Cardiotonic Steroids-Mediated Na^+/K^+-ATPase Targeting Could Circumvent Various Chemoresistance Pathways

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- Na^+/K^+-ATPase
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- multidrug resistance

Abstract

Many cancer patients fail to respond to chemotherapy because of the intrinsic resistance of their cancer to pro-apoptotic stimuli or the acquisition of the multidrug resistant phenotype during chronic treatment. Previous data from our groups and from others point to the sodium/potassium pump (the Na^+/K^+-ATPase, i.e., NaK) with its highly specific ligands (i.e., cardiotonic steroids) as a new target for combating cancers associated with dismal prognoses, including gliomas, melanomas, non-small cell lung cancers, renal cell carcinomas, and colon cancers. Cardiotonic steroid-mediated Na^+/K^+-ATPase targeting could circumvent various resistance pathways. The most probable pathways include the involvement of Na^+/K^+-ATPase β subunits in invasion features and Na^+/K^+-ATPase α subunits in chemosensitisation by specific cardiotonic steroid-mediated apoptosis and anoïkis-sensitisation; the regulation of the expression of multidrug resistant-related genes; post-translational regulation, including glycosylation and ubiquitinylation of multidrug resistant-related proteins; c-Myc downregulation; hypoxia-inducible factor downregulation; NF-κB downregulation and deactivation; the inhibition of the glycolytic pathway with a reduction of intra-cellular ATP levels and an induction of non-apoptotic cell death. The aims of this review are to examine the various molecular pathways by which the NaK targeting can be more deleterious to biologically aggressive cancer cells than to normal cells.

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Introduction

Resistance of cancer cells

Resistance to chemotherapy is the most important reason for treatment failure in cancer patients. Tumours may be intrinsically drug-resistant or develop resistance to chemotherapy during treatment [1]. It is well known that cancer cells are able to resist various cytotoxic agents because they possess a set of anti-cell death mechanisms that counteract chemotherapeutic responses. These protective mechanisms include the constitutive activation of the phosphatidylinositide 3-kinase (PI3-K)/Akt and the nuclear factor-kappa B (NF-κB) signalling pathways, which are interlinked [2,3]. Treatment can lead to the death of most tumour cells (drug-sensitive), but some cells (drug-resistant) survive and grow. Cancer has the ability to become resistant to many different types of drugs. Increased efflux of drug, enhanced repair and increased tolerance to DNA damage, high anti-apoptotic potential, decreased permeability and enzymatic deactivation allow cancer cells to survive chemotherapy. Acquired resistance is a particular problem, as tumours do not only become resistant to the drugs that are originally used to treat them but may also become cross-resistant to other drugs with different mechanisms of action.

A major obstacle to the effective treatment of cancer is the multidrug resistance (MDR) phenomenon exhibited by many cancers [4,5]. MDR can be an intrinsic characteristic of malignant cells or acquired during drug therapy [5]. The most prominent mechanisms mediating MDR to anti-neoplastic agents are (a) over-expression of members of three ATP-binding cassette (ABC) transporter sub-families, ABCB, ABCC, and ABCG, (b) lung resistance-related protein (LRP, identified as the major vault protein (MVP)), and (c) loss of genes, such as p53, that control DNA integrity [5–7]. Thus, targeting or circumventing these proteins' activities would have a major impact on cancer

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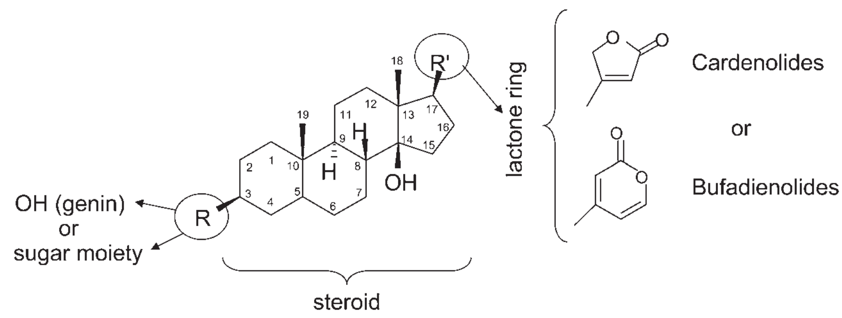

Fig. 1 Classification and chemical structures of cardiotonic steroids. Cardiotonic steroids are compounds presenting a steroid nucleus with a lactone moiety at position 17. The aglycone moiety is composed of the steroid nucleus and the R group (lactone ring) at position 17 that defines the class of cardiotonic steroid: the cardenolides (with an unsaturated butyrolactone ring) and the bufadienolides (with an *α*-pyrone ring). The steroid nucleus has a unique set of fused ring systems that makes the aglycone moiety structurally distinct from the other more common steroid ring systems. The steroidal skeleton can be substituted at position 3 by the third structural component, a sugar moiety (glycoside), leading to the chemical classification of sub-families as glycosylated cardenolides or glycosylated bufadienolides (depending on the lactone moiety). Up to 4 sugar molecules may be present in cardiac glycosides; attached in many via the 3*β*-OH group.

chemotherapy and cancer patients' survival [8]. Although many efforts to overcome MDR have been made, no outstanding breakthroughs have been achieved [8]. Consequently, there remains an urgent need to identify new biological targets associated with cancer cell chemoresistance as well as novel anti-cancer agents, with the goal of overcoming resistance to chemotherapy. Previous unsuccessful approaches indicate the need to target simultaneously multiple MDR-related targets and thus disable the cancer cells' ability to deploy escape strategies. Accordingly, a completely new way of attacking resistant cancer cells might rely on targeting the sodium/potassium pump (Na^+/K^+-ATPase; NaK) with its highly specific ligands, i.e., cardiotonic steroids (CS).

