Cardiac Glycosides, a Novel Treatment for Neuroblastoma: Efficacy and Mechanism

by

Paulo Clasio De Gouveia

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

© Copyright by Paulo De Gouveia 2010
Cardiac Glycosides, a Novel Treatment for Neuroblastoma: Efficacy and Mechanism

Paulo De Gouveia
Master’s of Science
Institute of Medical Science
University of Toronto
2010

Abstract

In an attempt to identify agents that specifically target neuroblastoma (NB) tumour-initiating cells (TIC) we performed drug screens using libraries of bioactive compounds. Cardiac glycosides (CGs) were the largest class of drugs identified with antitumour activity. At high CG doses inhibitory effects on the Na\(^+\)/K\(^+\)-ATPase induce cardiotoxicity; therefore, CG analogues were designed in an attempt to separate the effects on NB cells from cardiotoxicity. We identified RIDK34 as our lead compound from a structure-activity-relationship analysis (IC\(_{50}\) 8 nM). RIDK34 contains a unique oxime group and shows increasing potency against NB TICs. The Na\(^+\)/K\(^+\)-ATPase is a target for the apoptotic activity of digoxin and RIDK34, whereby a signaling cascade involving Src and ERK may induce apoptosis. Furthermore, we predict that signaling activation does not require inactivation of the Na\(^+\)/K\(^+\)-ATPase and subsequent deregulation of [Na\(^+\)]\(_i\) and [K\(^+\)]\(_i\) gradients. Thus CGs and particularly RIDK34 may be expected to display diminished cardiotoxicity and greater therapeutic potential.
Acknowledgments

I would like to thank everyone who has made this work possible, beginning with my family and friends who have supported my educational pursuits. Both my mother and brother have inspired me to work hard and to pursue what I enjoy most. They have also taught me invaluable lessons that I will carry on for the rest of my life. I would also like to thank my extended family; namely, my cousins who are the equivalent of brothers and sisters. They have reminded me not only to be dedicated to my work but also to enjoy what it is I get to do.

The members of the Irwin lab deserve credit for supporting this work, including Dan, Lynn, Fiona, Jacky, Jennifer, Joanne, Ian, Alvaro and Paola. At one point or another, I’ve come to each lab member with questions and their willingness to help me has been extraordinary. Also members of the Kaplan/Miller lab, particularly those in the neuroblastoma group have been instrumental in making this work possible. Of note, I would like to thank Kristen Smith and Mayumi Fujitani for their work that contributed to this project on cardiac glycosides. From working alongside members of both these lab groups, I’ve formed wonderful bonds and have acquired skills as a scientist that will stay with me to the next phase of my career.

I would also like to thank some of the people whom I’ve collaborated with including Jaimie and Myl from Dr. Clifford Lingwood’s laboratory. Both Jamie and Myl were instrumental in making the novel cardiac glycoside compounds and for generating interesting discussions about these compounds. Also, I would like to thank Clifford Lingwood and Aaron Schimmer- my two committee members for providing positive feedback and different perspectives within and outside of committee meetings. Lastly, I would like to thank David Kaplan and Meredith Irwin for allowing me to pursue a master’s in their respective labs. Individually, David has motivated me to think about the big picture and has encouraged me to be a well-rounded scientist. Also, as a supervisor, Meredith has been incredibly supportive over the past two years and I feel very fortunate to have had the opportunity to train under her guidance. Thank you everyone!
# Table of Contents

Acknowledgments ........................................................................................................ iii

List of Tables ................................................................................................................ vii

List of Figures ............................................................................................................... viii

List of Abbreviations .................................................................................................... x

Chapter 1 Review of Literature ...................................................................................... 1

1.1 Overview of Neuroblastoma ....................................................................................... 1

1.1.1 Introduction ........................................................................................................... 1

1.1.2 NB treatments, staging and prognosis ..................................................................... 1

1.1.3 Genetic Predisposition to NB ................................................................................ 2

1.1.4 Genetic Characteristics of NB .............................................................................. 3

1.1.5 NB Characteristics ............................................................................................... 5

1.1.6 Neuroblastoma Tumour-Initiating Cells ................................................................. 5

1.2 Sodium Potassium ATPase ....................................................................................... 8

1.2.1 Introduction ........................................................................................................... 8

1.2.2 Structure and Function of the Na⁺/K⁺-ATPase ......................................................... 9

1.2.3 Subunit Diversity and Expression of the Na⁺/K⁺-ATPase ......................................... 10

1.2.4 Regulation of the Na⁺/K⁺-ATPase ......................................................................... 12

1.2.5 Na⁺/K⁺-ATPase: a novel anticancer target .............................................................. 13

1.3 Cardiac Glycosides .................................................................................................. 15

1.3.1 Introduction ............................................................................................................ 15

1.3.2 Chemical structure ............................................................................................... 15

1.3.3 CG binding site on the Na⁺/K⁺-ATPase ................................................................. 16

1.3.4 CGs as inotropic and cardiotoxic agents .............................................................. 17

1.3.5 The role of the Na⁺/K⁺-ATPase and CG antitumour activity .................................. 20

1.3.6 CGs: Mechanism of antitumour activity ............................................................. 23

1.3.7 Cardiac Glycoside: epidemiological evidence of anticancer properties .............. 25

1.3.8 CGs: anti-tumour activity *in vivo* and in clinical studies ..................................... 25

1.3.9 Novel Strategies to circumvent Cardiac Glycoside induced toxicity .................... 27

Chapter 2 Rationale, Aims and Hypothesis .................................................................. 28
Chapter 3 Material and Methods ................................................................. 30

3.1 Cell Systems .............................................................................................. 30
3.2 Cell Culture ................................................................................................ 31
3.3 Cell proliferation and viability assays .......................................................... 31
3.4 Drug treatment ............................................................................................ 33
3.5 \( Na^+ / K^+ \)-ATPase activity screen ............................................................... 34
3.6 Immunoblotting (IB) .................................................................................... 34
3.7 Immunofluorescence (IF) ............................................................................ 35
3.8 Antibodies ................................................................................................... 35
3.9 Plasmids ....................................................................................................... 36
3.10 Reverse-transcriptase polymerase chain reaction ......................................... 36
3.11 Short-interfering RNA (siRNA) .................................................................. 37
3.12 Transfection of neuroblastoma cells ............................................................. 37
3.13 Statistical Analysis ...................................................................................... 38

Chapter 4 Results ............................................................................................. 39

4.1 Digoxin inhibits cell growth of NB TICs and immortalized adherent and non-adherent cell lines ........................................................................ 39
4.2 Digoxin induces apoptosis in a dose dependent manner in IMR-5 and NB88R2 ......................................................................................... 40
4.3 Structure-Activity-Relationship Analyses ..................................................... 41
4.4 RIDK34 inhibits cell survival and proliferation of NB TICs ................................ 45
4.5 RIDK34 induces apoptosis in a dose dependent manner in NB88R2 ................. 45
4.6 Exogenous expression of murine \( Na^+ / K^+ \)-ATPase alpha one protects against digoxin-induced inhibition of cell growth in IMR-5 ........................................................................ 46
4.7 Digoxin inhibits NB TIC growth independently of its effects on \([Na^+], \) and \([K^+]\) .......................................................... 47
4.8 \( Na^+ / K^+ \)-ATPase alpha one subunit knockdown sensitizes IMR-5 and NB88R2 to digoxin growth inhibitory effects .................................................. 48
4.9 \( Na^+ / K^+ \)-ATPase alpha one subunit knockdown sensitizes NB88R2 to RIDK34 growth inhibitory effects ................................................................. 49
4.10 RIDK34, Digitoxin and Convallatoxin inhibit the \( Na^+ / K^+ \)-ATPase at concentrations higher than the inhibitory concentrations for NB cell growth ........................................................................ 50
4.11 Digoxin increases phosphorylation status of Src and ERK1/2 ....................... 52
4.12 RIDK34 increases activation of ERK1/2 and at low doses Src kinase ............... 53
Chapter 5 Discussion ........................................................................................................... 54

5.1 Digoxin and the novel CG analogue RIDK34 inhibit growth of NB cells ....................... 54
5.2 Activity of CG-derived sugar groups .............................................................................. 55
5.3 The C19 substituent affects CG potency ......................................................................... 56
5.4 The lactone moiety is essential for the antitumour activity of CGs ................................. 57
5.5 Mechanism of CG antitumour activity and the role of the Na⁺/K⁺-ATPase ................. 57
5.6 Silencing of ATP1A1 increases susceptibility to digoxin and RIDK34 antitumour activity 59
5.7 RIDK34 is a novel CG analogue with higher potency and displays lower relative inhibition of Na⁺/K⁺-ATPase activity ...................................................................................... 60
5.8 Putative role of the α3 and α4 subunit in CG-induced antitumour activity .................... 63
5.9 Therapeutic rational for CGs in combinatorial therapy ................................................ 63

Chapter 6 Conclusion and Future Directions ...................................................................... 65

References ............................................................................................................................ 69

Appendix ................................................................................................................................ 81
List of Tables

Table 1  Children’s Oncology Group (COG) risk stratification schema, by stage  13
Table 2  Tissue distribution of Na⁺/K⁺-ATPase alpha isoforms in humans  23
Table 3  Cardiac glycosides and their respective inhibitory and affinity values for human Na⁺/K⁺-ATPase alpha isoforms expressed in P.Pastoris membranes  28
Table 4  Characteristics of cell lines  41
Table 5  Culturing conditions of cell lines  42
Table 6  Conventional CGs and chemotherapeutic agents used for drug efficacy studies  44
Table 7  List of Antibodies used in immunoblotting, and immunofluorescence studies  46
Table 8  Sequence of RT-PCR Primers  48
Table 9  ON-TARGETplus SMART pool sequences  48
Table 10  Structure-Activity-Relationship Analysis  54
Table 11  Comparison of CG serum toxicity and IC50 values for inhibition of NB88R2 growth and Na⁺/K⁺-ATPase activity  62
Table 12  Na⁺/K⁺-ATPase expression profile in Neuroblastoma Tumour-Initiating Cells  92
Table 13  Reported Mechanisms of CG antitumour activity  94
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Structure of the of Na⁺/K⁺-ATPase with the obligatory alpha and beta subunits</td>
<td>21</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Structure of Cardiac Glycosides</td>
<td>27</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Inotropic and Cardiotoxic effects of CGs</td>
<td>31</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Na⁺/K⁺-ATPase mediated signal transduction</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Digoxin inhibits neuroblastoma cell growth</td>
<td>51</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Digoxin induces apoptosis in a dose dependent manner in IMR-5 and NB88R</td>
<td>52</td>
</tr>
<tr>
<td>Figure 7</td>
<td>RIDK34 inhibits neuroblastoma cell growth with moderate selectivity</td>
<td>56</td>
</tr>
<tr>
<td>Figure 8</td>
<td>RIDK34 induces apoptosis in neuroblastoma tumour-initiating cells</td>
<td>57</td>
</tr>
<tr>
<td>Figure 9</td>
<td>High K⁺ medium does not protect against digoxin induced inhibition of cell growth</td>
<td>58</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Exogenous expression of cardiac glycoside resistant murine Atp1a1 protects IMR-5 from digoxin induced inhibition of cell growth</td>
<td>59</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Knockdown of the alpha one subunit of the Na⁺/K⁺-ATPase increases susceptibility to digoxin-induced inhibition of cell growth</td>
<td>60</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Knockdown of the alpha one subunit of the Na⁺/K⁺-ATPase increases susceptibility to RIDK34-induced inhibition of cell growth</td>
<td>61</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Curve fits, and IC50 determination of Na⁺/K⁺-ATPase inhibition in HEK-293 cells for Convallatoxin, RIDK34, and digitoxin</td>
<td>62</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Digoxin induces Src and ERK1/2 activation</td>
<td>63</td>
</tr>
<tr>
<td>Figure 15</td>
<td>RIDK34 induces Src and ERK1/2 activation</td>
<td>64</td>
</tr>
<tr>
<td>Figure 16</td>
<td>CGs possibly activate two parallel death pathways in NB TICs</td>
<td>70</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Crystal structure of Na(^+)/K(^+)-ATPase with bound ouabain</td>
<td>93</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Reaction scheme for RIDK34</td>
<td>93</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>Amp</td>
<td>ampere</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>human sodium potassium ATPase alpha 1 polypeptide gene</td>
</tr>
<tr>
<td>Atp1a1</td>
<td>murine sodium potassium ATPase alpha 1 polypeptide gene</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 antagonist of cell death</td>
</tr>
<tr>
<td>BIRC5</td>
<td>baculoviral IAP repeat-containing 5</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CG</td>
<td>cardiac glycoside</td>
</tr>
<tr>
<td>CHD5</td>
<td>chromatin helicase binding domain 5</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COG</td>
<td>children's oncology group</td>
</tr>
<tr>
<td>DAD</td>
<td>delayed after depolarization</td>
</tr>
<tr>
<td>DM</td>
<td>double minute</td>
</tr>
<tr>
<td>DOX</td>
<td>doxorubicin</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FISC</td>
<td>focus in synthetic chemistry</td>
</tr>
<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
</tr>
<tr>
<td>GRB2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>HEK-293</td>
<td>human embryonic kidney cell</td>
</tr>
<tr>
<td>Il2rg</td>
<td>interleukin-2 receptor subunit gamma</td>
</tr>
<tr>
<td>Jnk</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MKI</td>
<td>mitotic-karyorrhectic index</td>
</tr>
<tr>
<td>MYCN</td>
<td>myelocytomatosis viral related oncogene</td>
</tr>
<tr>
<td>Na(^+)/K(^+)-ATPase</td>
<td>sodium potassium adenosine triphosphatase/ sodium potassium pump</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide adenine (phosphate) dinucleotide</td>
</tr>
<tr>
<td>NB</td>
<td>neuroblastoma</td>
</tr>
<tr>
<td>NB TIC</td>
<td>neuroblastoma tumour-initiating cell</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium, calcium exchanger</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor-kappaB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NME1/2</td>
<td>non-metastatic cells 1/2</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung carcinoma</td>
</tr>
<tr>
<td>NTRK</td>
<td>neurotrophic tyrosine kinase receptor</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPM1D</td>
<td>protein phosphatase, Mg(^{2+})/Mn(^{2+}) dependent, 1D</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 domain containing</td>
</tr>
<tr>
<td>siRNA</td>
<td>short-interfering RNA</td>
</tr>
<tr>
<td>SKP</td>
<td>skin derived precursor</td>
</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Src</td>
<td>sarcoma kinase</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>WCE</td>
<td>whole cell extract</td>
</tr>
</tbody>
</table>
Chapter 1
Review of Literature

1.1 Overview of Neuroblastoma

1.1.1 Introduction

Neuroblastoma (NB) is the most common extra-cranial solid tumour of childhood and the age-dependent rate of incidence is 9.5 cases per million children (1, 2). NB comprises 8% to 10% of all childhood tumours and accounts for 15% of all paediatric-oncology related deaths (2, 3). It is a malignancy derived from neural crest cells that would normally give rise to cells of the sympathetic nervous system. Primary tumours are commonly found in the adrenal medulla and along various sites in the sympathetic chain. Enigmatically, NB is very aggressive in high-risk patients with disseminated disease, while in a unique subset of patients under one year with disseminated disease NB spontaneously regresses (2, 3). Survival rates, particularly for high-risk patients, have not improved over the past three decades despite intensive combination therapies (4, 5). Thus, currently applied modalities of treatment are not curative for the majority of high-risk patients and novel therapeutic strategies are required.

1.1.2 NB treatments, staging and prognosis

Approximately 40% of NB patients present with localized disease (stage 1 to 3 by the International Neuroblastoma Staging System [INSS]). Prognosis is generally good for localized disease with survival rates between 70% and 90% (6, 7). Treatment includes tumour excision and/or adjuvant chemotherapy depending upon the stage and biology of the tumour. However, more than half of NB patients are diagnosed with the high-risk form of the disease if they present with widespread metastasis [stage 4] or have localized disease with poor prognostic biological markers [stage 2 or 3] (Table 1). These patients require comprehensive treatment regimes that combine high-dose chemotherapy, radiation therapy, stem cell rescue as well as therapy for residual disease. Nonetheless, current five-year survival rate for high-risk patients with metastatic disease is below 35% (4, 5).
Table 1. Children’s Oncology Group (COG) risk stratification schema, by stage.

<table>
<thead>
<tr>
<th>Age</th>
<th>MYCN status</th>
<th>Ploidy</th>
<th>Histology</th>
<th>Other</th>
<th>Risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>2A/2B</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>&gt;50% resection</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>&lt;50% resection</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>Biopsy only</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Amplified</td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>3</td>
<td>&lt;547 days</td>
<td>Not amplified</td>
<td>Favorable</td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>≥547 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>≥547 days</td>
<td>Amplified</td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>4</td>
<td>&lt;365 days</td>
<td>Amplified</td>
<td>Unfavorable</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>365-547</td>
<td>Amplified</td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>365-547</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>DI &gt;1</td>
<td>Intermediate</td>
</tr>
<tr>
<td>365-547</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>≥547 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>DI &gt;1</td>
<td>High</td>
</tr>
<tr>
<td>4S</td>
<td>&lt;365 days</td>
<td>Not amplified</td>
<td>DI &gt;1</td>
<td>Favorable</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>DI=1</td>
<td>Intermediate</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Missing</td>
<td></td>
<td></td>
<td>Missing</td>
<td>Intermediate</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>Missing</td>
<td>Intermediate</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Not amplified</td>
<td></td>
<td></td>
<td>Unfavorable</td>
<td>Intermediate</td>
</tr>
<tr>
<td>&lt;365 days</td>
<td>Amplified</td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
</tbody>
</table>

Abbreviations: DI, diploid. Adapted from Neuroblastoma, Maris 2007 (2).

1.1.3 Genetic Predisposition to NB

Predisposition to NB has been demonstrated with a subset of patients and is consistent with the Knudson two-mutation hypothesis for the origin of childhood cancers (8). Approximately, 1-2% of cases are related to known hereditary NB (8). Patients with familial NB are much younger
with median age of diagnosis at 9 months compared to median age at diagnosis of 18 months for sporadic cases of NB (8). Familial NB patients often present with bilateral adrenal or multifocal primary tumours.

Many neuroblastoma predisposition genes are thought to play an important role in the normal development of the sympathetic nervous system. PHOX2B is a homeodomain-containing protein that is involved in the development of the peripheral nervous system. Inactivating mutations of PHOX2B is commonly associated with sporadic or familial neuroblastoma and with either congenital central hypoventilation syndrome, Hirschsprung's disease, or in conjunction with both diseases (9-11). In addition, inactivation of the NF1 gene has been reported in neuroblastoma (12, 13). Thus the genes implicated in the genesis of Hirschsprung disease (RET, EDNRB, EDN3, GDNF, ECE1, and ZFHXB), central hypoventilation (RET, GDNF, EDN3, BDNF and PHOX2B), and/or NF1 may be involved in the initiation or progression of human neuroblastoma, especially in cases of coincidence with these syndromes (12, 13).

Recently, activating mutations in the tyrosine kinase domain of the anaplastic lymphoma kinase (ALK) oncogene have been reported in neuroblastoma. These germ-line mutations are thought to account for most cases of hereditary neuroblastoma and encode a single-base substitution in critical regions of the kinase domain resulting in constitutive activation of the kinase (12). Single base pair mutations or amplifications that result in oncogenic activation of ALK are somatically acquired in 5% to 15% of neuroblastoma (12, 14, 15). The identification of these and other familial neuroblastoma predisposition genes will benefit affected families, as well as sporadic neuroblastoma patients by contributing to our understanding of the initiation and progression of NB. In addition, although ALK and PHOX2B mutations account for the majority of familial cases of neuroblastoma, additional familial genes may still be discovered.

1.1.4 Genetic Characteristics of NB

**Near diploid and triploid DNA content**

The DNA content of neuroblastoma cells can be classified into near-diploid and near-triploid (hyperdiploid). Near-diploid tumours are usually associated with unfavourable prognosis while hyperdiploidy is more likely to be detected in favourable tumours (16, 17). DNA ploidy is a useful prognostic marker for patients less than one year of age with advanced disease and is
currently used by the Children’s Oncology Group (COG) and other international groups for determining risk-adapted therapy.

