Inhibition of the Sodium Potassium Adenosine Triphosphatase Pump Sensitizes Cancer Cells to Anoikis and Prevents Distant Tumor Formation

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Abstract
Normal epithelial cells undergo apoptosis upon detachment from the extracellular matrix, a process termed “anoikis.” However, malignant epithelial cells with metastatic potential resist anoikis and can survive in an anchorage-independent fashion. Molecules that sensitize resistant cells to anoikis will be useful chemical probes to understand this pathway. To identify novel anoikis sensitizers in anoikis-resistant PPC-1 prostate adenocarcinoma cells, a library of 2,000 off-patent drugs and natural products was screened for their ability to preferentially induce cell death in suspension over adherent culture conditions. This screen identified five members of the family of cardiac glycosides as anoikis sensitizers, including ouabain, peruvoside, digoxin, digitoxin, and strophanthidin. We conducted further studies with ouabain to discern the mechanism of cardiac glycoside-induced anoikis sensitization. Ouabain initiated anoikis through the mitochondrial pathway of caspase activation. In addition, ouabain sensitized cells to anoikis by inhibiting its known target, the Na’/K’ ATPase pump, and inducing hypoosmotic stress. Resistance to anoikis permits cancer cells to survive in the circulation and facilitates their metastasis to distant organs, so we tested the effects of Na’/K’ ATPase inhibition on distant tumor formation in mouse models. In these mouse models, ouabain inhibited tumor metastases but did not alter the growth of subcutaneous tumors. Thus, we have identified a novel mechanism to sensitize resistant cells to anoikis and decrease tumor metastasis. These results suggest a potential mechanism for the observed clinical reduction in metastasis and relapse in breast cancer patients who have undergone treatments with cardiac glycosides.

Introduction
Apoptosis that results from the loss of cell-matrix interaction is termed anoikis and has been suggested to act as a physiologic barrier to metastasis (1–4). Anoikis resistance allows for matrix-independent survival of metastatic cancer cells (5). In addition, anoikis resistance permits malignant cells to survive in a loosely suspended state as they travel through the circulatory and lymphatic systems tumbling and mildly adhering to the vessel walls until they implant in a distant site (6, 7). Thus, molecules that sensitize resistant cells to anoikis could serve as useful probes to better understand this pathway and potentially could be leads for therapeutic adjuncts to control cancer metastasis.

The process of anoikis can involve activation of the death receptor and/or mitochondrial pathways of caspase activation. For example, upon detachment, nonmalignant human umbilical vascular endothelial cells undergo anoikis through a mechanism dependent on the Fas-associated death domain and activation of caspase-8 (8). Amplification of the death signal through the mitochondrial pathway of caspase activation is also required for anoikis in cell lines such as Madin-Darby canine kidney cells (9).

Previously, we showed that overexpression of the caspase-8 inhibitor FLIP confers resistance to anoikis in metastatic cells (10). Decreasing FLIP with siRNA or our chemical inhibitor 5809354 prevented distant tumor formation in a mouse model (10). To identify other mechanisms of anoikis resistance, we conducted a chemical screen for anoikis sensitizers and identified the cardiac glycoside family of Na’/K’ ATPase inhibitors. Subsequent studies showed that these compounds sensitized resistant cells to anoikis by inducing hypoosmotic stress and through a pathway related to disruption of mitochondrial activity. As anoikis resistance is required for tumor metastasis, we evaluated cardiac glycosides in mouse models of metastasis and showed that these agents blocked distant tumor formation.

Materials and Methods
Reagents. The Spectrum chemical library was purchased from Microsource. Ouabain, Peruvoside, and 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbocyanineiodide (JC-1) were purchased from Sigma-Aldrich. Z-VAD-fmk was purchased from Calbiochem.

Cell culture. PPC-1 and PC-3 prostate, HeLa cervical, OVCAR3 ovarian, and T47D breast cancer cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics. PPC-1 cells stably expressing dsRed (10), ATP1α1 (11), FLIP, CrmA, Bcl-2, and Bcl-X<sub>L</sub> were maintained in RPMI 1640 supplemented with 10% FBS, 0.8 mg/mL G418, and antibiotics. All cells were grown at 37°C, 5% CO<sub>2</sub> in humid conditions.

