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Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials

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SUMMARY

Cardiac glycosides have a long history in the treatment of cardiac disease. However, several preclinical studies as well as two phase I studies have shown that cardenolides may also possess anticancer effects. The mechanisms of these anticancer effects may include intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}; intracellular acidification; inhibition of IL-8 production and of the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway. To date three cardiac glycosides have been developed for treatment of cancer and were tested in a phase I clinical trial to determine dose-limiting toxicities and maximum tolerated dose. Future studies of this novel class of anticancer drugs are warranted to determine their possible role in cancer treatment.

INTRODUCTION

Cardiac glycosides have been used in the treatment of cardiac disease for more than 200 years and were already known to the ancient Egyptians over 3000 years ago.[1] Cardiac glycosides contain a common molecular structure comprised of a steroid nucleus, an unsaturated lactone ring at the C-17 position, and one or more glycosidic residues at the C-3 position.[2,3] Chemically, cardiac glycosides can be divided into two groups: cardenolides and bufadienolides. Cardenolides contain a lactone ring of five members and bufadienolides are characterized by a 6-membered unsaturated lactone ring.

Common cardenolides include digoxin, digitoxin, digitoxigenin, lantoside C and ouabain (Figure 8.1). From a therapeutic point of view, the most important cardiac glycosides are digoxin and digitoxin as they are both used for the treatment of cardiac congestion and some types of cardiac arrhythmias, such as atrial fibrillation.

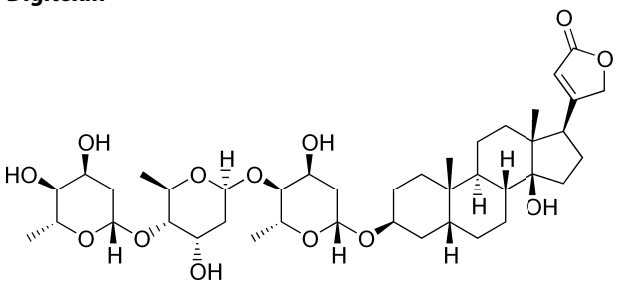
A variety of reports suggested that cardiac glycosides may have anticancer properties. In the 1960s clear inhibition of malignant cells of cardiac glycosides *in vitro* was reported. Almost two decades later, observation of the altered morphology of breast cancer cells from women on digitalis by Stenkvist *et al*. showed more benign characteristics than cancer cells from control patients not on digitalis.[4,5] Stenkvist *et al*. also showed that 5 years after the mastectomy, the recurrence among patients not taking digitalis was 9.6 times that in patients taking digitalis.[6]

In this manuscript, we will give an overview of the possible mechanisms involved in the anticancer activity of cardiac glycosides and discuss their early development in cancer therapeutics.

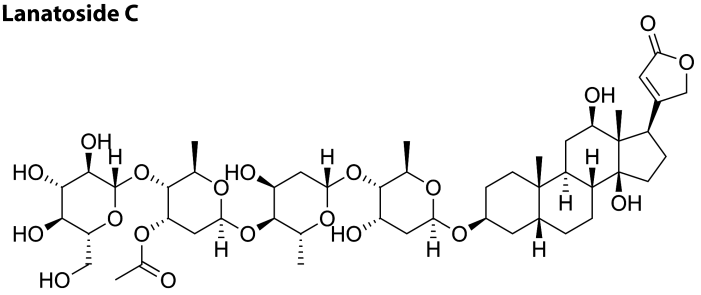
POSSIBLE CYTOTOXIC MECHANISMS OF ACTION

It is well known that cardiac glycosides, such as digitoxin, inhibit the activity of the Na^+/K^+-ATPase (also known as the Na^+ pump or Na^+/K^+ pump). This pump is a transmembrane enzyme that acts as an electrogenic ion transporter in the plasma membrane of all mammalian cells. Each cycle of the Na^+/K^+-ATPase activity extrudes three Na^+ from the cell, moves two K^+ into the cell and utilizes one ATP. The primary role of the Na^+/K^+-ATPase is therefore, to maintain high intracellular K^+ and low intracellular Na^+. This pump also has an important role in regulating cell volume, cytoplasmic pH and Ca^{2+} levels through the Na^+/H^+ and Na^+/Ca^{2+} exchangers, respectively, and in driving a variety secondary transport processes

