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Elucidating the Mechanism of Antimigratory Activity of Cardiac Glycosides

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Elucidating the Mechanism of Antimigratory Activity

of Cardiac Glycosides

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Spring 2012

Submitted in Fulfillment of the Honors Scholar Requirements
Abstract

The focus of this research is on cell migration and how it can be better understood through the use of small molecules that modulate cell migratory activity. The results have particular relevance in the realm of cancer pharmacology. Cardiac glycosides, which are known inhibitors of the eukaryotic Na⁺/K⁺-ATPase, have been determined to have antimigratory activities through the screening of several small molecule libraries. Here we investigate the antimigratory activities of the cardiac glycoside digitoxin as well as its analogs that we synthesized. Antimigratory activity was determined by conducting a wound closure assay with MDA-MB-231 human breast carcinoma cells. This antimigratory activity was compared to the inhibitory activity when Na⁺/K⁺-ATPase was treated with digitoxin and its analogs in a Na⁺/K⁺-ATPase assay. A coloration between wound closure activity Na⁺/K⁺-ATPase inhibitory activity provides evidence that cardiac glycosides inhibit cell migration through their interaction with the Na⁺/K⁺-ATPase protein. This research will provide the grounds to study the specific pathways in which Na⁺/K⁺-ATPase is linked to the regulation of cell migration.
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Finally, I would like to put forward a most heartfelt recognition to the late Dr. John Tanaka, I will miss you deeply.
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Chapter One: Introduction

1A. What are cardiac glycosides?

Cardiac glycosides are a class of naturally occurring small molecules that have been used as drugs to treat heart conditions, namely congestive heart failure and cardiac arrhythmias such as atrial fibrillation. Recorded medicinal use of cardiac glycosides dates back as far as 1785, when Sir William Withering first described using the foxglove plant as a treatment for “dropsy,” or edema associated with congestive heart failure.¹ ² The foxglove plant, Digitalis purpurea, is still used as a source for extracts containing the cardiac glycoside digoxin, which has well studied inotropic properties that have been shown to increase myocardial contractility.²

Some of the most well known and well studied cardiac glycosides include digoxin, digitoxin, ouabain, oleandrin, and bufalin. Although these natural molecules each have their own unique chemical structure, they all share a conserved core framework.
As the name cardiac glycoside implies, each molecule has a glycone or sugar portion. The glycone can be a monosaccharide such as the single L-rhamnose on ouabain or it can be a polysaccharide chain such as the 2-deoxy trisaccharide of digitoxin. The sugars tend to be the part of the chemical structure that has the least significant effects on the biological activities of the molecules.

The glycone portion of the cardiac glycoside can be cleaved by hydrolysis to leave the aglycone. The suffix –genin is used to indicate that the structure only includes the aglycone. So the aglycone of digitoxin is called digitoxigenin. The aglycone is composed of two important regions: the tetracyclic steroidal core, and the lactone on C-17. The steroid moiety consists of 3 six-membered rings and 1 three-membered ring for a total of four fused rings. The way in which these four rings are fused provides them with a three dimensional structure that is very important to the overall shape of the entire cardiac glycoside and its biological activity. Rings A and B are fused in a cis conformation and rings C and D are also fused in a cis conformation. However, the two center rings B and C are fused in trans conformation that gives the steroid core a slight twist and a curve into a crescent shape. The level of oxidation of the steroid core with hydroxyl group varies among the different cardiac glycosides. In fact, the only difference in the structures of digoxin and digitoxin is the hydroxyl group on the C-12 of digoxin. Additional hydroxyl groups on the tetracyclic steroidal core can increase the solubility of the molecule.

The lactone ring in cardiac glycosides is always on C-17 and determines which of two groups the cardiac glycoside falls into. If the lactone ring is a butenolide ring, like in ouabain and digoxin/digitoxin, then that cardiac glycoside is a cardenolide. If the lactone ring is an α-pyrone ring, such as in bufalin, then the molecule is classified as a bufadienolide. While cardenolides are extracted as secondary plant metabolites, bufadienolides are often produced by mammals and
amphibians as well as plants and are five times as toxic in humans\(^3\). Overall, bufadienolides are the much less common form of cardiac glycosides and due to their higher toxicity, they are not used in the same pharmacological capacity as cardenolides. With regard to the biological activities of cardenolides glycosides, the butenolide ring is thought to be more important than the sugars, but less important than the tetracyclic steroidal core.

The most well studied biological activity of cardiac glycosides is their interaction with the \(\text{Na}^+/\text{K}^+\) ATPase pump, an enzyme found in the plasma membrane of virtually all animal cells. This enzyme has four subunits; two \(\alpha\) subunits that extend into the interior surface of the cell and two \(\beta\) subunits that extend into the exterior space around the cell. There are different isoforms of these subunits that are found in different cells in the body.\(^4\) Four different isoforms of the \(\alpha\) subunit (\(\alpha_1, \alpha_2, \alpha_3, \) and \(\alpha_4\)) and three different isoforms of the \(\beta\) subunit (\(\beta_1, \beta_2, \beta_3\)) have been discovered\(^5\) Combinations of these subunits are found throughout different tissues. For instance, the \(\alpha_1\beta_1\) isoform is present in all cell types, while the \(\alpha_3\beta_2\) heterodimer isoform is less common, and found in the pineal gland and retinal photoreceptor cells.\(^5\) Although it is agreed upon that cardiac glycosides bind to the \(\alpha\) subunits of the \(\text{Na}^+/\text{K}^+\) ATPase pump, it is difficult to say whether cardiac glycosides have stronger affinity to a particular \(\alpha\) subunit isoform. Different studies have shown ouabain to have greater affinity for each \(\alpha_1, \alpha_2,\) and \(\alpha_3\) depending on the study and the type of animal tissue used.

