Cellular location and expression of the Na, K–ATPase α–3 subunit is associated with relative anti-proliferative activity of oleandrin

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Cardiac glycoside extracts from plants and animals as therapeutic agents

**Digitalis purpurea:**
Source of cardiac glycosides used for Rx of congestive heart failure
Cardiac glycoside extracts from plants and animals as therapeutic agents

But what about use of cardiac glycosides for treatment of cancer?

**Digitalis purpurea:** Source of cardiac glycosides used for Rx of congestive heart failure

**Bufo Toad**
Source of Chinese Medicine Huachansu from Anhui Jianchan Biochemical Inc.

**Nerium oleander:**
Source of the oral product PBI-05204 from Phoenix Biotechnology, Inc. and Anvirzel from Nerium Biotechnology, Inc.
Cardiac glycosides and Cancer


Cardiac glycosides and Cancer

In the last 15 years there have been over 1100 articles cited in PubMed that refer to cardiac glycosides and cancer. In addition in the past three years alone there have been four review articles regarding cardiac glycosides for cancer management:

Alghoul, et al., Ther. Drug Monit, 2008
Newman et al., Mol. Interv., 2008
Oleandrin, bufalin and lipid soluble cardiac glycosides

Na, K-ATPase

MAPK

PI3k

ROS

Glycolysis

NF-Kb

HIF1α

Apoptosis

Cell cycle arrest

Autophagic cell death

Red cross – Down-regulation; Blue arrow – Up-regulation; Dashed arrow – hypothesized down regulation
Na, K-ATPase

- Transmembrane protein
- Four isoforms of α subunits binding site for Na⁺, ATP, and cardiac glycosides
- Three isoforms of β subunits
- One γ subunit
- Binding affinity of α subunits to cardiac glycosides: α₂ and α₃ >> α₁ (250 fold greater binding to CGs)

- Na, K-ATPase α₃ subunit is over-expressed in colon cancer cells compared to normal colon cells, while α₁ expression was reduced (Sakai et al., FESEB Letter, 2004)
Oleandrin-mediated inhibition of human tumor cell proliferation: Importance of Na,K-ATPase α subunits as drug targets

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Abstract
Cardiac glycosides such as oleandrin are known to inhibit the Na,K-ATPase pump, resulting in a consequent increase in calcium influx in heart muscle. Here, we investigated the effect of oleandrin on the growth of human and mouse cancer cells in relation to Na,K-ATPase subunits. Oleandrin treatment resulted in selective inhibition of human cancer cell growth but not rodent cell proliferation, which corresponded to the relative level of Na,K-ATPase α3 subunit protein expression. Human pancreatic cancer cell lines were found to differentially express varying levels of α3 protein, but rodent cancer cells lacked discernable expression of this Na,K-ATPase isofom. A correlation was observed between the ratio of α3 to α1 isoforms and the level of oleandrin uptake during inhibition of cell growth and initiation of cell death; the higher the α3 expression relative to α1 expression, the more sensitive the cell was to treatment with oleandrin. Inhibition of proliferation of Panc-1 cells by oleandrin was significantly reduced when the relative expression of α3 was decreased by knocking down the expression of α3 isoform with α3 siRNA or increasing expression of the α1 isoform through transient transfection of α1 cDNA to the cells. Our data suggest that the relative lack of α3 (relative to α1) in rodent and some human tumor cells may explain their unresponsiveness to cardiac glycosides. In conclusion, the relatively higher expression of α3 with the limited expression of α1 may help predict which human tumors are likely to be responsive to treatment with potent lipid-soluble cardiac glycosides such as oleandrin. [Mol Cancer Ther 2009;8(8):2319–28]

Introduction
Cardiac glycosides are a class of compounds used to treat congestive heart failure by increasing myocardial contractile force (1). Oleandrin is a cardiac glycoside derived from Neriium oleander, which has been used for many years in Russia and China for this purpose. In contrast to its use for the treatment of heart failure, preclinical and retrospective patient data suggest that cardiac glycosides (e.g., digoxin, digitoxin, ouabain, and oleandrin), may reduce the growth of various cancers including breast, lung, prostate, and leukemia (2–7). Recent work from our laboratory and others has shown that these compounds induced selective cell death in certain human but not murine tumor cells (8, 9) or normal human cells (10). Previously, we reported that oleandrin and oleandrinogenin inhibited proliferation and induced apoptosis due to an increase in intracellular Ca2+ via inhibition of Na,K-ATPase (5). Oleandrin and oleandrinogenin also inhibited the export of fibroblast growth factor-2 through membrane interaction and inhibition of Na,K-ATPase activity (11). In addition, we reported that oleandrin inhibits the growth of human melanoma BRO cells due, in part, to the generation of reactive oxygen species that caused mitochondrial injury (12). Other investigators have reported that cardiac glycoside drugs, such as digitoxin and oleandrin, inhibit the constitutive hypersecretion of nuclear factor κB–dependent proinflammatory cytokine interleukin 8 from cystic fibrosis lung epithelial cells (13). These investigators also observed that oleandrin, as well as digoxin, suppressed the tumor necrosis factor α (tumor necrosis...
Relative Na,K-ATPase α3 subunit expression is associated with cell sensitivity to oleandrin.