**Amplification of MYCN Locus at 2p24**

MYCN amplification is the best-characterized genetic abnormality in neuroblastoma. Amplification occurs in approximately 20% of primary tumours and is strongly correlated with advanced stage disease and poor patient outcome (18-20). MYCN belongs to the MYC family of transcriptional factors and maps to chromosome position 2p24. In NB, MYCN is commonly amplified and found on extrachromosomal double minutes (DM) or in homogenously staining regions in the genome distinct from 2p24 (21, 22). In addition besides MYCN, large regions flanking either side of this gene including ALK are amplified. A recent study has suggested that amplification of a region proximal to MCYN and independent of both ALK and MYCN may also have prognostic significance (23).

**1p and 11q loss of heterozygosity**

Deletion at 1p36 and of 11q occurs in 25-30% and 35-45%, in primary neuroblastoma tumours respectively at diagnosis (24). Deletion at 1p36 is commonly observed in patients with advanced disease and is often associated with MYCN amplification and other poor prognostic features (2, 25, 26). Also it appears that 1p36 deletion is associated with increased risk of disease relapse in patients with localized tumors. Unlike 1p deletions, 11q loss of heterozygosity (LOH) is very rarely observed with MYCN amplification yet is still associated with high-risk features and is an independent indicator for tumour relapse. The prognostic value of 1p and 11q for predicting disease relapse has led to the incorporation of these markers in patient risk stratification and the determination of appropriate therapy. Although there are tumour suppressors located in these regions, including p73 and Chromatin Helicase Binding Domain 5 (CHD5), research is ongoing to identify novel tumour suppressors at 1p36 and 11q involved in the pathogenesis of neuroblastoma (27).

**17q addition and other cytogenetic sites**

Gain of 17q genetic material is a predictor of poor outcome and occurs in over half of the patients with NB, making it the most common cytogenetic abnormality in NB (28, 29). MYCN and/or 1p deletion occurs in 75% of tumours with 17q addition. Unbalanced translocation of 1p and 17q resulting in addition of distal 17q and loss of distal 1p genetic material frequently occurs.
in NB. 17q translocations are also thought to occur at other genetic regions in addition to 1p. Several candidate genes conferring a selective advantage at the distal 17q site for tumour cells include NME1/2 PPM1D, and BIRC5 (survivin); however, the role of these and other candidate genes in the initiation and progression of NB is still poorly understood (28).

1.1.5 NB Characteristics

**Neurotrophin Receptors: TrkA, TrkB and TrkC**

The neurotrophin receptors (NTRKA, NTRKB and NTRKC encoding TrkA, TrkB and TrkC) and their ligands (NGF, BDNF and neurotrophin-3, respectively) can induce survival and differentiation of neural cells including sympathetic neuroblasts (30). High expression levels of TrkA and its ligand NGF correlates with younger onset of NB, lower risk stage, the absence of MYCN amplification and is associated with favourable outcome (31). In addition, NB cells expressing high levels of TrkA undergo differentiation in the presence of NGF or programmed cell death when deprived of NGF; conversely, high levels of TrkB and its ligand BDNF is associated with MYCN amplification and poor outcome (2, 32). Evidence suggests that co-expression of TrkB and BDNF activate an autocrine or paracrine survival pathway that promotes chemotherapy resistance and metastases, possibly by suppressing anoikis (loss of cell/cell and cell/extra cellular matrix attachment) resulting in cell death (33, 34). Investigation into targeted therapies are ongoing and are promising such as with the Trk selective inhibitor Lestaurtinib, which is in preclinical and phase I clinical study (35).

**Tumour Histology**

The degree of neuroblastic maturation toward a mature ganglion phenotype correlates with favourable diagnosis. Tumours with largely undifferentiated morphology have poor prognosis, while tumours containing Schwann, chromaffin and neuronal cell types have more favourable prognosis (36). These findings allowed Shimada and colleagues to develop a schema that was incorporated into the International Neuroblastoma Pathology Classification System (37).

1.1.6 Neuroblastoma Tumour-Initiating Cells

**Support for Tumour-Initiating Cells in Neuroblastoma**

The clinical presentation and treatment response of neuroblastoma supports the likelihood of a
cell population with stem cell-like as well as tumour-initiating properties. In NB, histology is one method used to assess patient risk (see Section 1.1.5) and would suggest that NB is derived from a neural crest or sympathoadrenal precursor. NB tumors are comprised of both undifferentiated tumour cells that express neural stem cell markers such as nestin, and more differentiated cells with markers of mature neural cells and Schwann cells. This heterogeneity is consistent with the existence of a cancer stem cell model with hierarchical properties. Moreover, in vitro neuronal induction of immortalized neuroblastoma cells with retinoic acid (RA) and/or dibutyryl cyclic AMP gives rise to cells with neuronal (38), chromaffin (39) or Schwannian (40) phenotypes, further supporting the existence of a cell with cancer stem cell properties in NB.

**Cancer Stem and Tumour-Initiating Cell Theory**

One possible reason for the ineffectiveness of current treatments for high-risk NB is the lack of cell culture and in vivo models for metastatic NB that can be utilized for drug testing. Recently, NB cells with cancer stem cell and tumour-initiating properties have been identified that may be used towards better understanding and developing novel therapies against NB. Evidence suggests that cancer stem cells exist as a subpopulation and share essential features with normal stem cells such as the expression of stem cell genes and self-renewal capacity in cell culture (41-43). In addition, these cells exhibit enhanced tumour-initiating capacity as compared to most established-adherent cell lines grown in serum and are often organized as a hierarchy, with cancer stem cells able to generate both a differentiated daughter cell that is non-tumorigenic, and a tumorigenic copy of itself (41-43). Thus far, cancer stem cells have been described in adult leukemia (44), breast cancer, pediatric brain tumors (45, 46), melanoma (47), ependymomas (48), colon cancer (49), and head and neck squamous cell carcinoma (50).

**Characterization of NB Tumour-Initiating Cells**

NB tumour-initiating subpopulations may be isolated from established cell lines or cells taken directly from primary tumour or bone marrow metastasis samples (51-53). Sub-populations, have been identified by various groups through the use of sorting markers and functional assays (51-53). Mahller et al. were able identify tumour-initiating cells from the established adherent cell line LA-N-5, which in serum free conditions grows as spheres and express CD133+ (52). In contrast Das et al. were able to increase tumour stemness in the adherent established cell line SK-N-(BE-2) by identifying side-population (SP) cells, which are cells that negatively stain for Hoechst dye (53). This side-population exhibited even greater tumour-initiating capacity upon
further selecting SP cells that migrate towards the media of stressed bone marrow stromal cells (53). These cells were identified as SPm cells, which displayed further tumour stemness by subjecting these cells to oxidative stress and hypoxia followed by reoxygenation and were identified as SPm(hox) cells (53). These functional assays increased the tumour-initiating capacity of SK-N-(BE-2) derived cells, as only 100 SPm(hox) cells were required to form tumours in BALBc, nude/nude mice, unlike SPm and normal SK-N-(BE-2) that require $1 \times 10^4$ and $2.5 \times 10^5$ cells respectively to be injected to form tumours (53).

In contrast to the previously mentioned cell systems, the Kaplan laboratory used primary cells that were not grown at any point in serum conditions in order to identify neuroblastoma tumour-initiating cells (NB TICs). Given that the most common site of metastasis is the bone marrow, the majority of NB TICs were isolated from bone marrow aspirates of high-risk patients, using a filtration technique to enrich for NB cells that tend to form aggregates in this niche (51). The dissociated cells reformed spheres in neural crest stem cell media, expressed neuroblastoma markers such as NB84 and tyrosine hydroxylase, neural crest precursor markers such as nestin, and had chromosomal alterations consistent with NB (51). Similar to cancer stem cells, sphere-forming neuroblastoma cells were capable of self-renewal and differentiation into the cell types observed in NB (51). Also, sphere forming neuroblastoma cells, when injected orthotopically into the adrenal fat pad of immunocompromised mice, formed metastatic tumours that could serially reform spheres and could recapitulate the original phenotype of the tumour (51). In addition, the frequency of tumourigenic cells is much higher in NB TICs as only 10 NB TICs injected into SCID/Beige mice are required to form tumours which is several orders of magnitude less than immortalized NB cell lines (51-54).

Also unlike cancer stem cells from leukemia, colon and brain tumors, spheres from NB bone marrow metastasis do not appear to contain a hierarchical organization, in which a subpopulation of tumour cells express a distinct cell-surface marker and is capable of giving rise to all NB subtypes (51). This could be because a prospective marker has yet to be identified for the TIC population in NB, or that there is a fundamental difference between developmental tumours such as NB relative to adult tumours, or possibly because metastatic neural crest-derived cells are relatively homogenous regarding TIC potential (51). Recently, Morrison and colleagues demonstrated that single unsorted melanoma cells are able to form a tumour in SCID/Beige (Il2rg−/−) mice. They suggested that at least in melanoma, tumourigenic cells are very common with little evidence implicating hierarchical organization in these tumours (55). Similarly, it is
possible that neuroblastoma cells that metastasize to the bone marrow are highly enriched in tumour-initiating potential compared to the tumour from which they arose and do not exhibit hierarchical organization in this niche (51).

**Applications of NB TICs**
There are several potential applications for NB TICs. In one case, neuroblastoma tumour sphere cells isolated from a patient in remission and showing no morphologic evidence for disease was able to form tumours in immunocompromised mice. As anticipated from these results this patient clinically relapsed a few short months after the tumour spheres were isolated. Given that NB TICs can be isolated from the bone marrow of patients in relapse as well as in remission with a high chance of relapse, suggests that NB TICs may in the future be used as a predictor of clinical behaviour, treatment efficacy in high-risk patients, and in detecting minimal residual disease. By comparing the biology of NB TICs at different stages of treatment from newly diagnosed patients, patients in remission and to relapse patients, we may better understand the cellular and molecular events leading to relapse and disease progression in neuroblastoma. Lastly, current therapies in NB patients are largely ineffective, and while high-risk patients may go into remission, they invariably relapse. Given their potent tumour-initiating capacity, NB TICs likely play an integral role in treatment response and failure. Thus drugs that specifically target NB TICs are more likely to be effective agents for treating NB. As described in the proceeding sections, cardiac glycosides, which are inhibitors of the sodium potassium pump represent a novel class of agents with efficacy against NB TICs.

**1.2 Sodium Potassium ATPase**

**1.2.1 Introduction**

The sodium potassium pump (Na⁺/K⁺-ATPase or sodium pump) belongs to the P-type ATPase family and is responsible for the low intracellular ratio of Na⁺ to K⁺ ions. The Na⁺/K⁺-ATPase from the hydrolysis of ATP, transports three Na⁺ ions out of the cell in exchange for two K⁺ ions into the cell (56-58). In mammals, the Na⁺/K⁺-ATPase regulates several vital cellular functions; specifically, it regulates ion homeostasis, cell volume, acts as a signal transducer and maintains resting membrane potential, which is essential to the function of electrogenic cells such as cardiac myocytes and neurons (58-62). The sodium pump is also essential for the function of
secondary transporters that are coupled to the Na\(^+\) gradient across the plasma membrane such as the Na\(^+\)/Ca\(^{2+}\) exchanger (56-58). Thus, as a result of ion regulation and ATP hydrolysis, the Na\(^+\)/K\(^+\)-ATPase regulates much of the cellular metabolic rate and accounts for 1/3 to 2/3 of energy expenditure depending on the cell type (63-65).

### 1.2.2 Structure and Function of the Na\(^+\)/K\(^+\)-ATPase

The Na\(^+\)/K\(^+\)-ATPase consists of an \(\alpha\) and \(\beta\) subunit in equimolar ratio (Fig. 1). The \(\alpha\) subunit is the catalytic polypeptide and has ten transmembrane domains (63). The \(\alpha\) subunit contains the extracellular binding sites for K\(^+\) ions and cardiac glycosides (CGs). On the intracellular face of the \(\alpha\) subunit are the Na\(^+\) binding and phosphorylation sites (65). The activity and expression of the catalytic \(\alpha\) subunit is regulated by the \(\beta\) subunit, which is a single transmembrane polypeptide and contains a highly glycosylated extracellular loop (65). The \(\beta\) subunit is also thought to act as a chaperone protein for localization of the \(\alpha\) subunit to the plasma membrane (63-65). The third subunit is the FXYD subunit, which is not required for the function and localization of the Na\(^+\)/K\(^+\)-ATPase; however, the seven FXYD isoforms appear to alter the Na\(^+\)/K\(^+\)-ATPase affinity for K\(^+\), Na\(^+\) and ATP (66-68).

The Na\(^+\)/K\(^+\)-ATPase like other ion-transporting ATPases has two major conformations, denoted as \(E_1\) and \(E_2\) (69). According to the Albers-Post model, in the presence of K\(^+\), Na\(^+\), Mg\(^{2+}\) and ATP, the \(\alpha\) subunit becomes phosphorylated, which is followed by occlusion of three Na\(^+\) ions. This high energy \(E_1P\) state of the Na\(^+\)/K\(^+\)-ATPase undergoes a conformational change to the lower \(E_2P\) form, which translocates and releases Na\(^+\) ions into the extracellular milieu (69-71). The presence of K\(^+\) ions causes the \(E_2P\) form to become dephosphorylated followed by binding of K\(^+\) ions. K\(^+\) binding leads to the \(E_2\) to \(E_1\) transition, which is hastened by the presence of ATP (69-71). Upon transition to the \(E_1\) conformation, K\(^+\) is released from the Na\(^+\)/K\(^+\)-ATPase into the cell and ATP binds to repeat the cycle (69-71).
1.2.3 Subunit Diversity and Expression of the Na/K-ATPase

There are four α subunits, α1-α4, and three β subunits, β1-β3 (63-65, 72, 73). The primary sequences of the four α and three β subunits are highly conserved among species (63). The individual α and β isoforms in rat, mouse and human show greater than 90% sequence identity; the only exception is β3 with 75% similarity across species (as reviewed in 65, 66, 73). Despite this, very modest changes to the primary structure of the Na+/K+-ATPase can result in extraordinary changes in the pharmacological properties of the α and β isoforms across species. For example, sheep, rat and human sequences differ only slightly in the α1 subunit of the Na+/K+-ATPase; however, just two single residue substitutions (glutamine-118 to arginine and asparagine-129 to aspartic acid) in the α subunit increases ouabain resistance 1,000 fold in rodents relative to sheep and human (74, 75).
There is slightly less similarity among the four α or the three β subunits within a given species. In mammals, the α1, α2 and α3 share ~87% sequence homology, while the α4 is approximately 76%-78% similar to the other isoforms (65, 66, 73). The β isoforms are more divergent than the α subunits with β2 and β3 sharing with β1 34% and 39% similarity respectively, whereas β2 and β3 have 49% amino acid identity (64).

The Na⁺/K⁺-ATPase is most abundantly expressed in ion-transporting epithelia such as the kidney and in excitable tissues like the brain, skeletal muscle and cardiac muscle. Alternatively, the Na⁺/K⁺-ATPase is weakly expressed in vascular smooth muscle whereby electrochemical gradients are less important for physiological function. In addition to differential expression, the α and β isoforms of the Na⁺/K⁺-ATPase are expressed in a tissue-specific manner (Table 2). The α1 and β1 isoforms are expressed ubiquitously and the α1/β1 complex is the major isozyme in nearly every tissue (Table 2). Expression of α2 is mainly restricted to the adult heart, vascular smooth muscle, cartilage, bone adipocytes and various regions of the brain. Thus far, α3 is mostly localized to the conductive system of the heart and the central and peripheral nervous system (76-80). Unlike α1, α2 and α3, expression of α4 is mainly restricted to the testis (81). The β2 isoform expression is restricted to the heart, brain, cartilage and erythrocytes, whereas β3 isoform expression is restricted to lung, brain, cartilage and erythrocytes. Not surprisingly, the tissue restricted expression of the α and β isoforms and their respective affinities for Na⁺ ions, K⁺ ions and CGs in large part determines the physiologic response of tissues to these substrates (81-83).
Table 2. Tissue distribution of Na\(^+\)/K\(^+\)-ATPase alpha isoforms in humans.

<table>
<thead>
<tr>
<th>Na(^+)/K(^+)-ATPase isoform</th>
<th>Gene</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha 1</td>
<td>ATP1A1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Alpha 2</td>
<td>ATP1A2</td>
<td>Skeletal muscle, heart, brain, adipocytes, cartilage, bone, vascular smooth muscle, eye</td>
</tr>
<tr>
<td>Alpha 3</td>
<td>ATP1A3</td>
<td>Neurons (PNS and CNS), ovaries, white blood cells, heart (conduction system)</td>
</tr>
<tr>
<td>Alpha 4</td>
<td>ATP1A4</td>
<td>Testis</td>
</tr>
<tr>
<td>Beta 1</td>
<td>ATP1B1</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Beta 2</td>
<td>ATP1B3</td>
<td>Heart, brain, cartilage, erythrocytes</td>
</tr>
<tr>
<td>Beta 3</td>
<td>ATP1B3</td>
<td>Lung, brain, cartilage, erythrocytes</td>
</tr>
</tbody>
</table>

1.2.4 Regulation of the Sodium Potassium ATPase

Several hormones including endogenous cardiac glycosides (CGs), catecholamines and various other steroid or peptide hormones regulate the Na\(^+\)/K\(^+\)-ATPase through short-term or long-term regulatory mechanisms. Short-term regulation involves; 1) direct affects on the kinetic rates of the enzyme or 2) translocation of formed oligomers of the pump between intracellular compartments and the plasma membrane (83-86). Modulating its affinity for substrates in part regulates kinetic rates of the Na\(^+\)/K\(^+\)-ATPase. For example, Ser-16 phosphorylation of the \(\alpha\) subunit by PKC can increase the pump’s affinity for Na\(^+\) ions (87). In addition, in alveolar lung epithelium, dopamine regulates Na\(^+\)/K\(^+\)-ATPase activity by promoting recruitment of the pump to the basolateral membrane from intracellular stores (88).

Long-term regulation of the Na\(^+\)/K\(^+\)-ATPase occurs by transcriptional and post-transcriptional mechanisms including changes in transcription rate, mRNA stability, translation rate, protein degradation and isozyme expression (reviewed in 86, 89). Gene regulation occurs in an isoform specific manner as individual isoforms of the \(\alpha\) and \(\beta\) subunits are encoded by different genetic loci. During late neonatal stages cardiac tissue switches expression from \(\alpha1\) and \(\alpha3\) mRNA to high levels of mostly \(\alpha1\) and \(\alpha2\) transcripts due to isoform-specific regulation by thyroid
hormone and glucocorticoids (90). Also aldosterone regulates isoform expression, as it can induce α3 expression in the brain, while targeting α1 expression in the kidney (86, 90). Several hormones, including thyroid hormone, aldosterone and glucocorticoids, regulate the spatial-temporal expression of the Na⁺/K⁺-ATPase leading to its very complex isozyme expression, which likely explains the different physiologic response of tissues to Na⁺/K⁺-ATPase substrates, namely Na⁺ ions, K⁺ ions and CGs.

1.2.5 The Na⁺/K⁺-ATPase: A Novel Anticancer Target

Previous reports have correlated increases in Na⁺/K⁺-ATPase activity with tumour progression and in some cases increased Na⁺/K⁺-ATPase activity has been observed prior to morphological evidence of an established tumour (91-93). Correlation between elevated Na⁺/K⁺-ATPase and tumour growth would suggest that the Na⁺/K⁺-ATPase likely plays an important, yet poorly defined role in initiation and progression of tumours (91-93). Increased Na⁺/K⁺-ATPase activity has been theorized by Weidemann to be a compensatory mechanism to higher Na⁺ content that results from increased passive Na⁺ membrane permeability of neoplastic cells (94). Collectively, the higher intracellular Na⁺ content and higher Na⁺/K⁺-ATPase activity in tumour cells facilitate: 1) an increase in co-transport and accumulation of nutrients for the metabolic demands of transformed cells; and 2) support cell proliferation as a result of higher intracellular K⁺ concentration and lower cAMP from increased pump activity (94).

