

ANALYSIS

CARDIAC GLYCOSIDES IN CANCER RESEARCH
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Abstract: The well known and accepted mode of action of cardiac glycosides is inhibition of the ubiquitous plasma membrane Na⁺, K⁺-ATPase that leads to increased intracellular Ca²⁺ ion concentrations. Ca²⁺ ions play pivotal role in many signaling pathways including those regulating apoptosis. It has been suggested that some forms of cardiac glycosides inhibit proliferation and induce apoptosis in prostate cancer cells in clinically relevant concentrations. It was also found out that the degree to which cardiac glycosides inhibited cancer cell growth was correlated to topoisomerase II-inhibiting activity. Digitoxin at concentrations found in cardiac patients induced levels of DNA-topoisomerase II cleavable complexes similar to etoposide, a topoisomerase II poison widely used in cancer chemotherapy. Cardiac glycosides can also regulate one of the most potent angiogenesis promoting substances, fibroblast growth factor-2 (FGF-2), and may inhibit activation of the transcription factor NF-κB. FGF-2 and NF-κB are relevant targets for anticancer drugs. There is growing interest in evaluating the oleander products and possibly other cardiac glycosides as antineoplastic agents. The first of these therapies to be developed in the United States is a patented, water-soluble oleander extract called Anvirzel®.

Keywords: cardiac glycosides, anticancer drugs, apoptosis, cytotoxicity, topoisomerase

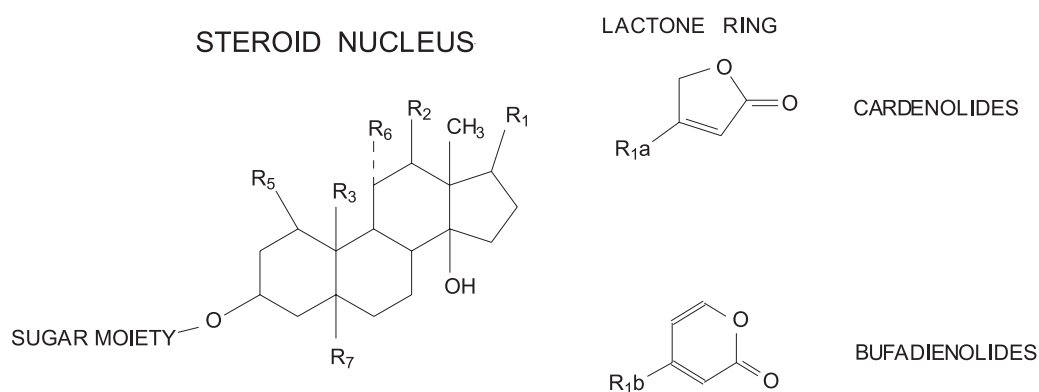
Cardiac glycosides are a class of natural products that are traditionally used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias (1-3). These glycosides are found as secondary metabolites in a diverse group of plants including *Strophanthus* (ouabain), *Digitalis lanata* and *Digitalis purpurea* (digoxin, digitoxin), *Scilla maritima* (proscillaridin A), *Nerium oleander* (oleandrin, oleandrogenin) but also in frogs (some frog-poisons contain bufadienolides – bufalin, marinobufagenin). Plants containing cardiac steroids have been used as heart drugs at least since 1500 B.C. Throughout history these plants or their extracts have been variously used as arrow poisons, emetics, diuretics, and heart tonics (2,4,5).

Cardiac glycosides represent a class of compounds that share a common structure consisting of a steroid (cyclopentanoperhydrophenanthrene) ring, substituted in position 3-OH with sugar moiety and in position 17β with an unsaturated lactone ring (Figure 1). The lactone at the C17 position defines the subgroups of cardiac glycosides (cardenolides and bufadienolides). The cardenolides have an unsaturated butyrolactone ring (5-membered unsaturated lactone) whereas the bufadienolides have a pyrone ring (6-membered unsaturated

lactone) (2,6). One to four sugars are found to be present in most cardiac glycosides attached to the 3β-OH group. The sugars most commonly found include L-rhamnose, D-glucose, D-digitoxose, and D-digitalose.

