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From Na^+/K^+-ATPase and Cardiac Glycosides to Cytotoxicity and Cancer Treatment

Petr Babula[1], Michal Masarik[2], Vojtech Adam[2], Ivo Provaznik[1] and Rene Kizek[2,*]

[1]*International Clinical Research Center, Center of Biomedical Engineering, St. Anne's University Hospital Brno, CZ-656 91 Brno, Czech Republic, European Union;* [2]*Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, CZ-613 00 Brno, Czech Republic, European Union*

Abstract: The cardiac glycosides are a group of compounds isolated from plants and some animals. They have been used in therapy for heart failure for many years. The cytotoxic effect of many cardiac glycosides has been demonstrated, but the mechanism of action is very complicated and complex, and Na^+/K^+-ATPase surely plays a crucial role in it. On the other hand, Na^+/K^+-ATPase is regulated by many endogenous factors, such as hormones or FXYD proteins, whose role in regulating the cell cycle has been studied intensively. This review focuses on the role of Na^+/K^+-ATPase in regulating the cell growth, the cell cycle and the cell proliferation and on the involvement of cardiac glycosides in regulating Na^+/K^+-ATPase. The cytotoxic effect of cardiac glycosides is discussed with respect to the apoptotic mechanisms possibly induced by these compounds. Novel strategies in cancer therapy based on cardiac glycosides are discussed as are possibilities for counteracting multidrug resistance by using cardiac glycosides. The aim of this review is to present cardiac glycosides not only as pharmaceuticals used in the management of heart failure, but also as potent cytotoxic agents with potential uses in cancer treatment.

Keywords: Cardiac glycosides, apoptosis, cancer, Na^+/K^+-ATPase, FXYD proteins, cytostatics.

INTRODUCTION

The cardiac glycosides (CGs, also referred to as cardiac steroid glycosides) are a diverse family of naturally derived compounds, C_{23} or C_{24} steroidal glycosides that have been found in many angiosperms. The most important CG-containing plant families are *Apocynaceae*, incl. *Asclepiadaceae* (*Adenium* [1], *Cerbera* [2], *Cryptostegia* [3], *Nerium* [4, 5], *Parepigynum* [6, 7], *Periploca* [8-10], *Strophanthus* [11-15], *Thevetia* [16-22]), *Brassicaceae* (*Erysimum* [23-29], and *Lepidium* [30]), *Celastraceae* (*Euonymus* [31, 32], and *Lophopetalum* [33]), *Convallariaceae* (*Convallaria* [34-45]), *Crassulaceae* (*Cotyledon* [46], and *Tylecodon* [47, 48]), *Hyacinthaceae* (*Schizobasis* [49], and *Urginea* [50, 51]), *Fabaceae* (*Coronilla* [52, 53]), *Malvaceae* (*Corchorus* [54-56], and *Mansonia* [57, 58]), *Moraceae* (*Antiaris* [59-61], *Castilla* [62], *Maquira* [63-66], and *Naucleopsis* [65]), *Ranunculaceae* (*Adonis* [67-71], *Eranthis* [72], and *Helleborus* [73]), *Scrophulariaceae* s.s. (*Digitalis* [13, 74-91]), and *Solanaceae* (*Nierembergia* [92]). CGs have also been found in some animals, such as members of the genus *Bufo* [7, 8, 15]. Endogenous cardiac glycosides have also been discovered [93, 94], the most important among them being ouabain; digoxin; 19-norbufalin and its peptide derivative; 3β-hydroxy-14α 20:21-bufenolide; proscillaridin A; marinobufagenin; and telocinobufagin (Fig. **1**). They have been found in different human tissues, in some cases related to pathological processes. The structure of the CGs allow two classes of them to be distinguished: to the cardenolides with a five-member lactone ring at the C17 position and the bufadienolides with a six-member lactone ring [95]. The sugar moieties attached to the aglycone by a C-3,β linkage are compounds consisting of one to four units. These units include glucose, rhamnose, and such deoxysugars as digitoxose and cymarose, which have been found only in this group of secondary metabolites (Fig. **1**). Especially, the sugar moiety at the C_3 position of the steroidal skeleton affects the pharmacological and pharmacokinetic properties of the cardiac glycosides.

*Address correspondence to this author at the Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Tel: +420-5-4513-3350; Fax: +420-5-4521-2044; E-mail: kizek@sci.muni.cz

PHARMACOLOGY AND USAGE OF CARDIAC GLYCOSIDES IN CONVENTIONAL THERAPY

The first plant introduced into Western medicine was foxglove (*Digitalis purpurea* L.), which was used by William Withering in 1785 to treat dropsy. The mechanism of action of the cardiac glycosides is based on binding and inhibiting Na^+/K^+-ATPase in the cardiac myocyte membrane. This increases the intracellular concentration of Na^+ and subsequently reduces the extrusion of calcium [96-100]. An increased concentration of calcium in the cytoplasm increases the uptake of calcium by the sarcoplasmic reticulum (SERCA2 transporter), which can finally cause increased contraction [101, 102]. On the other hand, an elevated concentration of Na^+ compromises the mitochondrial energetics and redox balance by blunting the mitochondrial accumulation of Ca^{2+}, thereby contributing to a possible cytotoxic effect of the CGs [103]. Cardiac glycosides have been used clinically for many years to treat heart failure and atrial arrhythmias [104-111]. The medicinally most important cardiac glycosides, which have been or still are used therapeutically are digoxin, digitoxin, lanatoside A, lanatoside C (*Digitalis lanata* Ehrh., *D. purpurea* L.), and thevetin (*Nerium oleander* L.). However, CGs are known to increase the levels of reactive oxygen species (ROS), which contribute to arrythmogenesis through the redox modification of cardiac ryanodine receptors [112, 113]. ROS may play a role in the cytotoxicity of CGs [114]. Direct blocking of the cardiac potassium channel hERG by CGs is another pro-arrytmogenic factor [115, 116].

Na^+/K^+-ATPASE

Na^+/K^+-ATPase is an integral membrane protein present in all mammalian cells (Fig. **2**). It transports Na+ and K+ ions across the plasma membrane, and is necessary for maintaining the electrochemical gradient which is important in the processes of electrical excitation and the transport of other ions. Na^+/K^+-ATPase is a heterodimer composed of two subunits. The alpha subunit, a catalytic subunit with 10 trans-membrane segments, couples ATP hydrolysis with ion transport. The beta subunit, with one trans-membrane segment, is involved in the processes of the structural and functional maturation of the enzyme and in trafficking to the plasma membrane [117]. Na^+/K^+-ATPase usually also contains

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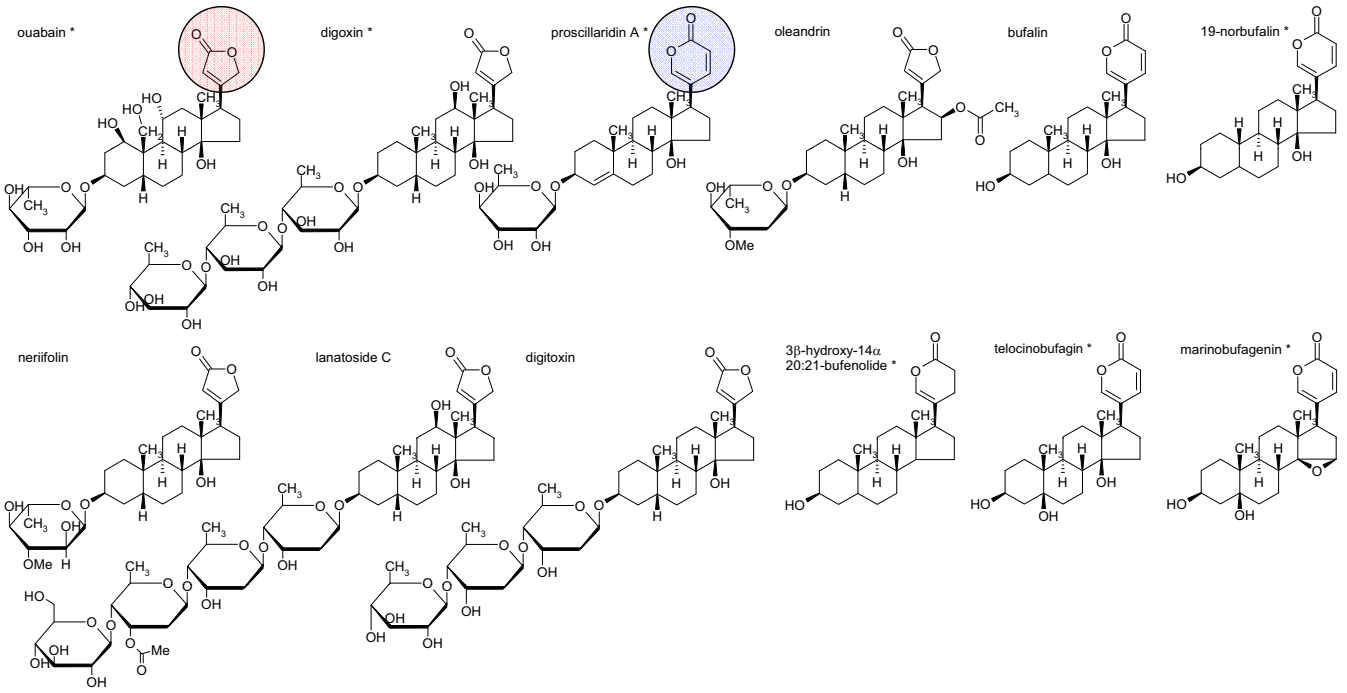

Fig. (1). Chemical structures of selected cardiac glycosides. * indicates endogenous presence in human tissues. The five-membered lactone ring of the cardenolides is indicated in a red circle, the six-membered lactone ring of bufadienolides is indicated in a blue circle.

