Novel therapeutic applications of cardiac glycosides

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Abstract | Cardiac glycosides are a diverse family of naturally derived compounds that bind to and inhibit Na^+/K^+-ATPase. Members of this family have been in clinical use for many years for the treatment of heart failure and atrial arrhythmia, and the mechanism of their positive inotropic effect is well characterized. Exciting recent findings have suggested additional signalling modes of action of Na^+/K^+-ATPase, implicating cardiac glycosides in the regulation of several important cellular processes and highlighting potential new therapeutic roles for these compounds in various diseases. Perhaps most notably, the increased susceptibility of cancer cells to these compounds supports their potential use as cancer therapies, and the first generation of glycoside-based anticancer drugs are currently in clinical trials.

Inotrope
Inotropic agents affect the force of muscular contractions.

Pharmacophore
A molecular framework that carries the essential features responsible for a drug's biological activity.

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The use of digitalis purpurea extracts containing cardiac glycosides for the treatment of heart disorders was first described by William Withering in 1785. The mechanism of cardiac glycoside action was delineated approximately 50 years ago when Schatzmann and colleagues identified these compounds as specific inhibitors of Na^+/K^+-ATPase[1] (EC 3.6.3.9). Na^+/K^+-ATPase is a ubiquitous membrane protein that uses energy derived from ATP hydrolysis to drive the active transport of potassium ions into cells and sodium ions out of cells. Extensive studies of the mode of action of these compounds has yielded one of the best-defined mechanisms attributed to a drug so far: inhibition of Na^+/K^+-ATPase[1] raises the level of sodium ions in cardiac myocytes, which leads to an increase in the level of calcium ions and an increase in cardiac contractile force. Further understanding of their positive inotropic effects established these molecules as effective drugs for heart failure, and members of this family (digoxin, digitoxin) are still in clinical use[2].

Recent studies have highlighted a new aspect of the biology of Na^+/K^+-ATPase as a versatile signal transducer, as well as additional modes of action for cardiac steroids[3–6]. This emerging evidence suggests that binding of these compounds to Na^+/K^+-ATPase activates multiple downstream signal transduction pathways, and implicates cardiac glycosides (endogenous and exogenous) in the regulation of many important physiological and pathological states[7–9]. Furthermore, unexpected results from epidemiological studies describing significantly lower mortality rates of patients with cancer receiving cardiac glycosides sparked new interest in the anticancer properties of these drugs. Numerous subsequent *in vitro* and *in vivo* studies verified these initial observations[10–12], and cardiac-glycoside-based drugs have now entered clinical trials for treating cancer[13–15]. In addition, the inclusion of several cardiac glycosides in large compound libraries for the increasing needs of hypothesis-neutral, high-throughput screening assays has uncovered further candidate therapeutic aspects of these drugs for a number of non-cancer pathologies. In this Review, we focus on these newer discoveries on the potential therapeutic roles of cardiac glycosides in various human diseases, in particular, cancer.

Characteristics of cardiac glycosides

Cardiac glycosides comprise a large family of naturally derived compounds. They show considerable structural diversity, but all members of this family share a common structural motif. The core structure consists of a steroidal framework, which is considered the pharmacophoric moiety responsible for the activity of these compounds[16]. This steroid core is double-substituted with an unsaturated lactone ring at position 17 and a sugar portion at position 3 (FIG. 1). The nature of the lactone moiety characterizes the subgroup of the glycosides. Cardenolides have a five-membered unsaturated butyrolactone ring, whereas bufadienolides contain a six-membered unsaturated pyrone ring (FIG. 1). Unlike sex hormones, mineralocorticoids and glycocorticoids, which are all *trans*-connected, cardiac glycosides show an A/B and C/D *cis*-conformation.

A wide variety of sugars are attached to natural cardiac glycosides; the most common are glucose, galactose, mannose, rhamnose and digitalose. Although sugars

Angiosperms
The largest phylum of living plants. They develop seeds from ovules contained in ovaries and the seeds are enclosed by fruits that develop from carpels.

themselves have no activity, the addition of sugars to the steroid affects the pharmacodynamic and pharmacokinetic profile of each glycoside. For example, free aglycones are absorbed more rapidly and are metabolized more easily than their glycosylated counterparts. Moreover, the type of the attached sugar influences the potency of the compound. For instance, the addition of rhamnose was shown to increase potency several times (6–35 times), whereas the addition of mannose had no significant effects[17]. Based on this, Langenhan and colleagues recently developed a powerful new tool, called neoglycorandomization, for the study of the relationship between attached sugars and biological activity. This high-throughput method allows rapid conversion of a single aglycone molecule into a library of analogues with diverse sugar moieties[18]. Techniques such as this could facilitate the discovery of novel cardiac glycoside analogues with improved therapeutic properties.

More than a hundred cardiac glycosides have been identified as secondary metabolites in plants, with most belonging to the angiosperms[17]. Recently, however, cardiac glycosides of the bufadienolide class were identified in the skin and the carotid gland of animals, and mainly in the venom of several toad species[19]. TABLE 1 summarizes the most extensively studied glycosides of plant and animal origin.

The ability of some animal species to synthesize cardiotonic steroids, together with the highly conserved nature of the digitalis binding site, has given rise to the speculation that humans can also produce these compounds. Indeed, advances in mass spectrometry led to the identification of mammalian endogenous cardiac glycosides, collectively termed as digitalis-like compounds[20,21]. Digitalis-like compounds are found in mammalian tissues (such as the brain and adrenals) and body fluids (such as plasma, urine and cerebrospinal fluid)[22].