Increased activity of the Na⁺/K⁺-ATPase may be accomplished by increased isozyme expression of the Na⁺/K⁺-ATPase at the plasma cell membrane. The α3 Na⁺/K⁺-ATPase has higher affinity for K⁺ ions and lower affinity for Na⁺ ions relative to other isoforms, and functions with lower occupancy of the intracellular Na⁺ sites (95). This may suggest that elevated expression of α3 in tumours can manifest high Na⁺ and K⁺ in transformed cells as predicted by Weidman. Indeed, reports have demonstrated an increase in α3 expression in kidney, pancreatic and bladder cancer with associated decreases in α1 expression in both colorectal and prostate cancers (96, 97). Alternatively, elevated α1 expression has been observed in both non-small cell lung cancer (NSCLC) and glioblastoma multiforme (GBM) (98, 99). In these tumours the expression of α2 and α3 subunits at the plasma membrane did not reveal any significant differences compared to respective controls (normal lung tissue and normal brain tissue). Elevated expression of α1 and/or α3 subunits in some tumours may account for overall increases in Na⁺/K⁺-ATPase
activity. How increased expression of α subunits may drive the development of tumours is still poorly understood and continued investigation is required to elucidate the effects of the α1 and α3 subunits on tumour growth.

Specific cancer related changes are also observed in the β subunit and FXYD domain of the Na⁺/K⁺-ATPase. Rajasekaran et al. demonstrated that decreased expression of the β1 subunit correlated with poorly differentiated cancer cell lines derived from colon, prostate, kidney and pancreatic cancer (100-103). Furthermore, they suggested that the Snail transcription factor inhibits gene expression of the β1 subunit and cadherin, leading potentially to an epithelial-mesenchymal transition permitting cancer cells to become more invasive (103).

Several FXYD polypeptides are specifically associated with cancer and almost exclusively expressed in cancer cells. For example, the FXYD3 (Mat-8: a mammary tumour marker) is highly up-regulated in prostate, colorectal, pancreatic, gastric (adenocarcinoma) and breast tumours (104-108). Yet, FXYD3 overall appears to be down-regulated and sometimes mutated in various types of lung cancers including, adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma (109). This down-regulation in lung cancer cells may be the resultant loss of an epithelial phenotype, as over-expression of wild type FXYD3 in tumour cells restores the well-demarcated distribution of cortical actin that separates the apical layer from the basolateral regions of epithelial lung cells (109). Furthermore, a recent study on intestinal suggested that the interaction of FXYD3 with the Na⁺/K⁺-ATPase may indirectly promote tight junctions and cortical actin leading to the polarization and differentiation of intestinal epithelia. Still the specific role of FXYD3 in cancer is poorly understood yet it appears that its loss may promote an undifferentiated state in tumour cells.

Several pieces of evidence show that targeting the Na⁺/K⁺-ATPase may have anticancer effects including; (1) its role in cell adhesion and polarity, (2) observations of increased expression in several cancer models and (3) observations of its role in signal transduction (see section 1.3.5). Given the potential role of the Na⁺/K⁺-ATPase in cancer, specific targets of the pump such as CGs may be predicted to have therapeutic value.
1.3 Cardiac Glycosides

1.3.1 Introduction

The membrane inserted Na\(^+\)/K\(^+\)-ATPase is the known target of cardiac glycosides (CGs). CGs or cardiotonic steroids have long been used for a variety of conditions. Its best described therapeutic application is for the treatment of cardiac dysfunction namely, congestive heart failure and atrial arrhythmias (110, 111). These inotropic agents are thought to increase intracellular calcium as an indirect effect of inhibiting the Na\(^+\)/K\(^+\)-ATPase. Retrospective, epidemiological studies of breast cancer patients would suggest that therapeutic concentrations of CGs could be achieved to treat breast carcinoma with minimal toxicity (112, 113). This and other findings thereafter has lead to the emergence of CGs as a possibly novel drug target for the treatment of cancers.

1.3.2 Chemical structure

CGs are compounds with three basic structures: 1) a tetracyclic steroid ring (steroid nucleus); 2) a sugar moiety at position C3 of the steroid nucleus and 3) a lactone moiety at the C17 position in the steroid nucleus (Fig. 2). The lactone moiety defines two classes of CGs. The lactone group of cardenolides is an unsaturated butyrolactone ring and for bufadienolides this is a \(\alpha\)-pyrone ring. CGs derived from *Digitalis* and *Nerium* plant species (digoxin, digitoxin) have A/B rings in cis conformation while the B/D rings are in trans conformation resulting in the ‘U’ shape of these compounds. A number of CGs produced by the milkweed family *Asclepiadacea*, including calactin, uscharin, 2″-oxovoruscharin and the derivative UNBS1450 contain A/B fused rings in trans confirmation giving rise to a flat shape of the nucleus structure as well as a unique dioxanoid attachment of the sugar moiety to the steroid nucleus (double link) (83, 114, 115).
Figure 2. Structure of CGs. CGs are composed of a tetracyclic steroid ring, a lactone ring and sugar moiety. CGs are classified as bufadienolides or cardenolides based on the structure of the lactone ring. Digitoxigenin is the aglycone of digitoxin as it lacks three digitoxose sugar groups and digitoxin lacks the hydroxyl group attached to the C12 carbon of digoxin. Cardenolides are shown with the exception of bufalin (bufadienolide) and oleandrin (enolide).

1.3.3 CG binding site on the Na\(^+\)/K\(^+\)-ATPase

Recently, the crystal structure of the Na\(^+\)/K\(^+\)-ATPase alone as well as with bound K\(^+\) and ouabain has been resolved from shark rectal tissue (see Appendix Fig. 16). The lactone ring and the tetracyclic steroid ring are thought facilitate binding to the M4-M6 helices of the Na\(^+\)/K\(^+\)-ATPase (116). The carbonyl group in the lactone ring is predicted to disrupt the K\(^+\) coordination site II of the Na\(^+\)/K\(^+\)-ATPase and is thought to cause a structural change in the Na\(^+\)/K\(^+\)-ATPase, leading to unwinding of the M4 helix creating a hydrophobic binding cavity for the steroid nucleus (116). The steroid nucleus and lactone group likely cooperate to stabilize binding interactions with the Na\(^+\)/K\(^+\)-ATPase; as extensive experiments with simple lactones alone lack inotropic activity of digoxin, which suggests that the lactone group alone is not sufficient for binding to the pump (117, 118).

The proximity and distribution of the CG binding site around the K\(^+\) coordination site, may in
part explain the ability of $K^+$ ions to antagonize the inhibitory effects of CGs on the $Na^+/K^+$-ATPase (116). One implication of this property is that CGs likely bind with high affinity to the $Na^+/K^+$-ATPase in unbound potassium states (e.g. $E_2P$) and bind with low affinity to potassium bound states of the $Na^+/K^+$-ATPase ($E_2-K+Pi$) (74, 116, 119).

Furthermore, Paula et al. suggested that the sugar moiety may play an important role in CG binding, with minimal effects on $Na^+/K^+$-ATPase inhibition. This is readily demonstrated with ouabain and ouabagenin which are similar in structure except for a hydroxyl group in ouabagenin where the rhamanose sugar binds to the tetracyclic steroid ring of ouabain (119). These two CGs have nearly identical inhibitory potencies on the $Na^+/K^+$-ATPase; however, the rhamnose sugar of ouabain is predicated to form hydrogen bonds with the M4 helix which confers ~300 times great binding affinity than ouabagenin (116, 119). In addition, digitoxin and its aglycone digitoxigenin (substitution of digitoxose sugar moiety for a hydroxyl group), displays a similar disparity between binding affinity and the $Na^+/K^+$-ATPase inhibitory values (Table 3).

### Table 3. CGs and their respective affinity and inhibitory values for human $Na^+/K^+$-ATPase alpha isoforms expressed in P. Pastoris membranes (from Katz et al. 120).

<table>
<thead>
<tr>
<th>Cardiac Glycoside</th>
<th>Binding affinity $K_d$ (nM)</th>
<th>Inhibitory values $K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha 1$</td>
<td>$\alpha 2$</td>
</tr>
<tr>
<td>Digoxin</td>
<td>87±6</td>
<td>25.6 ±2.8</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>38 ±3</td>
<td>18.3 ±4</td>
</tr>
<tr>
<td>Digitoxigenin</td>
<td>270±21</td>
<td>322±11</td>
</tr>
<tr>
<td>Ouabain</td>
<td>9.8 ± 33</td>
<td>21.9 ± 0.56</td>
</tr>
</tbody>
</table>

1.3.4 CGs: Inotropy and Adverse Effects

CGs, are characterized as inotropic agents because they increase the rate and force of cardiac contraction. These properties of CGs support the use of digoxin and digitoxin in the treatment of cardiac dysfunction. CGs are often used only when standard therapy including angiotensin-converting enzymes and β-blockers fail to relieve symptoms of heart failure (121). One reason for caution is that their upper therapeutic concentrations overlap with symptoms of CG-induced
toxicity of the central nervous system (CNS) namely depression, decreased vision acuity and psychosis (122). Also given the high levels of Na\(^+/K^+\)-ATPase expression in epithelia tissue, it is not surprising that there are detrimental gastrointestinal effects from CG therapy. Adverse effects, such as diarrhea, nausea and vomiting, are thought to arise from increased motility of the GI tract (123,124).

The most widely supported theory to explain the toxic and inotropic effects of CGs is the Na\(^+\)-lag hypothesis (Fig. 3). Under normal physiologic conditions, the Na\(^+/Ca^{2+}\) exchanger bound to the plasma membrane utilizes the Na\(^+\) gradient established by the Na\(^+/K^+\)-ATPase to drive the efflux of Ca\(^{2+}\) ions. The inhibitory effects of CGs on the Na\(^+/K^+\)-ATPase results in a slow increase of intracellular Na\(^+\) concentrations, resulting in a dampening of the Na\(^+\) gradient and an increase of intracellular Ca\(^{2+}\) due to diminished Na\(^+/Ca^{2+}\) exchanger activity. The excess intracellular Ca\(^{2+}\) is then taken up into the sarcoplasmic reticulum (SR) by the SR Ca\(^{2+}\)-ATPase, increasing intracellular stores, which when released, strengthens subsequent contractions of affected cardiac myocytes. In addition to mechanical effects, CGs affect the electrical properties of cardiac tissue through; 1) indirect autonomic action that decreases conduction and nerve impulses to the heart, and 2) direct actions on cardiac cells leading to shortening of the plateau phase and an overall shorter action potential (123). Partial pump inhibition in cardiomyocytes (roughly 30%) is believed to mediate the therapeutic effects of CGs, while greater than 50% inhibition leads to toxicity (122). At higher concentrations further decreases in the Na\(^+\) gradients reduces resting membrane potential. With a more neutral (less polar) membrane potential, delayed after depolarizations (DADs) are more likely to occur following a normal action potential. DADs are associated with overloading of the intracellular calcium stores in the SR and can lead to their own action potential and cardiac myocyte contraction. If DADs are coupled with normal action potentials in the Purkinje conduction system of the heart, bigeminy (paired heart contractions) will result. DAD evoked action potentials can themselves induce a DAD and ectopic beats causing tachycardia that may ultimately deteriorate into ventricular fibrillation, which can be fatal (123).

Although this theory is well accepted, there are questions with regards to the exact mechanism of Ca\(^{2+}\) regulation by the Na\(^+/K^+\)-ATPase and the specific roles of the \(\alpha1\), \(\alpha2\) and \(\alpha3\) isoforms expressed in the heart. Studies from \(\alpha1\)-/- and \(\alpha2\)-/- heterozygous mice as well as evidence of subcellular colocalization of \(\alpha2\) with the Na\(^+/Ca^{2+}\) exchanger in the plasma membrane nearby the
SR suggests that α2 sensitivity could mediate inotropic effects of CGs (124). Furthermore, in this model the α1 isoform, based on its uniform distribution in cardiac myocytes would be responsible for bulk transport of Na\(^{+}\) and K\(^{+}\) ions into and out of the cell. Inhibition of the α1 Na\(^{+}/K^{+}\)-ATPase isoform would therefore affect global cellular ion concentration giving rise to a diminished electrochemical gradients with possible toxic outcomes (124). The historical assumption is that partial inhibition versus complete inhibition of the Na\(^{+}/K^{+}\)-ATPase gives rise to the respective therapeutic and toxic effects of CGs; however, the differential expression of isoforms in the heart may also in fact explain these differences. In particular the α2 subunit may mediate inotropic effects of CGs by close association with the SR allowing for subcellular Ca\(^{2+}\) oscillations, whereas inhibition of all isoforms including α1 may result in whole cell increases of Na\(^{+}\) ions leading to cardiotoxicity (124). Previously, drugs designed to increase inotropic activity while minimizing cardiotoxicity attempted to increase the binding affinity of CGs and decrease inhibition of these drugs, based on the assumption that not all of the inotropic effects of CGs are the result of Na\(^{+}/K^{+}\)-ATPase inhibition (124). However, if true this relatively new theory for the therapeutic mechanism of CGs would shift the focus to design novel agents that preferentially target isoforms other than α1 that may improve inotropic effects and decrease cardiotoxicity. This same rationale may also be employed in drug development of CGs with anticancer potential (see Section 5.8).
Figure 3. Inotropic and cardiotoxic effects of CGs. According to the Na\(^+\)-lag hypothesis, partial inhibition of the Na\(^+\)/K\(^+\)-ATPase by CGs leads to a transient increase of [Na\(^+\)]. This in turn leads to an increase in [Ca\(^{2+}\)]\(_i\) via a reduction in Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) activity. Excess [Ca\(^{2+}\)]\(_i\) is taken up by the endoplasmic reticulum and upon stimulation, release of stored Ca\(^{2+}\) amplifies the normal force of contraction. Complete inhibition of the Na\(^+\)/K\(^+\)-ATPase by CGs evokes a constantly elevation in [Ca\(^{2+}\)]\(_i\) that overwhelms the sarcoplasmic reticulum and leads to deleterious cardiotoxic effects, including hypertrophy and tachycardia.

1.3.5 The role of Na\(^+\)/K\(^+\)-ATPase in CG Antitumour Activity

In comparing a series of compounds derived from the CG 2”-oxovoruscharin, Van Quaquebeke et al. found that compounds with very poor inhibition of the Na\(^+\)/K\(^+\)-ATPase (IC\(_{50}\) >50 µM) did not inhibit cancer cell growth (IC\(_{50}\) >10 µM) (114). This association might suggest that inhibition of cancer cell growth by CGs requires inhibition of the Na\(^+\)/K\(^+\)-ATPase. These conclusions were drawn by Winnicka et al. following 24-48 hour treatment with ouabain (100 nM and 200 nM), that induced apoptosis and a sustained increase in intracellular Ca\(^{2+}\) in human breast cancer cells (MDA-MB-435, see Appendix Table 13) (125). Furthermore, the doses used to induce apoptosis (1 µM and 10 µM) in prostate cancer cells (PC-3, LNCaP and DU145) also were associated with sustained elevation in intracellular Ca\(^{2+}\) (126). These studies in breast cancer and prostate cancer models support the following interpretation: inactivation of the Na\(^+\)/K\(^+\)-ATPase and inhibition of Na\(^+\)/K\(^+\)-ATPase ion regulation induces antitumour activity.
Although, Na\(^+\)/K\(^+\)-ATPase and cancer growth inhibition are associated, this alone does not completely explain the antitumour activity observed with CGs. In many cases the concentration of cardiac glycoside used to induce antitumour activity do not inhibit the Na\(^+\)/K\(^+\)-ATPase and pump-mediated intracellular concentrations of Na\(^+\), K\(^+\) and Ca\(^{2+}\). For example, ouabain (10-100 nM) induces antiproliferative effects with less than 25\% of pump inhibition (over a period of seven days) (127). In this same study, associated increases in levels of p21, Src, ERK and JNK signaling were reported (61). In SH-SY5Y neuroblastoma cells, apoptosis was observed with ouabain (<100nM) and was associated with activation of ERK, p53 and pBad at lower concentrations than is required to dramatically increase intracellular Ca\(^{2+}\) (128). Thus, evidence indicate that both an ionic pathway and a non-ionic signaling pathway play important roles and work possibly in parallel to induce anticancer activity in addition to likely other Na\(^+\)/K\(^+\)-ATPase-dependent functions such as inotropy, and internalization of the Na\(^+\)/K\(^+\)-ATPase itself.

**Na\(^+\)/K\(^+\)-ATPase-mediated Signal Transduction**

Recently recognized is the capacity of CGs to induce, downstream of binding to the Na\(^+\)/K\(^+\)-ATPase, a myriad of signaling proteins that leads to changes in gene expression, and the rate of proliferation in a manner that varies between cells, the concentration of CG and even with individual CGs (Fig. 4) (129-137). As described in the previous section, the signaling pathways activated in response to CGs do not necessarily induce large changes in cytosolic Na\(^+\) ions (61, 128, 138). In part this may be explained by two pools of the Na\(^+\)/K\(^+\)-ATPase within the plasma membrane; one pool being the classical energy transducing ion pump and the other, the signal transducing form of the pump restricted to mostly caveolae (134). Although the precise mechanism by which the Na\(^+\)/K\(^+\)-ATPase induces a signaling cascade has not been completely elucidated, the following sections describe the current knowledge of CG-induced signaling via the Na\(^+\)/K\(^+\)-ATPase.
Figure 4. Na\(^+\)/K\(^+\)-ATPase-mediated signal transduction. Upon binding to the Na\(^+\)/K\(^+\)-ATPase, CGs induce a signaling cascade. CGs release Src from the inhibitory restraint of the Na\(^+\)/K\(^+\)-ATPase, allowing Src to activate EGFR and the Ras-MEK-ERK1/2 pathway. Also CG binding activates a complex consisting of PLC tethered to the Na\(^+\)/K\(^+\)-ATPase and the IP3 receptor on the endoplasmic reticulum. By binding to the Na\(^+\)/K\(^+\)-ATPase, CGs increase IP3 formation, which in turn increases intracellular stores of Ca\(^{2+}\). These two signaling pathways can affect PI3K-Akt signaling, NF-κB signaling and a host of other signaling cascades leading to a proliferative or antiproliferative response depending on the cell type, CG concentration, and CG (129-137).

**Src-EGFR-Ras-ERK1/2 signaling**

The Na\(^+\)/K\(^+\)-ATPase undergoes a conformational change, following ouabain binding, that activates the cytoplasmic tyrosine kinase SRC in a complex that includes the sodium pump (131). Activation of Src requires phosphorylation at the activating Tyr418 site, allowing the kinase to induce autophosphorylation of neighbouring EGFR (137). Activated EGFR subsequently recruits SHC (Src homology 2 domain containing), GRB2 (growth factor receptor-bound protein 2) and SOS (Son of Sevenless), which induces the activation of the RAS-RAF and ERK1/2 protein cascade (66, 136, 137, 139-141).
Signaling of PLC IP3 and intracellular Ca\(^+\) changes

In parallel to Src activation and in a process not fully understood, PLC and IP3 in a complex with the Na\(^+\)/K\(^+\)-ATPase become activated. This complex of proteins brings the sodium pump in close proximity to the endoplasmic reticulum, inducing single and repeated transient changes of intracellular Ca\(^{2+}\) (142).

NF-κB signaling

Activation of NF-κB occurs downstream of Src activation and [Ca\(^{2+}\)]\(_i\) oscillations. In addition, activation of RAS by Src activation leads to the opening of ATP-sensitive mitochondrial potassium channels, resulting in a concomitant production of reactive oxygen species, triggering NF-κB (66, 136, 137, 139-141). In parallel to SRC activation, low frequency calcium oscillations (4-6 minute) in response to low concentrations of ouabain (0.1- 10 nM) also invokes NF-κB to induce gene programs which leads to differentiation or proliferation (133, 143).