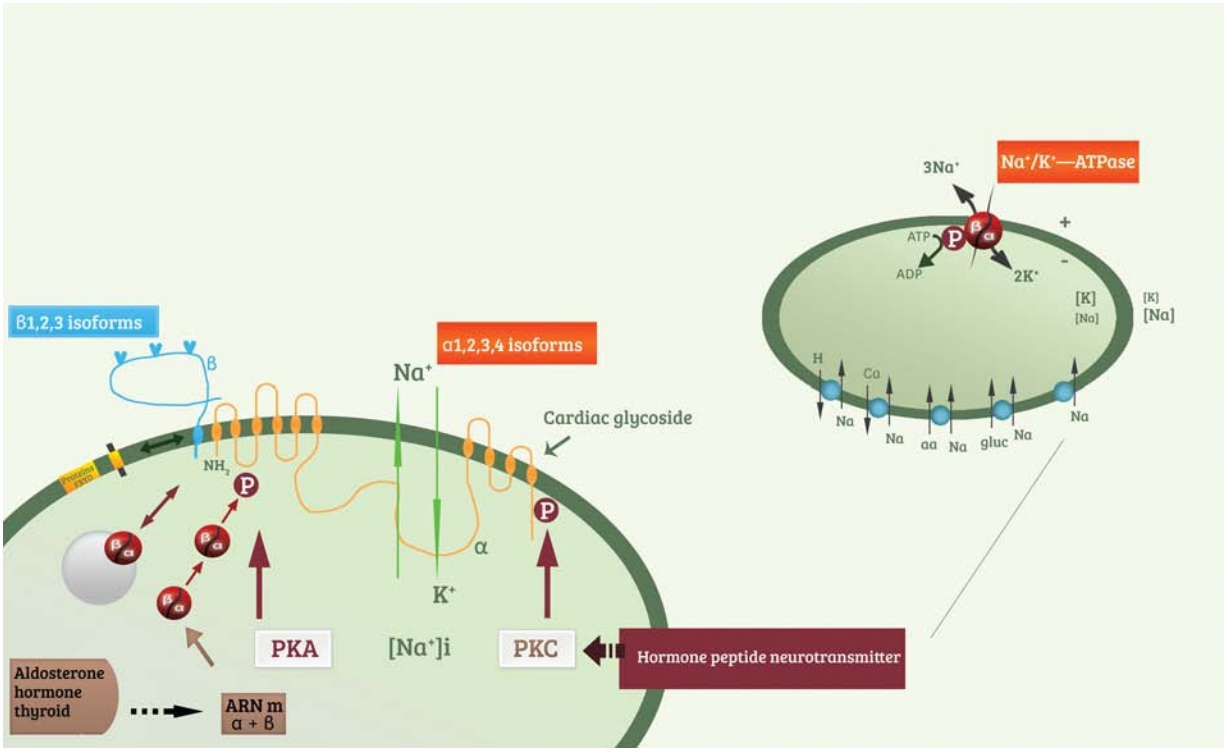

Fig. (2). Structure and regulation of Na^+, K^+-ATPase. Na^+-K^+-ATPase is a ubiquitous protein that carries two K^+ ions into the cell in exchange for three Na^+ ions, using the energy of ATP hydrolysis. Na^+, K^+-ATPase maintains the gradients of Na^+ and K^+ between the extracellular and intracellular space. The Na^+ gradient produces energy for the activity of the secondary transporters necessary for cellular homeostasis. Na^+, K^+-ATPase is composed of an α subunit with 10 transmembrane segments and a β subunit. The α subunit is the catalytic subunit that hydrolyzes ATP, transports cations, and binds cardiac glycosides. The β subunit is a glycoprotein that has functions as a molecular chaperone for the insertion of the α subunit into the membrane. Four isoforms α and 3 isoforms β are expressed in a tissue-specific manner and can form 12 different Na^+, K^+-ATPase isozymes with different transport properties. The expression of Na^+-K^+-ATPase on the cell surface is regulated by neurotransmitters or peptide hormones that activate protein kinase A (PKA) or protein kinase C (PKC). Na^+, K^+-ATPase is phosphorylated, which modulates the distribution of Na^+, K^+-ATPase between the plasma membrane and intracellular compartments. Furthermore, aldosterone and thyroid hormone influence the transcription of the genes encoding subunit α and β and produce an increase in Na^+, K^+-ATPase at the cell surface. Finally, the transport activity of Na^+, K^+-ATPase is modulated by tissue-specific interaction with members of the FXYD protein family. gluc: glucose, aa: amino acid.

an auxiliary subunit of the FXYD protein family. The alpha subunit also contains a functional site for cardiac glycoside inhibitors. Four alpha and three beta isoforms are expressed and regulated in tissue- and development-specific manners [118]. Some factors have been identified as modulators of Na^+/K^+-ATPase activity (Fig. **2**); the most important of these are the FXYD proteins. The distribution of the individual Na^+/K^+-ATPase subunits is probably regulated by hormones. Treatment of hypothyroid rats with T-3 increased the relative abundance of both alpha 1 and beta 1 subunits in the total membranes and led to a 1.9-fold increase in enzyme activity. Na^+/K^+-ATPase uses energy from the hydrolysis of ATP to drive the movement of K^+ ions into cells and exchange them for Na^+ ions. This process also transports other solutes, amino acids, sugars, and phosphates. The homeostasis of these ions is crucial in the processes of cell cycle regulation, cell proliferation, and apoptosis.

On the other hand, some hormones are able to affect processes closely connected with the activity and conformation of Na^+/K^+-ATPase [119], e.g., aldosterone [120], thyroid hormones [119, 121], glucocorticoids [122], catecholamines [123], and insulin [124]. But cAMP [125] also affects the expression of Na^+/K^+-ATPase in different tissues. It regulates the promoter activity of the alpha four isoform [126, 127], which is of great importance in light of the recently found role of Na^+/K^+-ATPase expression in regulating cell growth and proliferation [128]. In addition, Na^+/K^+-ATPase serves as a signal transducer. The above-mentioned hormones are also responsible for the translocation of Na^+/K^+-ATPase. Moreover, the changes in some hormonal systems are quite rapid, reflect both the endogenous and exogenous conditions. These changes are also involved in modifying the function of Na^+/K^+-ATPase, its eventual translocation, and changes in the promoter activity. In addition to the above-stated facts, the endogenous cardiac glycosides that have been described and will be discussed with respect to the regulation of the cell cycle in the following subchapter, are also involved in regulating the Na^+/K^+-ATPase activity. The regulatory role of hormones probably consists not only in inhibiting this enzyme, but also regulating the activation of signaling pathways, possibly by changing the conformation of the enzyme. It has been established that ouabain binds the phosphorylated E_2P conformation of Na^+/K^+-ATPase (E_2P-ouabain) with great affinity. This is followed by phosphorylation of the thyrosine 418 of Src kinase, which is required if the full catalytic activity of Src kinase is to be obtained. This activated form can enter signaling pathways. ADP as well as reduced level of ATP proved to have an inhibitory effect on the phosphorylation of Src, so the ATP/ADP ratio determines the extend of Src activation [129]. In the conclusion, Na^+/K^+-ATPase inhibits Src kinase, which suggests a possible role for endogenous cardiac glycosides in regulating the cell cycle.