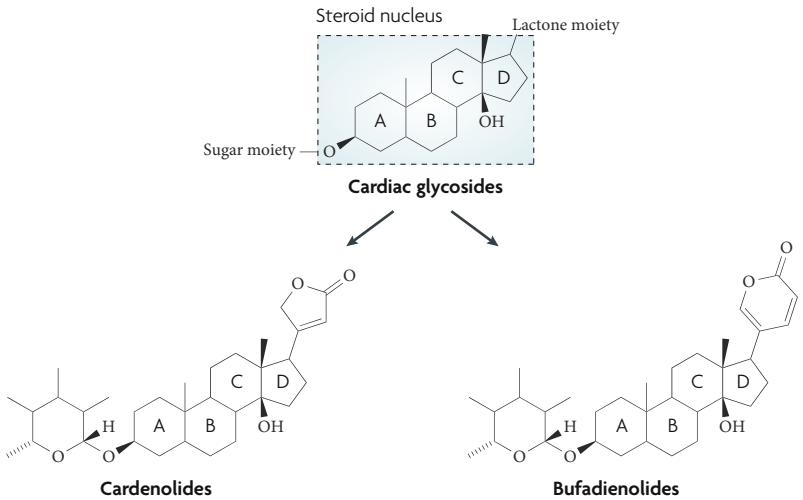


Figure 1 | **General structural characteristics of cardiac glycosides.** Each molecule of this family consists of three distinct structural motifs: a steroid nucleus, a sugar moiety and a lactone moiety. The lactone moiety defines the functional class of each compound. Cardenolides contain a five-membered unsaturated butyrolactone ring, whereas bufadienolides contain a six-membered unsaturated pyrone ring.

The growing list of endogenous glycosides identified so far includes several members of both the cardenolide and the bufadienolide class such as ouabain (human plasma, adrenal cortex and hypothalamus)[20,23–26]; digoxin (human urine)[21]; 19-nor bufalin (cataractus human lenses)[27]; marinobufagenin (human urine after acute myocardial infraction)[28]; and proscillaridin A (human plasma)[23,29,30]. The biosynthesis of these steroid hormones utilizes cholesterol and progesterone and is under tight regulation by other hormones, such as renin–angiotensin, endothelin and adrenaline[30].

The recent discovery of the signalling properties of Na^+/K^+-ATPase has helped in the assignment of new functional roles for cardiac glycosides (endogenous and exogenous) at both the molecular and cellular level. To better understand the physiological and pathological roles of cardiac glycosides and their extended therapeutic impact, we will first review briefly the latest findings related to Na^+/K^+-ATPase.

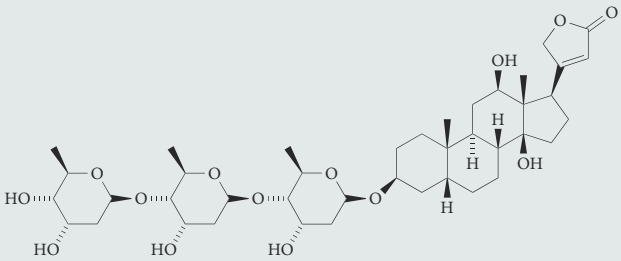
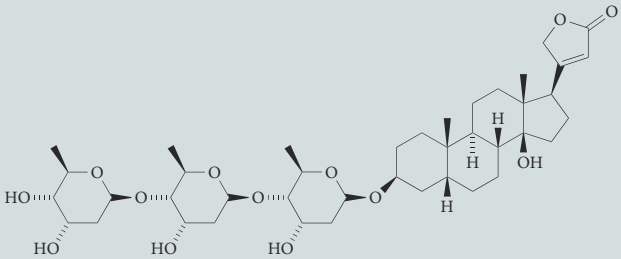
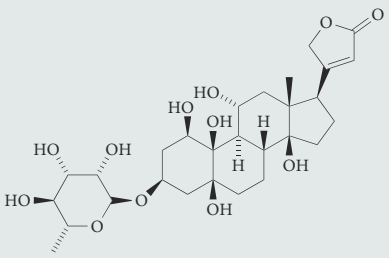
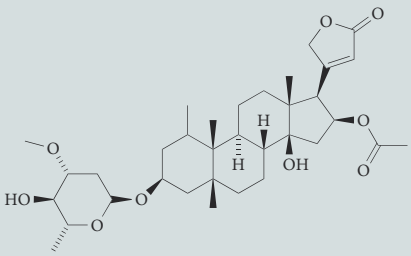
Na^+/K^+-ATPase structure and function

Na^+/K^+-ATPase, which is the largest protein complex of the P-type family of cation pumps, uses the energy derived from the hydrolysis of ATP to drive the active transport of potassium ions inside and sodium ions outside cells in a 2:3 stoichiometry. Its main physiological role is the establishment and maintenance of an electrochemical gradient across the plasma membrane[31], which is critical for physiological processes such as neuronal communication, osmotic regulation of cell volume and ion homeostasis. Moreover, this gradient force is coupled to the secondary transport of many organic and inorganic substrates[31]. It is estimated that the pumping activity of this enzyme accounts for approximately 30% of a cell's overall energy consumption at rest[5].

The inotropic effects following the interaction of cardiac glycosides with the sodium pump are well characterized[32]. In short, cardiac-glycoside-induced inhibition of Na^+/K^+-ATPase leads to an increase in intracellular levels of sodium ions. As a result, the activity of the Na^+/Ca^{2+} exchanger is reduced and therefore intracellular concentrations of calcium ions are increased, which accounts for the positive inotropic effects (for more details see REFS 31,33).