PI3K-Akt signaling

PI3-K is another part of the signaling complex of Na\(^+\)/K\(^+\)-ATPase-EGFR and Src. PI3-K is bound to a proline-rich region of the catalytic α subunit (144). CG binding to the Na\(^+\)/K\(^+\)-ATPase is thought to stimulate PI3-K, and Akt via phosphorylation by PI3-K (145).

1.3.6 CGs: Mechanism of Antitumour Activity

Anticancer effects have been reported in several types of carcinoma, including breast (113, 146, 147), prostate (148, 149), lung (NSCLC) (150-152), melanoma (153), pancreatic (154, 155) leukemia (156, 157), renal adenocarcinoma (158), colorectal adenocarcinoma (159, 160), glioblastoma (161), sarcoma (Kaposi sarcoma) (162), osteosarcoma (163), retinoblastoma (164) and neuroblastoma (128, 139, 165). As described below, CGs induces apoptosis and disrupts numerous cellular processes vital to tumour growth, such as inhibiting proliferation, angiogenesis, cell migration and invasion (see Appendix Table 13).

Cell death response to CGs

Cell death is the most commonly reported mechanism of CG anti-cancer effects. In neuroblastoma cells, namely SH-SY5Y, apoptosis is induced in response to ouabain at 10-100 nM concentrations. In addition, UNBS1450, a novel CG analogue preferentially induces autophagy in glioblastoma and NSCLC cell lines (98, 152). Oleandrin was also used in NSCLC
and sensitizes NSCLC cells to Apo2L/TRAIL induced apoptosis via up-regulation of Death Receptors 4 and 5. Interestingly, stabilizing DNA topoisomerase II was demonstrated as the mode of antitumour effects in MCF-7 breast cancer cells treated with 100 nM of digoxin, ouabain or proscillaridin (166). MCF-7 and other estrogen receptor positive breast cancer cells may be subjected to dual targeting of the Na⁺/K⁺-ATPase and estrogen receptor (ER) by CGs (167). Reports of computer based alignment analysis predict that the amino acid residues 323-395 of ERα and likely ERβ are similar to the ouabain binding site of the Na⁺/K⁺-ATPase and could allow for CGs to bind antagonistically to the estrogen receptor (167).

**CGs induce differentiation and cell cycle arrest**

In contrast to activating cell death, CGs have also been reported to induce differentiation and cell cycle arrest. Human breast cancer cells MDA-MB-435 (ER negative), following ouabain treatment (<100 nM) have increased levels of p21 and undergo cell cycle arrest (61). Also, Bufalin has been shown to induce differentiation of human leukemia cells (HL60, U937 an ML1) toward a Macrophage/Monocyte fate by cell cycle arrest in the G2-S phase (168-170). In addition, UNBS1450 is thought to induce cell cycle arrest in prostate cancer cells. Thus far only three reports have cited that CGs can induce cell cycle arrest or differentiation and there is no proposed mechanism. Lastly, to date there is no evidence supporting differentiation and cell cycle arrest in neuroblastoma cells in response to CG treatment.

**Antiangiogenic effects of CGs**

CGs can also regulate the secretion of FGF-2 (fibroblast growth factor-2) in prostate cancer cells (PC3 and DU145), which can induce angiogenesis and is involved in a variety of biological processes (171). The concentrations of ouabain, oleandrin, and Anvirzel used to inhibit FGF-2 release were shown not to inhibit the viability and proliferation of the prostate cancer cell lines, suggesting that the affect of CGs on angiogenesis was not due to antiproliferative effects (171). In addition, previously digoxin has been shown in neuroblastoma to have antiangiogenic effects. Specifically, Svensson *et al.* demonstrated that treatment of neuro-2a tumours in *vivo* with digoxin decreases vascularization induced by FGF-2 in when cells are injected into chorioallantoic membranes (165).

**CGs inhibit the metastatic potential of tumour cells**
In addition to antiproliferative effects, UNBS1450 (for structure see Fig. 2), impairs migration and actin cytoskeleton in glioblastoma cells (10 to 100 nM). The anticancer effects of CGs may also be explained by inhibition of N-glycan expression. Aberrant N-linked glycans promote tumour invasiveness; consistent with effects on N-glycan cell surface expression, digoxin inhibited tumour cell migration, invasion and distant tumour formation (172). CGs were also identified in a high-throughput screen for agents that restore sensitivity of tumour cells to anoikis. Anoikis occurs as epithelial cells undergo cell death in response to detachment from cell/cell and cell/extracellular matrix contacts. Cancer cells are thought to be resistant to anoikis allowing for cell migration and invasion (173). This screen identified five members of the family of CGs as anoikis sensitizers (ouabain, peruvoside, digoxin, digitoxin, and strophanthidin), suggesting in addition to the previous described studies that CG anticancer activity may also be in part due to inhibition of tumour cell migration and invasion.

1.3.7 Cardiac Glycoside: Epidemiological evidence of Anticancer Properties

Stenkvist et al. first reported the potential anticancer effects of CGs in a cohort of women receiving treatment for breast carcinoma. The study found that patients who were coincidentally receiving CGs to treat cardiac dysfunction had breast cancer cells that appeared morphologically more benign, relative to cancer cells from non-CG treated patients (174-176). Furthermore, 5 years following mastectomy, the rate of relapse for patients on digitalis treatment was 9.6 times lower compared with patients not receiving CG treatment. A 20-year retrospective study on the same patient group revealed that the death rate from breast carcinoma (excluding confounding factors) was 6% (two of 32) among patients on digitalis therapy (mainly digoxin or digitoxin), compared with 34% (48 of 143) among patients not on digitalis (113). Thus clinical evidence would support further clinical studies into CGs as a potential novel class of anticancer agents.

1.3.8 CGs: Anti-tumour Activity in vivo and in Clinical Studies

Although, there are in vitro and epidemiological studies supporting the anti-cancer properties of CGs, there is less in vivo evidence supporting these drugs as effective anticancer agents. The first study fifteen years ago, reported that digitoxin inhibited tumour formation of mouse skin and mouse pulmonary tumours (177). Recently, anticancer activity of UNBS1450 was demonstrated
in xenografts of glioblastoma, prostate cancer and NSCLC (151, 161, 178). Also studies have shown that digoxin can inhibit cell migration with \textit{in vivo} models of metastatic prostate cancer cells (172). In another model for prostate cancer, circulating prostate cancer cells developed fewer tumours when treated with ouabain relative to non-CG treated controls (173). In addition, Svensson \textit{et al.} reported that digoxin induced inhibition of neuroblastoma xenografts. Interestingly, inhibition of tumour cell growth was targeted against neuroblastoma cells (44\% inhibition of SH-SY5Y cells), whereas lung cancer cells in this study did not respond to digoxin treatment. This result would suggest that CGs have antitumour potential against only some tumours, including NB. These studies represent most if not all the \textit{in vivo} studies to date on the anticancer effects of CGs. One reason for the lack of \textit{in vivo} models is the lack of a well-accepted pre-clinical model. Most models for cancer study use rodent models; however, given the insensitivity to CGs of most rodents these models are unable to predict toxicity associated with drug treatment.

Thus far results from clinical trials on CGs are inconclusive as to the effectiveness of these compounds to treat cancer. One completed phase II clinical trial studied the effects of digoxin in combination with the EGFR targeting antibody erlontib in NSCLC. The rationale for this clinical study was based on the association of the Na$^+$/K$^+$-ATPase with the EGFR receptor. No improvement was detected in the response rate of patients with combined erlontib and digoxin therapy compared to single agent erlontib (179). More promising results were observed in treating melanoma patients with digoxin in combinatorial therapy that included cisplatin, vinblastine, dacarbazine, interleukin-2 and interferon. Preliminary evidence suggests that digoxin had a synergistic effect on tumours with minimal treatment related toxicities (180). There is one known documented clinical trial of CGs as a single agent. Anivirzel (a nerium oleander extract containing predominately oleanadrin) was safely administrated to patients in a phase I clinical trial (0.8 ml/m$^2$/day intramuscular injection). Unfortunately, these patients, all of whom had refractory disease and were heavily pre-treated did not display signs of tumour regression (181). The assumption that these drugs may lack antitumour activity at tolerated dose levels possibly explains why there are only a few clinical trials conducted with these compounds. Although traditional CGs have not been well-studied in clinical trials, \textit{in vitro} and \textit{in vivo} experiments have shown that novel CG analogues may be effective anticancer agents with predicted diminished toxicity and for these reasons may be superior to traditional CGs. UNBS1450 is the only known
novel CG analogue under phase I clinical trial and is currently being tested for efficacy against solid tumours and lymphomas in Belgium (182).

1.3.9 Novel Strategies to Circumvent Cardiac Glycoside Induced Toxicity

Stenkvist et al. found that in a retrospective analysis of patients receiving concurrent chemotherapy and digitalis, therapy resulted in increased survival for breast carcinoma patients. However, the risk of cardiotoxicity associated with anticancer doses of CGs compounds in part has stalled further clinical investigation into their anticancer potential. This is consistent with a narrow therapeutic window for digoxin of 0.6-1.9 nM and digitoxin of 13-33 nM to treat cardiac dysfunction.

Recent reports of novel CG derivatives suggest that these molecules have increased anti-proliferative effects with predicted decreases in related toxicity. For example, neoglycorandomization, the addition of novel glycosides to the C3 carbon of the tetracyclic steroid ring, showed the importance of the stereochemistry of the C2 carbon in the sugar moiety of CGs. In particular, a (S) configuration made CG analogues more potent against a panel of cancer cell lines with concurrent decrease in Na⁺/K⁺-ATPase inhibition relative to digitoxin (183). In addition, the modification of the C19 substituent to a sterically non-hindered polar group was important in maintaining anti-tumour effects while increasing in vivo tolerance of the novel cardiac glycoside UNBS1450 (114). In these studies in vivo tolerance was the maximum dose tolerated by mice that did not cause death over approximately one month(114). Although mice are not an appropriate preclinical model to assess toxicity, an increase of in vivo tolerance for UNBS1450 relative to the starting compound would suggest that modifications to the tetracyclic steroid ring may increase the therapeutic potential of these drugs (114). UNBS1450 also revealed that the Na⁺/K⁺-ATPase is a target for the anticancer properties of CGs, but that inhibition of the Na⁺/K⁺-ATPase by CGs do not necessarily correspond to the effects of antitumour activity (182). Therefore, this would suggest that modifications to the structure of CGs, in particular the steroid nucleus but also the lactone ring and sugar moiety may improve anticancer activity without Na⁺/K⁺-ATPase inhibition, which is thought to lead to cardiotoxicity.
2.1 Rationale

Neuroblastoma accounts for 15% of paediatric-oncology related deaths (2). Approximately half of all patients are diagnosed with high-risk NB (2-4). Over the past three decades survival rates of high-risk patients have not improved despite the intensification and combination of available therapies (2-4).

In a concerted effort to better understand the biology of NB and develop novel therapies to increase overall survival, the Kaplan laboratory has identified tumour-initiating cells (TICs) harvested from the metastases and primary tumours of NB patients. As few as ten NB TICs injected orthotopically into SCID/Beige mice form neuroblastoma tumours; thus, given their potent tumour-initiating capacity, NB TICs likely play an integral role in treatment response and treatment failure. Therefore, drugs that specifically target NB TICs may prove to be effective against NB.

A high-throughput screen was preformed to identify drugs that selectively target NB TICs compared to non-transformed control cells with neural crest cell properties termed skin derived precursors or SKPs (184). Four different chemical libraries, LOPAC, Prestwick, Spectrum and Biomol were used to test 4687 licensed drugs. The class of CGs was the largest group identified with 13 compounds showing moderate selectivity against NB TICs (184).

In vitro and in vivo studies would suggest that traditional CGs have anticancer potential against neuroblastoma. However, a major impediment to the clinical use of traditional CGs is the cardiotoxicity associated with these drugs. Cardiotoxicity results from inactivation of Na⁺/K⁺-ATPase leading to diminished gradients of Na⁺ and K⁺ ions across the cell membrane.

Studies suggest that the potential antitumour activity of CGs can be separated from inhibition of the Na⁺/K⁺-ATPase. To achieve a more potent drug with lower predicted cardiotoxicity, we tested novel CG compounds synthesized by the Focus in Synthetic Chemistry (FISC) group at the Hospital for Sick Children for antitumour activity on NB TICS.
2.2 Hypothesis

We hypothesize that CGs are potential treatment agents for NB and modifications to the functional groups will identify structural components of CGs and will lead to a compound with greater antitumour activity and lower predicted cardiotoxicity.

2.3 Project Aims

The **first aim** is to ascertain the effective inhibitory doses of CGs and novel CG analogues in NB cells. To test the efficacy of parent and novel CG analogues, sphere assays will be conducted on NB TICs and a metabolic activity assay (MTT assay) on adherent cell lines. A combination of NB TICs and NB adherent cell lines are being employed because NB TICs are thought to be a highly relevant model for drug studies and NB adherent cells are more commonly used and widely accepted in the NB field. As well, immunoblotting for cleaved-PARP will be used to assess induction of apoptosis following drug treatment.

The **second aim** is to assess whether the inhibitory activity on the Na⁺/K⁺-ATPase is divergent from potential anti-tumour activity of CGs on NB cells. To characterize the effects of CGs on the Na⁺/K⁺-ATPase, gene silencing and expression of an insensitive form of the Na⁺/K⁺-ATPase will be used with CG treatment. In addition, Na⁺/K⁺-ATPase inhibitory activity is thought to play a predominant role in cardiotoxicity of CGs, thus relative inhibition of the Na⁺/K⁺-ATPase in HEK-293 cells will be used as a surrogate test for potential cardiotoxicity induced by CGs.

The **third aim** is to identify potential signaling mechanisms involved in the antitumour activity of CGs. Reports suggest that several signaling proteins are up-regulated downstream of CG binding to the Na⁺/K⁺-ATPase in a Na⁺/K⁺-ATPase-mediated signaling transduction pathway. One such protein is ERK, which has been described to be up-regulated following CG treatment in neuroblastoma cells and will be the focus of this aim.
Chapter 3
Material and Methods

3.1 Cell Systems
Investigation of the antitumour activity of CGs on neuroblastoma involved the use of several NB cell lines including, NB TICs (NB12, NB67, NB88 and NB 122), established adherent NB lines (IMR-5 and SK-N-(BE-2)) and established NB cell lines that grow as spheres in NB TIC growing conditions (KCNR) (Table 4). Although preliminary screens used NB TICs there are several reasons for the incorporation of immortalized adherent cell lines in this project, including: 1) established adherent cell lines were used to optimize conditions for NB TICs, 2) to observe any specificity towards NB TICs verses established NB cells, and 3) because established adherent cells are commonly used and widely accepted in the NB field. Thus testing CGs against both NB established adherent cells and NB TICs increases the veracity of CGs as effective therapeutic agents against NB. Lastly, SKPs were used as the non-transformed control. SKPs are derived from the dermal papilla of foreskin and display neural crest cell properties (185, 186). Also similar to NB TICs, SKPs grow in serum free conditions and require SKP conditioned media for optimal growth (185, 186).

Table 4. Characteristics of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 status</th>
<th>MYCN amp</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Established adherent cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMR-5</td>
<td>wt</td>
<td>amp</td>
<td>Adherent cell line</td>
</tr>
<tr>
<td>KCNR</td>
<td>n/a</td>
<td>amp</td>
<td>Grows as spheres in neural crest stem cell media</td>
</tr>
<tr>
<td>SK-N-BE-2</td>
<td>C135F</td>
<td>amp</td>
<td>Adherent Cell line</td>
</tr>
<tr>
<td>NBTIC lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NB 12</td>
<td>wt</td>
<td>non-amp</td>
<td>Grows as dense spheroids</td>
</tr>
<tr>
<td>NB 67</td>
<td>n/a</td>
<td>non-amp</td>
<td>Grows as dense spheroids</td>
</tr>
<tr>
<td>NB 88</td>
<td>wt</td>
<td>non-amp</td>
<td>Grows as lose spheroids</td>
</tr>
<tr>
<td>NB 122</td>
<td>wt</td>
<td>non-amp</td>
<td>Semi-adherent</td>
</tr>
<tr>
<td>SKPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FS90</td>
<td>n/a</td>
<td>n/a</td>
<td>Grows as dense spheroids</td>
</tr>
<tr>
<td>FS99</td>
<td>n/a</td>
<td>n/a</td>
<td>Grows as dense spheroids</td>
</tr>
<tr>
<td>FS217</td>
<td>n/a</td>
<td>n/a</td>
<td>Grows as dense spheroids</td>
</tr>
</tbody>
</table>

Abbreviations: wt, wild-type; amp, amplification; NA, not available. (187-189).
3.2 Cell Culture

NB and SKP cells were incubated in 5% CO₂ at 37 °C in culture media listed in Table 5 for each cell line. NB TICs were isolated and cultured as previously described by Hansford and colleagues (51). Briefly, cells were isolated from the bone marrow metastasis of high-risk patients and cultured in vitro with neural crest stem cell media containing 1% penicillin/streptomycin (100 units/mL). Sphere lines were dissociated to passage cells into a new flask and for experiments early passages of sphere lines were used (< passage 12).

Table 5. Culturing conditions of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immortalized neuroblastoma cell line</td>
<td>DMEM (Invitrogen)</td>
<td>0.1 mM non-essential amino-acids, 1mM sodium pyruvate, 10% (FBS)</td>
</tr>
<tr>
<td>IMR-5, SK-N-NE-2</td>
<td>DMEM (Invitrogen)</td>
<td>0.1 mM non-essential amino-acids, 1mM sodium pyruvate, 10% (FBS)</td>
</tr>
<tr>
<td>SKPs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FS90 FS99 FS 217</td>
<td>1/2 conditioned media and 1/2 fresh media (DMEM: F12; 3:1 (Invitrogen))</td>
<td>Fresh media containing 2% B-27 supplement (Invitrogen), 40 ng/mL bFGF, (Collaborative), 20 ng/mL EGF (Collaborative), 100 U/mL penicillin/streptomycin</td>
</tr>
<tr>
<td>Neuroblastoma Tumour-Initiating Cells</td>
<td>DMEM: F12; 3:1 (Invitrogen)</td>
<td>2% B-27 supplement (Invitrogen), 40 ng/mL bFGF, (Collaborative), 20 ng/mL EGF (Collaborative), 100 U/mL penicillin/streptomycin</td>
</tr>
</tbody>
</table>

Abbreviations: bFGF, basic fibroblast growth factor DMEM, Dulbecco’s modified eagle’s medium; FBS, Fetal Bovine Serum; EGF, epidermal growth factor.

3.3 Cell proliferation and viability assays

Alamar Blue Assay

The alamarBlue® assay (Biosource International, Camarillo, CA, USA) indicates viability and proliferation of cells. Resazurin, a non-fluorescent indicator dye is converted to a red-fluorescent resorufin via reduction reactions of metabolically active cells. Cells were seeded in a 96-well plate and treated with drugs in triplicates. At the end of treatment, 10% alamar blue was added to each of the wells followed by 24 hour incubation in 5% CO₂ at 37 °C. Fluorescence was measured using the FLx 800 fluorescence plate reader (ELx800 Universal Plate Reader, Bio-Tek
Instruments) with excitation wavelength of 560 nm and emission wavelength of 590 nm. For each well the amount of fluorescence is directly proportional to the number of viable cells.

**MTT Assay**

To assess viability and proliferation MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Roche Applied Science, Laval, QC, Canada), a tertrazolium salt was used and is reduced in viable cells to purple formazan crystals. BioReduction of MTT occurs mostly by mitochondrial enzymes primarily succinate dehydrogenase and by NAD(P)H in the cytoplasm (190). Cells were seeded in a 96-well plate in triplicate. At post-treatment, for adherent cells old media was aspirated and cells were replenished with 100 µL fresh media containing 10% MTT reagent. Following, incubation in 5% CO₂ at 37 °C for 4 hours, 100 µL of solubilization solution was added to solubilize formazan crystals. After a second incubation at 37 °C for 24 hours, soluble formazan was measured at 560 nm absorbency using the ELISA plate reader (ELx800 Universal Plate Reader, Bio-Tek Instruments). Absorbency of formazan directly correlates to the number of viable cells.