CYTOTOXICITY OF CARDIAC GLYCOSIDES AND MECHANISMS OF ITS ACTION

Exciting recent findings have suggested additional signaling modes of action of Na^+/K^+-ATPase that implicate the cardiac glycosides in the regulation of several important cellular processes and highlight new therapeutic roles for these compounds in various diseases. During the past three decades, it has been suggested that cardiac glycosides inhibit cell proliferation and possess valuable cytotoxic activity against different tumour cell lines, usually by inducting apoptosis [63, 130-136]. Interferences between GCs and the corresponding transporters may be responsible for the toxicity of the GCs. The mechanisms of the cytotoxic action of the CGs have been widely discussed. The most important cytotoxic mechanisms of the CGs have been revealed as follows: i. disturbance of the Na^+, K^+, and Ca^{2+} homeostasis; ii. inhibition of proteosynthesis and DNA synthesis; iii. generation of reactive oxygen species (ROS); iv. changes in the fluidity of the biomembranes and the resultant influence on the integrity of the cell, v. inhibition of DNA toposiomerases I and II; and vi. modulation of the N-glycosylation. The above-mentioned cytotoxic mechanisms of CGs are not the only mechanisms studied in relation to the toxicity of CGs. Some CGs, namely digoxin, strophanthidin, and digoxigenin, have been found by Hundeshagen *et al*. to enhance autophagy, and autophagic flux[137]. Bufalin induces autophagy-mediated cell death in human colon cancer cells [138]. The role of autophagy and related signaling pathways in two human NSCLC cell lines, A549 and H460, upon treatment with the representative cardiac glycosides digoxin and ouabain, has been studied by Wang *et al*. [139]. The authors identified autophagy as a mechanism involved in the toxicity of both CGs. AMPK-mediated down-regulation of mTOR signaling, along with ERK1/2 activation, was observed to play a pivotal role in the autophagy induced by the chosen CGs. Similar results were obtained by Tsai *et al*., who studied the mechanism of autophagy in SK-HEP-1 human hepatocellular carcinoma cells induced by bufalin [140]. An overview of the most important mechanisms of the cytotoxicity of the CGs is presented in Fig. (**3**).

The inhibition of Na^+/K^+-ATPase leads Na^+ and Ca^{2+} ions to accumulate in tumour cells and reduce the membrane potential and the intracellular levels of K^+ [141]. It has been established that cytosolic Ca^{2+} signals regulate the mitochondrial proton gradient [142]. The depletion of ATP after exposure to the cardiac glycoside UNBS1450 has been studied by Lefranc and Kiss [143]. Depletion of ATP potentiates induction of the mitochondrial permeability transition [144]. It leads to failure of the Ca^{2+} homeostasis which in turn causes the mitochondrial permeability transition, changes in the energy metabolism, and eventually the death of cell [145]. Increased cytosolic calcium levels signal apoptosis *via* a mitochondria-caspase-mediated pathway [146, 147]. An increase in the cytoplasmic level of Ca^{2+} connected with the release of cytochrome c and apoptosis has been demonstrated in poorly metastatic PC3-M-Pro4 and highly metastatic PC-3 M-LN4 cell lines [148]. The reduction of membrane potentials and the increase in intracellular levels of Na^+ under induction of apoptosis by different compounds have been demonstrated in the esophageal cells JH-EsoAd1 and CP-A [149, 150]. All of the above-mentioned modes of apoptosis are based on mitochondria-caspase-mediated pathway. Diverse conclusions are presented in the work of Panayiotidis at al., who investigated the influence of ouabain treatment combined with Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), hydrogen peroxide, thapsigargin or UV-C on the apoptosis of Jurkat cells [151]. Only ouabain combined with FasL or TRAIL potentiated apoptosis. The authors thereby demonstrate that the role of impaired Na^+/K^+-ATPase activity during apoptosis is linked to the homeostasis of Ca^{2+} that modulates apoptosis as the Fas ligand activates the Fas receptor [151]. This inference is supported by the work of Winnicka *et al*., who demonstrated the dual effect of the cardiac glycosides ouabain, digoxin, and proscillaridin A on the activation of caspase-3 and apoptosis [152]. Caspase-3 is activated in both mechanisms of apoptosis – the extrinsic (death ligand) and the intrinsic (mitochondrial) pathways. This dual effect of the CGs bufalin and cinobufagin has also been found in hepatocellular carcinoma cells (HCC) [153]. Bufalin has also been shown to down-regulate the expression of heat shock protein 27 (HSP27), an important anti-apoptic protein, in the human osteosarcoma cell lines U2OS and U2OS/MTX300 [154]. The role of heat shock protein 60 (HSP60) in the digoxin-induced apoptosis of HUVEC cells has been studied by Qiu *et al*. [155]. These heat shock proteins protect cells against stress and help them to survive stress conditions. They represent important regulators of apoptosis in both normal and tumour cell lines [156, 157]. Treatment with olendrin facilitated the activation of Fas by the translocating the nuclear factor of activated T-lymphocytes (NF-AT) to the nucleus and expressing the NF-AT gene products that serve as FasL as described in the work of Raghavendra *et al*. [141]. An increased intracellular level of Ca^{2+} may lead to the activation of calcineurin. Calcineurin itself activates

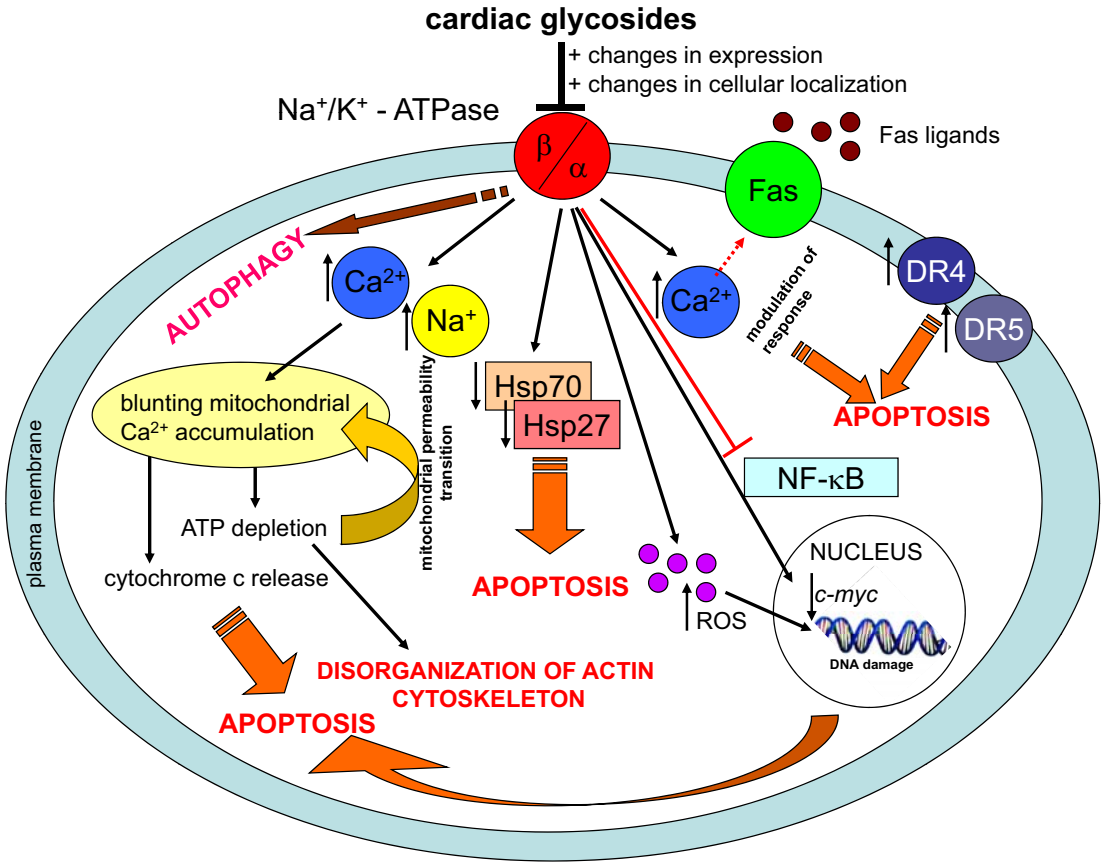

Fig. (3). The most important mechanisms of cytotoxic action of the cardiac glycosides. The first mechanism is based on changes in the homeostasis of Ca^{2+} and Na^{+}. This leads to mitochondrial dysfunction based on blunting the accumulation of mitochondrial Ca^{2+}. Depletion of ATP causes a mitochondrial permeability transition. These processes are completed by the release of cytochrome c and apoptosis. Disorganization of the actin cytoskeleton is induced by the depletion of ATP. Changes in the homeostasis of Ca^{2+} modulate the response of the Fas receptor to Fas ligands. CGs also up-regulate two cell death receptors – DR4 and DR5. CGs down-regulate heat shock proteins that are important in the survival of cells under stress conditions. An increased level of reactive oxygen species (ROS) damages biomolecules, including DNA. The down-regulation of the expression of *c-myc* is among the next mechanisms of CG action. Both the formation of ROS and the down-regulation of *c-myc* lead to apoptosis. The down-regulation of NF-κB and autophagy are the next processes that lead to cell death.