The X-ray crystal structure of Na^+/K^+-ATPase (at 3.5 Å resolution) has been recently resolved[34]. It is an oligomer composed of at least two polypeptides: the α-subunit and the β-subunit. The α-subunit is the catalytic moiety of the enzyme. Homologous to single-subunit P-type ATPases, it bears the binding sites for Na^+, K^+, Mg^{2+}, ATP and the highly conserved cardiac glycoside-binding site. The binding site is formed by the extracellular loops of the M1/M2, M3/M4 and M5/M6 moieties, as recently revealed by elegant functional studies[35–37]. Several additional regulatory sites are also found on the α-subunit, including phosphorylation sites for numerous signal transducing kinases (such as phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and PKA), caveolins and ankyrins. These sites are important for the formation of the Na^+/K^+-ATPase signalosome

Table 1 | **Selected cardiac glycosides (part 1)**

Name (functional class)	Structure	Properties*
Digoxin (cardenolide)		• Family: Scrophulariaceae • Species: *Digitalis purpurea* • M_r: 780.9 • Molecular formula: $C_{41}H_{64}O_{14}$
Digitoxin (cardenolide)		• Family: Scrophulariaceae • Species: *Digitalis purpurea* • M_r: 764.9 • Molecular formula: $C_{41}H_{64}O_{13}$
Ouabain (cardenolide)		• Family: Apocynaceae • Species: *Nerium oleander* • M_r: 584.7 • Molecular formula: $C_{29}H_{44}O_{12}$
Oleandrin (cardenolide)		• Family: Apocynaceae • Species: *Nerium oleander* • M_r: 576.7 • Molecular formula: $C_{32}H_{48}O_9$

*Data retrieved from ChemBank database (see Further information).

(described below). The regulatory β-subunit is a single-span glycoprotein with a chaperone-like activity that is unique to the K^+-counter-transporting P-type ATPases[34]. It is mainly important for the recruitment of the α-subunit to the plasma membrane and for the occlusion of potassium ions[34]. Finally, the FXYD proteins are single-span, type I transmembrane proteins, which are often associated with the αβ-complex and seem to act as modulators of the kinetic properties of the pump[38,39].

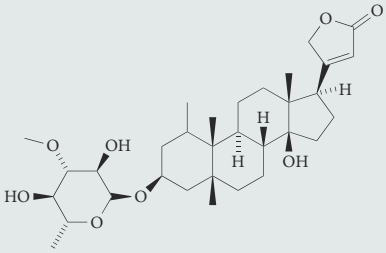
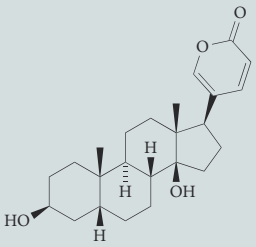
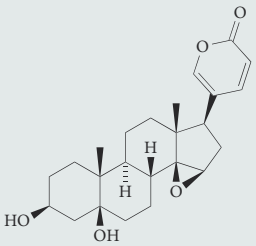
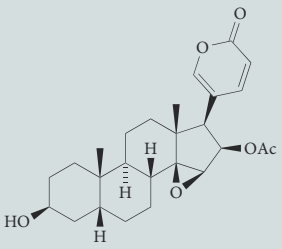
Notably, both the β-subunit and the FXYD subunit are found to affect the binding affinity of cardiac steroids to Na^+/K^+-ATPase. It is postulated that the tissue-specific expression of these subunits might account for the differential physiological responses of tissues to the effects of cardiac glycosides[40–44].

In addition to pumping ions, it is now established that Na^+/K^+-ATPase acts as a scaffold for the assembly of a multiple-protein signalling domain that transmits signals to various intracellular compartments[45–47]. Several members of this complex have now been identified, including SRC kinase, epidermal growth-factor receptor (EGFR), inositol 1,4,5-triphosphate (IP3) receptor and caveolins. These are all engaged in the formation of this signalling domain, which is localized in the coated pits of the plasma membrane. Conformational changes on binding of cardiac glycosides trigger a downstream protein interplay that ultimately results in the activation of intracellular signal transduction cascades.

Interestingly, the signal transduction activity of this enzyme occurs through properties that are independent of its function as an ion pump[48]. Indeed, doses of cardiac

Coated pits
A cell-surface depression that is coated with clathrin on its cytoplasmic side and functions mainly in receptor-mediated endocytosis.

Table 1 | **Selected cardiac glycosides (part 2)**

Name (functional class)	Structure	Properties*
Neriifolin (cardenolide)		• Family: Apocynaceae • Species: *Nerium oleander* • M_r: 534.7 • Molecular formula: $C_{30}H_{46}O_8$
Bufalin (bufadienolide)		• Family: Bufonidae • Species: *Bufo gargarizans* • M_r: 386.5 • Molecular formula: $C_{24}H_{34}O_4$
Marinobufagenin (bufadienolide)		• Family: Bufonidae • Species: *Bufo marinus* • M_r: 400.5 • Molecular formula: $C_{24}H_{32}O_5$
Cinobufagenin (bufadienolide)		• Family: Bufonidae • Species: *Bufo gargarizans* • M_r: 442.5 • Molecular formula: $C_{26}H_{34}O_6$

*Data retrieved from ChemBank database (see Further information).

steroids — at concentrations that result in only subtle changes to the pumping activity of Na^+/K^+-ATPase — activate downstream signal transduction cascades and regulate many cellular processes including cell growth[49], cell motility[50] and apoptosis[51]. The elucidation of the precise downstream signalling networks is still a subject of ongoing research; two of the most established signalling avenues are described below.