**Trypan Blue Assays**

Cell viability was measured using the trypan blue exclusion test to confirm results from alamar blue and MTT assay (Sigma-Aldrich). Non-viable cells stain blue, while viable cells with intact cell membranes do not absorb the trypan blue dye. Cells were first seeded in 24-well plates and treated with drugs in triplicate. Non-adherent cells were collected after washing once with PBS. Cells were centrifuged at 2600 rpm for 5 min. The supernatant was aspirated and 100 µl of 1:1 solution of trypan blue and PBS was added to the cell pellet. Cells were then resuspended, incubated in the solution for 2 min and counted using a hemocytometer. A viable cell appeared clear and a non-viable cell blue. At least 100 cells were counted and the results are expressed as a percentage of viable cells to the total number of cells (viable and non-viable cells).

**Sphere Formation Assay**

Drug efficacy was tested by sphere formation assay with the help of Mayumi Fujitani and Kristen Smith. NB TICs (NB12, NB88R2, NB67 and NB122R) and established NB cells (KCNR), as well as SKP cells were used to test CGs and novel CG analogues. Cell lines were triturated into single cells and seeded in triplicate into 96-well non-tissue culture treated plates (Unibioscreen) at 2000 cells per well (NB12, NB88, NB122R) and 3000 cells per well (KCNR,
SKPs, NB67) in 50 µl of media, which contained 50% cultured SKP media in experiments conducted with a SKP line. Upon treatment, 50 µl of media containing twice the indicated drug concentration was added to each well to 100 µl final volumes. Three days after seeding cells, wells were retreated with the indicated drug concentration. On day 7, wells were counted for number of spheres. Results are expressed as percent number of spheres relative to untreated vehicle controls (0.5% DMSO or H2O).

**Potassium Chloride Assay**

To assess the antagonistic effects of K+ on CG-mediated antitumour activity, NB88R2 were seeded in 96-well non-tissue culture treated plates with the indicated concentrations of KCl (10 mM, 15 mM and 20 mM). Cells were treated with indicated drug concentration on Day 0 and Day 3 as described with the sphere formation assay. On day 7, wells were incubated with 10% alamar blue and viability and proliferation was assessed as described with the alamar blue assay.

### 3.4 Drug treatment

Chemical suppliers and the stock concentration of novel agents, conventional CGs and chemotherapeutic agents are listed (Table 4). Novel CG analogues were synthesized by the Focus in Synthetic Chemistry at the Hospital for Sick Children. Drugs were either dissolved in dimethylsulfoxide (DMSO) for conventional CGs, H2O for novel CG analogues or in culture media for doxorubicin. For sphere assays and immunoblots drugs were serially diluted in the respective control media and further diluted in culture media to the indicated concentration. Final concentrations of DMSO or H2O in media for drug treatment was less than or equal to 0.05%.

**Table 6. Conventional CGs and chemotherapeutic agents used for drug efficacy studies**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conventional CGs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convallatoxin</td>
<td>Sigma-Aldrich</td>
<td>25 mM</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>Sigma-Aldrich</td>
<td>25 mM</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Sigma-Aldrich</td>
<td>25 mM</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Sigma-Aldrich</td>
<td>25 mM</td>
</tr>
<tr>
<td>Peruvoside</td>
<td>Sigma-Aldrich</td>
<td>25 mM</td>
</tr>
<tr>
<td><strong>Chemotherapeutic Agents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (DOX)</td>
<td>Sigma-Aldrich</td>
<td>1 mg/ml</td>
</tr>
</tbody>
</table>

Abbreviations: Conventional CGs are dissolved in DMSO. Doxorubicin is dissolved in media.
3.5 Na\(^+\)/K\(^+\)-ATPase activity screen

Na\(^+\)/K\(^+\)-ATPase activity was assayed by Aurora Biomed (Vancouver, B.C., Canada). Briefly, HEK-293 cells, which endogenously express Na\(^+\)/K\(^+\)-ATPase, were exposed to rubidium (Rb\(^+\)) and subsequently washed with SPA-Wash Buffer and lysed. Cellular uptake of Rb\(^+\) in response to drug treatment was measured using flame atomic absorption spectroscopy (ICR8000, Aurora Biomed). The Na\(^+\)/K\(^+\)-ATPase recognizes Rb\(^+\) ions as analogous to K\(^+\) ions and thus decreased Rb\(^+\) uptake is considered a marker for inhibition of Na\(^+\)/K\(^+\)-ATPase mediated ion influx.

3.6 Immunoblotting (IB)

For immunoblotting, spheres lines less than ten passages were dissociated into single cells and 3×10\(^6\) cells were seeded in 6.7 ml of media in a 25 cm\(^2\) culture flask. Upon treatment, 3.3 ml of drug-containing media at three times the indicated concentration was added to each culture flask to adjust for dilution. Cells were then pelleted by centrifugation at 4°C and washed with cold PBS. Subsequently, cells were lysed at 4°C for 30 min using EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5\% NP-40) supplemented with protease inhibitors consisting of aprotinin (11.5 µg/mL), leupeptin (1 mg/mL), sodium orthovanadate (0.2 mM), and phenylmethylsulfonyl fluoride (50 µg/mL PMSF; Roche Applied Science, Laval, QC, Canada). The protein concentration of whole cell extract (WCE) was measured using the Bradford reagent (Bio-Rad laboratories, Hercules, CA, USA). Protein was denatured by boiling in SDS-containing sample buffer at 100°C for 5 min. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 10\% Gels were used to resolve proteins larger than 30 kDa and 15\% gels for proteins smaller than 30 kDa. Proteins were transferred to nitrocellulose membrane at 0.1 Amp overnight at 4°C or 0.4 Amp for 6 hour at 4°C; afterwards, the membrane was blocked in Tris-buffered saline (TBS) with 5\% milk for 1 hour at room temperature (RT) or at 4°C overnight. The membrane was then probed with primary antibody overnight at 4°C, washed and incubated at RT for 1 hour with HRP-conjugated secondary antibody or infrared dye-tagged secondary antibodies (Table 7). Nitrocellulose membranes incubated with HRP-conjugated secondary antibody were washed 5 times with TBS with 0.1\% Tween. Protein-antibody complex was detected using an enhanced chemiluminescence system (Super Signal West Pico, Pierce, Rockford, IL, USA). For nitrocellulose membranes probed with infrared dye-tagged secondary antibodies, the protein-antibody complex was detected using the Li-Cor Odyssey infrared imaging system.
3.7 Immunofluorescence (IF)

100 µl of medium containing suspended spheres was removed from a flask and spun down onto coated slides using a Thermo Shandon Cytospin 4 apparatus (Thermo Shandon Inc., Pittsburgh, PA,). Afterwards, slides were air-dried for 5 minutes, washed three times with PBS, fixed with 4% paraformaldehyde in PBS for 15-20 minutes and then washed three times with PBS. Cells were permeabilized with 0.2% NP-40 for 5 minutes, washed three times for 5 minutes with HBS, and blocked for 1 hour at room temperature with 0.5% bovine serum albumin and 6% normal goat serum in HBS. Primary antibodies were then added in HBS containing 3% normal goat serum and left overnight at 4°C. Primary antibody was removed, cells were washed three times for 5 minutes with HBS, and incubated for 1 hour at room temperature with Alexa 488-conjugated goat-anti-mouse (1:1,000) or Alexa 555-conjugated goat-anti-rabbit (1:1,000; Molecular Probes) (Table 7). Cells were washed three times for 5 minutes with HBS, stained with DAPI, and visualized using a Zeiss Axioplan upright microscope.

3.8 Antibodies

Table 7. List of Antibodies used in immunoblotting, and immunofluorescence studies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Company</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>β actin</td>
<td>Sigma-Aldrich, Oakville, ON, Canada</td>
<td>1/30,000</td>
</tr>
<tr>
<td>cleaved-PARP</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/750</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase α1</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/500</td>
</tr>
<tr>
<td>Phospho-ERK 1/2 (Thr202/ Tyr204)</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/1,000</td>
</tr>
<tr>
<td>Phospho-Src (Tyr416)</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/1,000</td>
</tr>
<tr>
<td>Total-ERK1/2</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/1,000</td>
</tr>
<tr>
<td>Total-Src</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
<td>1/1,000</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Upstate, Lake Placid, NY, USA</td>
<td>1/20,000</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>Company</td>
<td>Dilution Factor</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>HRP-conjugated anti-mouse IgG (H + L)</td>
<td>Pierce Biotechnology, Rockford, IL, USA</td>
<td>1/5,000</td>
</tr>
<tr>
<td>HRP-conjugated anti-rabbit IgG (H + L)</td>
<td>Pierce Biotechnology, Rockford, IL, USA</td>
<td>1/5,000</td>
</tr>
<tr>
<td>IR Dye-tagged anti-mouse (520 nm)</td>
<td>Li-Cor Biosystems, Lincoln, NB, USA</td>
<td>1/5,000</td>
</tr>
<tr>
<td>IR Dye-tagged anti-rabbit (620nm)</td>
<td>Li-Cor Biosystems, Lincoln, NB, USA</td>
<td>1/5,000</td>
</tr>
</tbody>
</table>

### 3.9 Plasmids

The pcDNA3.1-*Atp1a1* (murine Na⁺/K⁺-ATPase) plasmid was kindly provided by Dr. Schimmer (Ontario Cancer Institute). The plasmid was amplified by transformation of competent *E. coli* bacteria and DNA from several colonies was purified by minipreparation (Qiagen). The plasmid was sequenced and PCR was performed using murine *Atp1a1* specific primers.

### 3.10 Reverse-transcriptase polymerase chain reaction

**RNA isolation and RT-PCR**

Total RNA was isolated using TRIzol®, a mono-phasic solution of phenol and guanidine isothiocyanate (Invitrogen), according to manufacturer’s instructions. RNA was then dissolved in 20-30 µl of DEPC treated water and the concentration of RNA was determined by spectrophotometry. Omniscript RT Kit (Qiagen) was used to generate cDNA; cDNA was then amplified for semi-quantitative rt-PCR using Taq DNA polymerase (Qiagen). *ATP1a1* and β-actin genes were amplified using the conventional protocol: (1) initial denaturation (95°C/3min), (2) 32 cycles of denaturation (94°C/1 min), annealing (55°C/ 1min) and extension (72°C/ 1min) and (3) final extension (72°C/ 10min). Primer sequences are shown in Table 8.
### Table 8. Sequence of RT-PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ATP1A1</em> set #1</td>
<td>F 5’- TGT GAT TCT GGC TGA GAA CG -3’</td>
</tr>
<tr>
<td></td>
<td>R 5’- TCT TGC AGA TGA CCA AGT CG -3’</td>
</tr>
<tr>
<td><em>ATP1A1</em> set #2</td>
<td>F 5’- TGGAGTTACCTGCACTACA -3’</td>
</tr>
<tr>
<td></td>
<td>R 5’- AGGTTTCCCTTCCACCCAG -3’</td>
</tr>
<tr>
<td><em>Atp1a1</em> (murine specific)</td>
<td>F 5’-ATA AAC CTT GCC CGC TGT CG -3’</td>
</tr>
<tr>
<td></td>
<td>R 5’- TCC CCT ACT CCC TTC TCA TC -3’</td>
</tr>
</tbody>
</table>

Abbreviations: F, forward; R, reverse.

### 3.11 Short-interfering RNA (siRNA)

siRNA oligonucleotides sequences were obtained from Dharmaco (Lafayette, CO, USA) and resuspended according to the manufacturer’s instructions. Cells were transfected with a siRNA pool of four oligonucleotides. Sequences are shown in Table 9.

### Table 9. ON-TARGETplus SMART pool sequences

<table>
<thead>
<tr>
<th>Human <em>ATP1a1</em>, NM_001001586</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ATP1a1</em> #1</td>
<td>UGA AUU UCC CUA UCG AUA A</td>
</tr>
<tr>
<td><em>ATP1a1</em> #2</td>
<td>GUU CAA AGA UCA UGG AAU C</td>
</tr>
<tr>
<td><em>ATP1a1</em> #3</td>
<td>GGU UGG ACG UGA UAA GUA U</td>
</tr>
<tr>
<td><em>ATP1a1</em> #4</td>
<td>GAA GGC ACC GCA CCG CAC GUG GUA</td>
</tr>
</tbody>
</table>

Abbreviations: F, forward; R, reverse

### 3.13 Transfection of neuroblastoma cells

Three transfection methods were used to insert nucleic acid into NB cells. Electroporation was utilized to transfect NB88R2 with siRNA. Lipofectamine was used to insert siRNA and PEI to insert plasmids into IMR-5 cells. Various transfection techniques were used based on transfection efficiency for each type of nucleic acid and cell type.

**Transfection with Electroporation**

NB TICs were electroporated with siRNA or plasmid using the pipette-type microporator MP-
100 (Digital Bio Seoul, Korea). Briefly, 2 x10^6 cells were suspended in 100 µl of R buffer (provided in microporation kit) and mixed with 100 nM of specific or control siRNA oligonucleotide prior to transfection. To transfect cells the following settings were used: 1350V, 2 ms pulse width and 2 pulses. Samples were then re-counted and seeded into either 6 or 96-well plates and were incubated for 24 hours in 2 times growth factor media prior to subjecting cells to any assays. Gene expression was analyzed at 24, 48, 72 and 96 hours following transfection. Drug treatments were started 24 hours following transfection and cell growth or protein levels assessed at 72 to 96 hours following transfection.

**Transfection with Lipofectamine™ 2000**

Adherent cells were transfected with oligonucleotide for gene silencing using Lipofectamine 2000 (Invitrogen) using 6-well plates. In one eppendorf tube 3µl Lipofectamine 2000 was incubated in 250 µl OptiMEM at RT for 20 seconds and in a separate eppendorf tube 3 µl of DNA was diluted in 250 µl OptiMEM. Lipofectamine was added drop-wise to the diluted DNA bringing the proportion of DNA to Lipofectamine to a 1 µg to 1 µl ratio. The mixture was gently mixed and incubated at RT for 20 min. Cells were washed with OptiMEM, and bathed in 2ml/well with OptiMEM. The DNA/Lipofectamine mixture was added to the cells drop-wise and gently in a spiral pattern. Four hours later, the contents of each well were aspirated and replaced with regular culture media and 24 hours later experimental assays were conducted.

**Transfection with PEI**

Adherent cells were transfected with DNA using PEI in 6-well plates. 3 µg of DNA was diluted in 400 µl of OptiMEM and PEI (9 µl) was added to the DNA solution to obtain a 3:1 ratio followed immediately by mixed the solution with a vortex for 10 seconds. The solution was then incubated for 15 min at RT. The solution was then added and mixed into 6-well plates containing cells and 2 ml of fresh complete media. The following day, the media was changed and approximately half a day after changing the media, experimental assays were conducted.

**3.12 Statistical Analysis**

Data were presented as mean ± standard error of the mean and ‘n’ indicates the number of independent experiments. For growth assays each independent experiment was performed in triplicate. Data were analyzed using two-tail paired t-test. Samples were statistically significant and denoted by * when p < 0.05 and by ** when p < 0.01.
Chapter 4
Results

4.1 Digoxin inhibits cell growth of NB TICs and immortalized adherent and non-adherent cell lines

As previously described, CGs were identified in a high-throughput screen for selective inhibitors of NB TIC growth relative to non-transformed control cells (SKPs) (184). One of the identified compounds was digoxin and we used digoxin in the following experiments as a CG comparison for novel CG analogues because it has been previously reported to display anticancer activity and its therapeutic and toxic effects are well-described (123, 172). Furthermore, we used the sphere formation assay to identify the effective inhibitory doses of digoxin on NB TICs. We reported the inhibitory dose as the IC$_{50}$ value, which is the drug concentration needed to decrease the overall number of spheres for NB TICS, KCNR and SKPs by 50% following 7 days of drug treatment. For two immortalized adherent cell lines IMR-5 and SK-N-(BE-2), we report the IC$_{50}$ value, as the drug concentration required to inhibit 50% growth using the MTT assay, following 3 days of drug treatment. The inhibitory growth curve of cells in response to digoxin treatment is shown in Figure 5. The IC$_{50}$ of digoxin on SK-N-(BE-2) and IMR-5 cell growth was 57.4 nM and 121.3 nM respectively (Fig. 5a). The IC$_{50}$ of digoxin on NB12, NB88R2 and NB122R was approximately 37.46 nM, 55.83 nM and 47.37 nM respectively. In addition, digoxin decreased overall sphere size, suggesting that it also inhibits proliferation of NB TICs, as shown in Figure 5b with NB88R2. The results depicted for NB88R2 are representative for all NB TICs tested and both sphere size and number decreased in a time dependent manner. For the KCNR cell line the IC$_{50}$ was 25.4 nM. For FS99 (non-transformed SKP control) the IC$_{50}$ was 83.3 nM, suggesting that digoxin inhibits SKP growth with very little selectivity towards NB TICs (Fig. 5c).
Figure 5. Digoxin inhibits neuroblastoma cell growth. A, Cells were treated with various concentrations of digoxin and cell growth of adherent NB cell lines, IMR-5 and SK-N-(BE-2) was assessed by MTT at 72 hours following drug treatment (n=2). NB TICS (NB12, NB88 and NB122R), KCNR and the SKP line FS 99 were treated with increasing concentrations of digoxin and sphere forming potential was assessed at day 7 (B-C).

4.2 Digoxin induces apoptosis in a dose dependent manner in IMR-5 and NB88R2

IMR-5 and NB88R2 were treated with 10 nM, 50 nM and 100 nM of digoxin for 48 hours. Poly (ADP-ribose) polymerase (PARP), a key substrate of effector caspases was used to assess cell death by immunoblotting cell lysates prepared from digoxin-treated cells with an antibody to cleaved-PARP. Levels of cleaved-PARP directly correlated with an increase in digoxin concentration in both IMR-5 and NB88R2, suggesting that at least in part digoxin induces apoptosis to reduce cell viability of neuroblastoma cells (Fig. 6).
Figure 6. **Digoxin induces apoptosis in a dose dependent manner in IMR-5 and NB88R2.** Apoptosis was assessed by immunoblotting for cleaved-PARP 48 hours following drug treatment (n=3).

### 4.3 Structure-Activity-Relationship Analyses

Digoxin has been described to induce cardiotoxicity at concentrations that overlap closely to the therapeutic doses for treating cardiac dysfunction. The doses that induce cardiotoxicity are also predicted to be close to effective doses on NB TICs; therefore, in an effort to circumvent cardiotoxicity we tested several novel CG analogues to identify potential compounds and structural groups that may contribute to potency (low IC_{50}) and to circumvent predicted cardiotoxicity. In order to do this we focused on modifications to the three components of a CG: the lactone ring, the tetracyclic steroid ring and the sugar moiety. The data in Table 10 shows the structure of compounds synthesized by FISC derived from ouabain, convallatoxin and peruvoside with their corresponding IC_{50} values in four NB TIC lines, NB12, NB67, NB88R2, NB122R; one immortalized NB line, KCNR; and three SKP lines FS 90, FS99 or FS217. The results highlight the following: (1) the lactone ring is necessary for inhibiting tumour sphere formation. This was demonstrated with the diminished antitumour efficacy of compounds containing open lactone rings (RIDK21 and RIDK32) or that exhibited steric hindrance with the addition of an ethyl derivative (RIDK21, RIDK28 and RIDK32). (2) The sugar moiety may be sufficient to inhibit sphere formation in TIC lines as displayed with RIDK 10 on NB88R2, RIDK11 with respect to NB12 and NB122R, and RIDK51 on all tested cell lines. (3) Modifications to the C19 substituent in the tetracyclic steroid ring affected the potency of CG analogues. This is evident in the peruvoside-derived (numbered in the 50s) and convallatoxin-derived compounds (numbered in the 30s) as increasing the negative charge of the C19 bonded group correlated with higher IC_{50} values in all cell lines. RIDK34 and RIDK54 were the most
potent compounds in their respective subsets (lowest IC_{50} values). Unlike RIDK54, RIDK34 may display slight selectivity for NB cells. With the exception of NB67, the IC_{50} of RIDK34 against NB cells is 2-3 times lower than the IC_{50} values for SKP cells (note SKPs n=2). However, this difference has not been shown to be significant. Alternatively, another compound with greater selectivity for NB TICs versus SKPs is RIDK52, which relative to NB12 and NB67 is 5 and 25 fold more selective for NB TICs and although promising this result does need to be repeated (n=1). Thus, on the basis of the observed trend at the C19 bonded position and reports from the literature that demonstrated the importance of this functional group (see Section 1.3.9), RIDK34 was chosen for further validation as a potential therapeutic agent against neuroblastoma.
Table 10. Structure-Activity-Relationship Analysis.