NF-AT, so the path to apoptosis is free [158]. The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) belongs to a TNF family known to transduce death signals *via* cell membrane receptors. The sensitization of non-small lung cancer cells to Apo2L/TRAIL brought about by cardiac glycosides is accompanied by up-regulation of the death receptors 4 and 5 (DR4 and DR5) [159]. Badr *et al*. have identified lanatoside C as sensitizing GBM cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cell death, partly by up-regulating death receptor 5 [160]. In addition, digitoxin and structurally related CGs are able to block the TNF-alpha/NF-kappa B signaling pathway [161]. Considering the fact that NF-kappa B is a nuclear transcription factor involved in the induction of apoptosis, processes of cancerogenesis, and inflammation, these results are promising and may possibly have implications in cancer therapy. These findings are very important in the search for possible new therapies for cancer. The overexpression of the Blc-xL leads to protection against apoptosis mediated by CG oleandrin [158]. Other pharmacological effects of CGs must be considered in specialized cells. The inhibition of brain-located glutamate transporter GLAST is responsible for the potential neurotoxicity of these secondary metabolites [162].

In addition, experiments on prostate cancer cell lines have demonstrated other possible cytotoxic actions of CGs, such as the induction of hypoxia-inducible factor-1 (HIF-1) and due to the increased cytoplasmic levels of Ca^{2+} also disturbances of the Ca^{2+} signaling [163, 164]. Digoxin has been identified as a potent inhibitor of the HIF-1 alpha signaling pathway in C4-2 castration-resistant prostate tumors at both the mRNA and protein levels. Whereas C4-2 tumours are resistant to the cytotoxic effect of digoxin, the formation of new blood vessels is significantly inhibited [165]. Zhang *et al*. considered the role of HIF-1 to be the most important of all the mechanisms of GCs cytotoxic action. These authors investigated the effect of digoxin, ouabain, and proscillaridin A on transfected cells with enhanced expression of HIF-1. The xenografts derived from these transfected cells were resistant to the cytotoxic effect of the CGs [166].

Cancer-related processes are connected not only with angiogenesis, but also with tumour invasion and growth. All of these processes are significantly affected by the group of serine proteases called kallikreins (KLK). Cardiac glycosides at relatively low concentrations of 10-50 nmol/L are able to regulate the above-mentioned processes by suppressing the transcription of KLK. However, the mechanism of action remains unknown. A role played by the expression of *c-myc*/*fos* prooncogene has been suggested [167]. Bufalin, a component of the bufadienolides, secondary plant metabolites present in some plants used in traditional medicine, can differentiate many cell lines. It induces apoptosis in HL60 and M1 leukemia cells by down-regulating the expression of the prooncogenes *c-myc* and *bcl-2* [168]. Bufalin also induces G0/G1

phase arrest and down-regulates the protein levels of cyclin D, CDK4, cyclin E, CDK2, phospho-Rb, phospho-AKT, and Bcl-2 while simultaneously up-regulating the expression of the cytochrome c, Apaf-1, AIF, caspases 3, 7, 9, and Sax proteins. Caspase activities were also observed in T24 cells [169]. Similar results have been determined in lung cancer cells [170, 171], human choriocarcinoma cells [172], prostate cancer cells [173], endometrial and ovarian cancer cells [174], gastric cancer cells [175], human osteosarcoma cells [176], and human bladder cancer cells, where the degradation of poly (ADP-ribose) polymerases (PARPs) and the collapse of the mitochondria membrane potential were also shown [177]. In addition, there are other mechanisms for the cytotoxic action of CGs. Significant inhibition of protein synthesis has been demonstrated in colorectal tumor cell lines moderately resistant to the cytotoxic effects of digoxin and digitoxin [178]. This mechanism of action has been proved by the work of Perne *et al*. [179]. The ability of the CGs to generate reactive oxygen species and influence membrane fluidity may represent other mechanisms for the cytotoxicity of CGs [138, 141, 180]. The effect on membrane fluidity probably reflects chemical modification of the membrane. This fact was showed in the work of Gasper *et al.*, who used Fourier transform infrared spectroscopy to investigate the modification of prostate cancer PC-3 lipidome upon exposure to sub-lethal concentrations of ouabain [181]. ROS are responsible for the translocation of Bax from cytosol to mitochondria, the transition of the mitochondrial permeability, and the activation of caspase-3 [180]. The inhibition of DNA topoisomerases I and II represents an important pro-apoptotic mechanism of the cardiac glycosides [132, 182, 183]. This fact has been established for ouabain, proscillaridin A, and digoxin [184]. Conjugates of proscillaridin A and digoxin with the polyamidoamine dendrimer G3-PAMAM-NH2 have been studied intensely as topoisomerase I and II inhibitors [185]. Aberrant N-linked glycans promote the malignant potential of cells by enhancing the epithelial-to-mesenchymal transition and the invasive phenotype. Dihydroouabain is the most potent modulator of N-glycosylation. In addition, the work of Zavareh *et al*. has proved the ability of digoxin to inhibit N-glycosylation-mediated processes of tumor cell migration and invasion [186]. Cardiac glycosides, tumor cell lines, xenograft models, mechanisms of action, and the corresponding references are summarized in Table **1**.

ANTI-APOPTOTIC EFFECT OF CGS AND POSSIBLE MECHANISMS OF ACTION

Despite the above-mentioned facts, low doses of GCs have anti-apoptotic effects. This is connected with the modulation of the subcellular levels of Bcl-2. Winnicka *et al*. have demonstrated this dual effect of three CGs: ouabain, digoxin, and proscillaridin A. While a high concentration (300 nM) led to the activation of caspase-3 and the induction of apoptosis in human fibroblasts, a low concentration of the CGs (30 nM) stimulated anti-apoptotic action by increasing the level of phosphorylated extracellular signal-regulated kinases (P-ERK 1/2) [152]. Similar data have been reported by Xu *et al*., who treated lymphocytic leukemia Jhhan cells and megakaryocytic leukemia M07e cells with ouabain. The concentrations of ouabain that were used (1 nM and 10 nM) promoted cell proliferation [187]. On the other hand, this phenomenon may be implicated in the prevention of cytotoxicity-mediated neuronal injury [188]. However, the chemical structure of the CGs may indicate possible interactions between CGs and estrogen receptors. An estrogenic effect of cardiac glycosides has been proposed and verified in the case of digoxin. Digoxin is able to bind to an estrogen receptor (ER) and can cause gynecomastia. Women using digoxin show an increased incidence of cancers of the breast and uterus, which are usually sensitive to estrogen [189, 190]. Biggar has suggested that digoxin works *via* ER- stimulated cell proliferation of the ductal or acinar cells to accelerate the growth of a nascent cancer [191]. The risk of cancer is minimized when digoxin treatment is stopped [190]. In addition, digoxin and ouabain therapy may lead to the induction of P-glycoprotein (Pgp), a transmembrane transporter that is responsible for extruding some drugs including the important cytostatic drug doxorubicin, as has been demonstrated on the human colon cancer HT29. The mechanism of this process remains almost completely unknown, but it seems that calmodulin kinase II and HIF-1 increase the expression of Pgp. Generally, this fact must be considered if patients are treated with digoxin as it may significantly reduce the pro-apoptotic effect of doxorubicin [192]. However, the connection of HIF-1 with the anti-apoptotic effect is surprising, especially in light of the role of HIF-1 in CG-mediated apoptosis.

CARDIAC GLYCOSIDES IN CANCER TREATMENT

Perhaps most notably, the increased susceptibility of cancer cells to these compounds supports their potential use in cancer therapy. The first generation of glycoside-based anticancer drugs is currently in clinical trials [193]. Accumulating preclinical and clinical data suggest that digitoxin might be used in cancer therapy [194]. Recent publications indicate its applicability for the treatment of prostate cancer [195] or retinoblastoma [196]. Recent reports have shown that digitoxin can inhibit growth and induce apoptosis in cancer cells at concentrations commonly found in the plasma of cardiac patients treated with this drug [194]. However, cytotoxic effect of the CGs significantly differs depending on how it is applied. Whereas orally administered digoxin has no effect on a retinoblastoma, applying it parenterally – intra-arterially –causes a cytotoxic effect [196]. However, it seems that a significant problem that has to be solved consists in achieving therapeutic levels of the CG in the target tissues of the tumor. The inhibition of glycolysis may be a key mechanism by which CGs selectively target cancer cells. This is one of the many possible cytotoxic mechanisms that have been discussed above. Finally, whether or not there is enough evidence to support the clinical evaluation of digitoxin in patients with cancer has been discussed [197]. *In vitro* tests have demonstrated the sensitivity of B-precursor and T-ALL cells to the CG digitoxin. However, further investigation is necessary. Some clinical trials are currently focused on the cytotoxicity and possible uses of cardiac glycosides in cancer treatment. A phase-I trial is testing ouabain for the treatment of acute myeloid leukemia [198]; PBI-05204 based on oleandrin, is in a phase-I trial testing its effect on solid tumours [199], and the combination of erlotinib with digoxin is being used in a phase-II trial with patients suffering from non-small cell lung cancer [200]. It seems that some cardiac glycoside-based compounds will undergo the clinical testing alone or in combination with approved anticancer agents. One example of the latter is promising combination of eribulin mesylate with digoxin [201]. The clinical trials are summarized in Table **2**.