High-throughput anoikis assay. Liquid handling was done by a Biomek FX Laboratory Automation Workstation (Beckman Coulter). PPC-1 cells...
A chemical screen identifies inhibitors of the Na+/K+ ATPase pump as anoikis sensitizers. A, PPC-1 cells were seeded in 96-well polystyrene (adherent) or hydrogel-coated (suspension) plates, respectively. Compounds (5 µmol/L) from the spectrum chemical library were added to cells in parallel. Twenty hours later, cell viability was assessed via MTS. Points, relative viability suspension cells divided by relative viability adherent cells. B, PPC-1 cells were seeded for 24 h in 96-well plates in adherent or suspension conditions. Cells were then cultured with increasing concentrations of ouabain or peruvoside for 18 h. After incubation, an MTS assay was used to assess cell viability. Points, mean percent of viable cells; bars, SE. C, PPC-1 cells were seeded in 96-well plates in adherent or suspension conditions. After 24 h, cells were cultured with increasing concentrations of ouabain for 18 h. After incubation, cell viability was measured by the MitoTox assay. Points, mean percent of viable cells; bars, SE. D, HeLa, T47D, PC-3, and OVCAR3 were seeded for 24 h in suspension and adherent conditions. Cells were cultured with increasing concentrations of ouabain for 18 h, and viability was assessed via the MTS assay. Points, mean percent of viable cells; bars, SE.
were seeded into 96-well polystyrene (adherent) and 96-well ultra-low binding hydrogel-coated (suspension) tissue culture plates (Corning). Compounds from the chemical libraries were added in parallel to cells in adherent and suspension conditions. After 20-h incubation, cell viability was assessed using the CellTiter 96 AQ One Solution Cell Proliferation Assay (MTS; Promega). Hits were defined empirically as compounds that preferentially reduced viability under suspension conditions but not adherent conditions and defined mathematically as (relative viability under suspension conditions)/(relative viability under adherent conditions) ≥ 3 SDs below the mean of the population of compounds tested (12).

Cell viability, cytotoxicity, and caspase assays. The MTS assay was used as a surrogate to measure cell viability as previously described (13). Cytotoxicity was assessed using the Multitox-Fluor cytotoxicity assay (Promega) according to the manufacturer's protocol (14). The ratio of the relative number of dead cells to relative number of viable cells was calculated and plotted. Caspase activation in intact cells was measured by flow cytometry using FITC-labeled peptides that bind to caspase-8 or caspase-9 (Cell Technologies), as previously described (12, 15).

Flow cytometric analysis of mitochondrial membrane potential. To measure differences in the mitochondrial membrane potential, cells were stained with 0.75 μg/mL JC-1 (Sigma). After incubation for 20 min at 37°C, 5% CO2, in the dark, cells were subjected to flow cytometric analysis. The excitation wavelength was 488 nm, and observation wavelengths were 530 nm for green and 585 nm for red fluorescence.

In vivo distant tumor formation. Male severe combined immunodeficient mice (SCID) were obtained from and housed at the Ontario Cancer Institute animal colony, kept in laminar-flow cage racks under standardized environmental conditions with access to food and water ad libitum. Distant tumor formation was assessed using our previously described model (10–12). A single common threshold was applied to identify and measure fluorescence in each organ. The number of fluorescent spots and the corresponding pixel area were recorded for each lung lobe. All quantification was performed on unmanipulated images (10, 12, 16).

Tumor spread and metastasis were also assessed through the BON1 neuroendocrine tumor model. Briefly, NOD/SCID mice were xenografted with an i.p. injection of 5 × 106 BON1 cells. Tumor growth was monitored weekly with MicroCT, and after 4 wk, animals were sacrificed and tumor weight, invasion, and metastasis were evaluated. All experiments were performed according to the regulations of the Canadian Council on Animal Care.

Figure 2. Inhibition of the Na+/K+ ATPase pump sensitizes cells to anoikis. A, PPC-1 cells stably expressing the ATP1a1 or vector control were seeded for 24 h in 96-well plates in suspension conditions. Cells were then treated with increasing concentrations of ouabain for 18 h. After incubation, an MTS assay was used to assess cell viability. Columns, mean percent of viable cells; bars, SE. ***, P < 0.0003 by the student’s t test. B, PPC-1 cells were seeded for 24 h in 96-well plates in adherent or suspension conditions. Cells were then cultured with increasing concentrations of dihydroouabain, digoxin, digitoxogenin, digoxin, or strophanthidin for 18 h. An MTS assay was used to assess cell viability. Points, mean percent of viable cells; bars, SE.
Results

Identification of small molecules that sensitize resistant cells to anoikis. Resistance to anoikis allows malignant cancer cells to survive in the absence of adhesion to the extracellular matrix, which facilitates metastases. To better understand the regulation of this pathway, we developed, and conducted a chemical screen to identify small molecules that could sensitize resistant cells to anoikis. Cells under suspension or adherent conditions were treated with aliquots from the Spectrum (n = 2,000) library of off patent drugs and natural products (final concentration 5 μmol/L and <0.2% DMSO). Twenty hours after incubation, the MTS assay was used as a surrogate to measure cell viability (Fig. 1A).