R1 = sugar moiety  
R2 = tetracyclic steroid ring  
R3 = lactone ring

<table>
<thead>
<tr>
<th>Compound</th>
<th>Starting Material</th>
<th>Reaction Site</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R1</td>
</tr>
<tr>
<td>Ouabain</td>
<td></td>
<td></td>
<td>2.86</td>
</tr>
<tr>
<td>RIDK10</td>
<td>Ouabain methylated</td>
<td>N/A</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>hamnose sugar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 11</td>
<td>Ouabain methylated</td>
<td>N/A</td>
<td>1.02x10⁶</td>
</tr>
<tr>
<td></td>
<td>hamnose sugar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 12</td>
<td>Ouabain dehydrated</td>
<td>N/A</td>
<td>5.69x10⁶</td>
</tr>
<tr>
<td></td>
<td>derivative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 21</td>
<td>Ouabain</td>
<td></td>
<td>8.23x10⁶</td>
</tr>
<tr>
<td>RIDK 22</td>
<td>Ouabain Ethyl</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>derivative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 25</td>
<td>Ouabain</td>
<td></td>
<td>3.15x10⁶</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>trifluoroacyetyl</td>
<td>derivative</td>
<td></td>
</tr>
<tr>
<td>RIDK 26</td>
<td>Ouabain</td>
<td></td>
<td>3.41x10³</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>trifluoroacyetyl</td>
<td>derivative</td>
<td></td>
</tr>
<tr>
<td>RIDK 27</td>
<td>Ouabain</td>
<td></td>
<td>17.13</td>
</tr>
<tr>
<td>Compound</td>
<td>Starting Material</td>
<td>Reaction Site</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>---------------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R1</td>
<td>R2</td>
</tr>
<tr>
<td>RIDK 28</td>
<td>Ouabain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convallatoxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 33</td>
<td>Convallatoxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 34</td>
<td>Convallatoxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peruvoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 51</td>
<td>Peruvoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peruvoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIDK 53</td>
<td>Peruvoside</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peruvoside</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: nd, not determined; --, did not inhibit sphere formation (no effect). The SKP line tested for a given CG compound is indicated in parentheses.
4.4 RIDK34 inhibits cell survival and proliferation of NB TICs

RIDK34 was the most promising compound identified from our structure-activity-relationship analysis (Table 10). RIDK34 has an oxime function bonded to the C19 carbon, whereas the starting material convallatoxin has an aldehyde group at this site (Fig. 2). To identify the effective inhibitory dose (shown also in Table 10), NB12, NB88R2, NB122R, KCNR and SKPs, were treated with increasing concentrations of RIDK34 and cell growth was assessed by sphere formation assay. RIDK34 reduced the number of spheres (survival) with IC$_{50}$ concentrations in NB12, NB88R2 and NB122R of 8.81 nM, 10.94 nM and 7.43 nM, respectively (Fig. 7a). As demonstrated with NB88R2 (Fig. 7b), RIDK34 decreased the overall size of NB TICs sphere, suggesting that proliferation was also inhibited. Lastly, RIDK34 inhibited the growth of the non-transformed SKP control line FS99 with an IC$_{50}$ of approximately 25 nM (Fig. 7).

![Figure 7](image.png)

**Figure 7. RIDK34 inhibits neuroblastoma cell growth.** Cells were treated with increasing concentrations of RIDK34 and sphere formation was assessed on day 7. A & B, sphere formation assay (n=3, except SKPs n=2).

4.5 RIDK34 induces apoptosis in a dose dependent manner in NB88R2

NB88R2 was treated with 5 nM, 10 nM and 20 nM of RIDK34 for 48 hours and apoptosis was assessed by immunoblotting for cleaved-PARP. RIDK34 induced apoptosis in NB88R2 at low
doses (5 nM) and at higher doses of 10nM and 20 nM (Fig. 8). In addition, the concentrations of RIDK34 that induce apoptosis directly correlate with observed inhibitory doses of RIDK34 in sphere formation assays, suggesting that at least in part RIDK34 induces apoptosis to reduce cell growth.

![NB88R2](image)

**Figure 8. RIDK34 induces apoptosis in NB88R2.** Apoptosis was assessed by immunoblotting for cleaved-PARP following 48-hour treatment with RIDK34 at 5 nM, 10 nM and 20 nM (n=3).

### 4.6 Exogenous expression of murine Na⁺/K⁺-ATPase alpha one protects against digoxin-induced inhibition of cell growth in IMR-5

To ask whether CGs exert their antitumour activity through a mechanism that requires the Na⁺/K⁺-ATPase, we expressed the murine alpha one subunit of the Na⁺/K⁺-ATPase in NB cells. Specifically, IMR-5 cells were transfected with pcDNA3.1 containing murine Atp1a1 or pcDNA3.1 without Atp1a1. Expression of the murine insensitive Na⁺/K⁺-ATPase partially blocked digoxin-mediated growth inhibition of IMR-5 cells compared to cells transfected with control plasmid (Fig. 9). These results suggest that the antitumour activity of digoxin is in part mediated by the Na⁺/K⁺-ATPase.
4.7 Digoxin inhibits NB TIC growth independently of its effects on $[Na^+]_i$ and $[K^+]_i$

Studies on digoxin and other CGs show that these compounds abolish transmembrane gradients of $Na^+$ and $K^+$ via inactivation of the catalytic alpha isoforms of the $Na^+/K^+$-ATPase. Thus to further elucidate the mechanism of CG-induced antitumour activity, increasing concentrations of KCl was used to study whether digoxin and likely other CGs affect cell growth through modulation of intracellular $Na^+$ and $K^+$ concentrations. For this experiment, cell growth of NB88R2 was observed in the presence of increasing concentrations of KCl and digoxin treatment. Unlike, normal physiological concentrations of extracellular $K^+$ (approximately 5 mM), elevated concentrations of KCl (>5 nM) are able to antagonize the inhibitory effects of CGs on $Na^+$ and $K^+$ gradients that occur as result of $Na^+/K^+$-ATPase inactivation (60). As shown in Figure 10, elevated concentrations of KCl (10 nM, 15 nM and 20 nM KCl) did not restore cell growth of digoxin treated NB88R2 cells. This result supports the theory that digoxin-induced inhibition of cell growth is not directly caused by deregulation of $Na^+$ and $K^+$ gradients. The findings from Figure 9 and 10 suggest that the anticancer effects of digoxin are in part mediated by the $Na^+/K^+$-ATPase, yet in a manner that does not necessarily require inactivation of the $Na^+/K^+$-ATPase, and diminished $Na^+$ and $K^+$ gradients.
Figure 10. High K$^+$ medium does not protect against digoxin-induced inhibition of cell growth. Cells were treated with digoxin in media containing normal physiological concentrations of KCl (5 mM) or increasing concentrations of KCl (10 nM, 15 nM and 20 mM KCl). On the seventh day, cell growth was assessed by Alamar Blue (n=3).

4.8 Na$^+$/K$^+$-ATPase alpha one subunit knockdown sensitizes IMR-5 and NB88R2 to digoxin growth inhibitory effects

NB TICs highly express the $\alpha_1$ subunit of the Na$^+$/K$^+$-ATPase relative to $\alpha_2$, $\alpha_3$ and $\alpha_4$ subunits (see Appendix Fig. 17). Also, to determine the effect of the Na$^+$/K$^+$-ATPase in CG-induced inhibition of cell growth, we also silenced the expression of the $\alpha_1$ subunit of the Na$^+$/K$^+$-ATPase in NB cells (IMR-5 and NB88R2). NB88R2 and IMR-5 cells were transfected with a siRNA pool targeting ATP1A1 transcripts (alpha one isoform of the human Na$^+$/K$^+$-ATPase) or with non-targeting siRNA. The siRNA to the Na$^+$/K$^+$-ATPase effectively reduced the expression of this gene (Fig. 11a and 11c). Silencing the expression of the Na$^+$/K$^+$-ATPase in the presence of digoxin treatment effectively increased the magnitude of digoxin-induced inhibition of cell growth in both IMR-5 and NB88R2 (Fig. 11b and 11d). This result suggests two possibilities; (1) the $\alpha_1$ subunit is required for survival such that drug binding and gene silencing have a concomitant effect; or (2) the $\alpha_1$ subunit sequesters CGs preventing the therapeutic agent from interacting with cell death effectors whereby in non-transfected cells, endogenous expression of the $\alpha_1$ subunit attenuates cell death from CG-treatment.
Knockdown of the alpha one subunit of the Na\(^+\)/K\(^+\)-ATPase increases the magnitude of digoxin-induced inhibition of cell growth. Knockdown was assessed by western blot and rt-PCR in IMR-5 and NB88R2 respectively (A & C). Viability and proliferation was assessed following drug treatment by either MTT at 36 hours for IMR-5 (n=2) or by alamar blue at 72 hours for NB88R2 (n=3) (B & D respectively). Data was analyzed by Student’s t-test and statistical significance of between RIDK34 treated siControl and siATP1a1 is indicated by * (p<0.05).

4.9 Na\(^+\)/K\(^+\)-ATPase alpha one subunit knockdown sensitizes NB88R2 to RIDK34 growth inhibitory effects

NB88R2 was transfected with siRNA targeting ATP1A1 and treated with RIDK34 as previously described for digoxin. Similar to results with digoxin treatment, knockdown of \(\alpha\) sensitizes NB88R2 to RIDK34 growth inhibitory effects, suggesting that \(\alpha\) is a target for the antitumour activity of RIDK34 in NB TICs (Fig. 12).
Figure 12. Knockdown of the alpha one subunit of the \( \text{Na}^+/K^+ \)-ATPase increases susceptibility to RIDK34-induced inhibition of cell growth. Knockdown was assessed by western blot, rt-PCR (Fig. 11) and immunofluorescence in NB88R2 (A). Viability and proliferation was assessed as previously described in Figure 11 (B). Data was analyzed by Student’s t-test and statistical significance between RIDK34 treated siControl and siATP1a1 is indicated by * (p<0.05) and ** (p<0.01), (n=3).

4.10 RIDK34, Digitoxin and Convallatoxin inhibit the \( \text{Na}^+/K^+ \)-ATPase at concentrations higher than the inhibitory concentrations for NB cell growth

\( \text{Na}^+/K^+ \)-ATPase activity was tested by Aurora Biomed. The \( \text{Na}^+/K^+ \)-ATPase recognizes \( \text{Rb}^+ \) ions as analogous to \( K^+ \) ions and decreased \( \text{Rb}^+ \) uptake is inferred as inhibition of \( \text{Na}^+/K^+ \)-ATPase activity. Moreover, inhibition of the \( \text{Na}^+/K^+ \)-ATPase mediated \( \text{Na}^+ \) and \( K^+ \) ion flux is associated with toxic effects, including cardiotoxicity; thus, \( \text{Na}^+/K^+ \)-ATPase inhibition was used as a predictor of CG-induced toxicity. The IC\textsubscript{50} for \( \text{Na}^+/K^+ \)-ATPase inhibition was 1.038 \( \mu \text{M} \), 0.749 \( \mu \text{M} \) and 1.042 \( \mu \text{M} \) for digitoxin, convallatoxin and RIDK34 respectively (Fig. 13). RIDK34, relative to convallatoxin and digitoxin has the lowest IC\textsubscript{50} on NB TIC cell growth yet the highest IC\textsubscript{50} for pump inhibition (Table 11). As a result, RIDK34 is predicted to be more effective and better tolerated than digitoxin and convallatoxin. From the \( \text{Na}^+/K^+ \)-ATPase activity assay, we predict that serum concentrations of RIDK34 that invoke toxicity are similar to digitoxin (approximately 33 nM), which is above the inhibitory concentration for RIDK34 on NB TIC cell
growth (approximately 8 nM). This compound may therefore have a wider therapeutic window than digitoxin.

**Figure 13.** Curve fits, and IC$_{50}$ determination of Na$^+$/K$^+$-ATPase activity inhibition in HEK-293 cells. Aurora biomed tested for Na$^+$/K$^+$-ATPase inhibition using the Rb$^+$ uptake assay for (A), Convallatoxin; (B), RIDK34; and (C), digitoxin.

**Table 11.** IC$_{50}$ values for inhibition of NB TIC growth, Na$^+$/K$^+$-ATPase activity and CG serum toxicity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>NB TIC cell growth (IC$_{50}$)</th>
<th>Na$^+$/K$^+$-ATPase inhibition (IC$_{50}$)</th>
<th>Serum concentration for threshold toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIDK 34</td>
<td>8.16 nM</td>
<td>1.042 uM (HEK-293)</td>
<td>nd</td>
</tr>
<tr>
<td>Convallatoxin</td>
<td>12.23 nM</td>
<td>0.749 uM (HEK-293)</td>
<td>nd</td>
</tr>
<tr>
<td>Digoxin</td>
<td>47 nM</td>
<td>nd</td>
<td>0.6 to 1.9 nM (125) or 2.15-3.16nM (123)</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>300 nM</td>
<td>1.038 uM (HEK-293)</td>
<td>13 to 33 nM (125)</td>
</tr>
</tbody>
</table>

Abbreviation: nd, not determined.
4.11 Digoxin increases phosphorylation status of Src and ERK1/2

The previous data indicates that in neuroblastoma cells the effects of digoxin and RIDK34 are in part mediated by the Na$^+$/K$^+$-ATPase (Fig. 9, 10 and 11). In addition, studies have demonstrated that digoxin, ouabain and other CGs activate a Na$^+$/K$^+$-ATPase-mediated signal transduction pathway involving Src kinase and ERK1/2 activation (129, 130, 138, 191). Kulikov et al. demonstrated in response to ouabain, ERK1/2 and programmed cell death are activated in SH-SY5Y NB cells (128), suggesting that the Na$^+$/K$^+$-ATPase-mediated signal pathway is activated in CG treated NB cells. To determine whether Src and ERK1/2 are activated in NB88R2 cells in response to CG-induced cell death, cell lysates were prepared from digoxin or vehicle treated cells and probed in western blots with activation-specific antibodies for Src (pSrc Y416) and ERK 1/2 (pERK T202/ Y204). Our results suggest that Src activity increased relative to vehicle at 50 nM of digoxin, while ERK 1/2 increased at 50 nM and 100 nM of digoxin (Fig. 14).

Figure 14. Digoxin induces Src and ERK1/2 activation. Cells were treated as described in Figure 2 with digoxin and Src as well as ERK phosphorylation was assessed via western blot using the appropriate antibody. The lower band in Src blots is non-specific for the protein (192, 193). For ERK blots, the upper band is ERK1 and the lower band is ERK2 (n=3).
4.12 RIDK34 increases activation of ERK1/2 and at low doses Src kinase

The activity of Src and ERK1/2 was assessed in NB TICs after treatment of cells with RIDK34 (Fig. 15). Src activation was observed only at low doses of RIDK34 treatment (5 nM and 10 nM), likely due to the lack of protein due to cell loss at 20nM. An increase in ERK1/2 was observed at 10nM RIDK34, with a much larger increase at 20 nM.

**Figure 15. RIDK34 induces Src and ERK1/2 activation.** Cells were treated with RIDK34 as described in Figure 14. Src kinase and ERK1/2 phosphorylation status was assessed via western blot using the appropriate antibody following treatment with RIDK34 (n=3). The lower band for the Src blot is non-specific for the protein (n=2). For ERK blots, the upper band is ERK1 and the lower band ERK2 (n=3).
Chapter 5
Discussion

Neuroblastoma is an enigmatic malignancy with a broad spectrum of clinical outcomes. In general, current available therapies for high-risk patients with metastatic neuroblastoma are ineffective and survival rates for these patients are less than 35% (4, 5). Despite intensification and combinatorial therapies to improve outcome, survival rates have changed very little over the past three decades for high-risk patients.

One likely reason for the lack of effective therapies is the lack of appropriate \textit{in vitro} and \textit{in vivo} NB models. In an effort to better understand and develop novel therapies to treat NB, NB TICs were isolated from patients samples. Consistent with tumour-initiating and cancer stem cell models, NB TICs share many properties with normal stem cells and are capable of self-renewal and differentiation into the cell types observed in NB (51). Also, NB TICs have enhanced tumour-initiating capacity and form tumours with as few as ten cells in SCID/Beige mice (51). Lastly, NB TICs are thought to play an integral role in treatment response, suggesting that therapies targeting NB TICs are likely to be effective against NB.

In an attempt to identify novel therapies to better treat NB, a high-throughput screen was performed to identify bioactive molecules that selectively inhibit tumour growth potential of NB TICs. Of the over 4,000 compounds tested, inhibitors of the Na\(^+\)/K\(^-\)-ATPase were the most commonly identified class of drugs (184). From the 13 CGs identified, 9 were cardenolides and 4 were bufadienolides. Two cardenolides: digoxin and ouabain were previously identified as potential anticancer agents against NB (128 &164), however, the drug screen completed in the laboratory of David Kaplan is the first to implicate a large number of CGs as potential agents that could be used for the treatment of NB.

5.1 Digoxin and the novel CG analogue RIDK34 inhibit growth of NB cells

Traditionally prescribed for the treatment of cardiac dysfunction, CGs also display antitumour activity in several \textit{in vitro} tumour models including NB (128). We demonstrated that CGs have anticancer activity \textit{in vitro} against immortalized adherent cells SK-N-(BE-2) and IMR-5, immortalized non-adherent cells KCNR and NB TICs. From modifications of the three parts that constitute a CG; the lactone ring, sugar moiety and tetracyclic steroid ring, we were able to
characterize the necessary functional groups in CG compounds for antitumour activity. As a result, the potency of CGs against NB was enhanced as evident with our lead compound RIDK34, which unlike conventional CGs displays anticancer effects at doses below 10 nM (see Appendix Fig. 19 for reaction scheme).

5.2 Activity of CG-derived sugar groups

In testing the effects of CGs and their derivatives on NB TICs, we observed that a few isolated sugar groups derived from CGs displayed antitumour activity. This was demonstrated with RIDK10 on NB88R2, RIDK11 with respect to NB12 and NB122R. RIDK 51, the L-thevetose sugar moiety of peruvoside, appeared to have antitumour activity on all tested TIC lines (Table 10). RIDK10 and RIDK11 are identical in structure and are the rhamnose sugar of ouabain. RIDK11 is derived from ouabain, while RIDK10 is a pure sugar compound purchased from Sigma-Aldrich. Interestingly, both these compounds displayed partial antitumour activity; thus, we can conclude that the remaining ouabain unconsumed in the production of RIDK11 did not account for the antitumor effects of these drugs. Very little is known about the effects of sugar compounds on tumour growth and whether the effective micromolar doses on NB TICS in vitro could be achieved in patients. Given that these CG analogues lack the lactone and tetracyclic steroid rings necessary for binding to the Na\(^+\)/K\(^+\)-ATPase, we expect that these compounds work through a novel mechanism. Unlike conventional CGs these sugar groups may not be restricted to the nanomolar range for therapeutic effects. One possible mechanism for the antitumour activity of RIDK51 is cytolysis resulting from hyperosmotic media incurred from enriched sugar concentrations. Although we did not test this prediction, cell culture media is typically supplemented with 5.5 mM glucose and the concentration of RIDK51 required to induce antitumour activity in NB TICs is 200-250 µM. To achieve this concentration of RIDK51 would require an increase of the concentration of sugar compounds in the media to 5.75 mM. This small increase in sugar concentration would likely not affect cell viability, suggesting that the antitumour response to RIDK51 is not due to hyperosmotic conditions.