One of the most widely used cardiac glycosides is *digitalis*, a powdered extract of *Digitalis purpurea* (foxglove) or *Digitalis lanata*. *Digitalis* itself consists of two major specific cardiac glycosides – digoxin and digitoxin. The structural difference between digitoxin and digoxin is just an extra hydroxyl group on digoxin (Figure 1), which changes the pharmacokinetics and pharmacodynamics of these substances. Digitoxin is more lipophilic, is mainly metabolized in the liver and has a longer half-life than digoxin (2).

The mechanism of action of cardiac steroids in the heart is well known and involves inhibition of the plasma membrane Na⁺, K⁺-ATPase, leading to increased intracellular Na⁺ and Ca²⁺ ions and decreased intracellular K⁺ ions. In the 1960s inhibition of malignant cells of cardiac glycosides *in vitro* was reported (7) and since then other anticancer effects of cardiac glycosides have been observed. Therapeutic effect of cardiac glycosides in breast cancer has been known from 1979 (8,9). Five years after the mastectomy, the recurrence



	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇
Digoxin	a	OH	CH ₃	(digitoxose) ₃	H	H	H
Digitoxin	a	H	CH ₃	(digitoxose) ₃	H	H	H
Oubain	a	H	CH ₂ OH	rhamnose	OH	OH	OH
Bufalin	b	H	CH ₃	H	H	H	H
Proscillaridin A	b	H	CH ₃	rhamnose	H	H	H

Figure 1. Chemical structures of the chosen cardiac glycosides.

among patients not taking cardiac glycosides was 9.6 times that in patients taking digitalis (10). These encouraging results, however, did not trigger researches at that time to evaluate a potential role of cardiac glycosides in cancer treatment. In 1999 Stenkvis made a reanalysis of a possible benefit for cardiac glycosides in cancer (11). A group of 175 patients with breast cancer, 32 of which were on digitalis glycoside therapy when the disease was diagnosed, were studied over a period of more than 22 years. Patients receiving digitalis showed a significantly lower death rate (6%) than those without digitalis treatment (34%) (11). The breast cancer patients on digitalis had tumors with lower proliferative capacity and a reduced recurrence rate (8,9,11). However, the number of women on digitalis was small so Stenkvis's results need to be confirmed in more studies. A clinical connection between Na⁺, K⁺-ATPase inhibition and cancer treatment might be tamoxifen. Repke et al. showed that just a part of the inhibition of malignant breast cancer cells is due to receptor interaction. Tamoxifen also works as Na⁺, K⁺-ATPase inhibitor and this stands for at least some of the anticancer effect not explained by receptor interaction (12).

Cytotoxicity of cardiac glycosides

It should be stressed, that there are marked differences in cytotoxicity between the cardiac glycosides, both in potency and selectivity. Of seven analyzed compounds, the most potent was proscillaridin A followed by digitoxin, and then ouabain, digoxin, lanatoside C, digitoxigenin and digitonin (13). Digitoxin and digoxin expressed selective toxicity against solid tumor cells from patients, while proscillaridin A expressed no selective toxicity against either solid or hematological tumor cells (13).

Johnson et al. have shown that the ability of cardiac glycosides to induce apoptosis in the human prostate cancer cell line PC-3 correlates with their activity in inhibiting expression of four of the prostate target genes including transcription factors Hoxb-13, hPSE/PDEF, hepatocyte nuclear factor-3 α , and the inhibitor of apoptosis, survivin (14). The finding that ouabain and digitoxin are potent inhibitors of several prostate target genes suggests that the membrane Na⁺, K⁺-ATPase may directly affect transcriptional regulation of these prostate transcription factors. Evaluating the effects of known drugs on target genes of relatively unknown

function can provide information linking the target genes to known cellular pathways and drug mechanisms. A large number of genomic alterations in a cancer cell appear early in sporadic tumor development (14). This results in different genes expression between the normal and cancer cells and may be a potential targets for effective anticancer drugs.