An analysis of a crystal structure of ouabain bound to the \(\text{Na}^+/\text{K}^+\) ATPase pump showed how each of the important structures (the lactone ring, the steroidal core, and the carbohydrate rhamnose) interact with the transmembrane helices of \(\text{Na}^+/\text{K}^+\) ATPase.\(^6\) The crystal structure agrees that the main binding sites for ouabain are the M5 and M6 helices on the \(\alpha\) subunit, but it challenges the previously held idea that ouabain binds to the extracellular surface portion of
these transmembrane helices. Instead, the crystal structure (Figure 1) shows that ouabain is bound deep within the helices and is held almost entirely within the phospholipid bilayer of the membrane.
The inotropic properties of cardiac glycosides are a result of inhibition of \( \text{Na}^+/\text{K}^+ \) ATPase. One of the important physiological functions of this pump is to regulate the ion concentrations across the cell’s plasma membrane. As the name implies, the \( \text{Na}^+/\text{K}^+ \) ATPase pump requires phosphorylation by ATP for each ion exchange to occur. The net result of each ion exchange is 3 \( \text{Na}^+ \) transported out of the cell, 2 \( \text{K}^+ \) transported into the cell, and one ATP consumed. When the cardiac glycoside binds to the \( \alpha \) subunit of \( \text{Na}^+/\text{K}^+ \) ATPase in the cardiac muscle, it stops this \( \text{Na}^+/\text{K}^+ \) ion exchange and allows for a greater force and velocity of contraction in the heart.\(^7\) In order to understand how this mechanism works, it may be helpful to briefly describe the normal mechanism of contraction in cardiac muscle.

Muscle contraction starts with the generation of an action potential, or depolarization of the surface of a cell. This action potential is a result of a rapid exchange of ions across the cell membrane. Once the action potential is created, there is a release of \( \text{Ca}^{2+} \) from the sacroplasmic reticulum. \( \text{Ca}^{2+} \) binds to troponin, the regulatory protein in the interaction between actin and myosin.\(^8\) Actin and myosin will interact and continue to cause muscle contractions until the \( \text{Ca}^{2+} \) is no longer present to bind troponin. The cell returns \( \text{Ca}^{2+} \) levels back to normal by pumping it out of the cell in exchange for \( \text{Na}^+ \) flowing down its concentration gradient into the cell.\(^8\)

When cardiac glycosides bind to \( \text{Na}^+/\text{K}^+ \) ATPase, they effectively inhibit the exchange of \( \text{Na}^+ \) and \( \text{K}^- \) ions. This disrupts the concentration gradient of \( \text{Na}^+ \), and prevents \( \text{Na}^+ \) from flowing down its gradient into the cell in exchange for \( \text{Ca}^{2+} \).\(^9\) Since there is no way to effectively remove \( \text{Ca}^{2+} \) from the cell, \( \text{Ca}^{2+} \) continues to binds troponin and allow for the interaction of actin and myosin. This results in greater force in the contractions of the cardiac muscle.\(^10\)

Although it is most well known for its ion exchange activity, the \( \text{Na}^+/\text{K}^+ \) ATPase pump is becoming increasingly thought of as an isoenzyme.\(^5\) Rather than accounting for the activity of
one biological process, it is a critical enzyme for an entire host of critical cell functions. Na⁺/K⁺ ATPase is responsible for creating the electrochemical gradient across the plasma membrane that allows for the secondary transport of various ions, substrates, and neurotransmitters via Na⁺-coupled transport systems. The electrochemical gradient created by Na⁺/K⁺ ATPase is also critical to renal physiology, as it facilitates the reabsorption of water in the proximal convoluted tubules of the nephrons to allow for the maintenance of homeostasis of electrolytes and fluids in the body. The ability of Na⁺/K⁺ ATPase to act as a receptor for a signal transduction pathway is also a very important part in its role as an isoenzyme.

Considering the many functions of Na⁺/K⁺ ATPase, it is not surprising that the interaction of cardiac glycosides with Na⁺/K⁺ ATPase effects several cell processes besides its most well studied relationship with cardiac muscle contraction. These cell processes include activation of cytoplasmic Ca²⁺ oscillation, cell growth, induction of apoptosis, glycogen synthesis, inhibition of endocytosed membrane traffic, and the production of high density microtubule granules. Some of these effects are a result of cardiac glycosides inhibiting the ion pumping of the ATPase. However, more studies are showing that these functions may the result of Na⁺/K⁺ ATPase acting as a receptor for cardiac glycosides and starting a signal cascade triggered by phosphorylation, and not necessarily correlated with the inhibition of the Na⁺/K⁺ ion pumping.

1B. The Importance of Cell Migration

The ability of cells to migrate is of great physiological significance. Cell migration is necessary for many normal cell processes, such as embryogenesis, immunoresponses, and wound closure. In normal cells, the inhibition of cell migration would create significant problems in growth, development, and maintenance of healthy tissues. However, there are times when the
blocking of cell migration would be beneficial. Inhibiting cell migration could help to obstruct
diseased states that rely on the movement of cells, including tumor formation, tumor metastasis,
and vascularization.

Cell migration is a very complex process involving the components of the cytoskeleton;
actin, microtubules, and intermediate filaments. Microtubules are the primary facilitators of
ciliary and flagellar movement and they also interact with actin and create cell polarity in regular
cell movement. Intermediate filaments are more important for structural support and they do
not play as significant of a role in cell movement other than signal transduction. This leaves
actin as the primary driving force in the cytoskeleton that allows for cell migration.