Signalling through alterations in intracellular calcium oscillations. In 2001, a new signalling mechanism for cardiac glycosides was revealed by the exciting finding from Aizman and colleagues that ouabain at concentrations that confer only partial or no inhibition of Na^+/K^+-ATPase can trigger intracellular calcium oscillations in renal proximal tubule cells[52]. More recently, similar oscillations were reported in human endothelial cells[53] and in COS-7 cells[54].

It is now established that the binding of nanomolar concentrations of ouabain to Na^+/K^+-ATPase triggers an allosteric conformational change at the N-terminal tail of the catalytic α-subunit, which activates the neighbouring SRC protein. In parallel, in a way that is not yet fully defined, phospholipase C (PLC) and IP3 are also recruited, resulting in the formation of a functional microdomain[54] that brings the cytosolic part of the sodium pump in direct contact with the IP3 receptor of the endoplasmic reticulum[47,55]. At this point, single or repeated transient increases in levels of intracellular calcium are produced.

Calcium oscillations are a universal mode of signalling that mediate a diverse range of cellular functions such as cell proliferation, differentiation and apoptosis[56]. The ultimate response of the cell is dependent on the periodicity of the calcium oscillations; depending on the stimulus they can vary from seconds to hours[54]. It is established that low concentrations of ouabain trigger low-frequency calcium oscillations (~4–6 min). In this range, the calcium-dependent transcription factor nuclear factor-κB (NF-κB) is activated and mediates transcription of several anti-apoptotic and proliferation-inducing genes. Indeed, ouabain (0.1–10 nM) was reported to induce the proliferation of and protected kidney cells from serum deprivation-induced apoptosis in an NF-κB-dependent manner[57].

Abnormal calcium homeostasis is linked to the pathogenesis of many diseases, and a plethora of therapeutic approaches aim to re-establish normal calcium homeostasis. G-protein-coupled receptors (GPCRs) are common drug targets owing to their ability to activate intracellular calcium release through the activation of IP3 receptors. The new findings on the signalling properties of Na^+/K^+-ATPase qualify this molecule as an alternative mediator of IP3-receptor-mediated calcium release and a potential new therapeutic target for calcium-related pathologies[5].

Signalling through Ras activation. Na^+/K^+-ATPase can also relay signals through activation of other multiple protein–protein interactions. The initial event, following binding of cardiac glycosides, is the release of the cytoplasmic tyrosine kinase SRC from the complex signalosome[45]. SRC kinase is activated upon phosphorylation at Tyr418 and, in turn, activates the proximal EGFR. Activated EGFR sequentially recruits the adaptor proteins SHC, growth factor receptor-bound protein 2 (GRB2) and SOS until eventually the signal activates the Ras–RAF–MAPK (mitogen-activated protein kinase) cascade[3,58].

Activation of Ras stimulates several downstream signalling cascades. In cardiac myocytes, ouabain-induced activation of Ras triggers the opening of the ATP-sensitive mitochondrial potassium channels, resulting in a concomitant production of mitochondrial reactive oxygen species (ROS)[59]. ROS, in turn, activate NF-κB, which stimulates the transcription of several cell-growth-related and differentiation genes, in parallel with the calcium-induced NF-κB activation. ROS production is also the result of a third described pathway,