Digitoxin



Lanatoside C



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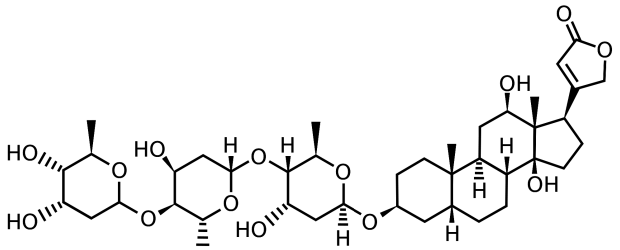
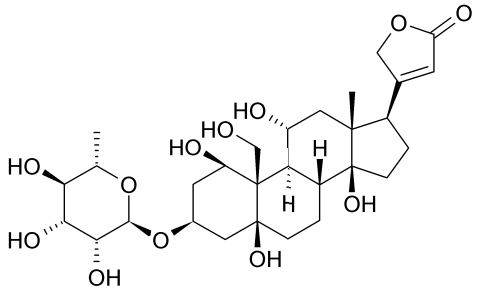


Ouabain



Digitoxigenin

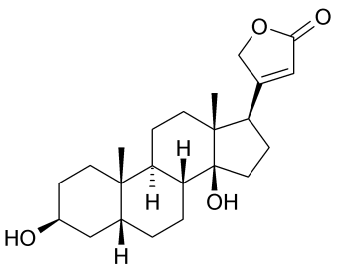


Figure 8.1 Chemical structures of common cardenolides.

such as Na^+ dependent glucose and amino acid transport.[7,8] Inhibiting Na^+/K^+-ATPase by cardiac glycosides leads to higher levels of intracellular Ca^{2+}, which leads to a decrease in heart rate and an increase in contractility of the heart. However, the decrease in intracellular K^+ and increase in intracellular Na^+ and Ca^{2+} following inhibition of the Na^+/K^+-ATPase may also induce apoptosis.[9-14] Inhibition of the Na^+/K^+-ATPase by digitoxin and subsequent increase in intracellular Ca^{2+} led to the induction of apoptosis of prostate cancer cells.[15,16]

Besides inducing apoptosis by intracellular decrease of K^+ and of Na^+ and intracellular Ca^{2+}, cytotoxic mechanisms of action include intracellular acidification; inhibition of IL-8 production and the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway (Figure 8.2). Whether the Na^+/K^+-TPase is the primary target of cardiac glycosides or not is actually a matter of intense debate.[17]

Intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}

Inducing apoptosis by excessive K^+ efflux and intracellular K^+ depletion are early key steps in apoptosis.[9] Physiological concentration of intracellular K^+ acts as a repressor of apoptotic effectors. Loss of cellular K^+, a common event in apoptosis of many cell types, may trigger the apoptotic cascade including caspase cleavage, cytochrome c release, and

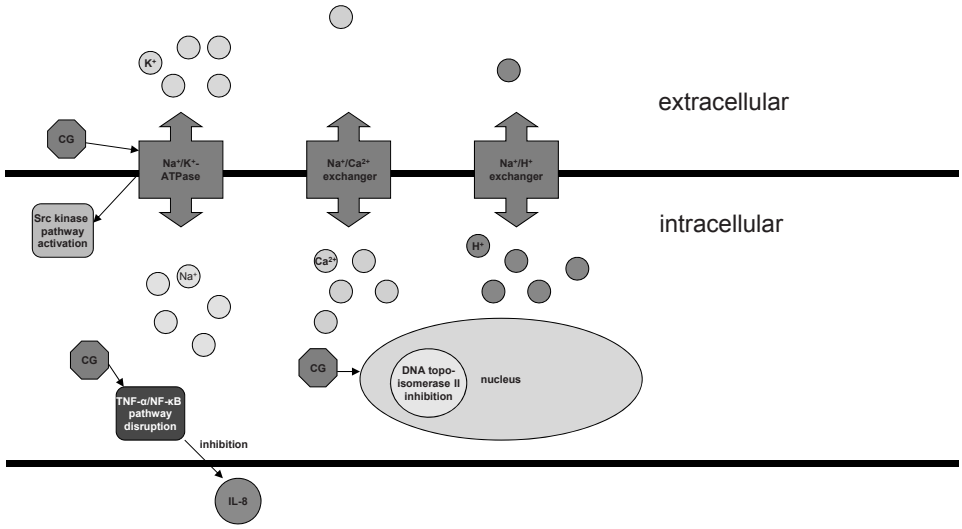


Figure 8.2 Proposed mode of action of cardiac glycosides. Cardiac glycosides (CG) induce apoptosis by intracellular decrease of K^+ and of Na^+ and intracellular Ca^{2+}. Other cytotoxic mechanisms of action include intracellular acidification; inhibition of IL-8 production and the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway.