The start of cell migration requires a protrusion of the cell membrane in direction of
movement. This protrusion is caused by a change in the structure of actin network within the
cell. The actin network is dynamic and can change as a result of lengthening actin chains by
polymerization, disassembly of actin filaments into free monomers, crosslinking of actin
filaments, and restructuring of actin density and organizational geometry. This dynamic actin
network begins to change once directionality and polarity has been established along the plane of
motion. Then the actin polymerizes to form filaments that create a protrusion of the outer
membrane, called the leading edge, in the direction of migration. Actin polymerization is the
assembly of free actin monomers (G-actin) into a polar polymer (F-actin). The F-actin is
considered polar because it has two different ends, a barbed end and a pointed end. The barbed
end of F-actin is the growing end, because it is easily accessible to rapid polymerization by the
free G-actin with the help of ATP, Mg^{2+}, and a G-actin binding protein. After polymerization
of G-actin into F-actin has created a protrusion or leading edge, actin and myosin work together
to create contractions and provide the force to pull the cell body forward, retract the lagging
portion of the cell, and cause detachment from the substratum.\textsuperscript{12} Integrins also play a very important role in attachment and detachment from the substratum. Integrins are surface proteins that serve as adhesion receptors for the extracellular matrix.\textsuperscript{13} They are regulated by intracellular signaling mechanisms and they also receive extracellular signals which they relay as intracellular signals themselves.\textsuperscript{13}

**Figure 2** (reproduced with permission)\textsuperscript{12} provides a very basic representation of the process of cell migration of animal cells. Although the process may appear simple in the diagram, it is actually a very complicated process full of intricate signaling cascades and regulatory pathways. With so many different molecules and pathways involved, there are many potential targets for inhibition of cell migration. Inhibitors can target either free or polymerized actin or even the capping protein that regulates binding to the barbed end of F-actin.\textsuperscript{12} Inhibitors of cell migration can also target other known, important structures such as microtubules or integrins. Then there is the host of upstream signaling molecules that all provide targets for the inhibition of cell migration.

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Fig. 2. A migrating animal cell, showing a lamellipodial membrane protrusion, polarity and orientation of actin filaments (F-actin), addition of actin monomer (G-actin) at the barbed end, dissociation of G-actin at the pointed end, and cell-substratum adhesion sites (the linkage to the actin cytoskeleton is not explicitly shown). Nucleation of new actin filaments, actin polymerization at the barbed end, filament crosslinking, network disassembly, actin depolymerization at the pointed end, cell-substratum adhesion, cell body contraction, and tail retraction occur concurrently in different parts of the cell and are controlled by proteins discussed in the text.
There are remarkably few known small molecule inhibitors of specific proteins that regulate cytoskeleton dynamics in cell migration. Small molecules mimic the much larger naturally occurring molecules that regulate cytoskeleton dynamics and act as highly useful tools for understanding cell migration. All of the mechanisms by which cells migrate and methods by which migration is regulated are still not fully understood, yet cell migration is still certainly an appealing target for drugs.

1C. Significance to Cancer Pharmacology

Small molecules that are able to effectively target specific proteins and inhibit cell migration would have potential application to cancerous cell invasion, the early stages of cancerous tumor development, as well as metastasis. Inhibition of cell invasion and metastasis would be particularly useful for controlling the formation of new tumors after unsuccessful treatment or surgery. This type of inhibition would also amplify the effectiveness of other anti-cancer drugs targeting containment of solid tumors by stopping cell growth and division.
Chapter Two: Synthesis and characterization of digitoxin analogs

2A. Purchase and characterization of commercial digitoxin

Digitoxin 1 was purchased commercially from Acros Organics (CAS number: 71-63-6) and was used as the starting structure for the synthesis of the rest of the digitoxin analogs. Digitoxin was soluble in water, methanol, ethyl acetate, and chloroform. In most of the reactions with digitoxin, methanol was the solvent used. Ethyl acetate was used as part of the solvent system during column chromatography to separate reaction products. Both deuterated chloroform and deuterated methanol were used as solvent for NMR spectroscopy.

Characterization of 1 was performed using $^1$H and $^{13}$C NMR spectroscopy. The observed NMR peak values matched those found in the literature. When synthesizing analogs of 1, these NMR data were used for comparison. The emergence of new peaks or the loss of peaks were helpful in confirming structural changes.

2B. Synthesis and characterization of digitoxigenin

Digitoxigenin 2 is the aglycone of digitoxin, and it was synthesized using two different procedures. The first method for synthesizing 2 was an established procedure for the deglycosylation of ouabain, a cardiac glycoside very similar in structure to digitoxin. Hong describes the reaction of ouabain octahydrate with acetone-12 N hydrochloric acid (0.4 mL HCl/100 mL acetone) at 23°C, with the aglycone forming as a precipitate after 10 days under reaction conditions. As shown by reaction A in scheme 1, we used digitoxin 1 as the starting
material under the same these same reaction conditions for the synthesis of the digitoxin aglycone, digitoxigenin 2. The reaction mixture was then separated by column chromatography to purify product 2. This procedure that Hong described for the deglycosylation of ouabain was successful for digitoxin, however the 10 to 14 days required for the reaction to reach completion was not ideal. So we switched to a different procedure that also used acid catalyzed hydrolysis, but under different reaction conditions allowing for the synthesis of 2 in several hours instead of 10 to 14 days.\textsuperscript{17} The reaction conditions described by Pádua are shown by reaction B in scheme 1. Under this procedure, 1 was dissolved in methanol and 1M aqueous HCl. The solution was then refluxed at 55°C for 35 minutes. The reaction products were then extracted with CHCl\textsubscript{3} and neutralized with 3% NaHCO\textsubscript{3}. Product 2 was purified by column chromatography.

Characterization was performed by \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy. The experimental peak values matched the peak assignments reported by Pádua for both \textsuperscript{1}H and \textsuperscript{13}C.
The synthesis of 2 is important because it results in an analog that is identical to digitoxin, minus the sugar moiety. Including this analog in the wound closure assay (Chapter Three) provides valuable information about the way in which cardiac glycosides inhibit cell migration.