Role of Na^+/K^+-ATPase in Tumor Cells and Its Direction

Changes in Na^+/K^+-ATPase expression (including both down- and up-regulation) have been found in numerous cancer cell lines (Table **3**). The mechanisms responsible for regulating the expression of Na^+/K^+-ATPase and its degradation in both normal and tumor cell lines are being studied intensely. As has been demonstrated on LLC-PK1, human breast BT20, and prostate DU145 cancer cells, where endocytosis and the degradation of Na^+/K^+-ATPase were observed under ouabain treatment [128]. TNF-alpha is probably the most important factor responsible for the down-regulation of Na^+/K^+-ATPase as demonstrated on HepG2 cells by Dakroub *et al*. [202]. This pathway is closely connected with sphingosine-1 phosphate, which is responsible for down-regulatory TNF alpha and ceramide effects. Exogenous sphingosine-1 phosphate has an inhibitory effect on Na^+/K^+-ATPase [202]. The oncogenic transcription factors of some genes involved in the cell proliferation beta-catenin stimulate Na^+/K^+-ATPase and sodium-glucose transport protein SGLT1[203]. This fact may be closely connected with the stimulation and overexpressionof Na^+/K^+-ATPase in some tumor

Table 1. Overview of cytotoxic cardiac glycosides, tumor cell lines (xenograft models), mechanisms of action, and corresponding references. The recent and the most important publications are considered.

Cardiac Glycoside	Cell Line/Model	Mechanism of Action	Reference(s)
Bufalin	T24 human bladder cancer cells	G(0)/G(1) arrest *via* inhibition of cyclin D, cyclin E, CDK2 and CDK4, induction of mitochondrial pathway of apoptosis	[169]
	A549 human lung adenocarcinoma epithelial cell line (a non-small cell lung cancer cell line)	Up-regulation of Bax expression, down-regulation of Bcl-2 and livin expression – inhibition of the PI3/Akt pathway	[171]
	HT-29 and Caco-2 human colon cancer cells	Generation of ROS, autophagy *via* the c-Jun NH_2-terminal kinase activation	[138]
	Hepatocellular carcinoma cell line HepG(2)	Both mitochondrial and Fas (death ligand) pathway of apoptosis	[153]
	Human non-small cell lung cancer (NSCLC) cells	Affecting cell proliferation *via* VEGFR1/ VEGFR2/EGFR/c-Met-Akt/p44/42/p38-NF-kappa B signaling pathway	[170]
	LNCaP, DU145, and PC3 prostate cells	p53 and Fas mediators in androgen-dependent LNCaP cells and Fas mediator in androgen-independent DU145 and PC3 cells	[173]
	HL-6D, ML1, and U937 leukemia cells	Inhibition of DNA synthesis and inhibition of topoisomerase II activity	[308]
	Gastric cancer MGC803 cells	Inhibition of the PI3/Akt pathway	[175]
Cinobufagin	Hepatocellular carcinoma cell line HepG(2)	Both mitochondrial and Fas (death ligand) pathway of apoptosis	[153]
	LNCaP, DU145, and PC3 prostate cells	p53 and Fas mediators in androgen-dependent LNCaP cells and Fas mediator in androgen-independent DU145 and PC3 cells	[173]
	Rabbit lens epithelial cells	Decreasing the rate of bcl-2	[309]
Digoxin	Subcutaneous C4-2 castration-resistant prostate tumors in athymic male nude mice	Down-regulation of HIF-1 expression, inhibition of angiogenesis	[165]
	Acute T-leukemic Jurkat cell line, myelogenous leukemia K-562 cell line, and non-pathologic human peripheral blood mononuclear cells	Induction of apoptosis with mammalian-derived digoxin-like immunoreactive factor (DLIF) *-only in Jurkat cells ** - ouabain has similar effect	[310]
	Lymph node carcinoma of the prostate (LNCaP) and DU-145 cells	p25 accumulation, CDK5 activation	[311]
	Mice grafted with neuroblastoma cell lines SH-SY5Y, Neuro-2a, colonic cancer cells LS174T or Lewis lung cancer cells	Inhibition of angiogenesis * - colonic cancer cells LS174T and Lewis lung cancer cells xenografts are less susceptible	[312]
Digitoxin	BxPC-3 pancreatic cancer cells	Kinase and interferon signaling network	[304]
	Human hepatoma SK-Hep-1 cells	Generation of ROS, specifically hydrogen peroxide	[313]
	Renal adenocarcinoma cancer cell line TK-10	DNA-topoisomerase II cleavable complexes induction	[182]
Lanatoside C	Human glioblastoma (GBM) cells resistant to apoptosis-inducing therapeutics	TRAIL-induced cell death	[160]
Neriifolin	Hepatocellular carcinoma cell line HepG(2)	Activation of caspases 3, 8, and 9, and up-regulated expression of Fas and FasL proteins * - similar effect of 2'-epi-2'-O-Acetylthevetin B	[136, 314]
Ouabain	Human fibroblasts	Activation of caspase-3 and apoptosis at low concentration (30 nM) * - together with digoxin and proscillaridin A	[152]
	Human colon cancer HT29 cells	Enhancement the activity of the calmodulin kinase II enzyme, which in turn activated the transcription factor HIF-1 * - together with digoxin	[192]
	HepG2, SMMC-7721 and Bel-7402 human hepatocellular carcinoma cell lines	Generation of ROS, cell cycle S phase arresting by decreasing the CyclinA1/cyclin-dependent kinase 2 (CDK2)	[114]
	Prostate cancer PC-3 cells	Modification of cell membrane lipidome	[181]
	Human hormone-independent prostate cancer PC-3 cells	Changes in mitochondrial potential, generation of ROS and induction of apoptosis	[315]
	Breast cancer MCF-7 cells	Stabilization of DNA-topoisomerase II complexes * - together with proscillaridin A and digoxin	[184]
Oleandrin	Human pancreatic cancer cell lines	Generally apoptosis * - higher sensitivity of cells expressing alpha 3 subunit of Na+/K+-ATPase	[316]
	PANC-1 human pancreatic cancer cell line	Changes in mitochondrial structure and their translocation, inhibition of pAkt formation and up-regulation of pERK	[284]
	Jurkat, HL-60, HuT-78, HeLa, SKOv3, MCF-7, and U-937 cells	Inhibition of NF-kappa B activation in tumor cells * - only in tumour cells	[317]
	Prostate cancer cell lines PC3 and DU145	Inhibition of export of fibroblast growth factor-2 (FGF-2) in a concentration- and time-dependent manner	[318]

Table 1. contd….

Cardiac Glycoside	Cell Line/Model	Mechanism of Action	Reference(s)
Proscillaridin A	Estrogen independent MDA-MB-231 breast cancer cells	Inhibition of thymidine incorporation into DNA, increase of cytosolic Ca^{2+} level, activation of caspase-3	[319]
	Breast cancer MCF-7 cells	Stabilization of DNA-topoisomerase II complexes * - together with ouabain and digoxin	[184]

Table 2. Clinical trials of cardiac glycosides in anticancer therapy.

Cardiac Glycoside (Combination)	Conditions	Trial Phase	Identifier	Reference
Digoxin+lapatinib	Breast cancer that overexpresses ErbB2	Phase I	NCT00650910	[320]
Digoxin	Recurrent prostate cancer	Phase II	NCT01162135	[321]
Digoxin+erlotinib	Non-small cell lung carcinoma	Phase II	NCT00281021	[200, 322]
PBI-05204	Solid tumors	Phase I	NCT00554268	[199, 323]
UNBS1450	Advanced solid tumours	Phase I	NCT00415038	[289]
Huachansu (= extract from bufo toad skin) +gemcitabine	Unresectable pancreatic adenocarcinoma	Phase II	NCT00837239	[324]

Table 3. Changes in Na+/K+-ATPase expression in different cancer tissues.