endonuclease activation. Pro-apoptotic disruption of K^+ homeostasis can be mediated by over-activated K^+ channels or ionotropic glutamate receptor channels, and most likely, accompanied by reduced K^+ uptake due to dysfunction of Na^+/K^+-ATPase. Studies indicate that also mitochondrial K^+ channels and K^+ homeostasis play important roles in apoptosis.[9-11]

During apoptosis, there is compelling evidence indicating an early increase in intracellular Na^+ followed by a decrease in both intracellular K^+ and Na^+ suggesting a regulatory role for these cations during both the initial signaling, and the execution phase of apoptosis. Studies have shown that the Na^+/K^+-ATPase is involved in controlling perturbations of Na^+ and K^+ homeostasis during apoptosis.[14]

Also cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartmentalization, can cause cytotoxicity and trigger either apoptotic or necrotic cell death.[15]

Intracellular acidification

Published data suggests that intracellular alkalinisation can produce malignant transformation.[18-25] It is also suggested that alkalinisation may be required for the development and maintenance of the transformed phenotype cancer cells and may be implicated in key cancer related processes.[18-25] In contrast, it has been observed that intracellular acidification can induce apoptosis in cancer cells and play an important role in the induction of apoptosis by different stimuli.[24,26-32] For example, Rich *et al*. demonstrated that apoptosis of leukemic cells accompanies reduction of intracellular pH after targeted inhibition of the Na^+/H^+ exchanger.[24] Moreover stress-activated protein kinase pathway activation and mitochondrial-derived hydrogen peroxide acts as an effector mechanism leading to induction of apoptosis by intracellular acidification.[26,27]

These observations indicate that induction of intracellular acidification possesses anticancer effects. Interestingly, cardiac glycosides induce intracellular acidification in cancer cells as the inhibition of the Na^+/K^+-ATPase may increase intracellular concentrations of Na^+, reduce the activity of the Na^+/H^+ exchanger and trigger intracellular acidification.

Inhibition of IL-8 production and the TNF-α/NF-κB pathway

Inhibition of IL-8 production and the TNF-α/NF-κB pathway is another mechanism of cardiac glycosides to produce anticancer effects. As production of IL-8 has been associated with important processes involved in tumor progression such as apoptosis resistance, angiogenesis or metastasis, inhibition of its expression is therefore thought to produce

anticancer effects.[33-35] Juncker *et al*. demonstrated that the hemi-synthetic cardenolide UNBS1450 leads to inhibition of IL-8 synthesis via NF-κB pathway disruption leading to apoptotic cell death.[36] Srivastava *et al*. showed similar results for digitoxin[37] whereas Yang *et al*. demonstrated that cardiac glycosides were potent blockers of the TNF-α/NF-κB pathway, which results in apoptosis, as NF-κB induces the expression of genes that are inhibitors of apoptosis.[38]

Inhibition of DNA topoisomerase II

Recently published data suggest that digitoxin may inhibit topoisomerase II. Because of their central role in DNA replication, transcription and repair processes, topoisomerase II inhibitors are a category of drugs commonly used in the treatment of malignancies by inducing apoptosis.[39,40] López-Lázaro *et al*. demonstrated that a renal adenocarcinoma cancer cell line was hypersensitive to digitoxin and died by apoptosis. *In vitro* experiments showed that digitoxin induced levels of DNA-topoisomerase II cleavable complexes comparable to etoposide, a topoisomerase II poison widely used in cancer chemotherapy. Cells exposed to digitoxin for 30 min showed low but statistically significant levels of DNA-topoisomerase II cleavable complexes; however these complexes disappeared after 24 h exposure.[39] The same research group also showed that digitoxin, at concentrations commonly found in the plasma of cardiac patients, significantly reduced etoposide and idarubicin-induced topoisomerase II cleavable complexes in leukemia cells.[40] Also other cardiac glycosides, such as ouabain, digoxin, proscillaridin and bufalin, have shown to inhibit topoisomerase II.[41,42] Bielawski *et al*. demonstrated that digoxin, ouabain and proscillaridin A exerted significant inhibitory effects on the proliferation of breast cancer cells. Of the two cardiac glycosides, proscillaridin A was more effective at inhibiting the proliferation of breast cancer cells than digoxin or ouabain.[41] Hashimoto *et al*. showed that bufalin caused a marked decrease in the steady-state level of topo II alpha mRNA in human leukemia cells, which led to a decrease in the amount and activity of the enzyme and to the induction of apoptosis.[42]