2C. Synthesis and characterization of elimination aglycone side product

Analogs 4a and 4b were at first unintentionally synthesized as side products of the deglycosylation reaction in an attempt to synthesize 2 under reaction conditions A in scheme 2.

They appeared as one spot on thin layer chromatography, and they were separated from the reaction mixture by column chromatography. At the time, I thought that I was likely separating product 2 from the reaction mixture. However, characterization by $^1$H and $^{13}$C NMR chromatography revealed that the product I purified was not digitoxigenin. The $^{13}$C NMR showed four downstream peaks that did not match the predicted chemical shifts for 2. These peaks were in the typical range for alkene carbons at 154.6, 138.5, 129.9, and 116.5 δ (ppm). At
this point, I did not know the identity of the compound I had isolated, but I had concluded that it was not 2.

It was not until reading the Pádua et al. study on the biotransformation of digitoxigenin\textsuperscript{17} that we realized that the substance we isolated from the deglycosylation reaction mixture must be a mixture of 4a and 4b. This accounted for the downstream $^{13}$C signals indicating the presence of alkenes. Upon comparison of the $^1$H and the $^{13}$C data to the data published by Pádua et al.,\textsuperscript{17} we were sure that the reaction product we had isolated from the reaction products was a mixture of 4a and 4b.

Attempts to separate 4a and 4b, by column chromatography were unsuccessful. The Rf values were identical in every solvent system that was used. They could have been separated by using a preparative RP-HPLC system,\textsuperscript{17} but that equipment was not available. For our purposes we decided that it would be acceptable to use a mixture of 4a and 4b in the wound closure assay to determine the importance of the steroid core to cell migration inhibitory activity. Additional 4a/4b was synthesized as a side product during the synthesis of 2 under the Pádua reactions conditions shown in B of Scheme 2.

2D. Synthesis and characterization of 20,22-dihydrodigitoxin

20,22-dihydrodigitoxin 3 was synthesized by reducing the double bond between C20 and C22 of digitoxin. The starting material, 1, was added to 10\% Pd on carbon dissolved in methanol. The solution in the round bottom flask was sealed with a rubber septum and flushed first with nitrogen and then with hydrogen gas. The solution was treated with excess hydrogen gas overnight. The reaction mixture was purified by filtering through celite to remove the carbon. Thin layer chromatography showed only one compound, with an Rf value very close to
that of the starting material. Complete conversion of 1 to 3 was assumed, so no further purification was performed.

![Scheme 3](image.png)

The product 3 was characterized by $^1$H and $^{13}$C NMR chromatography, which confirmed complete conversion of 1 to 3. In the $^{13}$C NMR of 1, there is a peak with a chemical shift of 117.8 $\delta$ (ppm) marking the presence of C22. In the $^{13}$C NMR of 3, this peak at 117.8 $\delta$ (ppm) is absent, indicating that the reduction of double bond between C20 and C22 has occurred.

3 is an important analog for providing information about role of the butenolide moiety in the inhibition of cell migration by cardiac glycosides. The reduction of the double bond changes the stereochemistry of the lactone ring, which could potentially affect the bond between 3 and its target protein.

**2E. Synthesis and characterization of 20,22-dihydrodigitoxigenin**

20,22-dihydrodigitoxigenin was synthesized in two steps under the reaction conditions shown in Scheme 4. The C20, C22 double bond in 2 proved to be significantly more difficult to
reduce than the same bond in 1. Several unsuccessful hydrogenation attempts were made using the same 10% Pd on carbon as in Scheme 3. Then, we switched to 20% palladium hydroxide on carbon, and the reaction was successful. The product 5 was characterized by $^1$H and $^{13}$C NMR chromatography and the reduction of the C20, C22 double bond was confirmed by the absence of the peak at 117.8 δ (ppm).

Since both the sugar moiety and the butenolide moiety were modified in this synthesis, a wound closure assay on 5 would not provide as valuable information about the structure-function relationship between digitoxin and cell migration inhibitory activity. It is better to modify only one moiety at a time, to see how that specific moiety affects cell migration inhibition. So after an error occurred during the first wound closure assay of 5, a second assay has not yet been completed.
2F. Attempted synthesis of open butenolide ring analog

The wound closure assay data from 3 gives some information about the butenolide ring and its effects on cell migration. However, a complete opening of the butenolide ring would allow for a clearer picture of its importance in the cell migration inhibition process.

The first method we used to try opening the butenolide ring was ozonolysis of digitoxin. Several unsuccessful ozonolysis attempts were made, using both methanol and methylchloride as solvents. In several attempts, the spectroscopy showed that the reaction had not gone to completion and only starting material present. Then in a few attempts, a product was formed, but the spectroscopy suggested that it was not the product we were looking for.

Ring opening aminolysis was also used to try to open the butenolide ring. We used the procedure described by Liu for opening lactone rings with benzylamine and sodium 2-ethylhexanoate, which acts as a base and a catalyst.\(^\text{18}\) However this reaction was unsuccessful when performed on both 1 and 2. In all attempts, there was no addition of the benzylamine, which would have been visible under UV light and also would have been noticeable in the spectroscopy.