Digitoxin compared to its aglycone digitoxigenin (sugar moiety is replaced by a hydroxyl group in digitoxigenin) exhibits higher CG binding affinity to the Na\(^+\)/K\(^+\)-ATPase, which does not correspond to an increase inhibitory effects on the Na\(^+\)/K\(^+\)-ATPase (see Table 3). Also, Langenhan et al. demonstrated that modifications to the sugar moiety could increase the potency of CG antitumour activity with no change or reduced inhibition of Na\(^+\)/K\(^+\)-ATPase activity.
These observations were the basis for the formulation of compounds such as RIDK27, which we hypothesized has a diminished inhibitory effect on Na\(^+\)/K\(^+\)-ATPase activity. Unfortunately, we did not test compounds such as RIKDK27 for inhibition of Na\(^+\)/K\(^+\)-ATPase activity or binding affinity using competitive [\(^3\)H] ouabain binding assays. Thus, future work may include structure-activity assays to identify modifications on the sugar moiety that increase antitumour activity, binding affinity and decrease inhibition of Na\(^+\)/K\(^+\)-ATPase activity. Following this analysis, the results could be used to create a RIDK34 derivative that displays a wider therapeutic window.

5.3 The C19 substituent affects CG potency

CG analogues demonstrated an increase in antitumour activity corresponding to C19 bonded groups that were sterically non-hindered and polar. This was exhibited with the antitumour activity of RIDK52, RIDK53 and RIDK54. The groups bonded to the C19 carbon were an oxime group for RIDK54, a hydroxyl group for RIDK53 and an amine group for RIDK52. The rank of antitumour activity in NB12 was RIDK54 > RIDK53 > RIDK52. Of the three functional groups, the oxime group has the greatest number of hydrogen bond acceptors and donors and was least sterically hindered by the tetracyclic steroid ring followed by the hydroxyl group and lastly the amine group. The convallatoxin-derived compounds RIDK 33, 35 and 34 have identical modifications to RIDK52, RIDK53 and RIDK54 respectively. These compounds also demonstrated that a sterically non-hindered polar group bonded to the C19 carbon increases antitumour activity of CGs.

The contribution of the C19 bonded group to CG antitumour activity is poorly understood, yet the potency of RIDK34 and RIDK54 may in part be due to increased binding affinity to the Na\(^+\)/K\(^+\)-ATPase. The amino acids glutamine-118 (M1 helix) and asparagine-129 (M2 helix) appear to be critical to CG binding of the Na\(^+\)/K\(^+\)-ATPase because the substitution of these two residues with arginine and aspartic acid respectively, causes a substantial reduction in CG inhibition of the rodent \(\alpha\)1 subunit (116). The crystal structure of bound ouabain to the Na\(^+\)/K\(^+\)-ATPase, predicts that these two residues potentially form hydrogen bonds with the C19 bonded groups (116). This model may also support the increased potential of the oxime group with a high polar character to form stronger and/or a greater number of hydrogen bonds with the at glutamine-118, asparagine-129 and possibly other residues in the M1-M2 helix. Alternatively, quantitative-structure-activity-relationship analysis suggest that a hydroxyl group at the C19 carbon, reduces binding affinity because it diminishes hydrophobic interactions.
between the β face of CGs (see Fig. 2) and complementary residues on the Na\(^+\)/K\(^+\)-ATPase (119). Although a C19 bonded oxime group would be predicted to diminish hydrophobic interactions, the added distance of hydrogen bond acceptors and donors from the steroid nucleus may diminish negative effects on hydrophobic interactions of the oxime group. The structure-activity-relationship analysis that lead to the identification of UNBS1450, suggests that a sterically non-hindered polar group, in the case of UNBS1450 a hydroxyl group attached to the C19 carbon is thought to increase its maximum tolerated dose in rats without decreasing its potency against tumour cells of NSCLC, GBM and prostate cancer. This would suggest that RIDK34 with a similar C19 bonded group may be well tolerated and may also exhibit efficacy against multiple types of cancer.

5.4 The lactone moiety is essential for the antitumour activity of CGs

Modifications to the lactone moiety of the starting compounds ouabain, peruvoside and convallatoxin negated the antitumour effects of the precursor molecule. This was observed with the open lactones RIDK21 and RIDK32 or with steric hindrance by the addition of an ethyl derivative in RIDK21, RIDK28 and RIDK32. Our results suggest that the lactone moiety is necessary for antitumour activity and is consistent with reports showing decreased activity of CGs with open-ring lactones (118). However, modifications to the lactone ring may still yield analogues with antitumour activity. The most promising compounds would be CG analogues that maintain binding affinity yet display diminished Na\(^+\)/K\(^+\)-ATPase inhibition. Reports suggest that CGs with 5-membered lactone rings have higher binding affinity and lower Na\(^+\)/K\(^+\)-ATPase inhibition compared to a 6-membered lactone ring (119). Possibly, CGs with a 4-membered lactone ring or another slight lactone ring modification will enhance the discrepancy between binding affinity and inhibition of the Na\(^+\)/K\(^+\)-ATPase. Such a molecule would be predicted to have a wider therapeutic window and to be a useful therapeutic agent against NB (119).

5.5 Mechanism of CG antitumour activity and the role of the Na\(^+\)/K\(^+\)-ATPase

To assess the mechanism of CG-induced antitumour activity on NB cells we choose digoxin because it displayed potency against NB TIC growth and because it is the most widely reported CG in the literature with well described therapeutic and toxic effects. In sphere formation assays, we saw a fewer number of spheres with digoxin treatment suggesting that it inhibits cell survival. Also CGs are widely reported to induce apoptosis (see Appendix Table 13), thus we treated
IMR-5 and NB88R2 with digoxin and observed induction of cleaved-PARP in a dose dependent manner.

To study the role of the Na\(^+\)/K\(^+\)-ATPase in CG antitumour activity we used increasing KCl concentrations, expression of murine Na\(^+\)/K\(^+\)-ATPase\(\alpha\)I and siATP1A1 to silence the expression of the Na\(^+\)/K\(^+\)-ATPase \(\alpha\)1 subunit. When expressing the CG-insensitive murine Na\(^+\)/K\(^+\)-ATPase\(\alpha\)1 we observed partial protection against digoxin relative to transfection control. Also with siATP1a1 we saw an increase in the magnitude of digoxin-induced inhibition of cell growth relative to siControl (see Section 5.6 for more explanation). These two assays suggested that in NB cells the Na\(^+\)/K\(^+\)-ATPase is a target for CG antitumour activity. Lastly, to assess the role of the Na\(^+\)/K\(^+\)-ATPase in CG antitumour activity, we used increasing KCl concentrations. The literature reports that >5 mM KCl, partially reduces the binding and inhibition of CGs to the Na\(^+\)/K\(^+\)-ATPase (60, 194). However, increasing [K\(^+\)] had no affect on the antitumour activity of digoxin (Fig. 10). To explain this, studies by Orlov et al. showed [K\(^+\)] lower than 5 mM and ouabain in 5 mM [K\(^+\)] both increase [Na\(^+\)], yet that only ouabain and not low extracellular [K\(^+\)] induces apoptosis. Interestingly, ouabain treatment in low [K\(^+\)] induces cell death without any changes in [Na\(^+\)], relative to untreated low [K\(^+\)] control, suggesting that changes in intracellular Na\(^+\) and K\(^+\) gradients downstream of Na\(^+\)/K\(^+\)-ATPase inactivation is not necessary for antitumour effects (195, 196). In our assay, we did not test for changes in intracellular gradients, yet we would expect that increased extracellular K\(^+\) would maintain low levels of [Na\(^+\)], in the presence of CG drugs. In addition, recent reports on NB cells show that intracellular [Ca\(^{2+}\)], [Na\(^+\)] and [K\(^+\)] are not perturbed by ouabain at concentrations that we show inhibit cell growth of NB TICs (128), which would further support a mechanism of action that may not require Na\(^+\)/K\(^+\)-ATPase inactivation (Fig. 17).

Although we suggest a mechanism of CG antitumour activity that does not require Na\(^+\)/K\(^+\)-ATPase inactivation, it is still likely that in parallel, Na\(^+\)/K\(^+\)-ATPase inactivation affects cell viability. We propose that the antitumour activity in NB cells in part requires interaction with the Na\(^+\)/K\(^+\)-ATPase, but may not directly require inactivation of Na\(^+\)/K\(^+\)-ATPase function and subsequent deregulation of [Na\(^+\)]\(_i\) and [K\(^-\)]\(_i\). This is in part supported by the observation of the binding affinity of some CGs to the Na\(^+\)/K\(^+\)-ATPase, which do not appear to directly correspond with Na\(^+\)/K\(^+\)-ATPase inhibition, suggesting the possibility of disparate affects resulting from CG binding to the Na\(^+\)/K\(^+\)-ATPase. Furthermore, Xie et al. previously reported that Na\(^+\)/K\(^+\)-ATPase
mutants that do not regulate ion flux, still respond to CGs by activating a signaling cascade that included Src and ERK (136, 137). Therefore, we looked to see if Src and ERK activity increases with digoxin concentration and found that both ERK and Src kinase overall appear to increase with digoxin treatment. These combined results implicate that the activation of a signaling pathway, involving Src and ERK, is associated with cell death in response to targeting the Na\(^+\)/K\(^+\)-ATPase, possibly independent of inactivation of Na\(^+\)/K\(^+\)-ATPase activity.

Figure 16. CGs possibly activate two parallel death pathways in NB TICs. CGs activate an ionic pathway that require Na\(^+\)/K\(^+\)-ATPase inactivation and deregulation of ionic gradients resulting in cell death. Also a second pathway called the Na\(^+\)/K\(^+\)-ATPase-mediated signaling pathway causes cell death in a manner possibly independent of Na\(^+\)/K\(^+\)-ATPase inactivation. Expression of murine insensitive ATP1a1 and silencing endogenous ATP1A1 likely affect both of these pathways because they predominately alter CG-target binding. Although the KCl assay likely affects the ionic pathway to offset deregulation of ion gradients, it is less likely to prevent the activation of a Na\(^+\)/K\(^+\)-ATPase-mediated signaling pathway that also induces cell death. The Na\(^+\)/K\(^+\)-ATPase-mediated signaling pathway may involve ERK and Src signaling, yet the role of these signaling targets in CG antitumour activity is still poorly understood.

5.6 Silencing of ATP1A1 increases susceptibility to digoxin and RIDK34 antitumour activity

Decreasing the expression of Na\(^+\)/K\(^+\)-ATPase\(\alpha\)1 in NB88R2 increased the magnitude of digoxin and RIDK34 antitumour activity and emphasized the role of the \(\alpha\)1 subunit in sustaining viability and proliferation of NB TICs. Given its essential role in cell physiology, cells likely compensate for reduced ATP1A1 by trafficking formed oligomers of the pump from intracellular membranes.
to the plasma membrane and decreasing degradation of the ATPase. Also the redundant roles of the α1-α4 subunits likely offset the knockdown of ATP1A1.

Presumably, silencing ATP1A1 reduces the overall plasma membrane pool of the Na+/K+-ATPase without inducing cell death; however, ATP1A1 silencing likely increased cell susceptibility to further insults targeting the Na+/K+-ATPase explaining increased antitumour activity in response to digoxin and RIDK34. An alternative interpretation purports that decreasing the levels of the α1 subunit frees up digoxin and RIDK34 to bind to a greater proportion of α3 and α4 subunits or an unknown target that more effectively propagates the antitumour effects of CGs. Evidence suggests that α3 is an important target of CG-induced antitumour activity, and was demonstrated in cells with high α3 expression, yet given that NB TICS weakly express α3 and α4 this may suggest a diminutive role of these α subunits in NB TICs. Alternatively, there may be cellular targets of CGs other than the α subunits of the Na+/K+-ATPase. Zhang et al. suggested that HIF-1α may represent an additional target of CGs. CGs are thought to inhibit HIF-1α dependent gene transcription, yet it is unclear whether this function of CGs may be in part mediated by the Na+/K+-ATPase (170). Additionally, like other steroid molecules such as estrogen, CGs may cross the plasma membrane and act directly on steroid receptors in the nucleus to affect tumour cell growth; again this mechanism is still poorly understood and requires further testing (167). Although we cannot dismiss the role of the α3 and α4 subunits as well as an alternative target, the results presented here with the expression of murine Na+/K+-ATPaseα1 and siATP1A1 experiments do indicate that the α1 Na+/K+-ATPase is a target for CG antitumour activity.

5.7 RIDK34 is a novel CG analogue with higher potency and displays lower relative inhibition of Na+/K+-ATPase activity

We observed that RIDK34 in a series of convallatoxin-derived compounds displayed the greatest potency against NB TICs. We suggest that this is due to an increase in favourable binding interactions with its cellular target(s). We also demonstrated that silencing the expression of the α1 subunit of the Na+/K+-ATPase resulted in an increase in the magnitude of cell growth inhibition from RIDK34 treatment. Similar results were observed when silencing the expression of α1 subunit with digoxin treatment. Thus, as with digoxin we believe that this is because the Na+/K+-ATPase is a target for RIDK34.
We observed with RIDK34 treatment a decrease in the total number of spheres with increasing RIDK34 concentration, thus as with digoxin, we assessed cleaved-PARP to observe if apoptosis was the mechanism of RIDK34 growth inhibition. We found that cleaved-PARP was activated by RIDK34 in a dose-dependent manner. Also, the dose of cleaved-PARP induction parallels the dose of growth inhibitory effects of RIDK34 suggesting that in part RIDK34 induces apoptosis to reduce cell growth.

Manunta et al. suggested that CG activity (to induce hypertension) does not strictly correlate with Na\(^+/K^+\)-ATPase inhibition (197). This may be true in our model whereby increasing the possible number of interactions to the M1-M2 region of the Na\(^+/K^+\)-ATPase may have little impact on K\(^+\) binding (and inhibition) that likely occurs when the lactone ring of CGs bind to the M4-M6 regions. Thus, the C19 bonded oxime group may increase binding affinity and the magnitude of cell death through its interactions with the M1-M2 helix without a corresponding increase in Na\(^+/K^+\)-ATPase inhibition. This prediction is supported by comparing growth inhibition and Na\(^+/K^+\)-ATPase inhibition of RIDK34 and its starting material convallatoxin, as the doses of growth inhibition of RIDK34 are slightly lower than convallatoxin for NB TICs (Table 11), yet the doses for Na\(^+/K^+\)-ATPase activity inhibition is slightly higher for RIDK34 (Table11). This would suggest as predicted by Manunta that inhibition of Na\(^+/K^+\)-ATPase ion pumping does not directly impact activity of CGs, namely antitumour activity of RIDK34; however, the two may still be closely related as binding to the Na\(^+/K^+\)-ATPase may impact both CG antitumour activity and Na\(^+/K^+\)-ATPase inhibition.

Suggesting that RIDK34 has a higher potency and lower Na\(^+/K^+\)-ATPase activity inhibition implies that \textit{in vivo} it will have a wider therapeutic window. This would include reduced adverse effects on the gastrointestinal tract that would normally lead to nausea and vomiting as well as reduced vision impairments normally associated with CG treatment such as decreased sharpness of vision and decreased colour perception (110-112). In addition, inhibiting the Na\(^+/K^+\)-ATPase leads to Ca\(^{2+}\) overload and ectopic beats, which can cause ventricular arrhythmias and may result in death if left untreated (202). Thus with RIDK34, we may have slightly decreased Na\(^+/K^+\)-ATPase activity inhibition and increased antitumour activity. Lastly, if we compare toxic serum doses in humans of RIDK34 with digitoxin, we find that the effective concentration of RIDK34 (8 nM) is below this range (13-33 nM). Thus, these results would justify further preclinical studies to more accurately determine the toxicity of RIDK34 with respect to cardiac function.
**The role of Src and ERK kinase in CG-induced cell death**

Our results suggest that the activation of a death effector pathway occurs following CG-targeting of the Na\(^+\)/K\(^-\)-ATPase. One such pathway could be the putative Na\(^+\)/K\(^-\)-ATPase-mediated signaling transduction pathway involving both Src and ERK, which when activated promote proliferation and survival or cell death depending on the specific CG, the type of cell and the effective CG concentration (130-138). In regards to cell death, Newman et al. demonstrated that pancreatic cancer cells treated with oleandrin have high levels of activated ERK associated with cell death and when ERK activation was abolished with a MEK1 inhibitor or a specific peptide inhibitor, cells were partially protected from oleandrin-induced cell death (154). In addition, studies in NB cells demonstrated that CGs upregulate ERK activity in possibly an α isoform specific manner as demonstrated by selective knockdown of the α1 and α3 isoforms (128). This may suggest an integral role for ERK in CG-induced cell death of NB TICs. This prediction is supported by our observations of ERK activation following digoxin and RIDK34 treatment of NB88R2. In general, ERK activation is important in cell survival, proliferation and differentiation; thus, our results generally conflict with published data regarding the role of ERK on cell viability and proliferation. Interestingly, unpublished data using chemical inhibitors on NB TICs demonstrated that in a normal setting the survival and proliferation of NB TICs does not depend on the activity of ERK, JNK or p38 MAPK. Regardless, whether ERK signaling is necessary for CG-induced cell death of NB TICs is not known and needs to be thoroughly tested (see Chapter 6).

Also in response to CG-treatment we assessed Src kinase activation and generally observed an increase in the levels of activated Src, which is consistent with the Na\(^+\)/K\(^-\)-ATPase-mediated signaling hypothesis. This finding is supported with reports showing abrogation of ouabain-induced ERK activation and neuronal cell death in cell cultures treated with the PP2 Src family kinase inhibitor (198). However, there are contrary reports that have suggested that Src activation is not necessary for CG-induced cell death and that the role of Src may be different depending on mostly the cell type, but also the CG tested and the CG concentration (194, 196). Generally, Src is thought to promote proliferation in cancer cells and unpublished data demonstrated that inhibition of Src activity by Src family inhibitors or knockdown reduces survival and proliferation of NB TICs. This may suggest that Src itself is not crucial for CG-induced cell death of NB TICs. If ERK activation is involved in CG-induced cell death we could
expect an alternative mode of ERK activation. One possible mechanism is through an increase in ROS production which has been previously described in NB cells in response to CG treatment (refer to Chapter 6 for experiments to test this prediction) (128).

5.8 Putative role of the α3 and α4 subunit in CG-induced antitumour activity

Despite weak expression of the α3 subunit, this form of the Na⁺/K⁺-ATPase may still partially be involved in the growth inhibitory response of NB TICs to CGs. Recently, Hauck et al. showed that the binding to human isozyme combinations of α1/β1, α2/β1 and α3/β1 was CG specific (200). In physiologically relevant conditions of K⁺, digoxin showed higher binding affinity for α2/β1 and α3/β1 than for α1/β1 isozymes (200). Thus isozymes more sensitive than α1/β1 may respond better to CGs to induce antitumour effects. Also weakly expressed α subunits may respond more effectively to CGs than higher abundance α subunits possibly through localization in the plasma membrane in caveolar pits (or other key regions) and/or association with important protein signaling complexes (130-137). This is suggested in SK-N-AS cells, in which ERK signaling was shown to require only α3 and not the α1 subunit (128). In addition to low α3 expression, NB TICs express low levels of the α4 subunit (see Appendix Fig. 17). Temporal-spatial expression of α4 in the nervous system has not been described before. Normally expressed during spermatogenesis in spermatocytes, spermatids and spermatozoa, α4 localizes to the mid-region of the flagella and its selective inhibition reduces motility as well as intracellular Ca²⁺ and pH (65, 81, 199). It is possible that α4 also plays an important role in NB TICs based on its unusual expression; however, what that role may be and the role of the α3 subunit in CG inhibition of NB TICs is not known.