Activation of the Src kinase pathway

Multiple studies have established that the binding of cardiac glycosides to Na^+/K^+-ATPase not only inhibits the ATPase activity but also stimulates protein tyrosine kinases such as Src. This process is the consequence of an additional function played by Na^+/K^+-ATPase besides its control of ionic cellular homeostasis, which is already the trigger of complex intracellular signalization pathway forming a signalosome. Accordingly, pools of non-

pumping Na^+/K^+-ATPase are localized in plasma membrane caveolae, where it clusters with other plasma membrane proteins and receptors, including growth factor receptors (i.e., the epidermal growth factor receptor EGFR).[43] Binding of Na^+/K^+-ATPase by cardiac glycosides may in turn unleash several kinase-dependent cascades, which are implicated in cell proliferation. Activated Src in turn transactivates EGFR, resulting in the assembly and activation of multiple signaling cascades controlled by the extracellular signal-regulated kinase (ERK)1/2 and phospholipase C-γ/protein kinase C pathways.[44] Liang *et al*. suggested that cells contain a pool of Src-interacting Na^+/K^+-ATPase that not only regulate Src activity but also serve as receptors for ouabain to activate protein kinases.[44] One year before, in 2005, Kometiani *et al*. showed in breast cancer cell lines that ouabain-induced cell growth inhibition may be mediated by activation/transactivation of Src/EGFR by Na^+/K^+-ATPase, which leads to activation of ERK1/2, increase in the levels of the cell cycle inhibitor $P21^{Cip1}$ and subsequent growth arrest.[45] Kometiana *et al*. also demonstrated that digoxin and digitoxin concentrations close to or at therapeutic plasma levels had effects both on proliferation and ERK1/2 similar to those of ouabain, supporting the proposed potential value of digitalis drugs for the treatment of breast cancer.[45] The existence of signalosomes where Na^+/K^+-ATPase plays a non-ionic activity has highlighted an endogenous activity of cardiac glycosides. Ouabain is endogenously produced[46] and circulating in the plasma, it acts in a paracrine/endocrine fashion and its levels are considered critical to determine several physio-pathological responses.[47-49] Interestingly, these endogenous biological effects correlate with a complex signaling cascade involving kinases.[50] The discovery of these non-canonical functions has very recently suggested a role for Na^+/K^+-ATPase as hormone receptor.[51] Altogether, these findings suggest in a very next future important hints in the elucidation of anticancer effects ascribed to cardiac glycosides and help in the explanation of preventive effects observed in patients under treatment with digitalis especially towards forms of hormonal cancer.

IMPACT OF CARDIAC GLYCOSIDES ON CANCER CELLS

Cardiac glycosides exert anti-proliferative and cytocidal effects on different cancer cell models.[17,52] Their ability to impair cancer cell viability represents a main hallmark of their anticancer activities. Nevertheless, multiple types of cell death are triggered by cardenolides and bufadienolides. The induction of apoptosis has been frequently reported. Both extrinsic and intrinsic apoptosis pathways were triggered. Moreover, the sensitization to other therapeutic agents has been also described. In a consistent number of reports, cardiac

glycosides led to the accumulation of cells essentially in the S phase[53,54] and G2/M[55-58] phase. This event has been correlated to the elicitation of intracellular reactive oxygen species.[55,57] Besides, in adherent cancer cell models, cardiac glycosides have been shown able to activate an autophagic cell death.[17] This dual cytocidal ability underlines the promising use of cardiac glycosides especially for the treatment of those forms of cancer that are resistant to agents inducing apoptosis. Nevertheless, the mechanisms determining the kind of cell death accomplished upon treatment with cardiac glycosides remain still unclear and debated. One possibility is that sustained autophagy may be commonly activated as a first response by the cells followed by a switch to apoptosis in cancer cells prone to activate programmed forms of cell death. In contrast, autophagic cell death may be undertaken as a kind of final backup cell death modality whenever apoptosis cannot take place. This hypothesis implies that cardiac glycosides may induce stress conditions that potentially lead to alterations of metabolic activities. Finally, very recently clinically used cardiac glycosides, as digoxin and digitoxin, have been shown to induce immunogenic cell death.[59] Interestingly, among the parameters determining immunogenic cell death is the autophagy-dependent secretion of ATP.[60]