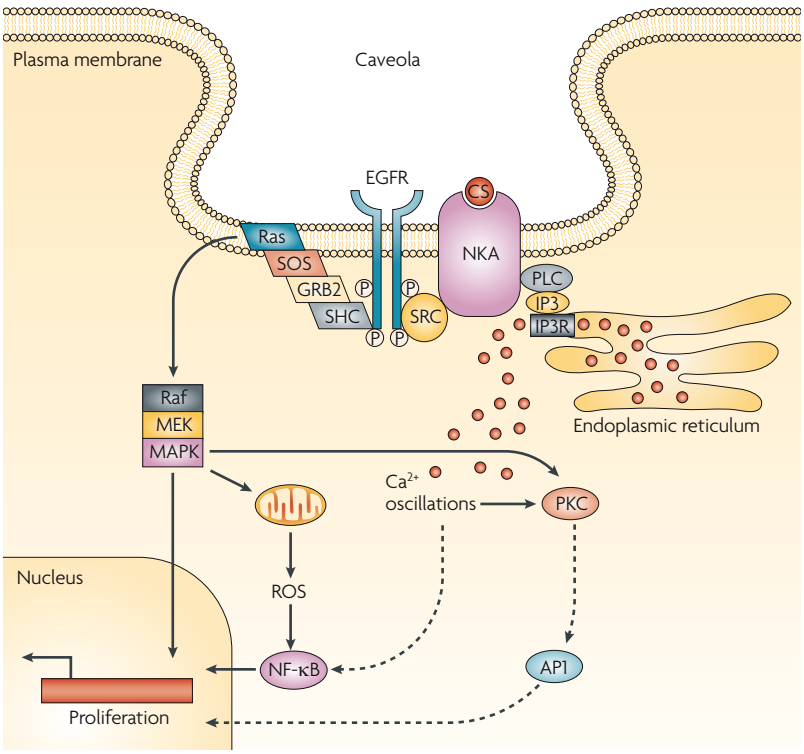


Figure 2 | **Na^+/K^+-ATPase as a versatile signal transducer.** Binding of cardiac glycosides to the preassembled Na^+/K^+-ATPase (NKA) signalosome activates multiple signal transduction cascades that inhibit cell death and trigger proliferation in myocytes, endothelial cells and epithelial cells. To summarize briefly, following cardiac glycoside binding to NKA, the tyrosine kinase SRC is activated and in turn activates the proximal epidermal growth-factor receptor (EGFR). Activated EGFR sequentially recruits the adaptors SHC, growth factor receptor-bound protein 2 (GRB2) and SOS, which ultimately leads to activation of the mitogen-activated protein kinase (MAPK) cascade. In parallel, phospholipase C (PLC) and inositol 1,4,5-triphosphate (IP3) also participate in the formation of a functional microdomain that brings NKA into direct contact with the IP3 receptor (IP3R) of the endoplasmic reticulum. At this point, single or repeated transient increases in intracellular Ca^{2+} are produced. Ca^{2+} oscillations are a universal mode of signalling that mediate a diverse range of cell functions including cell proliferation, differentiation and apoptosis. AP1, activating protein 1; CS, cardiac steroids; MEK, MAPK kinase; NF-κB, nuclear factor-κB; PKC, protein kinase C; ROS, reactive oxygen species. Adapted from REF. 58.

which is based on PI3K–Akt activation. PI3K is another part of the signalosome and is bound to a proline-rich region of the catalytic α-subunit of Na^+/K^+-ATPase[60]. In a similar way, stimulated PI3K mediates proliferation signals through nitric oxide-induced production of ROS[61]. FIGURE 2 summarizes some of the signalling cascades that are activated in myocytes, endothelial cells and epithelial cells when cardiac glycosides bind to Na^+/K^+-ATPase. Mitochondrial ROS and calcium ions seem to be the crucial downstream secondary messengers that mediate signals to the nucleus[62].

Collectively, these new findings indicate that cardiac glycosides can regulate several cellular processes, which are beyond their well-established role in ion homeostasis. Indeed, a growing body of recent publications describe the effects of these compounds on the regulation of gene expression[63], cell attachment[64], orientation of polarity[65], protein trafficking[66] and induction of proliferation[3,53,67,68]. Overall, it is now clear that the ultimate response to cardiac glycoside treatment is dependent on the tissue, exposure time and dose[10]. Notably, responses of cancer cells to cardiac glycoside treatment seem to be different, which has stimulated interest in their potential as anticancer drugs, as described in the following section.

Cardiac glycosides and cancer therapy

In the 1980s, Stenkvist and colleagues reported that breast cancer cells obtained from women on digitalis therapy were characterized by a series of more benign features compared with cancer cells from control patients[69–71]. Moreover, 5 years after mastectomy, the recurrence rate of breast cancer among patients on digitalis treatment was 9.6-times lower compared with patients not on digitalis[71]. Around the same time, a second confirmation came from Goldin and colleagues, who studied the effects of digitalis treatment in 127 patients with cancer. Among the 21 deaths attributed to cancer, only one patient belonged to the digitalis group[72]. However, these encouraging results did not trigger much attention at the time. More recently, Stenkvist reported on 20 years of follow-up data and demonstrated that patients receiving digitalis had a significantly reduced mortality rate (6%, 2/32) compared with the control group (34%, 48/143)[73]. However, the small number of patients in this study did not allow strong conclusions to be made regarding the anticancer effects of these drugs. In response to this need, Haux and colleagues conducted an internal dose–response analysis of 9,271 patients on digitoxin treatment and investigated the potential anticancer effects of this compound. Although no significant anticancer effects were reported, a link between high plasma concentration of digitoxin and reduced risk for leukaemia and for cancers of the urinary tract were proposed[74,75].

These data paved the way for numerous follow-up studies that established the anticancer properties of cardiac glycosides. More than a thousand papers are now published, with most describing the anticancer properties of these compounds *in vitro*.

In vitro *evidence for the anticancer properties of cardiac glycosides.* The first *in vitro* evidence for the inhibition of malignant cell proliferation by cardiac glycosides dates back to 1967 (REF. 76). Since then, numerous other reports have confirmed the antiproliferative and apoptotic effects of these compounds in several cancer cell lines, including breast[6,77,78], prostate[79–81], melanoma[82], pancreatic[83], lung[84,85], leukaemia[86–91], neuroblastoma[92] and renal adenocarcinoma[78] (TABLE 2). The exact mechanisms underlying these effects of cardiac glycosides are not yet fully elucidated; a summary of possible mechanisms is provided in BOX 1.

Interestingly, marked differences characterize the potencies of these structurally similar compounds. For instance, Johansson and colleagues evaluated the cytotoxic profile (IC_{50}) of seven cardiac glycosides in primary cultures of tumour cells from patients and in a panel of human cell lines[93]. They found that proscillaridin A was the most potent, followed by digitoxin, ouabain, digoxin, lanatoside C, digitoxigenin and digitonin[93]. Furthermore,

Table 2 | ***In vitro* antiproliferative and/or apoptotic effects of cardiac glycosides in cancer cells**