Hits were compounds that preferentially induced cell death under suspension conditions and were empirically defined as compounds where the relative viability ratio was three SDs below the mean relative viability ratio of all 2,000 compounds tested. The top reproducible hits from this screen were the cardiac glycosides ouabain and peruvoside. Members of this family of Na⁺/K⁺ ATPase inhibitors, namely digoxin and digitoxin, are used to treat congestive heart failure and cardiac arrhythmias. Interestingly, the screen also identified three other cardiac glycosides, as weaker anoikis sensitizers.

Ouabain sensitizes resistant cells to anoikis. To validate ouabain and peruvoside as anoikis sensitizers, PPC-1 cells were treated overnight with increasing concentrations of the compounds under adherent and suspension conditions, and viability was measured by the MTS assay. Both ouabain and peruvoside sensitized PPC-1 cells to anoikis with LD₅₀ of 163 ± 45 nmol/L and 148 ± 46 nmol/L, respectively, under suspension conditions. In contrast, the LD₅₀ of both compounds under adherent conditions were >50 μmol/L (Fig. 1B). As ouabain and peruvoside had similar results, we chose to carry ouabain forward for further analysis. Increasing the treatment time to 48 hours lowered the LD₅₀ to 24 ± 15 nmol/L in suspension conditions (data not shown). Ouabain sensitized PPC-1 cells to anoikis by the MitoTox assay, which directly measures the number of viable and dead cells (Fig. 1C). The spectrum of activity of ouabain as an anoikis sensitizer was observed in the PC-3, OVCAR3, HeLa, and T47D cell lines (Fig. 1D).

Ouabain sensitizes to anoikis through the inhibition of the Na⁺/K⁺ ATPase pump. Ouabain is a known inhibitor of the Na⁺/K⁺ ATPase pump (17). To determine whether the observed anoikis

Figure 3. Ouabain initiates anoikis via a mitochondrial, caspase-9–dependent mechanism. A, PPC-1 cells were seeded in 96-well plates in adherent or suspension conditions. Cells were then treated for 18 h with and without Z-VAD (200 μmol/L). In the presence of increasing concentrations of ouabain. After incubation, an MTS assay was used to assess cell viability. Points, mean percent of viable cells; bars, SE. B, PPC-1 cells stably expressing c-FLIP, CrmA, Bcl-2, Bcl-XL, or vector alone were seeded in 96-well plates under suspension conditions for 24 h. Cells were then cultured in the presence or absence of 100 nmol/L ouabain for 18 h. After incubation, an MTS assay was used to assess cell viability. Columns, mean percent of viable cells; bars, SE. *, P < 0.001 by the Student’s t test. C, PPC-1 cells were seeded in 24-well plates under suspension conditions and treated with ouabain (100 nmol/L). At increasing times after treatment, caspase activation was detected with FITC-labeled peptides that bind to caspase-8 or caspase-9. Caspase activation was monitored by flow cytometry to quantify the percentage of fluorescently labeled cells. Columns, fold increase in activity; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by the Student’s t test. D, PPC-1 cells were seeded in 24-well plates in suspension conditions for 24 h. Cells were then cultured in the presence of ouabain (100 nmol/L). At increasing times after incubation, cells were harvested and stained with 0.75 μg/mL JC-1 and monitored by flow cytometry to quantify the percentage of fluorescently labeled cells. Loss of fluorescent signal in red channel represents mitochondrial depolarization. A representative experiment is shown.
The Na+/K+ ATPase Pump and Anoikis

sensitization was due to inhibition of this pump, genetic and chemical approaches were used. As a genetic approach, we stably overexpressed the murine Na+/K+ ATPase pump (ATP1a1), as this pump is resistant to inhibition by ouabain (18). Here, PPC-1 cells stably expressing ATP1a1 or vector control were treated with increasing concentrations of ouabain under suspension and adherent conditions for 20 hours. ATP1a1 protected cells against ouabain-induced anoikis (Fig. 2A), consistent with a mechanism of action related to the Na+/K+ ATPase pump.

