in which Hexose is D-Mannose (M) or unidentified (probably D-Galactose), HexNAc is N-Acetyl-D-glucosamine (Gn), and Deoxyhexose is l-Fucose.
prostate cancer cells the α1 subunit of murine Na\(^+\)/K\(^+\)-ATPase that is less sensitive to cardiac glycosides. Increased expression of the Na\(^+\)/K\(^+\)-ATPase was confirmed by reverse-transcriptase real-time PCR (data not shown). Overexpression of the α1 subunit of murine Na\(^+\)/K\(^+\)-ATPase blocked the effects of digoxin and prevented the increase in surface ConA staining (Fig. 3C). Likewise, we transfected PPC-1 cells with siRNA against the human Na\(^+\)/K\(^+\)-ATPase and knockdown of the Na\(^+\)/K\(^+\)-ATPase was confirmed by Q-RT-PCR (data not shown). Compared with cells treated with control siRNA, PPC-1 cells transfected with siRNA against the Na\(^+\)/K\(^+\)-ATPase displayed increased ConA-binding proteins on the cell surface (Fig. 3D). Likewise, knockdown of the Na\(^+\)/K\(^+\)-ATPase with siRNA in HeLa cells showed a similar effect on ConA surface–binding proteins (data not shown). Thus, taken together, these results show that inhibition of the Na\(^+\)/K\(^+\)-ATPase has a significant modifying effect on the N-glycosylation pathway.

MALDI-TOF mass spectrometry shows an alteration of the N-glycosylation pathway down stream of α-mannosidase II. To identify the site in the N-glycan pathway blocked by Na\(^+\)/K\(^+\)-ATPase inhibitors, Jurkat cells were treated with digoxin, the known Golgi α-mannosidase II inhibitor swainsonine, or buffer control. After treatment, the expression profile of intracellular oligosaccharides was measured by MALDI-TOF mass spectrometry (Table 1). Swainsonine increased the abundance of M9Gn2, M8Gn2, M7Gn2, M6Gn2, and M5Gn2, which is consistent with a block at Golgi α-mannosidase II and accumulation of these precursors. In contrast, treatment with digoxin decreased the abundance of these oligosaccharides but increased the levels of Gn1M3Gn2F1 hybrid and Gn2M3Gn2F1 biantennary complex N-glycans. Slight increases in Gn3M3Gn2F1 triantennary and Gn4M3Gn2F1 tetraantennary complex N-glycans were also seen. These results suggest that inhibition of the Na\(^+\)/K\(^+\)-ATPase by digoxin impairs the N-glycan pathway, thereby promoting the accumulation of hybrid and biantennary complex N-glycans. Digoxin therefore inhibits the N-glycan remodeling through a mechanism distinct from swainsonine.

Digoxin decreases migration and invasion of malignant cells. Increased GlcNAc branching of N-glycans on the cell surface promotes the malignant and metastatic potential of cells by altering cell migration and invasion (1, 28). As treatment with digoxin altered the N-glycan remodeling, we evaluated the effects of this compound on the N-glycosylation–mediated processes of cell migration and invasion. To determine the effects on cell migration, we evaluated the effect of digoxin in a scratch wound-healing assay. HT1080 human fibrosarcoma cells were treated with digoxin or buffer control and seeded in fibronectin-coated 4-well chamber slides. After adhering overnight, the monolayer was scratched to create a wound. Migration of cells to heal the wound was measured over time. Treatment with digoxin impaired cell migration and delayed wound healing (Fig. 4A). Of note, at the concentrations tested in these assays, digoxin-treated cells were >90% viable as measured by the MTS assay. Furthermore, the treated cells migrated sufficiently to completely heal the wound by 24 hours. Thus, the effects of digoxin on cell migration cannot be attributed to simple reductions in cell viability.

To further evaluate the effects of digoxin on cell migration, WRO human thyroid carcinoma cells were treated with digoxin, and migration through uncoated invasion chambers with an 8-μm pore size was measured (Fig. 4B). Again, digoxin inhibited cell migration but did not reduce cell viability as measured by MTS assay.
Finally, we assessed the effects of digoxin on cell invasion (Fig. 4C). WRO cells were treated with digoxin and seeded into Matrigel-containing invasion chambers in serum-free medium. Medium with 5% FBS was placed in the lower chamber as a chemo-attractant. Twenty four hours after seeding, cell invasion through the Matrigel was measured. Digoxin treatment decreased cell invasion but did not reduce cell viability as measured by the MTS assay.