We would still like to have an analog of digitoxin where the butenolide ring has been opened. We are hoping that we will be able to reduce the lactone of 3 or 5 to a diol by treating with DIBAL or lithium aluminum hydride.
Chapter Three: Inhibition of Cell Migration, Measured by a Wound Closure Assay

3A. Description of cell line

MDA-MB-231 human breast carcinoma cells were obtained from the American Tissue Culture Collection (ATCC) or Kam C. Yeung of the University of Toledo. This particular cell line is one of the most commonly used breast cancer cell lines in research labs across the country. The MDA-MB-231 cell line is well studied, characterized, and cited in the literature.\textsuperscript{19} It was originally taken from a 51 year-old, Caucasian woman in October of 1973 at the M. D. Anderson Cancer Center.\textsuperscript{20} The malignant MDA-MB-231 carcinoma cells were taken from a pleural effusion that matched the primary tumor of her breast.\textsuperscript{20} Systemic treatment for the patient included fluorouracil and prednisone, while chemotherapeutic treatment was a combination of cyclophosphamide, Adriamycin, amethopterin. The cells were resistant to both systemic and chemotherapeutic treatment at the time and the patient died several months later.\textsuperscript{20} MDA-MD-231 cells have a mean chromosome number of 64, making them nearly triploid.\textsuperscript{21} They also have several identifying markers and they are capable of forming tumors in nude mice.\textsuperscript{21}

The MDA-MD-231 cell line has several characteristics that make it a good choice for the wound closure and cell migration assay. First of all, this cell line grows fairly rapidly. This helped to expedite the process of growing the cells from frozen samples, culturing the cells, and subculturing the cells to create different passages. This also allows for efficient use of materials and growth medium, as compared to cell lines that would take weeks to culture into a confluent group and use up considerably more resources. A 1:10 dilution of MDA-MB-231 cells takes approximately four to five days to reach confluence,\textsuperscript{21} which was optimal for this research.
The growth properties of the MDA-MB-231 carcinoma cell line are essential for the cell migration assay used in our research. Some cell lines used in cancer research do not have this monolayer growth pattern and tend to grow in masses or clumps that do not adhere well to a surface. However, MDA-MB-231 cells grow into a confluent monolayer on glass and treated plates as shown in Figure 3. Formation of a monolayer with uniform cell growth allows for the wound to close evenly on all sides during the wound closure assay to determine cell migration. If the cells did not adhere well to the plate, they could easily be dislodged during the process of moving the plates from the incubator, to the laminar flow cabinet, to the microscope. This would of course completely disrupt the uniform wound closure and cell migration.

The MDA-MB-231 cell line also falls under the category of “triple-negative” cancer cells. Triple negative breast cancer cells are defined by their lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER2). Only about 10-17% of breast carcinomas can be categorized as triple negative. Triple negative cancer cell lines such as MDA-MB-231 are more aggressive and are not susceptible to some of the typical systemic therapy options that target the estrogen receptor or HER2. Instead, chemotherapy remains the only option for the treatment of triple negative cancers. This makes the study of triple negative cell lines and their interactions with small molecules relevant from a medicinal, pharmacological, and scientific standpoint.
3B. Procedure for screening of small molecule libraries for antimigratory activity

The wound closure assay used to screen libraries of small molecules was developed by the Fenteany lab, and the same procedures were essentially used in this screening. The small molecule libraries were obtained from Chembridge and AnalytiCon. The screening was conducted using BT-20, T47D, MDA-MB-231, MDA-MB-435, 4T1, and MDCK cells, which were obtained from either the ATCC or the Yeung lab. The cells were shipped and stored frozen at -80°C in cryovials. Cells were grown in a tissue culture incubator at 37°C with 5% CO₂ in minimum essential medium with additional Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS). The medium was changed every two days. Cells were plated on 24-well tissue culture-treated plates. When the cells reached confluence as depicted in Figure 4, the medium was changed a final time and the experiment was started the next day.

Fig 4. MDA-MB-231 cell growth and proliferation. A.) The MDA-MB-231 cells are first plated at a lower density in preparation for the wound closure/cell migration assay. B.) As the cells are fed, they grow and proliferate until they become a confluent monolayer, ready for the assay.
The carrier solvent for each compound was DMSO, used at less than 1%. Cells were treated with 100 µM of each compound or 1% DMSO alone. The compounds to be screened or the 1% DMSO control were mixed with fresh medium and added to the cells as a medium change. The cells were exposed to the compounds or 1% DMSO for 30 minutes before the cell monolayers were scratched with a micropipette tip, creating wounds of about 0.5–1.0 mm² in area as in Fig 5. After the 30 minute incubation, the cells were observed for wound closure every 12 hours for the next 72 hours with an inverted microscope. At each observation, the wounds were deemed either open or closed. Wounds that were still open after the control 1% DMSO wounds had closed indicated that the compounds had antimigratory activity. However, it had to be verified that the inhibition of wound closure was not due to cytotoxicity. As has often been quoted, “dead cells don’t move.” The trypan blue dye exclusion assay was used to determine cytotoxicity.²⁶

Several members of the cardiac glycoside group of natural products were among the small molecules that displayed antimigratory activity in the screening of the two libraries. They were deemed interesting choices for further investigation because, as explained in the introduction, they have already been well studied as inhibitors of the protein Na,K-ATPase, but not as inhibitors of cell migration. Analogs of digitoxin and ouabain were synthesized (see
Chapter 2) and used for additional antimigratory wound closure assays to further analyze the antimigratory activities of these cardiac glycosides.

3C. Procedure for conducting wound closure assay with a target molecule

For the assay of digitoxin and its analogues, the cell line used was the previously described MDA-MB-231 human breast carcinoma cells. Each wound closure assay was done in triplicate, with three different passages. In order to achieve these different passages, the cells had to be subcultured. The cells were removed from the -80° freezer and thawed by rolling the cryovial between one’s hands. The thawed cells in storage medium were then transferred to a conical tube containing growth medium and centrifuged at 1000rpm for five minutes. The supernatant was removed by aspiration and the remaining mass of cells was resuspended in 15mL of new growth medium. The cell suspension was then transferred and divided between three tissue culture-treated plates and incubated at 37°C with 5% CO₂. The cells were monitored daily and the growth medium was changed every 24 to 48 hours.