Tumor (Cell Line)	Na^+/K^+-ATPase Subunit	Down-/up-regulation	Reference(s)
One half of medulloblastoma and atypical teratoid/rhabdoid tumors	Alpha 1/alpha 3, in one third both alpha 1/alpha 3	Overexpression	[325]
Hepatocellular carcinoma	Alpha 3	Overexpression	[326]
Renal epithelial cells	Beta 1	The surface down-regulation of expression	[327]
Mammary epithelium tumors * - dogs	Alpha 1 * - together with glucose transporter GLUT1 and aquaporin 1	Overexpression, especially in neoplastic mammary epithelium	[204]
Cisplatin-sensitive squamous carcinoma cell lines	Both alpha and beta	Overexpression	[296]
Moloney sarcoma virus-transformed Madin-Darby canine kidney cells	Beta 1	Reduced expression	[328]
Bladder cancer (transitional cell carcinomas)	Both alpha and beta	Both down- and up-regulation, overexpression with high risk of recurrence	[329]
Clear-cell renal carcinoma cells	Both alpha and beta	Down-regulation of beta, normal, eventually slight overexpression of alpha	[330]

cells and their resultant demands for an increased uptake of the glucose needed for cell growth and proliferation. This would confirm the study of Freeman *et al*., who detected increased expression of Na^+/K^+-ATPase and the glucose transporter GLUT1 in tumors of canine mammary glands [204]. However, a closer relationship between Na^+/K^+-ATPase and glucose transporters and the regulation and importance of their expression remain uncertain and require follow-up studies. Not only does the Na^+/K^+-ATPase expression change in cancer cells, but also its locality is also altered. Yang *et al*. investigated the cellular distribution of Na^+/K^+-ATPase α3 isoform in paired normal and cancerous mucosa biopsy samples from patients with lung and colorectal cancers. While Na^+/K^+-ATPase α3 isoform was predominantly located near the cytoplasmic membrane in normal human colon and lung epithelia, the expression of this subunit in their paired cancer epithelia was shifted to a perinuclear position in both qualitatively and quantitatively. Similarly, the distribution of α3 isoform was shifted from the cytoplasmic membrane location in differentiated human colon cancer CaCO-2 cells to a perinuclear position in undifferentiated CaCO-2 cells. This fact points up the significance of changes in the locality of Na^+/K^+-ATPase and their possible use in cancer therapy [205].

Nevertheless, Na^+/K^+-ATPase plays a significant role in signal transduction and the regulation of Src-related signaling processes. This fact was demonstrated by Li *et al*., who supplemented different cell lines with pNaKtide, a peptide derived from the α1 subunit of Na^+/K^+-ATPase [206]. Application of pNaKtide significantly inhibited the growth and stimulated the apoptosis of the tumor cell lines. In addition, its administration inhibited the growth and angiogenesis of tumor xenografts. The activation of Src kinase under the application of GCs depends on the concentrations of the specific ligands of Na^+/K^+-ATPase, such as Na^+, K^+, ATP, and ADP [129]. The ATP/ADP ratio in particular determines the extent of Src activation. The depletion of ATP has been studied by Lefranc and Kiss, who observed ATP depletion in glioma cells after exposure to UNBS1450 [143]. This fact points to the possible involvement of a cell-energetic mechanism in the cytotoxicity of CGs.

Yin *et al*. have demonstrated the impairment of Na^+/K^+-ATPase in human T-cell leukemia cells followed by the depletion of the intracellular level of glutathione and the generation of reactive oxygen species [207]. These findings suggest that new anticancer therapeutic agents based on Na^+/K^+-ATPase can be developed. These compounds would be based not only on signal transduction,

with CGs and their derivatives as possible candidates, but also on therapeutic agents that directly affect the expression of Na^+/K^+-ATPase subunits [208]. In addition, some examples of Na^+/K^+-ATPase use in anticancer therapy are available; for example the drug monoterpene perillyl alcohol (POH) is used in the treatment of several malignant tumors, including gliomas [209]. On the other hand, it plays a significant role not only in regulating signal transduction and the processes of apoptosis, but also in the resistance of tumor cells to cytostatics.

Role of Small Proteins Involved in the Direction of Na^+/K^+-ATPase in Cancer

Despite the discussions about the role of Na^+/K^+-ATPase in cancer cells, there are some proteins that regulate its activity. The most important are the FXYD proteins that have been found in many tissues. This group of small proteins all share a 35-amino acid signature sequence domain – the invariant extracellular motif FXYD [210]. They have one trans-membrane segment, an extracellular N-terminus, and a cytoplasmic C-terminus. At least seven members (FXYD1-7) found in mammals with different organ/tissue expression have been identified (predominantly in excitable organs and in tissues involved in the transport of fluids and solutes) [211]. They are preferably associated with Na^+/K^+-ATPase alpha 1-beta isoenzymes and significantly affect their functions [212]. They are not essential for these functions, but they modulate the kinetic properties of Na^+/K^+-ATPase in directing the affinities and cation rates of Na^+ and K^+ transport, according to the demands of the different cells [118]. A stabilizating function of FXYD proteins has also been recognized [118].

FXYD-1 (PLM, phospholemman) significantly increases the affinity of the human alpha 1 and beta 1 isoforms of Na^+/K^+-ATPase for sodium ions [117] and regulates L-type cardiac calcium channels. In the heart, FXYD-1 serves as a substrate for protein kinases A and C. Its localization is not restricted only to cardiac and skeletal muscle (interactions with $\alpha_1\beta$ and $\alpha_2\beta$ isoenzymes). It has been found in extraglomerular mesangial cells (interaction with β_2 subunit), in renal vessels (interaction with $\alpha_2\beta_2$), and in the cerebellum and choroid plexus (interactions with $\alpha_1\beta$, $\alpha_2\beta$, and $\alpha_3\beta$) [213]. The inhibition of Na^+/K^+-ATPase by FXYD-1 is directed by its phosphorylation by protein kinases A and C [214-216]. However, no connection between FXYD-1 and cancer has been demonstrated.

FXYD-2 is known as a gamma-subunit of the Na^+/K^+-ATPase. There are two alternative splice variants – FXYD-2a and FXYD-2b. FXYD-2 expression has been reported in kidney [217]. However, the work of Venteo *et al*. has brought new knowledge about its expression in dorsal root ganglia and discussed its role in the sensory neurons responsible for relaying nociceptive, thermoceptive, mechanoceptive, and proprioceptive information from peripheral tissues toward the central nervous system [218]. FXYD-2 plays an important role in stress processes; its induction has been demonstrated in both normal and tumor cell lines, such as C6 (glioma) and PC12 (pheochromocytoma), during hypertonicity, heat shock, exogenous oxidation, or chemical stress [219]. On the other hand, its expression has recently been discussed with respect to chromophobe renal cell carcinoma and renal oncocytoma [220]. However, this investigation is only beginning.

FXYD-3 (Mat-8, mammary tumor protein) is involved in many physiological and pathological processes, including cancer. Its function is closely connected with the regulation of Na^+/K^+-ATPase [221], the up-regulated expression of which has been detected in gastric, breast, and prostate cancer cells [222-225]. In addition, strong FXYD-3 expression has been observed in the gliomas, especially in female patients [226], and in pancreatic ductal carcinomas [227]. Higher FXYD-3 expression is correlated with infiltrative tumor growth and a generally unfavorable prognosis in rectal cancer [228] and may serve as an important marker of adenomas in the colon [229, 230]. Suppression of FXYD-3 leads to significantly reduced cell proliferation in prostate cell lines [224]. Expression of FXYD-3 in the lymph nodes of patients suffering from bladder urothelial carcinoma serves as a sensitive marker of the spread of the disease [231]. On the other hand, its down-regulated expression in many other tumors has been proved. The best examples are lung tumors [232, 233] and the role of FXYD-3 in the epithelial-to-mesenchymal transition.The process that being investigated in the metastatic process and cell proliferation is discussed in the papers cited. the work of Yamamoto has shown that FXYD-3 is a gene targeted by the transforming growth factor beta signaling in human breast cancer MCF-7 cells but it is not directly involved in the process of epithelial-to-mesenchymal transition [234]. However, further investigation is quite necessary to understand the role of FXYD proteins in connection with Na^+/K^+-ATPase in cancer. It seems that FXYDs are involved in proper Na^+ and K^+ homeostasis in cancer cells and in the expression of the Na^+/K^+-ATPase isoenzymes necessary for the cell differentiation processes. This fact was demonstrated in Caco-2 (colon adenocarcinoma) cells, where the inhibition of cell differentiation in FXYD-3-deficient cells has been observed [235].