Thus, taken together, concentrations of digoxin that blocked N-glycan remodeling also inhibited the N-glycosylation–mediated processes dependent on GlcNAc-branched N-glycans: cell migration and invasion. These results suggest that the effects of digoxin on N-glycan expression are functionally important.

**Digoxin decreases distant tumor formation in vivo.** As aberrant GlcNAc branching of N-glycans on the cell surface promotes metastases, we tested the effects of digoxin on distant tumor formation in mouse models of metastatic prostate cancer. In the first model, dsRed-labeled PPC-1 cells were treated with digoxin (100 nmol/L), or buffer control in culture. After 20 hours of treatment, cells were injected i.v. into sublethally irradiated SCID mice. Three weeks after injection, mice were sacrificed and distant tumor formation in the organs was imaged with fluorescent microscopy.

Invasion of the prostate cancer cells was detected in the lung, bone, and liver, clinically relevant sites of metastases in prostate cancer. In particular, metastasis of dsRed-PPC-1 cells to the lung was readily quantifiable using image-based analysis (10, 17, 29, 30). Compared with buffer control, mice injected with digoxin-treated cells had decreased mean tumor number (142 ± 71 tumors versus 20 ± 19 tumors; \( P = 0.0004 \) by Student’s \( t \) test) within the lung (Fig. 5A). Median tumor number was also significantly attenuated by drug treatment (data not shown). It is important to note that both treated and control cells were >90% viable at the time of injection. To determine whether the decreased distant tumor formation was simply due to decreased proliferation, DsRed-PPC-1 cells were treated in culture with digoxin or buffer control and injected s.c. into mice. In contrast to its effects on distant tumor formation, digoxin did not significantly alter the growth of dsRedPPC-1 cells injected s.c. compared with cells treated with buffer control (227 ± 50 mg versus 331 ± 81 mg; \( P = 0.24 \) by Student’s \( t \) test; Fig. 5B). Therefore, the reduction in tumor burden after digoxin treatment is not solely due to reductions in cellular proliferation.

To further evaluate the effects of digoxin on distant tumor formation, sublethally irradiated SCID mice were injected i.v. with dsRed-PPC-1 cells and then treated i.p. with digoxin (1.35 and 0.675 mg/kg) or buffer control daily for 14 days. Four weeks after the injection of the cells, mice were sacrificed and analyzed as above. Compared with buffer control, both concentrations of digoxin decreased the mean number of tumors (48 ± 32 tumors (0.65 mg/kg) versus 28 ± 19 tumors (1.35 mg/kg) versus 171 ± 83 tumors (control)) in the lungs (Fig. 5C). Similar reductions in median tumor number were also observed (data not shown). Thus, taken together, digoxin inhibits distant tumor formation in vivo.

**Discussion**

To better understand the effect of modulating N-glycosylation in malignant cells, we used a chemical biology screen for inhibitors of L-PHA–induced cell death to identify novel regulators of glycosylation. From this screen, we identified the CG dihydroouabain and determined that structurally related CGs also blocked L-PHA–induced cell death. Of the tested CGs, digoxin was the most potent inhibitor. These results, coupled with the lack of effect on murine cells that suggest that CGs alter N-glycosylation by inhibiting their known target, the Na+/K+-ATPase. Further supporting a mechanism linked to the known binding target, overexpression of the murine Na+/K+-ATPase that is less sensitive to CG inhibition abrogated the effects of digoxin on surface ConA-binding N-glycoproteins. Moreover, knockdown of the Na+/K+-ATPase using siRNA recapitulated the effects of digoxin. Thus, taken together, we conclude that inhibiting the Na+/K+-ATPase alters the N-glycosylation pathway.

In this report, we used four independent approaches to show the ability of CGs to modify the N-glycosylation pathway. First, L-PHA induces cell death by binding primarily to cell surface N-glycans.
that occur downstream of the GnTI in the N-glycan synthesis pathway, and CGs blocked L-PHA–induced cell death. Second, CGs increased total cellular ConA-binding glycoproteins. Third, CGs increased cell surface ConA–binding N-glycoproteins. Finally, mass spectrometry revealed that digoxin increased the ConA-binding Glycogen synthase II hybrid and Gn2M3Gn2F1 biantennary N-glycans.