When the MDA-MB-231 cells reached confluence on the tissue culture plates, they were passaged, or “split.” The first step in splitting the cells was to remove the growth medium. This was done by aspiration. A phosphate buffered saline (PBS) solution was used to wash or rinse the plates three times in succession. This ensured the removal of any dead cells, cell wastes, spent medium, or serum in the medium which inhibits trypsin. After washing the cells, they were treated with trypsin, a protease that cleaves the bonds holding the cells to the surface of the treated culture plate. After incubating the cells with trypsin for 15 minutes, the cells were no longer attached to the plate so an equal volume of growth medium was added to quench the trypsin reaction. These suspended cells were transferred to a conical tube and centrifuged at
1000rpm for five minutes. The supernatant was removed and the cells were resuspended in growth medium. The new solution of cells was then plated onto treated tissue culture plates at different concentrations in order to create new passages that would take different amount of time to reach confluence. After each of these new passages was split several times in the same manner, they were split a final time. In this final split, they were diluted and plated in 96 well plates, with 100μL in each well. These cells took about four days to reach confluence, during which the medium was changed regularly. Once confluence was achieved in the final passage, the cells were ready for the wound closure assay.

The glycoside analog to be tested was diluted with four-fold serial dilution, with concentrations ranging from 1mM to 15nM. Once the glycoside solution was diluted in DMSO and mixed with fresh medium, it was added to the cell culture. The MDA-MB-231 cells were allowed to incubate with the glycoside solution for 30 minutes. After the incubation, the monolayer of MDA-MB-231 cells was wounded using micropipette tip to produce a small oval shape wound of about 0.5 to 1.0 mm\(^2\) in area. During this assay, an initial picture of the wound was taken, and then taken again every three hours, for 24 hours, or as long as it took for the control wounds to close entirely.

**Fig 6. Comparison of wound closure in cells treated with glycoside and cells treated with DMSO control.** The pictures of the wounded cell monolayers were taken using an inverted microscope and camera attached to a computer. The top panel of pictures represents one of the nine wells containing 1mM DMSO, and how the cells migrated to completely close the wound by 33 hours. The bottom panel of pictures represents one of the nine wells containing 250μM digitoxin, where the percent closure of the initial wound is significantly less than that of the DMSO control. This indicates inhibition of cell migration by digitoxin.
Under ideal timing, the final set of pictures was taken just before the DMSO control wounds were completely closed. This allowed for the most accurate measurement of percent closure of the wounds treated with the glycoside compared to the DMSO control.

The cardiac glycosides were tested at each concentration in triplicate and using three different passages of MDA-MB-231 cells. Each passage of cells was on its own 96 well plate. This gave a total of nine trials for each concentration of the target glycoside analog. After the assay was complete, the digital images were transferred to a PC to be processed. The NIH software ImageJ (free download at http://rsb.info.nih.gov/ij/) was used to trace the perimeter of the wound in each picture. The area enclosed by this tracing was computed in units of pixels. Once the areas of the wounds had been measured at each time interval, it was possible to calculate the percent closure by comparing the area of the wound at each time interval to the initial area at hour 0. These values were compared to the percent closure of the wounds treated with DMSO control. Minimum inhibitory values (MIC) and IC\textsubscript{50} values were calculated with GraphPad Prism software from concentration–response data.

**3D. Relating results of antimigratory activities for digitoxin and analogs to structure**

In the screening of the small molecule libraries, the cardiac glycosides ouabain, digitoxin, and oleandrin all displayed antimigratory activity. So it was expected that the digitoxin analogs synthesized would all have at least some antimigratory activity. The antimigratory activities are expressed with two different values. The first is the minimum inhibitory concentration or MIC. This is the lowest concentration of the cardiac glycoside that still displays antimigratory activity. The second value is the half maximal inhibitory concentration, or the IC\textsubscript{50}. This is the
concentration of cardiac glycoside that reduces cell migratory activity by half. Both the MIC and the IC₅₀ for digitoxin and its analogs are reported in Table 1.

Table 1. Measured activities for cell migration inhibition and Na⁺/K⁺-ATPase inhibition. These values were determined by wound closure assays and Na⁺/K⁺-ATPase inhibition assays.

<table>
<thead>
<tr>
<th>Cardiac Glycosides</th>
<th>Wound Closure Assay</th>
<th>Na⁺/K⁺ – ATPase Inhibition Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>Percent Inhibition (at highest concentration)</td>
</tr>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>51.5% (250 µM)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>31.8% (250 µM)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>36.8% (250 µM)</td>
</tr>
<tr>
<td>4a and 4b</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>43.2% (62.5 µM)</td>
</tr>
</tbody>
</table>
Digitoxin 1 proved to have greater antimigratory activity than any of the synthesized analogs. 1 inhibited wound closure with MIC and IC$_{50}$ values that were each on a nanomolar scale at 15 nM and 46 nM respectively. Activity on a nanomolar scale is a characteristic common to many of the most successful pharmaceutical drugs. It allows the drug to be used at low dosages that do not come near its minimum lethal concentration (MLC). For digitoxin, the trypan blue stain was performed at the conclusion of each wound closure assay to determine cytotoxicity. However, it was a rather subjective test, which made it difficult to determine an exact MLC value with any certainty. A large MLC and a small MIC is optimal for potential drugs, which are rated using a therapeutic index (MLC/MIC). However, without an MLC for digitoxin and its analogs, the calculation of therapeutic indices was not possible.

MIC and IC$_{50}$ values provide information about the activity of a compound at lower concentrations. The nanomolar MIC and IC$_{50}$ values for 1 show that it has the strongest antimigratory activity in terms of its ability to inhibit cell migration at low concentrations. However, 1 also has the strongest antimigratory activity as a measurement of percent inhibition at the highest concentrations. At 250 μM, 1 had 51.5% inhibition of cell migration, measured by the wound closure assay. This is a significantly greater value than any of the digitoxin analogs that were tested.