OBSERVATIONAL STUDIES

In the last decades observational studies have shown that digitalis may have an anticancer effect. In 1979, Stenkvist *et al*. reported that breast cancer cells from patients while taking digitalis for chronic heart disease were smaller and more uniform in morphology than breast cancer cells not exposed to cardiac glycosides.[5] Also the tumor mass was smaller at diagnosis in patients taking digitalis compared to patients not taking digitalis. The risk of recurrence was 9.6 times higher in the group of patients who were not taking digitalis.[6] Later, Goldin *et al*. conducted a retrospective trial of 127 cancer patients. They found only one cancer death (of a total of 21 deaths) within patients taking digitalis, suggesting that the use of cardiac glycosides may also prevent the development of cancer.[61]

Two large case control studies could nevertheless not show a significant protective benefit.[62,63] The authors of the large case-control study in Norway concluded that elevated morbidity and mortality in the digitoxin population disturbed efforts to isolate eventual anticancer effects of digitoxin.[62]

However, in 2008, Ahern *et al*. suggested in their case control study that digoxin treatment moderately increases the risk of invasive breast cancer among postmenopausal women instead of reducing it.[64]

PRECLINICAL STUDIES IN CANCER

The unusual species-dependent sensitivity to growth inhibition of cardiac glycosides across a broad spectrum of tumor cells is the reason for the paucity of animal data.

In the past decade there has been a substantial increase in the number of *in vitro* and *in vivo* studies regarding the effects of cardiac glycosides on the growth of human malignant tumor cells. In 1967 Shiratori already reported about the growth inhibitory effect of cardiac glycosides on neoplastic cells[65] and many research reports followed.

CARDIAC GLYCOSIDES IN PHASE I CLINICAL TRIAL

To date, there are three cardiac glycosides or derivatives that have been developed for treatment of cancer and were assessed in a phase I clinical trial. The initial product was Anvirzel™, an aqueous extract of Nerium oleander, the second was PBI-02504, a super critical CO_2 extract of Nerium oleander and the third UNBS1450, a semisynthetic cardenolide derivate of 2″-oxovoruscharin extracted from Calotropis procera, a desert shrub.[36,52]

In 2000, Manna *et al*. demonstrated that oleandrin inhibits the activation of NF-κB and AP-1 and their associated kinases.[66] Smith *et al*. showed that Anvirzel™, like oleandrin, inhibits fibroblast growth factor (FGF)-2 export *in vitro* from prostate cancer cells in a concentration- and time-dependent fashion and may, therefore, contribute to the antitumor activity of this treatment for cancer.[67]

Based on these preclinical data, a phase I study started and Mekhail *et al*. reported in 2006 the results of this study of Anvirzel™.[68] The study reported a phase I trial to determine the maximum tolerated dose (MTD) and safety of Anvirzel™ in 18 patients with advanced, refractory solid tumors. Patients were randomized to receive this agent by intramuscular injection at doses of 0.1, 0.2 and 0.4 ml/m^2/day with subsequent patients receiving 0.8 or 1.2 ml/m^2/day sequentially. Eighteen patients were enrolled and completed at least one treatment cycle of 3 weeks. Most patients developed mild injection site pain (78%). Other toxicities included fatigue, nausea, and dyspnea. Traditional dose-limiting toxicity was not seen, but the MTD was defined by injection volume as 0.8 ml/m^2/day. No objective antitumor responses were seen. They concluded that Anvirzel™ can be safely administered at doses up to 1.2 ml/m^2/day, with the amount administered intramuscularly limited by volume. The recommended phase II dose level is 0.8 ml/m^2/day.

PBI-05204 has recently completed testing for safety in Phase I clinical trial.[69] The publication of conclusions is in process and the initial findings were presented at the annual meeting of the American Society of Clinical Oncology (ASCO) in June 2011. PBI-05204 (Oleandrin), inhibits the α-3 subunit Na^+/K^+-ATPase pump. Relative expression of the α-3 subunit in tumor cells correlates with proliferation. Oleandrin inhibits FGF-2 export, activation of NF-κB, phosphorylation of Akt, p70S6K and decreases mTOR activity. In this first-in-human study, the authors sought to determine the MTD/recommended phase II dose and to define the pharmacokinetics (PK) and pharmacodynamics (PD) of PBI-05204 in advanced cancer patients. Forty-six patients were dosed at 8 dose levels (DL) of PBI-05204 (0.6 to 10.2 mg/day). Two dose-limiting toxicities occurred at DL 8 (grade 3 proteinuria, fatigue) thus the MTD was DL 7. Most common adverse events (AEs) were fatigue (56.1%), abdominal pain (41.5%), constipation (41.5%), nausea (41.5%), and diarrhea (39.0%). Cardiac disorders were reported in 10 patients (24.4%), all grade 1, except for one patient with grade 2 supraventricular tachycardias (SVT). Of the 45 evaluable patients, 7 showed a stable disease for > 4 months, with bladder, colorectal, fallopian tube, breast, appendical and pancreatic carcinoma (2 patients). They concluded that PBI-05204 is well tolerated up to 10.2 mg/day with very little AEs or cardiotoxicity.