FXYD-4 (CHIF, corticosteroid hormone-induced factor) is a small epithelial-specific protein regulated by aldosterone and the intake of K^+. It shares 50 % homology with the gamma subunit of Na^+/K^+-ATPase [236, 237]. This protein interacts with the subunit of Na^+/K^+-ATPase and increases its affinity for cellular Na^+. However, the role of FXYD-4 in different ion transport mechanisms has also been discussed [238]. In addition, there are no data showing a connection between FXYD-4 expression and cancer. The enhanced expression of FXYD-5 (dysadherin, RIC) has been demonstrated during metastatic processes - it affects the processes of cell adhesion and cell motility [239]. Its over-expression in different cell lines, such as renal carcinoma cells [240], cells of differentiated gastric carcinoma with submucosal invasion [241], head and neck cancer cells [242], non-small lung carcinoma cells [243], breast ductal carcinoma cells [244] has been discussed. Knockdown of FXYD-5 expression has been correlated with decreased cell motility, whereas transfection of FXYD-5 into liver cells led to decreased cell-cell adhesion, increased cell motility, and diminished expression of E-cadherin, a calcium-dependent cell-cell adhesion glycoprotein [245]. E-cadherin serves as an important marker of the progression of a cancer, especially in combination with FXYD-5, which serves as a marker for predicting aggressive tumor behavior [246, 247]. In this connection, a decrease in the glycosylation of beta 1 Na^+/K^+-ATPase under FXYD-5 expression in mouse kidney collecting duct cells M1 has been demonstrated. This fact indicates the role of normal beta 1 Na^+/K^+-ATPase glycosylation in cell-cell contact [239, 248]. FXYD-5 (as well as other FXYD family members) regulates Na^+/K^+-ATPase *via* the negative charge of S163, a highly conserved residue located in the C-terminus of all FXYD family members. S163A mutants inhibit cell migration [249].

FXYD-6 (phosphohippolin) is mainly expressed in the cochlea, the brain, and to a lesser extent in the colon, the lungs and the testes. In addition, its presence has been demonstrated in type II taste cells [250]. This member of the FXYD protein family is co-localized with Na^+/K^+-ATPase, specifically with its $\alpha_1\beta_2$ isoforms, and apparently increases its affinity for K^+ and Na^+. In the light of these facts, FXYD-6 expression in the production of endolymph in the cochlea has been studied [251, 252]. Its overexpression has been detected in bile duct tumors and in some types of pancreatic tumors [253]. However, further investigation is still needed.

FXYD-7 is expressed exclusively in the brain, where it is associated with the alpha 1-beta Na^+/K^+-ATPase isoform. Especially on threonine residues, FXYD-7 undergoes post-translational modifications, probably O-glycosylations, which are important for stabilizating the protein [254]. It reduces the apparent

K^+ affinity almost two-fold , and thus may play a crucial role in neuronal excitability and brain functions [255, 256]. However, this protein has not been found to be involved in cancer.

Endogenous CGs and Cancer

Some CGs, both cardenolides and bufadienolides, have been identified in human tissues [93, 257]. Two important cardenolides have been isolated from human tissues and identified: ouabain in blood plasma [258] and digoxin in the hypothalamus [259]. Whereas the presence of cardenolides in the human body is still being discussed, there is no doubt about the presence of bufadienolides. Generally, five different compounds have been reported – 19-norbufalin (in human cataractous lenses) [260, 261], and its peptide derivative 3β-hydroxy-14α 20:21-bufenolide (in human placenta) [262], proscillaridin A (in human plasma) [263], marinobufagenin (in human urine after acute myocardial infarction) [264, 265], and telocinobufagin also presented as telocibufagenin (in blood plasma of patients with renal failure) [266]. They are synthesized in the adrenal cortex and the hypothalamus [267]. The role of endogenous CGs is still rather unclear. Nesher *et al*. proposed some different effects, both systemic and molecular, of endogenous CGs. These include regulation of blood pressure [268] and heart contractility and rhythm [269], regulation of carbohydrate metabolism [270], and behavior and brain function [271-273]. Regulation of cell growth, differentiation, proliferation, and adhesion are closely connected with the inhibition of Na^+/K^+-ATPase activity and subsequent processes, including activation of the signal transduction mechanisms [274]. In addition, these CGs probably affect the recycling of endocytosed membrane proteins [275]. The physiological levels of endogenous CGs have a pro-proliferative effect on smooth muscle and on endothelial and renal epithelial cells. Trophic levels of endogenous ouabain are important for the survival of ganglion cells in the retina; an indication of the regulatory role of endogenous ouabain in cell viability [276]. Dvela *et al*. have cultivated neuronal NT2, rat neuroendocrine PC12, and monkey kidney COS-7 cells in the presence of endogenous ouabain [277]. Application of a specific anti-ouabain antibody led to a decrease in level of ouabain and reduced the viability of the cultured cells. NT2 cells were the most sensitive to this diminished level of ouabain levels under the subsequent affection of the ERK1/2 signaling pathway. In addition, these authors observed significant differences in sensitivity to ouabain depletion among different cell lines. Endogenous ouabain is probably involved in regulating the amount of Na^+/K^+-ATPase in cells. This has been demonstrated using LLC-PK1 cells; ouabain stimulated the PI3K/Akt/mTOR pathway and consequently up-regulated the Na/K-ATPase expression in these cells [128]. This fact indicates the ability of endogenous CGs to selectively regulate the processes of cell growth, differentiation, and apoptosis [277]. Telocinobufagin exhibits a potential regulatory effect on the immune system. Moreover, this compound markedly enhances natural killer cells and peritoneal macrophage activation. *Via* these mechanisms telocinobufagin can control the presence of unwanted cancer cells [278]. A significant cytotoxic effect of this compound has also been established [279]. Valente *et al*. have proposed a novel role of endogenous ouabain [280]. These authors suppose modulation of both the expression and the activity of proteins belonging to the ATP binding cassette family of transporters, such as ABCC7 (CFTR), ABCB1 (P-glycoprotein), and ABCC1 (MRP1). This finding may be very important in their putative role in the secretion of xenobiotics, including drugs. Moreover, ABCC1 is expressed in several other tissues, such as those of the brain, the testes, and the immune system, and is related to the transport of glutathione. Thus, it is possible that the release of ouabain may control a number of functions within these organs and tissues by modulating both the expression and the activity of ABCC1 [280]. In conclusion, the role of endogenous CGs in the processes of cell growth, proliferation, and apoptosis should be studied intensively. However, despite the fact that plenty of publications are focused on individual CGs described as endogenous, their role is often interpreted in light of *in vitro* studies, and further investigation is therefore quite necessary.

Structure-activity Relationship of CGs and Possible Synthesis of New CG Derivatives with Enhanced Cytotoxic Properties

An effort has been made to modify the structure of CGs to increase their cytotoxicity [95]. There are different approaches to enhancing the cytotoxic properties of CGs. The most important are based on modifying the CGs basic skeleton modifying the existing hydroxyl groups, which significantly affect the biological activity of the CGs offers an especially attractive possibility. A second approach is based on modifying the sugar moiety, and combining CGs with other drugs used in anticancer therapy, such as platinum-based derivatives, is a third possibility.

Modification of the CGs skeleton represents an important way to enhance cytotoxic activity. Ye *et al*. have reported groundbreaking work demonstrating the most important structural modifications of cinobufagin and bufalin, bufadienolides isolated from the skin and parotid venom gland of *Bufo gargarizans* Cantor [281]. These secondary metabolites, which are the most important components of some traditional Chinese plant drugs, represent the bufadienolide structure. Hydroxylation at different sites of the bufalin skeleton (Fig. **4**) may significantly increase the cytotoxicity and thus represent a way to develop new cytotoxic agents.

The cardenolides have not been studied similarly to such an extent. The work of Staroske *et al*. focused on the possibility of modifying the digitoxigenine lactone moiety; however, derivatives were discussed only as applied to the inhibition of Na^+/K^+-ATPase [282]. Three derivatives of ouabain, digoxin, and proscillaridin A containing a carboxylic group instead of a lactone moiety have been synthesized and examined for cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells by Winnicka *et al*. [183]. The derivative of proscillaridin A was the most potent, but its cytotoxicity was slightly lower than that of proscillaridin A.

CGs as well as semisynthetic derivatives of GCs have been further tested not only for cytotoxic properties, but also for their mechanisms of action [283-286]. The semi-synthetic cardenolide 19-hydroxy-2"-oxovoruscharin (derived from 2´´-oxovoruscharin isolated from *Calotropis procera*, *Apocynaceae*) is active in cancer cells that express diverse forms of multi-drug resistance (MDR), either conferred by the over-expression of selected drug-transporter proteins or induced by a range of chemotherapeutic agents. The data suggest that this novel compound might be especially applicable to notoriously drug-resistant cancers [208]. UNBS1450 is a molecule with very potent cytotoxic and antiproliferative properties [287-289]. Its mechanism of action is based on the disintegration of the actin cytoskeleton. Compared to other reference compounds, such as taxol, irinotecan, oxaliplatin, mitoxanthron, and temozolomide, UNBS1450 is more potent against aggressive and metastatic orthotopic NSCLC, refractory prostate cancer, and glioma [289, 290]. This semi-synthetic cardiac glycoside induces apoptosis in human leukemia cell lines (by reducing the expression of anti-apoptotic Mcl-1 and by recruiting pro-apoptotic Bak and Bax proteins) [286], and in A549, the human lung adenocarcinoma epithelial cell line, a non-small cell lung cancer (NSCLC) cell line that displays highly activated cytoprotective NF-kappa B signaling pathways (the mechanism of action consists in reducing both the DNA-binding capacity of the p65 subunit and the NF-kappa B transcriptional activity) [291]. UNBS1450 also strongly inhibits Na^+/K^+-ATPase, indicating a close connection between this enzyme and regulation of the cell cycle, proliferation and apoptosis [288, 292].