The only structural difference between 1 and 2 is the presence or absence of the three sugars. So the antimigratory activities of 2 provide information on the importance of the sugar moiety to the biological activity of cardiac glycosides. However, out of the digitoxin analogs tested, 2 had the smallest drop in antimigratory activity, with MIC and IC$_{50}$ values still at the upper end of the nanomolar scale at 240 nM and 260 nM respectively. The percent inhibition at a concentration of 250 μM also drops from 51.5% in 1 to 31.8% in 2.
The wound closure assays conducted with 3 provide information about the butenolide ring on cardiac glycosides and its role in the inhibition of cell migration. The reduction of the C20-C22 double bond in the lactone ring of digitoxin is the only structural difference between compounds 3 and 1, and it resulted in a decrease in antimigratory activity. The wound closure assay for 2 resulted in the MIC and IC\textsubscript{50} values for cell migration inhibition dropping out of the nanomolar range and into the micromolar range at 1.0 \(\mu\)M and 2.15 \(\mu\)M. Although the reduction of the C20-C22 double bond alters the structure of the butenolide ring and introduces a new chirality center, it does not entirely compromise the conformational integrity of the lactone. It would be very interesting to see if a complete opening of the butenolide ring would result in a much more pronounced decrease in antimigratory activity. Unfortunately, the synthesis of this type of analog has proved challenging and is still in progress as described in Chapter 2.

Compounds 4\textsubscript{a} and 4\textsubscript{b} had the lowest antimigratory activities among the digitoxin analogs tested in the wound closure assay. The measured MIC value was 9.2 \(\mu\)M and the IC\textsubscript{50} value was 10.8 \(\mu\)M. Two out of the three fundamental structures of cardiac glycosides described in Chapter 1, have been compromised in 4\textsubscript{a}/4\textsubscript{b}. The sugars have been removed from C3 and the curved shape of the steroid core has been altered by an elimination reaction. Direct comparison of 4\textsubscript{a}/4\textsubscript{b} to 1 would leave uncertainty as to whether the differences in antimigratory activities are as a result of the glycolysis or the elimination reaction that occurred in rings C and D. In order to determine the loss of antimigratory activity as a direct result of the changes to the steroid core, it is necessary to compare the MIC and IC\textsubscript{50} values of 2 and 4\textsubscript{a}/4\textsubscript{b}. As shown in Table 1, the IC\textsubscript{50} value increased from very significantly from 0.260 \(\mu\)M in 2 to 10.8 \(\mu\)M in 4\textsubscript{a}/4\textsubscript{b}. Although 4\textsubscript{a}/4\textsubscript{b} displayed the lowest antimigratory activity as expressed by MIC and IC\textsubscript{50}, they did have a 43.2% inhibition at the highest concentration (62.5 \(\mu\)M). This shows that at higher concentration,
**4a/4b** have greater antimigratory activity than **2** and **3**, which have percent inhibitions of 31.8% and 36.8% respectively at a concentration of 250 μM. Again, biological activity at higher concentrations is not as important pharmacologically because of concern about approaching the MLC, but for our purposes it is worth noting.

### 3E. Correlation between antimigratory activities and Na⁺/K⁺ - ATPase inhibition for digitoxin and analogs

After digitoxin and its analogs were tested for antimigratory activity with the wound closure assay, Na⁺/K⁺ -ATPase inhibition assays were conducted to measure the capacity of these molecules to inhibit the enzymatic activity of the Na⁺/K⁺ -ATPase protein. These assays were conducted by Anniefer Magpusao of the Peczuh Group at the University of Connecticut. **Figure 7** provides an overview of the experimental procedure for the Na⁺/K⁺ -ATPase inhibition assay performed by Magpusao.

The Na⁺/K⁺ -ATPase enzyme actively exchanges sodium and potassium across the plasma membrane against their concentration gradients. In order to provide energy for the ion exchange, the Na⁺/K⁺ -ATPase hydrolyzes ATP into ADP and phosphate. In this Na⁺/K⁺ -ATPase inhibition assay, the concentration of the newly generated phosphate is used as a direct measurement of the activity of the Na⁺/K⁺ -ATPase pump. The phosphate is measured using Taussky-Shorr reagent (1% w/v ammonium molybdate, 2.7% v/v sulfuric acid, and 5% w/v ferrous sulfate hexahydrate). The ammonium molybdate in Taussky-Shorr reagent reacts with the inorganic phosphate to form PMO$_{12}$O$_{40}^{3-}$, which is in turn reduced by the ferrous sulfate hexahydrate to form PMO$_{12}$O$_{40}^{7-}$, a blue colored ion that absorbs light at a wavelength of...
This absorbance is directly proportional to the concentration of phosphate in the solution, which is in turn proportional to the enzymatic activity of Na\(^+\)/K\(^+\)-ATPase pump.

Treating the Na\(^+\)/K\(^+\)-ATPase solution with digitoxin and analogs effectively inhibited Na\(^+\)/K\(^+\)-ATPase and resulted in decreased enzymatic activity. IC\(_{50}\) values were generated for each of these compounds and they are included in Table 1.

Data from the wound closure assays and the Na\(^+\)/K\(^+\)-ATPase inhibition assays were compared to look for a correlation between the antimigratory activity and the Na\(^+\)/K\(^+\)-ATPase
inhibition activity of the cardiac glycosides digitoxin and its analogs. Figure 8 illustrates how these data provide support for a positive correlation between these two activities.

**Digitoxin 1**, the most potent inhibitor of Na⁺/K⁺-ATPase, was also the most potent inhibitor of cell migration. 2 was second strongest inhibitor of both Na⁺/K⁺-ATPase and cell migration. This trend continued for 3 and 4a/4b, with the IC₅₀ values increasing proportionally for each type of activity. Additionally, none of the cardiac glycosides inhibited cell migration below the concentration at which they inhibited the Na⁺/K⁺-ATPase pump. The correlation was observed by Magpusao when the same assays were done with the cardiac glycoside ouabain and its analogs as part of current research that is not yet published.