UNBS1450, has also been tested in an open-label, dose escalation study to evaluate the safety, tolerability and pharmacokinetics of this single agent, administered once every 3 weeks in separate cohorts of patients with advanced solid tumors or lymphoma. Chemical modifications of 2″-oxovoruscharin (a novel cardenolide extracted from Calotropis procera) has led to the identification of UNBS1450.[70] The activity of the compound in preclinical cancer models, independent of cell type, has been tested *in vitro* on 57 human cancer models from 11 distinct histological types.[70] In aggressive and metastatic orthotopic NSCLC,[71,72] refractory prostate cancer[73] and glioma[74] models, UNBS1450 was more potent than tested reference compounds, including paclitaxel, irinotecan, oxaliplatin, mitoxantrone and temozolomide.[71-75] UNBS1450 was the most potent inhibitor of all three isozymes (α3β1, α2β1 and α1β1) with a potency ~6 to > 200 times greater than that ouabain (another cardenolide) and digoxin[74] The general mechanism of action associated with UNBS1450-mediated anticancer effects relates to the compound's propensity in disorganizing the actin cytoskeleton and thus non ATPase-mediated effects.[73-75] UNBS1450 can thus be considered both anti-proliferative (cytotoxic) and anti-migratory.[75,76] given that the actin cytoskeleton is essential to cytokinesis and to cancer cell migration.[77] In sharp contrast to digitalis-like cardenolides, UNBS1450 does not induce intracellular Ca^{2+} or Na^+ increase at concentrations at which it induces potent antitumor effects.[74,75] UNBS1450 induces both apoptotic and

non-apoptotic cell death processes depending on the cellular environment. Non-apoptotic cell death mechanisms such as lysosome membrane permeabilisation[71] and autophagy[74] were observed in solid tumors and thus may overcome major apoptosis resistance pathways responsible in part for the failure of therapeutics in certain cancers. Canonical intrinsic apoptosis was demonstrated by Juncker *et al*. in leukemia and lymphoma cellular models with an early degradation of anti-apoptotic Mcl-1, Bak and Bax activation leading to cytochrome C release, caspase-9, -7 and -3.[36] Experimental data involving NF-κB inhibition/deactivation evidenced it as an important new approach to the treatment of various malignancies was shown by the same authors.[36] UNBS1450 deactivates the cytoprotective NF-κB pathways at several points, in sharp contrast to specifically designed NF-κB inhibitors acting at one precise point.[72] In leukemia cells, UNBS1450 inhibits degradation of the IκB inhibitor of p50/p65 NF-κB heterodimers thus preventing transcription factor translocation in the nucleus. Using genomic and proteomic approaches, it was possible to evidence UNBS1450-mediated down-regulation of c-MYC gene, MYC oncoprotein-related genes, and genes with nucleolar functions.[15] UNBS1450-induced marked down-regulation of c-MYC expression in a number of human cancer cell lines lead to nucleolar disorganization resulting in impairment of cancer cell survival.[15] Unfortunately the phase I study was closed in 2011 by the sponsor because of bankruptcy before reaching the MTD after including 23 patients. Preliminary data will be published elsewhere.

CONCLUSION

Cardiac glycosides have a long history in the treatment of cardiac diseases, but several preclinical studies have shown that cardenolides have also anticancer effects. Two cardiac glycosides, Anvirzel™ and PBI-02504, completed testing for safety in a phase I clinical trial. Another phase I trial with UNBS1450 was closed early. Several mechanisms seem to participate in these anticancer effects. Additional in-depth preclinical research is required to find out the possible role for cardiac glycosides as primary anticancer agents as well as bona fide biological markers. As the pharmacological and safety profile of compounds like digitoxin is well known future clinical investigations should be accelerated.[78]

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