As a chemical approach to determine whether ouabain was acting through a mechanism related to the inhibition of the Na+/K+ ATPase pump, we tested a panel of known Na+/K+ ATPase inhibitors for their ability to sensitize PPC-1 cells to anoikis. Digoxin, digitoxin, digitoxigenin, dihydroouabain, and strophanthidin all sensitized cells to anoikis (Fig. 2B). Thus, taken together, these results show that the inhibition of the Na+/K+ ATPase pump sensitizes resistant cells to anoikis.

**Ouabain sensitization to anoikis involves caspase activation.** We and others have reported that anoikis is a caspase-dependent process (9, 10, 12, 19–21). To determine whether anoikis sensitization by ouabain involved the activation of caspases, PPC-1 cells were treated under suspension conditions with increasing concentrations of ouabain in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. z-VAD-fmk inhibited the ability of ouabain to promote anoikis (Supplementary Fig. S1; Fig. 3A). To further examine the role of caspases in ouabain-induced anoikis, we examined the cleavage of PARP, a downstream target of the effector caspases. PPC-1 cells were treated in suspension conditions with 100 nmol/L of ouabain. At increasing times after initiation, cells were harvested and PARP cleavage was measured by immunoblotting. Ouabain increased the cleavage of PARP in a time-dependent manner (Supplementary Fig. S2). Thus, taken together, these results show that caspase activation plays a role in ouabain-induced anoikis.

**Ouabain anoikis sensitization proceeds through the mitochondrial pathway of caspase activation.** Defects in the death receptor pathway of caspase activation can render malignant cells resistant to anoikis, and targeting these defects can restore anoikis sensitivity (10, 12, 22). To determine whether inhibition of the Na+/K+ ATPase pump sensitized cells to anoikis by affecting targets in the death receptor pathway, PPC-1 cells were seeded in adherent conditions in the presence or absence of the FAS activating antibody CH-11 (FAS) and treated with increasing concentrations of ouabain. Despite its ability to sensitize cells to anoikis, ouabain treatment increased the viability of FAS-treated cells (Supplementary Fig. S3A). Given these findings, we measured levels of Fas ligand mRNA expression by quantitative reverse transcriptase-PCR after ouabain treatment because we have previously shown that Fas ligand expression is increased in suspension conditions (10). Under both adherent and suspension conditions, ouabain decreased levels of FAS ligand mRNA in a time-dependent manner (Supplementary Fig. S3B). Taken together, these results suggest that inhibition of the Na+/K+ ATPase pump sensitizes cells to anoikis through mechanisms independent of the death receptor pathway.

To further assess whether ouabain sensitized cells to anoikis through mechanisms independent of the death receptor pathway of caspase activation, PPC-1 cells overexpressing Bcl-2, Bcl-XL, FLIP, or CrmA were treated with increasing concentrations of ouabain under suspension conditions. Compared with vector control, overexpression of Bcl-2 or Bcl-XL blocked ouabain mediated sensitization to anoikis, unlike the caspase-8 inhibitors CrmA or FLIP (Fig. 3B). Thus, these results suggest that ouabain is acting by stimulating the mitochondrial pathway of caspase activation. To test this hypothesis, PPC-1 cells were treated with 100 nmol/L ouabain or buffer control under suspension conditions. At increasing times after treatment, activation of caspase 8 and 9 was measured using cell-permeable FITC-labeled peptides that bind preferentially and irreversibly to active caspases. Active caspase-9 was detected before the activation of caspase-8 (Fig. 3C). Given this dependence on the mitochondrial pathway of caspase activation, we tested whether inhibition of the Na+/K+ ATPase pump sensitized cells to anoikis by disrupting the mitochondria. Here, PPC-1 cells were treated with 100 nmol/L ouabain in suspension conditions. At increasing times after treatment, mitochondrial membrane potential was measured by staining cells with the JC-1 cationic dye and flow cytometry (23). Treatment of PPC-1 cells with ouabain under suspension conditions induced a rapid loss of mitochondrial membrane potential (Fig. 3D). Thus, taken together, these results indicate that inhibition of the Na+/K+ ATPase pump sensitizes cells to anoikis by activating the mitochondrial pathway of apoptosis.