**Fig 8. Correlating antimigratory activity with Na⁺/K⁺ - ATPase inhibitory activity in digitoxin and its analogs.** The cardiac glycoside analogs that had lower IC₅₀ concentrations for inhibiting cell migration also had lower IC₅₀ concentrations for inhibiting the Na⁺/K⁺ - ATPase enzyme. As cell migration inhibition decreased from analog to analog, Na⁺/K⁺ - ATPase inhibition also decreased.
Chapter Four: Discussion

A. Summary of Results

The IC_{50} values of the wound closure data in Table 1 clearly show that digitoxin (1) is the most potent inhibitor of cell migration. When the sugars were cleaved from digitoxin to give digitoxigenin (2), there was a decrease in cell migration inhibition, but it still had an IC_{50} in the nanomolar range. A much more significant decrease in cell migration inhibition came when the the C20-C22 double bond in the butenolide ring was reduced in the analog 20,22-dihydrodigitoxin (3). Then the most drastic decrease in cell migration inhibition was for the elimination side product analog (4a/4b), where the structure of the steroid core was compromised. This trend was exactly the same for the Na\(^+\)/K\(^+\) -ATPase inhibition assay. Figure 8 presents this trend in a way that is easy to see. These results provide information as to which of the three moieties of cardiac glycosides is most important for interaction with the Na\(^+\)/K\(^+\) -ATPase. The sugar is least important, flowed by the butenolide ring, and then the steroid core is the moiety that is most crucial in the interaction between cardiac glycosides and the Na\(^+\)/K\(^+\) -ATPase protein.

The strong correlation between the IC_{50} values of the cell migration assay and the Na\(^+\)/K\(^+\) -ATPase inhibition assay is another key result of this research. This correlation strongly suggest that the cardiac glycoside digitoxin and its analogs are inhibiting cell migration through their inhibition of the Na\(^+\)/K\(^+\) -ATPase protein. This conclusion is further supported by the result that no glycoside analog inhibited cell migration at a lower concentration than was necessary to inhibit the Na\(^+\)/K\(^+\) -ATPase pump.
4B. Perspectives and Conclusions

Currently there are three main modes of action proposed for the observed effects of the inhibition of the Na\(^+\)/K\(^+\) -ATPase by cardiac glycosides. The classical Na\(^+\) lag hypothesis explains the positive inotropic effect of cardiac glycosides on cardiac monocytes. In this mechanism, inhibition of Na\(^+\)/K\(^+\) -ATPase decreases the sodium ion concentration inside the cells, which then leads to an increase in intracellular calcium concentration through the reverse mode Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). This increase in calcium concentration eventually leads to muscle contraction. The second mechanism of action proposes the existence of Na\(^+\)/K\(^+\) -ATPase signalosome. In this mechanism, it was proposed that certain populations of Na\(^+\)/K\(^+\) -ATPase exist in a caveolae along with a number of proteins such as Src, PLC, and P13K and forms a signalosome. Binding of cardiac glycosides in the Na\(^+\)/K\(^+\) -ATPase in this signalosome activates these proteins and triggers various signal transduction cascades leading to the activation of MAPK (ERK 1/2) pathway. Some of the signaling events were reported to occur even at concentrations of cardiac glycosides that are too low to affect the ion homeostasis, thus suggesting that the binding of cardiac glycosides to Na\(^+\)/K\(^+\) -ATPase can initiate a signaling cascade without affecting the pumping action of this ion transporter. The third method of action was proposed in 2008, which calls into action the energy-sensing kinase AMPK. Since Na\(^+\)/K\(^+\) -ATPase is responsible for the consumption of approximately 40% of the cellular ATP, its inhibition greatly affects the total AMP/ATP ratio in the cell. This in turn is a signal for the activation of AMPK. Soltoff and Hedden showed that inhibition of Na\(^+\)/K\(^+\) -ATPase blocks the phosphorylation of AMPK, which in turn leads to the activation of ERK 1/2.

These different mechanism were observed using different experimental conditions and different cell lines. In our study, we used triple negative MDA-MB-231 human breast cancer
cells and looked into the correlation of IC\textsubscript{50} values of different analogs of digitoxin with respect to both cell migration and Na\textsuperscript{+}/K\textsuperscript{+} -ATPase inhibition assays. We found that the IC\textsubscript{50} values for digitoxin and its analogs for the cell migration assay correlate very well with the IC\textsubscript{50} values for the Na\textsuperscript{+}/K\textsuperscript{+} -ATPase inhibition assay. Also, we did not observe inhibition of cell migration below the concentration of cardiac glycoside that inhibits Na\textsuperscript{+}/K\textsuperscript{+} -ATPase. Based on these results, it is more likely that the mechanism of the inhibition of cell migration and its analogs involves the perturbation of the Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} ion homeostasis caused by the inhibition of Na\textsuperscript{+}/K\textsuperscript{+} -ATPase. The next interesting step then to explore for project would be to evaluate how digitoxin, its analogs, and other cardiac glycosides and other Na\textsuperscript{+}/K\textsuperscript{+} -ATPase inhibitors affect the concentration of these three key ions. Correlating this with the observed inhibition of cell migration might provide further information on how cardiac glycosides inhibit cell migration.
13C NMR of Digitoxin 1
13C NMR of Digitoxigenin 2
13C NMR of Elimination Side Product 4a/4b
13C NMR of
20,22-dihydrodigitoxigenin 5
References:

1. Withering W. An account of the foxglove and some of its medical uses, with practical remarks on dropsy, and other diseases. 1941.