Digitalis can directly inhibit the proliferation of the androgen dependent prostate cancer cell line LNCaP and the androgen independent prostate cancer cell lines DU145 and PC3. This effect may be achieved by an elevation in intracellular Ca^{2+} and by apoptosis (15). The PC3 prostate cancer cell lines showed various degrees of sensitivity to digitalis treatment. According to the study done by Huang et al. (16), it is suggested that ouabain inhibit the cell growth in androgen-independent prostate cancer cells with the threshold concentration around nanomolar level, which is similar to the therapeutic plasma concentration in patients treated with cardiac glycosides. Since cardiac glycosides are drugs with a narrow range of therapeutic safety, more detailed *in vitro* intoxication trials in various normal human cells are needed before wide range *in vivo* studies are started.

There is growing interest in evaluating the oleander products and possibly other cardiac glycosides as antineoplastic agents. The first of these therapies to be developed in the United States is a patented, water-soluble oleander extract called Anvirzel®. Anvirzel® consists of several compounds, including complex polysaccharides, proteins and individual sugars (17). It contains non-water soluble compounds, and two of these have been specifically identified by molecular weight and fragmentation characterization as oleandrin (Figure 2) and oleandrigenin (oleandrin without the sugar moiety – oleandrose). These two compounds of Anvirzel® are cytotoxic (17). Oleandrin has structural similarity to cardiac glycoside digoxin and is known to cross react with various digoxin immunoassays.

Preclinical studies have demonstrated that this oleander extract has high activity against a variety of human malignant cell lines including melanoma, breast, and lung cancer (18). Pathak et al. results from tests on cultures of two human prostate cancer cell lines (PC-3M, C4-2), treated with Anvirzel®, indicate that human prostate cancer cell lines showed significant susceptibility to cell killing (17). The cell killing seems to be mediated through the loss of telomeric DNA, followed by the arrest of cells in G2/M phase, induction of endomitosis, extensive DNA fragmentation and reduced levels of

TRF2. Newman et al. have demonstrated that Anvirzel®, like oleandrin, inhibited FGF-2 export *in vitro* from PC3 and DU145 prostate cancer cell lines in a concentration- and time-dependent manner (19). Newman and coworkers have also shown that oleandrin is a potent inhibitor of NF- κ B in a wide variety of different cell types (20). Interestingly, ouabain (the most commonly used inhibitor of Na^+ , K^+ -ATPase), had no effect on blocking NF- κ B activation (20). These results suggest that oleandrin blocks NF- κ B activation through some other mechanism. It must be stressed that concentrations of Anvirzel® required to achieve cytotoxicity are relatively non-toxic, whereas the active implementation of clinically available cardiac glycosides into cancer therapy has been hampered by their concomitant cardiotoxic action. The description of compounds without cardiac activity but with tumor-specific cytotoxicity might be a milestone in cancer therapy.

Cardiac glycosides and apoptosis

Recently, digitalis and other cardiac glycosides in nontoxic concentrations have been shown to induce apoptosis in different malignant cell lines *in vitro* (21,22). Cytotoxicity induced by cardiac steroids includes a series of morphological and biochemical changes that are characteristic for apoptosis, such as phosphatidylserine externalization, internucleosomal DNA fragmentation and mitochondrial membrane potential disruption (22). Apoptosis, also known as programmed cell death, is distinct from necrosis in that it is an active process that leads to cell death. It plays an important role in embryogenesis, carcinogenesis, regulation of the immune system, and the killing of virally infected cells, serving as a physiological process that regulates cell number and eliminates damaged cells. In light of the pivotal role of apoptosis in cancer development and progression, and this new experimental finding concerning cardiac glycosides, it seems probable that the apoptosis-inducing