**Ouabain sensitizes cells to anoikis by inducing hypoosmotic stress.** Inhibition of the Na+/K+ ATPase pump increases intracellular Na+ (24). Consistent with this effect, cells treated with ouabain had morphologic evidence of increased cell size (data not shown). To quantify this effect, PPC-1 cells were treated with increasing...
concentrations of ouabain in adherent and suspension conditions, and cell size was measured by flow cytometry. After 20 hours of ouabain treatment, cell size was increased in suspension and adherent conditions when compared with cells treated with buffer control (data not shown; Fig. 4A). To determine whether the increased cell size induced by inhibition of the Na⁺/K⁺ ATPase was associated with anoikis sensitization, PPC-1 cells were treated under adherent or suspension conditions in media with decreasing osmolality for 1 hour. Hypoosmotic culture conditions increased cell size and sensitized cells to anoikis (Fig. 4B). Thus, taken together, these results suggest that inhibition of the Na⁺/K⁺ ATPase pump sensitizes cells to anoikis by increasing intracellular volume and inducing hypoosmotic stress.

Ouabain decreases the in vivo survival of circulating prostate cancer cells. Resistance to anoikis promotes metastasis by allowing normally adherent cancer cells to survive after detachment from the primary tumor site in the circulatory and lymphatic systems. Since we have shown that inhibition of the Na⁺/K⁺ ATPase pump is able to sensitize resistant PPC-1 cells to anoikis, we wished to test whether inhibition of this pump would decrease the in vivo survival of cells in the circulatory system of mice. PPC-1 cells labeled with dsRed2 red fluorescent protein (PPC-1 dsRed) were either treated with buffer control or increasing concentrations of ouabain in adherent conditions for 18 hours. After treatment, cells were injected i.v. into SCID mice that had been sublethally irradiated to reduce residual natural killer cell function (25). Five weeks after injection, mice were sacrificed and each organ was imaged using fluorescent microscopy. Retention and growth of the tumor cells were detected in the lung, bone, liver, and lymph nodes, all of which are clinically relevant sites of metastases in prostate cancer. Interestingly, i.v. injection of PPC-1 dsRed cells into SCID mice that had not been sublethally irradiated...
developed tumors in the lymph node but not other organ systems (data not shown). Therefore, the effects of ouabain on distant tumor formation were assessed in sublethally irradiated SCID mice. Mice injected with cells treated with ouabain developed fewer tumors in a dose-dependent manner in these organs when compared with mice injected with cells treated with buffer control (Fig. 5A). Because the resolution in the lungs was sufficient for quantitative image analysis, the total tumor number and tumor area was calculated. In a dose-dependent manner, significant reductions in tumor size and number were observed after injecting mice with cells treated with ouabain compared with controls (Fig. 5B and C). It is important to note that >94% of both treated and control cells were viable at the time of injection and remained viable for an extended period of time under adherent conditions, as tumor weight was not significantly altered after s.c. injection of PPC-1 dsRed cells treated with ouabain or buffer control (data not shown). Thus, these results further show that reductions in tumor burden were not simply related to injection of nonviable cells.

Ouabain treatment of mice decreases the in vivo survival of circulating prostate cancer cells. As a more rigorous measure of the effects of ouabain on distant tumor formation, mice were injected i.v. with dsRed-labeled PPC-1 cells followed by daily i.p. injections of ouabain (0.67 mg/kg) or PBS for 2 weeks. Treatment of mice with ouabain resulted in decreased tumor formation in the lungs, liver, bones, and lymph nodes when compared with mice treated with PBS 5 weeks after injection of cells. Ouabain decreased the size and number of tumors in the lungs compared with buffer-treated animals (Fig. 6A and B).

To determine whether ouabain decreased distant tumor formation simply by decreasing cellular proliferation, PPC-1 dsRed cells were injected s.c. into sublethally irradiated SCID mice. In contrast to its effects on distant tumor formation, ouabain did not alter the growth of the subcutaneous tumors (Fig. 6C). Thus, reduction in distant tumor formation after treatment with ouabain is consistent with sensitization to anoikis and is not solely due to reductions in cellular proliferation.

Similar results were also achieved in a mouse model of pancreatic neuroendocrine tumor metastasis. i.p. injection of BON1 cells results in peritoneal spread and liver metastasis. Treatment of mice with ouabain (0.67 mg/kg) via daily s.c. injections for 4 weeks decreased tumor weight and spread (data not shown). Thus, taken together, inhibition of the Na⁺/K⁺ ATPase inhibits distant tumor formation in multiple mouse models of metastasis.