Cancer type	Compounds tested	Cancer cell lines	Refs
Breast	Digitoxin, digoxin, proscillaridin A, ouabain, digoxigenin, gitoxin, gitoxigenin	MCF-7, MDA-MD-435	77,78
Prostate	Oleandrin, ouabain, digoxin, bufalin, cinobufagenin	PC-3, LNCaP, DU145	79–81
Melanoma	Digoxin, oleandrin, digitoxin, proscillaridin A, ouabain, digitonin	UACC-62, BRO	78,82
Lung	Digitoxin, digoxin, ouabain, UNBS1450, oleandrin	A549, NCI-H-358, Calu1, Sklu1, NCI-H6, H69AR	15,84, 85,93
Leukaemia	Bufalin, oleandrin, digitoxin, proscillaridin A, ouabain	HL60, U-937, CCRF-CEM, CEM-VM-1	78,87, 89–91,93
Neuroblastoma	Digoxin, ouabain	SH-SY5Y, Neuro-2a	92
Renal	Digitoxin, digoxin, digitoxigenin, proscillaridin A, ouabain	TK-10, ACHN	78,93
Myeloma	Digitoxin, digoxin, proscillaridin A, digitoxigenin, ouabain, digitonin, lanatocide C	8226-S, 8226-LR5, 8226-DOX-40	86,93
Pancreatic	Oleandrin	PANC-1	83

Van Quaquebeke *et al.*[94] semi-synthesized a library of 27 novel cardenolides and studied their structure–activity profile against a panel of 57 cancer cell lines. One of these compounds, UNBS1450, displayed better anti-tumour properties *in vitro* compared with commonly used chemotherapeutic drugs, and was best tolerated *in vivo* by mice compared with digitoxin and ouabain[94]. Such structure–activity relationship analyses highlight the structural characteristics that are important for the activity of these compounds, and lay the foundations for the development of novel, more active compounds with higher *in vivo* tolerance and improved therapeutic potential as anticancer agents.

In parallel, several recent publications highlight the effects of these compounds in the regulation of the gene-expression profiles of many cancer cells. Johnson *et al.*[95] screened 9,000 compounds for their ability to simultaneously inhibit expression of six commonly over-expressed genes in prostate cancer cells. Interestingly, digitoxin and ouabain were the only compounds that could confer significant inhibition in the expression of four of the target genes, including transcription factors *HOXB13*, *PDEF* (also known as *SPDEF*), hepatocyte nuclear factor 3α (*HNF3A*; also known as *FOXOA1*) and the apoptosis inhibitor survivin[95]. Moreover, oleandrin was shown to inhibit export of fibroblast growth factor 2 (FGF2) from PC-3 and DU145 prostate cancer cells in a concentration-dependent and time-dependent manner[96]. Manna *et al.*[97] found that oleandrin inhibits interleukin 8 (IL8)-mediated biological responses by altering the plasma-membrane fluidity. IL8 is highly expressed in many cancers, where it acts as a chemo-attractant and is a principal angiogenic stimulus for neovascularization.

Finally, cardiac steroids can also be used to improve the therapeutic index of radiation therapy. It was shown that when human lung adenocarcinoma cells were treated with low concentrations of ouabain, they become radiosensitized, unlike normal human lung fibroblasts[98]. In support of this, Verheye-Dua *et al.*[99] showed that ouabain enhances irradiation damage in a panel of cancer cells. It is suggested that cells treated with these compounds accumulate in the G2M phase, in which they are more sensitive to radiation. Another glycoside, oleandrin, has been found to enhance the sensitivity of PC-3 human prostate cells to radiation. Susceptibility of PC-3 cells to oleandrin and radiation-induced apoptosis was dependent on activation of caspase 3 (CASP3)[100].

Ex vivo *and* in vivo *data on the anticancer effects of cardiac glycosides.* More than a decade ago, Inada *et al.*[101] first reported the ability of digitoxin to inhibit tumour formation in a two-stage carcinogenesis model of mouse skin papillomas induced by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and in a mouse pulmonary tumour model induced by 4-nitroquinoline-*N*-oxide (4NQO) and glycerol. In agreement with this, a study by Afaq *et al.*[102] investigating the tumour growth-inhibitory effects of oleandrin after TPA induction of skin carcinogenesis found that topical application of oleandrin (2 mg per mouse) half an hour before TPA induction (3.2 nmol per mouse) significantly inhibited skin carcinogenesis in a time-dependent manner. Furthermore, significant anticarcinogenic effects of cardiac glycosides were evident in human neuroblastoma tumours. Svensson *et al.*[103] demonstrated that digoxin is a specific neuroblastoma growth inhibitor in mice grafted with the neuroblastoma cell lines SH-SY5Y and Neuro-2a. Moreover, Han *et al.*[104] investigated the antitumour activities of bufalin in an orthotropic transplantation tumour model of human hepatocellular carcinoma in nude mice and found that non-toxic concentrations of bufalin can induce specific apoptosis of transplanted tumour cells.

Box 1 | **Potential mechanisms for the anticancer effects of cardiac glycosides**

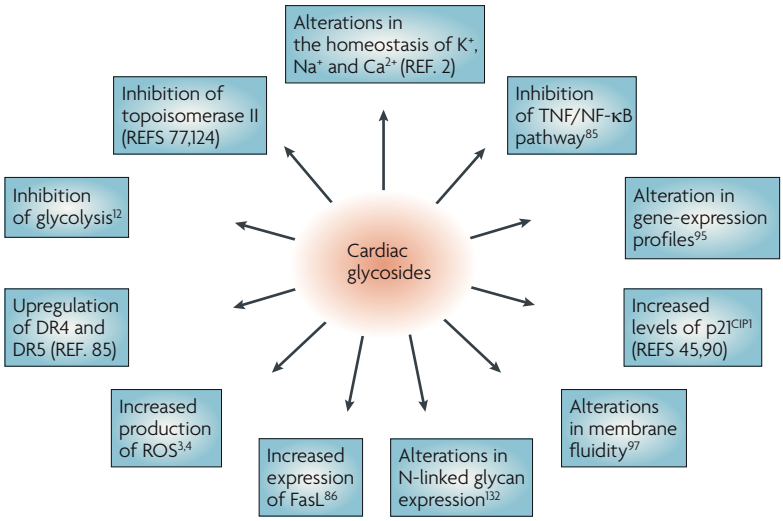
The molecular mechanisms underlying the increased susceptibility of cancer cells to cardiac glycosides are not yet fully elucidated; possible mechanisms are summarized in the figure below.