**Mouse**

- Panc-02
- BxPC3
- MiaPaca
- PANC-1

**Human**

- α1
- α3
- β-Actin

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>α3/β-actin</th>
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</thead>
<tbody>
<tr>
<td>Panc-1 (HUMAN)</td>
<td>71</td>
</tr>
<tr>
<td>MiaPaca (HUMAN)</td>
<td>32</td>
</tr>
<tr>
<td>BxPC3 (HUMAN)</td>
<td>4</td>
</tr>
<tr>
<td>Panc-02 (MOUSE)</td>
<td>0</td>
</tr>
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</table>

The expression of Na,K-ATPase α3 subunit is localized in or near the plasma membrane in normal colon tissues … but has a distinct perinuclear position in colon cancer tissues.
Expression of Na,K-ATPase $\alpha_3$ isoform in human lung tissues

**Tumor tissue**: Increased $\alpha_3$ expression overall; Shift in expression to a perinuclear/nuclear location
Can the change in relative expression of $\alpha_3$ subunit between normal and malignant cell types be shown \textit{in vitro}? How?

If so, what are the consequences of differential expression of the Na,K–ATPase $\alpha_3$ subunit?

What does treatment of malignant cells with oleandrin do to location and expression of the $\alpha_3$ subunit?

Can the differential expression of the $\alpha_3$ subunit be used as a ‘molecular target’ and predictor of sensitivity to lipid soluble CGs?
The distribution of $\alpha_3$ (fluorescence staining) depends on CaCo-2 cell phenotype:

Wild type/undifferentiated vs. differentiated

Green staining = $\alpha_3$; blue staining = nuclei. Note decreased $\alpha_3$ subunit presence near nuclei in differentiated cells.
Na,K–ATPase α3 subunit distribution in CaCO–2 cells human colon cells treated with oleandrin
Oleandrin induces autophagic cell death in undifferentiated (malignant phenotype) but not in differentiated (normal phenotype) CaCO-2 cells.
Undifferentiated CaCO–2 cells are more sensitive to oleandrin treatment than are differentiated CaCO–2 cells.
Oleandrin differentially affects the expression of pERK protein in differentiated and undifferentiated cells.

**Undifferentiated CaCO-2 cells**

<table>
<thead>
<tr>
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<th>Oleandrin (nM)</th>
<th>PBI-05204 (nM)</th>
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</thead>
<tbody>
<tr>
<td>ERK</td>
<td>0, 10, 20</td>
<td>10, 20</td>
</tr>
<tr>
<td>pERK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
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**Differentiated CaCO-2 cells**

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As expected, immunofluorescence analysis showed NaK-β₁ and NaK-α₁ predominantly localized to sites of cell-cell contact at the plasma membrane in control cells. In contrast, these subunits were localized to distinct intracellular vesicles surrounding the nucleus in TGF-β₁–treated cells (Fig. 1C). More importantly, the redistribution of both Na,K-ATPase subunits from the plasma membrane to intracellular vesicles was observed early on, after 18 hours of TGF-β₁ treatment, suggesting that altered Na,K-ATPase localization is an early event in the TGF-β₁–mediated induction of EMT. Consistent
Na,K-ATPase plays a significant role in the formation and maintenance of epithelial phenotype in mammalian cells.
Normal cell architecture:
Limited $\alpha_3$ expression
Location is near cell membrane

Malignant /abnormal cell architecture:
Abundant $\alpha_3$ expression-
Location is perinuclear

Oleandrin: Anvirzel, PBI-05204

TGF-B1
Na, K–ATPase α3 subunit is located primarily on the plasma membrane of cells in normal human colon and lung tissues, but translocates to an intracellular compartment surrounding the nucleus in colon or lung tumor tissues.

Cellular distribution of the Na,K–ATPase α3 subunit in CaCo–2 cells is altered when the phenotype is changed from an undifferentiated to a differentiated state. A perinuclear location is associated with adverse cell events such as cancer.

The sensitivity of undifferentiated and differentiated CaCO–2 cells to oleandrin appears to be associated with the relative cellular expression and distribution of Na,K–ATPase α3 subunit.

Among molecular events oleandrin enhances ERK phosphorylation in undifferentiated CaCO–2 cells, while no such change is observed in oleandrin treated differentiated CaCO–2 cells.
Na,K-ATPase must now be recognized as more than a simple membrane bound ion pump responsible for Na and K exchange.

The $\alpha_3$ subunit and perhaps other components of the enzyme show a differential distribution in normal as opposed to malignant cells.

Because lipid soluble cardiac glycoside compounds such as oleandrin bind selectively to the $\alpha_3$ subunit and such binding is correlated with antiproliferative activity, the relative expression of this subunit might serve as a marker of tumor sensitivity to therapeutic products containing oleandrin such as Anvirzel and PBI-05204.
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