The profile of oligosaccharides after digoxin treatment indicates that digoxin alters N-glycosylation. These results also indicate that digoxin alters the pathway through a mechanism distinct from the α-mannosidase II inhibitor swainsonine.

In this report, we showed that digoxin inhibited cellular migration and invasion, which are known functional consequence of blocking the N-glycan branching. For example, Mga5−/− mice have reduced cancer growth and metastasis (31). Likewise, siRNA knockdown of GlnAc-IV in malignant cells impairs cell migration and invasion (28). Although digoxin’s inhibition of cell migration and invasion are consistent with its effects on the N-glycan remodeling, we cannot exclude that these effects are related to other pathways effected by the molecule.

CGs can induce cell death in malignant cells (23, 24) through multiple mechanisms including activation of Cdk5 (32), Src kinase (33), and p21 (33). However, the effects of CG on N-glycan pathway and cellular processes of migration and invasion were not artifacts of cell death, as the concentrations of digoxin and times of incubation required to alter N-glycan remodeling were lower than those required to induce cell death. Furthermore, as the concentrations of CG required to alter the N-glycan pathway are lower than the concentrations associated with activation of these other pathways, we suspect that the effects of CG on N-glycosylation are not related to activation of these other pathways.

Serum concentrations of 2 nmol/L digoxin can be achieved in humans without significant toxicity (34). Although the concentration of digoxin required to inhibit N-glycosylation exceeded 2 nmol/L, potentially, chronic daily dosing of digoxin could sufficiently reduce N-glycan branching to affect the function of downstream effectors. Interestingly, patients with breast carcinoma who were coincidentally receiving CGs for cardiac dysfunction had a lower rate of relapse and metastasis than patients not receiving CGs (20, 21). Our results suggest that CGs could have an antitumor effect in patients by altering the N-glycosylation profile in malignant cells. To assess the effects of digoxin on distant tumor formation and mimic some of the processes of metastasis, we tested digoxin in two mouse models. In both of these models, digoxin decreased distant tumor formation. Thus, the decreased metastases supports a mechanism of action linked to the inhibition of glycosylation. Thus, digoxin could be a lead for a novel therapeutic agent for the treatment of malignancy and an adjunct to prevent metastases. A limitation to the xenograft studies, however, is that we cannot be certain that digoxin prevented metastases through a mechanism related to ability to inhibit glycosylation. Potentially inhibition of the Na+/K+-ATPase may have antitumor effects through mechanisms distinct from impairing glycosylation.

Interestingly, other glycosylation inhibitors have entered clinical trials for the treatment of malignancy. Swainsonine, a small molecule inhibitor of Golgi α-mannosidase II, has antitumor activity in preclinical models (27, 35). Given these results, swainsonine was advanced into a phase I clinical trial for patients with refractory malignancy. In the context of this trial, tumor regression was noted in one patient with head and neck cancer (36, 37). Thus, inhibiting α-mannosidase II may be a clinically effective anticancer strategy and inhibiting targets downstream of α-mannosidase II might also produce antitumor effects without untoward toxicity.

In summary, we used a chemical biology approach to investigate the Golgi N-glycan pathway. Our high-throughput screen identified Na+/K+-ATPase inhibitors that altered the N-glycan branching at a point distinct from the α-mannosidase II inhibitor. Na+/K+-ATPase inhibitors also blocked the N-glycan–dependent processes of cell migration and invasion of cancer cells as well as distant tumor formation in mouse models. Thus, our results help explain previously reported anticancer effects of these compounds and highlight new strategies for the development of anticancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Cardiac Glycosides Impair the N-Glycosylation Pathway


Correction: Cardiac Glycosides Impair the N-Glycosylation Pathway

In the article on how cardiac glycosides impair the N-glycosylation pathway in the August 15, 2008 issue of Cancer Research (1), there is an error in Table 1. In the second section, titled "Oligosaccharides after α-mannosidase II–hybrid structures", the third column should be labelled "Ctrl" and the fourth column should be labelled "Digoxin".

Inhibition of the Sodium/Potassium ATPase Impairs N-Glycan Expression and Function

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