Interestingly, despite the fact that the same, or closely related signalling cascades, seem to be implicated, the final responses of cancer cells and normal cells to cardiac glycosides differ. It is postulated that the differential expression and activity of the Na^+/K^+-ATPase subunits in tumour tissues compared with their normal counterparts might play a major role in this difference in response. Indeed, it is established that malignant transformation is characterized by a significant increase in the activity of Na^+/K^+-ATPase (leakage theory)[118]. Moreover, alterations in the expression profile of the Na^+/K^+-ATPase subunits were evident in various cancers, including bladder[119], gastric[120], colorectal[121] and non-small-cell lung cancer[122]. More details on the role of Na^+/K^+-ATPase in cancer can be found in recent reviews[10,123].

Unlike in normal cells, it has been demonstrated that activation of the SRC–epidermal growth-factor receptor (EGFR)–mitogen-activated protein kinase (MAPK) pathway in cancer cells by cardiac glycosides results in growth arrest through an increased expression of the cyclin-dependent kinase inhibitor 1A ($p21^{CIP1}$)[6]. Moreover, inhibition of DNA topoisomerase activity might largely explain the apoptotic effects of these compounds[124]. In fact, it was reported that digitoxin, at concentrations found in patients with cardiac conditions, induces levels of DNA topoisomerase II cleavable complexes similar to etoposide (a topoisomerase poison currently used clinically)[78]. Increased expression of prostate apoptosis response 4 (PAR4)[80], T-cell lymphoma invasion and metastasis 1 (TIAM1)[90] and death receptors 4 and 5 (DR4 and DR5; also known as TNFRSF10A and TNFRSF10B, respectively)[85] have also been shown to be involved in the apoptotic effects of these drugs.

Providing a different perspective, Lopez-Lazaro recently raised the interesting hypothesis that inhibition of Na^+/K^+-ATPase and concomitant inhibition of glycolysis might explain the anticancer effects of these compounds[12]. It is known that cancer cells are characterized by increased rates of aerobic glycolysis (Warburg effect), and that the constitutive activation of glycolysis is essential for cancer progression[125–128]. Additionally, it is recognized that cancer cells cannot generate enough ATP via oxidative phosphorylation (due to alterations in proteins required for this process) and therefore inhibition of glycolysis may result in ATP depletion and cell death[129]. In addition to this, inhibition of glycolysis reduces the capacity of cancer cells to eliminate H_2O_2 and therefore cell death mechanisms are activated (unlike cancer cells, normal cells do not produce high levels of H_2O_2)[130]. Given that cardiac glycosides have long been known to be able to inhibit aerobic glycolysis[131], it is suggested that attenuation of aerobic glycolysis following interaction of cardiac glycosides with cancer cells might account for their ability to selectively kill them[12]. Finally, in a recent study, prevention of distant tumour formation by digoxin in two mouse models of metastatic prostate cancer was attributed to the impairment of N-linked glycan expression and function of cancer cells[132].

FasL, Fas ligand; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; TNF, tumour-necrosis factor.



Glycoside-based anticancer drugs in the clinic. The already well-established pharmacodynamics and pharmacokinetics of these compounds provide a shorter pathway to clinical trials. In April 2000, the US Food and Drug Administration (FDA) approved a Phase I study of Anvirzel in patients with advanced solid tumours. Anvirzel is an aqueous extract of the plant *Nerium oleander*. It contains a variety of compounds including polysaccharides, proteins, sugars and cardiac glycosides — mainly oleandrin and its aglycone oleandrigenin. It has been demonstrated that Anvirzel inhibits the export of FGF2 from prostate cancer cells through sodium-pump inhibition by oleandrin[96]. Furthermore, Pathak *et al.*[105] investigated the mechanisms of Anvirzel-induced cancer cell death in various cancer cell lines of human, murine and canine origin and found that human cells are more susceptible to the effects of this drug. The results of Phase I trials show that Anvirzel can be safely administered to patients with solid tumours. Overall, this agent appears to be well tolerated as patients in the trial experienced only mild-to-moderate side effects. No evidence of significant antitumour activity was detected, but this might be due to the fact that the patient group consisted exclusively of individuals who had refractory cancers. To our knowledge, no Phase II clinical trials of Anvirzel have been conducted; however, another supercritical CO_2 extract of *N. oleander* recently entered Phase I clinical trials at the University of Texas, M.D. Anderson Cancer Center.

In parallel, UNBS1450, a semi-synthetic derivative of the novel cardenolide 2′′-oxovoruscharin, entered Phase I clinical trials in Belgium in 2006. This promising novel cardenolide has been shown to deactivate NF-κB-mediated cytoprotective effects in human non-small-cell lung cancer (NSCLC) cells[15,84]. The modifications induced by UNBS1450 led to a decrease in both the DNA-binding capacity of the p65 subunit and the NF-κB transcriptional activity[15,84]. UNBS1450 was as potent as taxol and SN38 (the active metabolite of irinotecan) in reducing the overall growth levels of the human A549 NSCLC cell line, and was more efficient than platin derivatives, including cisplatin, carboplatin and oxaliplatin[15,84].