The labile trisaccharide of digitoxin is the weakest part of its structure. Derivatives in which the labile trisaccharide has been replaced by a stable chain (a surrogate, ethylene glycol chain) also show cytotoxic effects. It seems that cardiac glycoside mimics

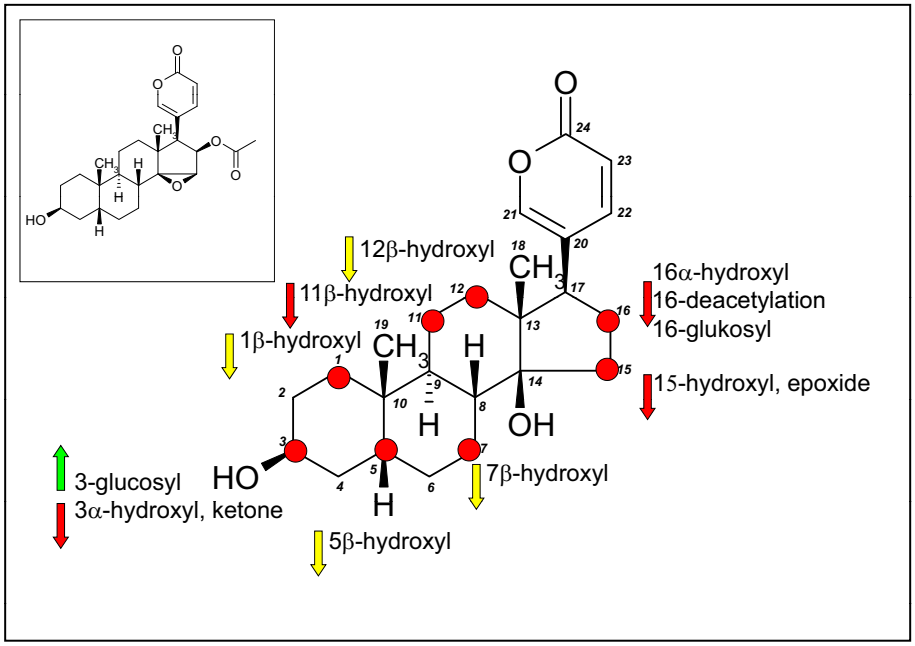

Fig. (4). Possible modifications of bufalin modification with focus on the reduction (red arrow), slight reduction (yellow arrow), or enhancement (green arrow) of cytotoxic properties. The sites of the modifications in the bufalin skeleton are marked by red circles. The chemical structure of bufalin is shown in the inset.

represent a possible way to design cytotoxic agents [293]. Iyer *et al.* investigated the role of the sugar moiety in the cytotoxic action of some CGs. They prepared mono-, di-, and tri-O-digitoxoside derivatives and the corresponding MeON-neoglycosides. The MeON-neoglycosides demonstrated effects similar to those of the O-glycosides in non-small cell lung cancer cells (NCI-H460) [294, 295].

Combination of CGs with Conventionally Used Cytostatics

Cisplatin is one of the most widely used cytostatics and has a high rate of acquired resistance, which limits its therapeutic potential. The role of the proteins involved in cisplatin resistance has been discussed in several papers. However, it seems that the accumulation of cisplatin depends on the Na+ /K+-ATPase activity. This has been demonstrated by Ahmed *et al.*, who detected the highest levels of cisplatin in squamous carcinoma tumor cell lines accompanied by high Na^+/K^+-ATPase activity and overexpression of alpha and beta Na^+/K^+-ATPase subunits [296]. This fact can be attributed to the synergic cytotoxic effect of digitoxin and oxaliplatin, demonstrated in the work of Felth and al. [297]. Digitoxin and oxaliplatin had synergic effects on different colorectal cancer cell lines [297]. On the other hand, Tummala *et al.* have proved that oxaliplatin has a diminished effect on C10B cells that express greatly reduced levels of Na^+/K^+-ATPase–beta 1 subunit [298]. This finding together with the reduced accumulation of oxaliplatin in tumor cells indicates not only a possible role of Na^+/K^+-ATPase in the accumulation of cytostatics in tumor cells, but also a prognostic role for the expression of the beta 1 subunit in tumor progression and response to anticancer therapy. Synergic effects have also been observed in HepG2 cells subjected to a combined dosage of bufalin and sorafenib [299]. In addition, the finding that CGs sensitize tumor cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) by up-regulating death receptors 4 and 5 may represent a new anticancer treatment strategy [159].

Radiosensitization of Tumor Cells by CGs

CGs also enhance radiosensitivity in some tumor cells, as demonstrated in lung tumor cell lines H460 and A549. On the other hand, no radiosensitization was observed in the H1299 (p53 null) cell line [300]. Oleandrin has demonstrated radiosensitozation in PC-3 cells [301]. The radiosensitization of tumor cells by CGs may be brought about by an effect on the mechanisms that repair DNA breaks. This effect is described and discussed in the work of Pastor and Cortes [302]. This process is closely connected with the regulation of the cell cycle and the induction of apoptosis. The crucial role of p53 in the cytotoxicity of GCs can be explained by the work of Wang *et al.*, which proved that initiating the Src/MAPK signaling pathway inhibited the synthesis of p53 [303, 304].

VIEW INTO THE FUTURE AND FURTHER PERSPECTIVES

The further investigation of CGs in the treatment of cancer will surely be connected with studying i. the role of Na^+/K^+-ATPase in tumor cells, ii. the role of endogenous CGs in the regulation of cell processes and cancerogenesis, iii. the structure-activity relationships of CGs and the possible synthesis of new CG derivatives with enhanced cytotoxic properties, iv. the possibile combinations of CGs with conventionally used cytostatics, and v. the further investigation of the role of small proteins involved in the direction of Na^+/K^+-ATPase.

CONCLUSIONS

Cardiac glycosides represent a group of plant secondary metabolites. They have also been found in animal products, endogenous CGs have been found in different human tissues. The role of CGs in cell growth, proliferation, and apoptosis is still being discussed. The cytotoxic effect of CGs is connected with their effect on Na^+/K^+-ATPase, both the expression of its subunits and its function, which affects the intracellular homeostasis of Na^+, K^+ and Ca^{2+}. However, its function is also regulated by other factors, of which the FXYD proteins are the most important. In light of the above-mentioned facts summarizing the expression of the FXYD family of proteins in individual types of cancer cells or tumor cell lines, scientists can be expected to be interested in questions focused on the role that the FXYD proteins play in the Na^+/K^+-ATPase function. However, there are only a limited number of works connecting these problems. The work of Arimorichi at al. is

pioneering among these [305]. The authors show the association of FXYD-3 with Na^+/K^+-ATPase in colorectal cancer cells and discuss the possible implications for cell growth. The alpha 1 Na^+/K^+-ATPase subunit is considered to be a modern anti-cancer target. Its inhibition by the cardiac glycoside ouabain has been established in HepG2 cells [114]. Similar results have been established, e.g., in the work of Li *et al*., who demonstrated that bufalin inhibited the growth of hepatocellular carcinoma (HCC) cells in a dose-dependent manner correlated with the expression level of alpha 3 Na^+/K^+-ATPase subunit in HCC cells [306]. Whereas the role of disturbances by intracellular Ca^{2+} in CG-induced apoptosis is well known, the possible implications of an increase in intracellular Na^+ must be further investigated. The possible role of Na^+/K^+-ATPase in Na^+ homeostasis and the possible implications in apoptosis remain almost unknown. On the other hand, the demands of tumor cells on the Na^+/K^+ homeostasis are discussed especially in connection with the maintenance of pH and the regulation of increases in volume *via* Na^+/H^+ exchangers [307]. In conclusion, further investigation is needed in order to understand the Na^+/K^+-ATPase function and its regulation by FXYD proteins. These findings would enable to design new derivatives of cardiac glycosides with possible applications in anticancer therapy. All of these findings aim towards proposing new CG-based cytostatics, which will overcome some of the problems with anticancer drugs, especially the resistance of tumor cells.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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