5.9 Therapeutic rational for CGs in combinatorial therapy

Retinoic acid (RA) is known to induce terminal differentiation of NB cells in vitro (2). In addition, after successful phase I and II clinical trials the retinoid, Fenretinide is currently being formulated for oral administration to young children (2). Similar to its effects on NB cells, RA in ES cells induces neuronal differentiation, which coincides with increased expression of the α3 subunit (155). Also, CGs are thought to be more potent against cells with higher expression of α3 to α1 subunits (155, 200). Thus if RA increases expression of α3 in NB TICs as with ES cells then combinational treatment of RA and RIDK34 (or another CG) may exhibit synergistic
antitumour activity due to RA induced differentiation but also due to sensitization of NB cells to CGs.

Conversely, RA appears to down-regulate expression of α3 in myeloid cells (HL-60), suggesting that these and possibly other immune cells might become less susceptible to CGs in combinatorial treatment with RA (201). However, one impediment to this treatment strategy is that RA also induces the expression of the α3 subunit in cardiac myocytes which could potentially increase sensitivity of cardiac myocytes and in children who may already have decreased cardiac function this may increased the risk of cardiotoxicity. Thus this may present one obstacle in combinatorial therapy of CGs with RA or any agent that may compromise cardiac function. (202).

Synergism of CGs with other therapeutic agents would likely result in lower doses of CGs required for antitumour activity thus reducing the potential for toxicity. Recently, several CGs have been identified to have synergistic effects with drugs currently used to treat NB or are currently under clinical trial, including oxalipatin, irinotecan, cisplatin and 5-fluoruracil. In particular, digoxin with oxalipatin or irinotecan showed synergistic effects (159). Reports also suggest combining selected CGs with radiotherapy as a possible therapeutic modality. Specifically ouabain and oleandrin conferred sensitivity to radiation therapy in lung adenocarcinoma cells (PC-3 cells) (203, 204). Furthermore, ouabain appears to selectively increase radiosensitivity of tumour cells as it was ineffective in modifying radioresponse of normal human lung fibroblasts (203). RIDK34 may also be a radiosensitizer such as other CGs and may be useful in several combinational therapies. However, except with RA there is very little literature on combination therapy of CGs in NB and may be an important future aspect to consider when determining the utility of RIDK34 and other CGs in treating NB.
Chapter 6
Conclusion and Future Directions

We hypothesized that CGs are potential treatment agents for NB and by testing several CGs in vitro including digoxin, ouabain, peruvoside and convallatoxin, we can conclude that CGs are effective agents against NB in vitro. Also, our hypothesis was that greater antitumour activity and lower predicted cardiotoxicity could be achieved by the modification of structural groups on CG compounds. We proved that it is possible to increase the potency of these compounds through different structural modifications of primarily the tetracyclic steroid ring. Our findings demonstrate a trend at the C19 bonded group that associated drug potency against NB TICs with polar character and steric hindrance at this site. This was demonstrated with the convallatoxin derived series of compounds RIDK33, RIDK34 and RIDK35, as well as with the peruvoside derived series of compounds RIDK52, RIDK53 and RIDK54. The most potent compounds in their respective series were RIDK34 and RIDK54; both contained an oxime group bonded to the C19 carbon. In addition to increasing polar character at this site and possibly the number of potential hydrogen bonds, the polar character of this group is further away from the tetracyclic steroid core than the alternative groups added to the C19 carbon, which may allow RIDK34 and RIDK54 to have more favourable interactions with its cellular target. Structure-activity-relationship analysis of 2\(^\text{o}\)-oxovoruscharin compounds leading to the identification of UNBS1450, showed that the addition of a hydroxyl group to the C19 carbon, in part is associated with higher in vivo tolerance of UNBS1450 relative to its precursor compound (114). In regards to this, RIDK34, not only displayed higher potency relative to its starting compound, but may also be better tolerated in vivo relative to its starting compound.

Further preclinical studies are required to test the in vivo efficacy of CGs, namely RIDK34 to treat NB. To test for in vivo efficacy, NB TICs would be injected subcutaneously into NOD/SCID mice and after tumour formation, drug treatment would commence and tumour response to RIDK34 would be observed. In addition, orthotropic models using NB TICs form metastases to the spleen, adrenal gland, liver, lymph node and kidney, which would allow for further study of the effects of CGs on migration and distant tumour formation (51). Also the orthotropic NB model may enable studies on the effects of the microenvironment on primary tumour and metastatic tumour response to RIDK34 and other CGs. Additionally, growing NB
TICs over an adherent layer of bone marrow stromal cells would allow in vitro investigation of tumour microenvironment. Investigating the in vivo contribution to NB TIC response to CGs would be an additional component that may inform future studies of these therapeutic agents.

Murine models could also be used to study how RIDK34 is metabolized. The parent compound of RIDK34, convallatoxin is readily metabolized in the liver at the C19 group from an aldehyde to an alcohol (112). Given the relative stability of the oxime group, we would suspect that RIDK34 would persist longer in the body (112); however, this would need to be confirmed by an in vivo study. Lastly, we suspect that similar to convallatoxin, RIDK34 is readily excreted by the kidneys and in feces. The total dose of convallatoxin is excreted in two days in healthy patients and longer in patients with impaired renal function (112). Decreased excretion of convallatoxin and RIDK34 may hasten CG-induced toxicity if combined with inappropriate chemotherapeutics and will be an important consideration for future potential combinatorial drug strategies.

We lastly hypothesized that lower predicted toxicity, particularly cardiotoxicity could be achieved by structural modification of CGs. Towards this end, Na\(^+/\)/K\(^+/\)-ATPase activity was tested in HEK-293 cells in response to treatment with RIDK34, convallatoxin and digitoxin. Again, we used RIDK34, our most promising compound and compared its inhibitory effect against its parent compound convallatoxin as a surrogate measure of relative toxicity associated with inactivation of Na\(^+/\)/K\(^+/\)-ATPase function and subsequent deregulation of [Na\(^+\)]\(_i\) and [K\(^+\)]\(_i\]. We found that higher concentrations of RIDK34 relative to convallatoxin was required to inhibit the Na\(^+/\)/K\(^+/\)-ATPase suggesting that it would be better tolerated in vivo and possibly display diminished cardiotoxicity. Although informative, testing for Na\(^+/\)/K\(^+/\)-ATPase activity is not the ideal model to assess toxic effects of RIDK34; however, given the lack of appropriate rodent models to test this hypothesis we used in vitro Na\(^+/\)/K\(^+/\)-ATPase activity assays. In the future, to test for adverse effects of CGs, porcine in vivo models would likely identify potential toxicity on the gastrointestinal system, CNS and cardiovascular system. In addition to test for cardiotoxicity, in vitro models using human cardiomyocyte progenitor cells from surgical waste that differentiate into functional beating, and mature cardiomyocytes might be the most reliable means to assess potential cardiotoxic effects of RIDK34 (205). These proposed toxicity models would require testing several CGs to determine the relative concentration of RIDK34 (or other novel CG analogues) required to induce cardiotoxicity. Lastly, this assay would be a crucial future experiment for the development of CG drugs for further preclinical or clinical trials.
Evidence supports the role of the Na\(^+\)/K\(^+\)-ATPase as a target for CG activity, including antitumour activity. To characterize the role of the Na\(^+\)/K\(^+\)-ATPase in NB cells treated with CGs, we used three different assays. From both silencing the expression of the Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\) subunit and expression of a CG-insensitive Na\(^+\)/K\(^+\)-ATPase, our findings suggest that the Na\(^+\)/K\(^+\)-ATPase plays a necessary role in CG-induced antitumour activity. However, experiments testing the effects of increasing [K\(^+\)] suggested that CGs may work through a means that does not require the inactivation of the Na\(^+\)/K\(^+\)-ATPase. Therefore, we predict that CGs target the Na\(^+\)/K\(^+\)-ATPase to induce antitumour activity in a manner that may not require inactivation of the Na\(^+\)/K\(^+\)-ATPase. To confirm this, we would need to test if CG treatment affects the levels of [Na\(^+\)], [Ca\(^{2+}\)], and [K\(^+\)]. Additionally knockdown experiments used a pool of siRNA targeting Na\(^+\)/K\(^+\)-ATPase\(\alpha_1\). In the future, we would likely use two siRNA sequences, separately to test the role of the \(\alpha_1\) subunit in CG-induced antitumour activity. Lastly, knockdown of the \(\alpha_1\) subunit is complicated by the expression of \(\alpha_3\) and \(\alpha_4\) subunits in NB TICs. The sensitivity of \(\alpha_3\) and \(\alpha_4\) towards CGs, as well as subunit localization, and association with signaling proteins in NB TICs may increase the role of \(\alpha_3\) and \(\alpha_4\) in CG-induced antitumour activity. To assess the contribution of these \(\alpha\) isoforms to CG antitumour activity, selective knockdown of each may be informative. Potentially more informative experiments in the future would also include knockdown of the \(\beta_1\) subunit. This subunit associates with all four \(\alpha\) subunits and selectively knocking down the \(\beta_1\) would reduce the expression of each of these isoforms.

We observed activation of Src and ERK kinase in response to CG treatment. We speculated that Src and ERK kinase activation does not necessarily require inactivation of Na\(^+\)/K\(^+\)-ATPase activity. This was supported in the literature with reports showing that Src and ERK signal independently of Na\(^+\)/K\(^+\)-ATPase inactivation (136-138, 191). To further understand the role of Src and ERK kinase, we would need to use peptide inhibitors or drug inhibitors for these proteins to assess whether Src and ERK signaling is necessary in NB TICs for the observed response to digoxin and RIDK34. Alternatively, ERK1/2 may also be activated by the generation of ROS in response to CGs. To test this theory we would need to use inhibitors of ROS (e.g. diphenylene iodonium) followed by assessing cell death and ERK activation to elucidate the role of ROS production in CG-induced cell death (206-208). In addition, cross talk amongst protein kinase signaling cascades is possible and has been observed prior in several cell systems in response to
CG treatment (112). Thus as a starting point we would also need to look at the contribution of JNK and p38MAPK signaling in mediating the antitumour activity of CG in NB cells.

Lastly, we observed induction of c-PARP in response to digoxin and RIDK34 treatment. Unfortunately, we were unable to assess the process by which CG binding to the Na\(^+\)/K\(^+\)-ATPase may lead to activation of c-PARP and cell death. Previous reports in the literature suggest that CGs can induce a host of death effectors that may increase c-PARP and induce apoptosis. One interesting target is p53, which Kulikov et al. demonstrated to be upregulated following ouabain treatment in NB cells (128). To properly assess the role of p53 in CG-induced cell death would require knockdown of the tumour suppressor as well as other assays that inhibit death effectors downstream of p53. Whether potential p53 activation is downstream of Src and ERK activation is another interesting question for further investigation. Unknown effectors of cell death are likely to play an important role in CG-induced antitumour activity; thus, a future experiment will be to perform a screen, possibly a phosphoproteomic screen, to identify signaling cascades that are preferentially upregulated with CG treatment. Understanding the mechanism(s) behind CG-induced antitumour activity will be important for future preclinical studies that may eventually lead to clinical trials with CG derivatives to treat NB.
References

3. Pizzo PA. Principles & Practice of Pediatric Oncology: Lippincott Williams & Wilkins.
69. Skou JC. The influence of some cations on an adenosine triphosphatase from peripheral nerves. BBA - Biochimica et Biophysica Acta 1957;23(C):394-401.


149. Simpson CD, Mawji IA, Williams MA, Hurren R, Beheshti-Zavareh R, Schimmer AD. A high throughput cell-based screen identifies inhibitors of the Na+/K+ ATPase pump as sensitizers to anoikis and inhibitors of metastasis Cancer Res. 2009 Apr 1;69(7):2739-47.


161. Lefranc F, Mijatovic T, Camby I, et al. The binding of the UNBS1450 cardenolide to the sodium pump in human glioblastoma (GBM) cells dramatically impairs both their migration and proliferation properties 2006.


78


190. Bopp SK, Lettieri T. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC pharmacology 2008;8:8.


Appendix

Table 12. Na\(^+\)/K\(^+\)-ATPase expression profile in Neuroblastoma Tumour-Initiating Cells.

<table>
<thead>
<tr>
<th>NB TIC</th>
<th>mRNA ΔC(_t) of Na(^+)/K(^+)-ATPase subunits</th>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>α4</th>
<th>β1</th>
<th>β2</th>
<th>β3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB12, p7</td>
<td></td>
<td>4.4</td>
<td>nd</td>
<td>13.7</td>
<td>15.1</td>
<td>5.2</td>
<td>15.8</td>
<td>4.4</td>
</tr>
<tr>
<td>NB88 R2, p6</td>
<td></td>
<td>4.9</td>
<td>nd</td>
<td>14</td>
<td>16.1</td>
<td>4.2</td>
<td>16.2</td>
<td>4.9</td>
</tr>
<tr>
<td>NB122R, p3</td>
<td></td>
<td>5</td>
<td>nd</td>
<td>11.2</td>
<td>11</td>
<td>4</td>
<td>12.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Data was generously provided by from Michael Andang’s lab. Subunit levels are expressed as ΔC\(_t\) target gene - ΔC\(_t\) housekeeping gene β2 microglobulin. Abbreviation: nd, expression levels too low to determine.

Expression profile α and β subunits in NB TICs

Neuroblastoma is thought to be derived from a neural crest or sympathoadrenal precursor. This is consistent with neuronal markers, Schwann cell markers and neuronal stem cell markers present on NB tumour cells, suggesting a hierarchical structure analogous to that of neuronal stem cells or sympathoadrenal precursors. In NB TICs the morphology is more consistent with an undifferentiated cell type than a differentiated cell type. Also, the expression profile of α and β subunits in NB TICs is consistent with the undifferentiated embryonic stem cells E3-ES. Similar to E3-ES cells, NB TICs express high levels of α1 and β1, and express relatively low levels of α2 (not expressed in E3-ES), α3 and β2; the one exception is β3 which is high in NB TICs compared to E3-ES cells. Interestingly, mature neurons, NB tumour samples and the NB lines SK-N-AS and SY-SY5Y all express robust levels of α3 (77, 128, 139, 209). Also, the expression of the α3 subunit coincides temporally with neuronal maturation of E3-ES cells into neurons following exposure to RA. This may suggest a possible role of the α3 subunit in determining a neuronal cell fate and the association of the α3 subunit with neuronal maturation would agree with the theory that NB TICs contain many features analogous to normal stem cells.
Figure 17. Crystal structure of Na⁺/K⁺-ATPase with bound ouabain. Superimposition of Cα traces of the amino acid chain of Na⁺/K⁺-ATPase from shark rectal gland. Cα traces are indicated by bound ouabain in yellow and unbound ouabain in cyan. The structure is viewed perpendicular to the surface of the cell membrane. Na⁺/K⁺-ATPase is fixed in a state analogous to E₂-2K⁺-Pi, with MgF₄²⁻ as a stable phosphate analogue. Ouabain (OBN; green and red) and K⁺ ions (I, II, and c; purple) are shown in space fill. The β-subunit, FXYD protein and several transmembrane helices are marked. From Ogawa et al., 2009 (116).

Figure 18. Reaction scheme for RIDK34. Convallatoxin (10 mg, 0.02 mmol) was dissolved in water (4 mL), added Hydroxylamine Hydrochloride (50 mg, 0.72 mmol) and stirred at RT for 24 hrs. Upon completion, the reaction-mixture was neutralized with NaHCO₃. Crude product was desalted on a reverse phase column and purified on a silica column to give RIDK34. ESMS, (ion) (expected, found); (M+Na⁺) (588.2788, 588.2844).
Table 13. Reported Mechanisms of CG antitumour activity.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Compound Tested</th>
<th>Cancer Model</th>
<th>Reported Mechanism of Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Ouabain</td>
<td>MDA-MB-468</td>
<td>Inhibits kallikrein expression</td>
<td>(210)</td>
</tr>
<tr>
<td></td>
<td>Peruvoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>proscillaridin A derivative</td>
<td>MDA-MB-231, MCF-7</td>
<td>Inhibits topoisomerase II</td>
<td>(147)</td>
</tr>
<tr>
<td></td>
<td>ouabain</td>
<td>MDA-MB-435s</td>
<td>Cell cycle arrest</td>
<td>(127)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T47D</td>
<td>Sensitizes cells to anoikis</td>
<td>(173)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Oleandrin</td>
<td>PC-3</td>
<td>Inhibition of FGF-2 &amp; apoptosis and autophagy</td>
<td>(211), (154)</td>
</tr>
<tr>
<td></td>
<td>Ouabain</td>
<td>PC-3</td>
<td>Apoptosis</td>
<td>(148)</td>
</tr>
<tr>
<td></td>
<td>Ouabain, Peruvoside</td>
<td>PPC-1 ouabain in vivo, PC-3</td>
<td>Sensitizes cells to anoikis</td>
<td>(173)</td>
</tr>
<tr>
<td></td>
<td>Dihydroouabain digitoxigenin digitoxinin strophanthidin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Digoxin</td>
<td>PPC-1 in vivo</td>
<td>Inhibits distant tumour formation</td>
<td>(172)</td>
</tr>
<tr>
<td></td>
<td>Bufalin</td>
<td>LNCaP, DU145, and PC3</td>
<td>Apoptosis</td>
<td>(212)</td>
</tr>
<tr>
<td></td>
<td>Cinobufagenin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UNBS1450</td>
<td>LNCaP, DU145, and PC3 in vitro &amp; in vivo</td>
<td>Nucleolar disorganization apoptosis; cell cycle arrest</td>
<td>(178)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>UNBS1450</td>
<td>U373-MG in vitro &amp; in vivo</td>
<td>Autophagy and inhibits cell migration</td>
<td>(98)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Oleandrin</td>
<td>BRO</td>
<td>Induces oxidative stress</td>
<td>(153)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>digoxin</td>
<td>Jurkat Cells</td>
<td>Inhibits N-glycan biosynthesis</td>
<td>(172)</td>
</tr>
<tr>
<td></td>
<td>Bufalin</td>
<td>HL-60, U937, ML1, K567</td>
<td>Apoptosis and differentiation</td>
<td>(213)</td>
</tr>
<tr>
<td>Lung (NCLSC)</td>
<td>UNBS1450</td>
<td>A549, A427; tested in vivo</td>
<td>Autophagy</td>
<td>(151, 214)</td>
</tr>
<tr>
<td>Cancer Type</td>
<td>Compound 1</td>
<td>Cell Lines</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------</td>
<td>------------</td>
<td>----------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Ouabain</td>
<td>SH-SY-5Y</td>
<td>Apoptosis</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>Digoxin</td>
<td>Neuro-2a</td>
<td>Angiogenesis &amp; tumour growth In mice</td>
<td>(165)</td>
</tr>
<tr>
<td>Kaposi Sarcoma</td>
<td>Cardenolide Glycosides from Pergularia tomentosa</td>
<td>primary cells</td>
<td>apoptosis</td>
<td>(162)</td>
</tr>
<tr>
<td>Fibrosarcoma (human)</td>
<td>Digoxin</td>
<td>HT1080</td>
<td>Inhibits cell migration/invasion and alters N-glycan biosynthesis</td>
<td>(172)</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma bladder and thyroid carcinoma</td>
<td>Digoxin</td>
<td>Colo320, WRO, 5637 (respectively)</td>
<td>Inhibits N-glycan biosynthesis</td>
<td>(172)</td>
</tr>
<tr>
<td>Ovarian Cancer</td>
<td>Bufalin</td>
<td>HHUA, HEC-1B, SK-OV-3, OMC-3</td>
<td>Differentiation</td>
<td>(169)</td>
</tr>
<tr>
<td></td>
<td>Ouabain</td>
<td>OVCAR3</td>
<td>Sensitizes cells to anoikis</td>
<td>(173)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>Bufalin</td>
<td>U2OS</td>
<td>Apoptosis</td>
<td>(163)</td>
</tr>
<tr>
<td></td>
<td>Digitoxin</td>
<td></td>
<td>Inhibits general protein synthesis</td>
<td>(215)</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>ouabain</td>
<td>Y79, RB355</td>
<td>Apoptosis</td>
<td>(164)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>Ouabain</td>
<td>Hela</td>
<td>Sensitizes cells to anoikis</td>
<td>(173)</td>
</tr>
</tbody>
</table>