Cardiac glycosides and other diseases

Cardiac glycosides, in particular digoxin and digitoxin, have been a cornerstone of the treatment of heart diseases for more than two centuries. However, the identification of angiotensin-converting enzyme inhibitors, β-adrenergic blockers and angiotensin-receptor blockers has significantly reduced their clinical use. Nevertheless, recent analysis of the large-scale randomized Digitalis Investigation Group trial reported that digoxin at low-serum concentrations significantly reduced mortality and hospitalizations in ambulatory patients with chronic systolic and diastolic heart failure[106]. However, whether digoxin should be considered a drug of the past for the treatment of heart diseases is still a controversial issue[107–109]. Moreover, recent findings regarding the signalling properties of Na^+/K^+-ATPase suggest improved therapeutic aspects of these compounds for the treatment

N-linked glycan
Sugars attached to the R-group nitrogen (N) of asparagine in the sequence Asn-X-Ser or Asn-X-Thr (X = all amino acids except for proline).

of heart diseases. As described above, these drugs trigger cardiac contraction through calcium oscillations at concentrations that do not interfere with the pumping activity of Na^+/K^+-ATPase. So, novel cardiac-glycoside-based drugs that can preferentially activate the signalling properties of Na^+/K^+-ATPase (improved inotropy-to-toxicity ratio) might represent better drugs for the treatment of cardiac pathologies.

Primary data also reveal potential applications of cardiac glycosides for the treatment of cystic fibrosis. The profound lung inflammation that characterizes cystic fibrosis is mainly attributed to an overproduction of IL8 in the lung. Interestingly, oleandrin has been shown to inhibit IL8-mediated biological responses in diverse cell types by modulating IL8 receptors through altering membrane fluidity and microviscosity[97]. In agreement with this, therapeutic concentrations of digitoxin were enough to not only suppress hypersecretion of IL8 from cystic fibrosis lung cells *in vitro*, but to potentially mimic gene therapy with wild-type *CFTR*. Indeed, comparative gene-expression analysis showed that the majority (62%) of the 'informative' genes affected by *CFTR* gene therapy were similarly affected upon treatment with non-toxic doses of digitoxin[110].

As already noted above, low concentrations of cardiac glycosides trigger downstream signalling cascades that can serve to prevent cell death and induce proliferation[57]. These effects underlie possible therapeutic uses of cardiac glycosides in the context of ischaemic stroke. Indeed, in a recent chemical genetic screen, Wang *et al.*[111] identified cardiac glycosides — neriifolin, digoxin, digitoxin and ouabain — as the molecules with the most potent neuroprotective effects in two *ex vivo* brain explant-based experimental models of ischaemic stroke, as well as in two independent animal models for clinical stroke. At the same time, studies by Pierre *et al.*[112] investigating the cardioprotective effects of diaxozide reported that certain compounds, including cardiac glycosides, that can cause opening of the mitochondrial ATP-sensitive potassium channels (K_{ATP}) might have therapeutic potential for the protection of ischaemic heart tissue. Indeed, ouabain was shown to protect rat hearts against ischaemia–reperfusion injury[112]. Taken together, these new findings suggest that cardiac-glycoside-based agents might have potential as novel therapies for stroke and heart ischaemia.

Finally, Piccioni *et al.*[113] recently suggested a new link between cardiac glycosides and neurodegenerative diseases. They screened 1,040 FDA-approved drugs for their ability to inhibit polyglutamine-dependent CASP3 activation. Interestingly, three of the four hits identified belong to the cardiac glycoside family — digitoxin, neriifolin and peruvoside — which suggests new therapeutic roles of these drugs for spinobulbar muscular atrophy and other polyglutamine-related diseases[113].

Collectively, these data highlight a potential multi-therapeutic character for these compounds. However, it should also be noted that increased levels of endogenous cardiac glycosides are implicated in numerous pathological states. For instance, high levels of endogenous glycosides are associated with high blood pressure and hypertension[114–117]. Indeed, rostafuroxin, an endogenous ouabain antagonist, is undergoing Phase II clinical trials for the treatment of essential (primary) hypertension.

Conclusions

Cardiac glycosides have a long history of therapeutic application. The early understanding of their positive inotropic effects facilitated their use as effective drugs for the treatment of heart-related pathologies. More recently, considerable *in vitro*, *in vivo* and epidemiological data support novel roles for such drugs for the treatment of several diseases.

Most notably, it is now established that cardiac glycosides can induce apoptosis and inhibit the growth of cancer cell lines at concentrations close to those found in the plasma of patients with cardiac conditions. Furthermore, on the basis of the increased susceptibility of cancer cells to cardiac glycosides, the potential use of cardiac glycosides as anticancer agents might be associated with fewer side effects than traditional cytotoxic therapies. Studies in animal models have validated the anticancer effects of these compounds and the first cardiac-glycoside-based anticancer drugs are now undergoing clinical trials.

In addition, in contrast to the apoptotic effects of these drugs on cancer cells, low concentrations of cardiac glycosides have been shown to stimulate proliferation and inhibit cell death in normal cells. These cytoprotective effects might form the basis for novel cardiac-glycoside-based future therapies for the treatment of ischaemic stroke and neurodegenerative diseases.

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