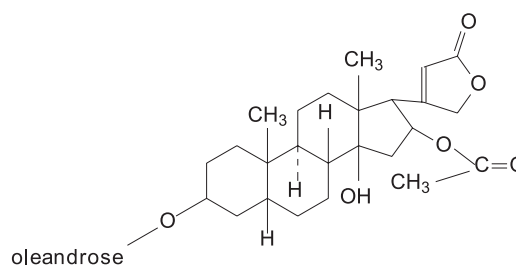


Figure 2. Chemical structures of oleandrin – the main component of Anvirzel®.

capability is explained by mechanisms other than just only Na^+ , K^+ -ATPase inhibition.

It is well known that digitalis has the ability to inhibit the activity of Na^+ , K^+ -ATPase and lead to increased intracellular Ca^{2+} . Deregulation of these ions results in activation of a number of intracellular pathways, such as a change in cellular structure or gene expression. A sustained elevation in intracellular Ca^{2+} may also activate various hydrolytic enzymes, including proteases, nucleases and lipases, which have been implicated as effectors of Ca^{2+} elicited toxicity. Thus, these changes may have a critical role in cellular toxicity. Activated hydrolytic enzymes may lead to exaggerated energy expenditure, impair energy production, initiate cytoskeletal degradation, decrease phospholipids, disrupt membrane and cytoskeletal proteins, and nuclear chromatin, and ultimately result in cell death.

The increase in cytosolic Ca^{2+} caused by cardiac glycosides might, in part, explain the anti-tumor effects of cardiac glycosides in cancer patients (18). Intracellular or extracellular Ca^{2+} chelators, Ca^{2+} channel blockers, and calmodulin antagonists can all delay or abolish apoptosis in several model systems. Disruption of intracellular Ca^{2+} homeostasis (e.g. via inhibition of the Na^+ , K^+ -ATPase by cardiac glycosides) might result in the induction of apoptosis in diverse type of cells, including tumor cell lines (18). Furthermore, thapsigargin (Ca^{2+} ionophore), which selectively inhibits Ca^{2+} -dependent ATPase pumps in sarcoplasmic and endoplasmic reticulum and directly stimulates an intracellular Ca^{2+} increase, induces prominently the apoptosis in androgen-sensitive and -insensitive prostate cancer cells (23). Most prostate cancers are mixtures of androgen-dependent and androgen-independent cells. The major problem in androgen-independent prostate cancer is that, as yet, an effective therapeutic regimen is still lacking. However, it is well known that Ca^{2+} plays a crucial role in stimulating endonucleases and in cleaving internucleosomal DNA in cells responsive to apoptotic stimuli (24). Thus, the modulation of intracellular Ca^{2+} level would be a strategy for the treatment of hormone-resistant prostate cancers. It has been suggested that some forms of cardiac glycosides inhibit proliferation and induce apoptosis in prostate cancer cells in clinically relevant concentrations (13,25). These reports suggest that the modulation of intracellular Ca^{2+} level may induce or enhance the apoptotic response in human cancer cells and provide a new target for therapeutic strategies in cancer chemotherapies. Yeh's et al.

results have also shown that digitalis inhibits the proliferation of prostate cancer cell lines at least partially through a mechanism of cytotoxicity effects induced by a sustained elevation of intracellular Ca^{2+} (26). However, they suggest that in addition to sustained intracellular concentration of Ca^{2+} , another possible mechanism involved in the apoptotic effect induced by bufalin or cinobufagin might be the change in intracellular Na^+ concentration (26,27).

It is worth to note, that malignant cells in general are more susceptible to the effects of cardiac glycosides than normal cells. It may be due to the fact that in many cases, Na^+ , K^+ -ATPase activity is different in tumor or transformed cells compared to their normal counterparts (28,29). One of the cardiac steroids, bufalin, in nontoxic concentrations, was able to induce apoptosis in human leukemia HL60 and ML1 cells but not in normal leukocytes (30). Therefore, bufalin seems to act as a potent differentiation- and apoptosis-inducing agent in cancer cells. Although the signal-transduction pathway from Na^+ , K^+ -ATPase is unknown, it appears that the signal is transmitted sequentially from Ras, Raf-1, and MAP kinase (31).