Discussion

Anoikis serves as a physiologic barrier to metastasis. Resistance to anoikis permits cancer cells to survive in circulation and facilitates their metastasis to distant organs (10, 22, 26, 27). Clinically, the presence of circulating tumor cells in the peripheral blood of patients afflicted with solid malignancies correlates with worse clinical outcome in some but not all studies (28–31). Thus, understanding the mechanism of anoikis will improve our understanding of the process of metastasis. In addition, these results highlight the potential therapeutic adjunct to decrease metastasis and improve patient outcome.

To further our understanding of anoikis, we developed, automated, and conducted a chemical screen for anoikis sensitizers. From this screen, we identified a family of cardiac glycosides, two of which, digoxin and digitoxin are used to treat patients with congestive heart failure and cardiac arrhythmias (32). These compounds exert a positive inotropic effect on the heart due to their ability to inhibit the Na⁺/K⁺ ATPase pump which leads to a concomitant increase in intracellular calcium (33).

Using genetic and chemical approaches, we showed that cardiac glycosides sensitize cells to anoikis by inhibiting their known target the Na⁺/K⁺ ATPase pump. The Na⁺/K⁺ ATPase pump is a transmembrane protein that transports potassium ions into and sodium ions out of eukaryotic cells against their natural gradients (34). Cardiac glycosides bind to the pump at a regulatory site that is located on the extracellular side on the α-subunit of the enzyme (35). This inhibition results in an increase in intracellular Na⁺, and a decrease in intracellular K⁺ (24). These results support the Na⁺/K⁺ ATPase pump as the target for the effects of ouabain on anoikis. However, we concede that ouabain may also bind additional unidentified targets that influence its effects on anoikis.

Increased intracellular Na⁺ causes an influx of water through osmosis to balance the ionic concentrations of the cytoplasm to the extracellular environment. Consistent with this process,
inhibition of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump increased cell size. Furthermore, culturing cells in hypotonic medium also sensitized them to anoikis. However, our results suggest that the osmotic stress produced by ouabain is functionally important for anoikis sensitization because cells cultured in hypotonic medium were also sensitized to anoikis. We also showed that ouabain sensitized cells to anoikis by disrupting the mitochondrial membrane potential and activating the mitochondrial pathway of caspase activation. Consistent with a connection to the induction of osmotic stress, prior studies have reported that cells cultured in hypotonic media experience loss of mitochondrial membrane potential (36). Thus, we highlight a new strategy to restore sensitization to anoikis that targets the Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump and the mitochondrial pathway of caspase activation.

Cardiac glycosides can directly induce cell death in cells through multiple and complex mechanisms including, increasing Ca\textsuperscript{2+} concentration (37), decreasing nuclear factor-\textbeta DNA binding (38) and through the up-regulation of the death receptors DR4 and DR5 (39). However, in the cells tested in this study, the concentrations of cardiac glycosides required to sensitize cells to anoikis were lower than the concentrations required to induce cell death.

Cardiac glycosides may also have anticancer effects in patients (40–42). Five years after mastectomy, patients receiving cardiac glycosides had a ~10-fold lower rate of relapse and improved survival, than patient not receiving these drugs (43–46). Despite these clinical findings, the mechanism(s) by which cardiac glycosides exert an anticancer effect and prevent metastasis are unclear. Based on our study, it is tempting to suggest that the antimetastatic effect of cardiac glycosides could be partly attributed to their effect on anoikis. However, caution must be taken in accepting this conclusion. The maximal therapeutic plasma concentrations of digitoxin and digoxin are 30 and 2 mmol/L, respectively (47). These concentrations are lower than the concentration of ouabain required to sensitize cells to anoikis. However, it is unknown what concentration of ouabain can be achieved in humans. In addition, the results in our mouse models of distant tumor formation must also be interpreted with caution. The rodent Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump is less sensitive to inhibition by cardiac glycosides and the mouse heart is less sensitive to arrhythmias that could be produced by high doses of cardiac glycosides. Therefore, the antimetastatic effects we observed in mice after ouabain treatment may not be relevant to humans. Another limitation to our study is that the studies of distant tumor formation and retention were conducted in sublethally irradiated SCID mice. Radiation was used to reduce residual immune function known to be present in SCID mice (25) and improved distant tumor formation. However, we concede that the radiation may have altered the integrity of the endothelium that may have skewed our results. Nonetheless, this work identifies a novel mechanism that influences anoikis. In addition, it suggests that Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump inhibitors that have less cardiac effects could be leads for anticancer agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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