Terness et al. found out that cardiac glycosides and their derivatives have a strong antiproliferative action and a tumor-specific, apoptosis-mediated cytotoxic effect (22,32). Interestingly, their results show that removal of the chemical groups that are responsible for inhibition of the Na^+ , K^+ -ATPase weakens but does not abrogate the apoptotic effect of cardiac glycosides (22). This process involves the classical caspase-dependent pathway with damage of mitochondria and internucleosomal DNA fragmentation (22). Lopez-Lazaro et al. have also shown the apoptotic activity of extracts obtained from the leaves of *Digitalis purpurea* on three human cancer cell lines: TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma) (33).

More recent works have shown that caspase activation and DNA fragmentation are preceded by a drop in intracellular K^+ levels (22,34), and that inhibition of this drop blocks caspase activation and cell death (34). Importantly, cardiac glycosides induce both an increase in Ca^{2+} and a decrease in K^+ ions. In addition, parallel studies have shown that oleandrin suppresses NF- κ B (nuclear factor- κ B) activation (35), which could also contribute to cell death induction (36). However, the cell death-promoting activity of cardiac glycosides appears cell type specific, because other work has shown that they inhibit multiple pathways of apoptosis in vascular smooth muscle cells (37).

McConkey et al. have provided the evidence that oleandrin, ouabain and digoxin are potent inducers of apoptosis in androgen-independent human prostate cancer cell lines (PC-3) (18). Cell death was associated with early release of cytochrome c from mitochondria, followed by proteolytic processing of caspases 8 and 3 (18). Interestingly, the effects of oleandrin on cell cycle arrest appear to be much more pronounced than those of thapsigargin (18). These findings may suggest that the oleandrin-induced intracellular Ca^{2+} elevation is not principally responsible for the antitumor effect and that alterations in K^{+} and/or Na^{+} may also be involved.

Cardiac glycosides and topoisomerases

There are several facts that suggest that DNA topoisomerases might be involved in the anticancer activity of cardiac glycosides. For instance, like cardiac steroids, several topoisomerase poisons currently used in the clinic (e.g. etoposide and camptothecin derivatives) have a lactone moiety which seems to be crucial for their anti-cancer effects. Steroid substances such as corticosteroids have been extensively used for a long time in medical oncology in the treatment of lymphoproliferative cancers or prostate and breast cancer. Cardiac glycosides have also shown a radiosensitizing effect on malignant cancer cell lines but not on normal ones, and interestingly, malfunction of topoisomerases has been proposed to be involved in the radiosensitization processes (38).

It was found that bufalin selectively inhibited the growth of various lines of human cancer cells and induced apoptosis (30,39), due at least in part to its specific effect on topoisomerase II (34). Hashimoto et al. have demonstrated that bufalin caused a marked decrease in the steady-state level of topoisomerase II in human leukemia cells, which led to the fragmentation of DNA, a typical feature of apoptosis (34). Recently, Lopez-Lazaro et al. have shown that digitoxin at concentrations found in cardiac patients induced levels of DNA-topoisomerase II cleavable complexes similar to etoposide, a topoisomerase II poison widely used in cancer chemotherapy (40).

It was also found out that the degree to which cardiac glycosides inhibited cell growth was correlated to topoisomerase II-inhibiting activity (33,34,40). While topo I is the specific target for only a limited group of drugs acting as poisons of the enzyme, such as camptothecin and derivatives, topoisomerase II is the primary target of poisoning by an increasing number of cytotoxic drugs of diverse nature currently available for the clinical

treatment of human cancers (41-43). Because of their central role in DNA replication, transcription and repair processes, topoisomerases II enzymes are important in both current and future strategies for cancer chemotherapy especially since overexpression of these proteins has been demonstrated in many human tumor types, such as breast cancer. While both ouabain and digoxin inhibited topoisomerase II catalytic activity at nanomolar concentrations, neither agent inhibited topoisomerase I catalytic activity even at concentrations as high as 100 μM . On the other hand, proscillaridin A was a potent poison of topoisomerase I and II activity at nanomolar drug concentrations, suggesting that this agent may produce its cytotoxic activity by targeting both enzymes simultaneously. There has been substantial interest in compounds that may act against both type I and type II topoisomerases. Dual topoisomerase poisons are of interest for their use in the treatment of cells exhibiting multi-drug resistance (MDR).

Chemotherapeutic agents that target topoisomerases I and II can set in motion a series of biochemical changes that culminate in cell death, but only under certain conditions. Whilst a range of signalling molecules have been implicated in cell death mediated by topoisomerase-interacting agents, generally their roles remain undefined. The realization that stabilization of covalent topoisomerase-DNA complexes was insufficient to insure this ultimate fate has prompted major research in this area. One might speculate, therefore, that the abilities of cardiac glycosides to compromise the overall catalytic activities of topoisomerase II impact on their cytotoxic potential via some as yet to be defined activation of an apoptotic program. Thus, in addition to inhibition of topoisomerase II, the cytotoxicity of cardiac glycosides is associated with multiple mechanisms that may be responsible for inhibition of growth of tumor cells.

Cardiac glycosides and angiogenesis

Cardiac glycosides can also regulate one of the most potent angiogenesis promoting substances, fibroblast growth factor-2 (FGF-2), and may inhibit activation of the transcription factor NF- κB (nuclear factor- κB) (19,20). FGF-2 and NF- κB are relevant targets for anticancer drugs. FGF-2, a regulatory peptide secreted from cells, is involved in a variety of biological processes including cell differentiation, cell growth and migration, angiogenesis, and tumor formation (44). Unlike other proteins, FGF-2 lacks the signal peptide sequence required for export from the cell by the endoplasmic reticulum for

protein secretion (35). Hence, the mechanism of FGF-2 release from the cell was previously believed to require disruption of the cell membrane. It was, however, reported that FGF-2 export occurs through an ATP-dependent pathway (35). Furthermore, investigators have demonstrated that FGF-2 binds to the α_1 -subunit of Na^+ , K^+ -ATPase and have hypothesized that inhibition of this enzyme activity would decrease FGF-2 release from the cell (35,45). Therefore, cardiac glycosides that inhibit Na^+ , K^+ -ATPase activity may also inhibit FGF-2 release from the cell. The findings of Newman's study support the hypothesis that cardiac glycosides inhibit FGF-2 export from the cell in a concentration- and time-dependent manner (19).

NF- κ B regulates the expression of various genes that play critical roles in apoptosis, viral replication, tumorigenesis, various autoimmune diseases, and inflammation (46). NF- κ B seems to be an ideal target for novel anticancer drugs. Activation of NF- κ B has been shown to block apoptosis, promote proliferation, and to induce resistance to chemotherapeutic agents (47,48).

More recently, it was found out that the Na^+ , K^+ -ATPase might also play a role in the regulation of cell growth and expression of various genes (49,50). It was only newly discovered that cardiac glycosides might affect cells at the concentrations lower than that required for the inhibition of the sodium pump (51). Dmitrieva suggested that the Na^+ , K^+ -ATPase might act as a cell signaling receptor activated by a cardiac glycosides binding (51). It is thought that this signaling may influence cytoskeletal reorganization as well as cell survival, its growth and differentiation (49,51). This pathway is still unresolved form of cardiac glycosides action. Interestingly, studies have recently shown that the Na^+ , K^+ -ATPase pump is also involved in membrane transport of selected cellular proteins and cationic substances important to tumor cell growth (35).

These all findings provide novel insight into the mechanism of action of cardiac glycosides and raise new questions regarding functions of these compounds in the cell.

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