The sodium/potassium pump (Na^+/K^+-ATPase; NaK)

NaK is an integral membrane protein composed of catalytic *α* and regulatory *β* subunits; it is responsible for translocating sodium and potassium ions across the cell membrane utilising ATP as the driving force [9]. Although the transport function of the Na^+/K^+-ATPase has been investigated extensively in the past, during the last decade multiple lines of evidence have suggested a number of other functions for the sodium pump, revealing NaK as (i) a multifunctional protein with key roles in the formation and maintenance of adhesion complexes, induction of epithelial cell tight junctions and polarity, cell adhesion, motility, and actin dynamics [10–18], (ii) a signalling protein [19–26], and (iii) a valuable novel target in anti-cancer therapy because its aberrant expression and activity are implicated in the development and progression of a growing number of cancers [27–38].

In addition to the growing number of scientific publications, a number of inventions (recently reviewed in [39]) have also emphasised the potential usefulness of considering NaK expression for future anti-cancer therapy by using it as a diagnostic and prognostic tool, as a biomarker of a therapeutic response in cancer chemotherapy with CS, and as a valuable new target. A recent, in-depth analysis of patent literature [39] revealed a large increase in the number of inventions focusing on new NaK inhibitors and ligands designed or selected as potential anti-cancer agents.

Cardiotonic steroids

The CS, which include cardenolides and bufadienolides (**Fig. 1**), are compounds that are able to bind to the extracellular surface of the NaK [27] and are its natural ligands. The best-known naturally occurring CS are digoxin, digitoxin, ouabain, and oleandrin as cardenolides as well as bufalin, hellebrin, and marinobufagenin as bufadienolides. The CS have long been used as positive inotropic agents in the treatment of congestive heart failure [40]. Retrospective epidemiological studies conducted during the late 20th century revealed some intriguing results: very few patients that underwent CS treatment for heart problems died from cancer [41]. Over the last 20 years, interest in developing the CS as anti-cancer agents has grown progressively. CS were identified to be among the most potent inhibitors (out of 9000 screened chemicals) of the prostate cancer target genes investigated [42]. Furthermore, in a large investigation that searched for new natural, cytotoxic anti-cancer compounds, Lindholm et al. [43] screened extracts from 100 different plants and obtained seven plants with strong evidence of anti-tumour potential, among which were three CS-enriched plants, *Digitalis lanata, Digitalis purpurea*, and *Helleborus cyclophyllus*. By binding to the sodium pump, CS elicit marked effects on cancer cell behaviour, and a number of studies have emphasised their potential use in oncology [27,37,44,45]. Some recent reviews [27,37,45–48] summarise the anti-tumour properties of this class of compounds as well as their multiple mechanisms of action (briefly summarized in **Fig. 2**). We recently reviewed the scientific literature to perform an in-depth structure-activity relationship (SAR) analysis with respect to cardenolide- versus bufadienolide-mediated anti-cancer effects [47]. In that review, we described the SAR of the CS based on a molecular model of the NaK pump bound to ouabain [47]. After an analysis of the anti-cancer potency of the most representative CS, we determined the key structural features that lead to powerful cytotoxic agents and those that are deleterious for anti-tumour activity.

It is interesting that the CS tested *in vitro* induced potent antiproliferative effects in all of the human cancer cell lines examined; consequently, there is no particularly resistant human cancer type. Indeed, the cancer cell lines in the NCI 60 panel (http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp) display similar sensitivities to the CS tested (ouabain, digitoxin, and hellebrin), and this effect was further confirmed with 19-hy-

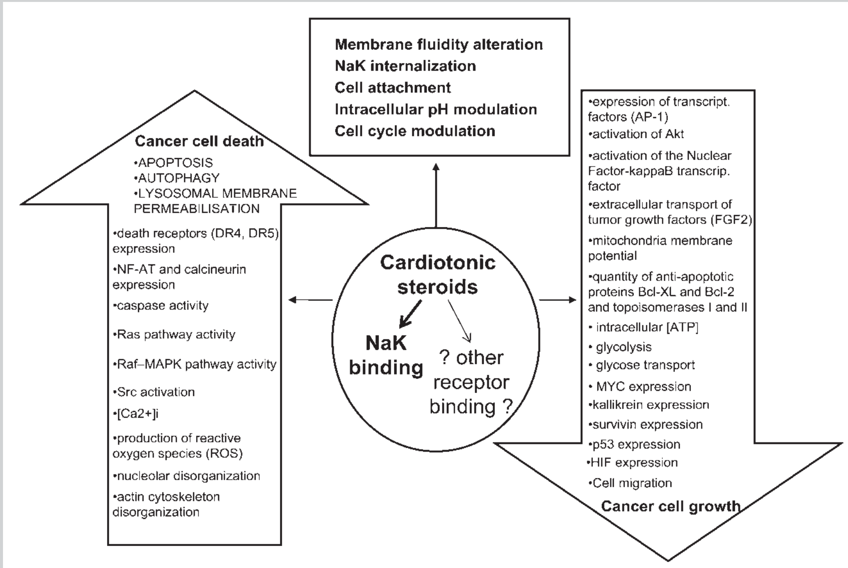

Fig. 2 Summary of postulated mechanisms of CS-mediated anti-cancer activity. Summary of the multiplicity of suggested molecular targets for the action of most studied CS in human cancer cells. For more details see [19, 20, 26, 27, 37, 45–49, 93].

droxy-2″-oxovoruscharin (also known as UNBS1450) [27, 37, 44, 49]. A growing number of reports document the ability of some CS to circumvent cancer cell chemoresistance [50–54], making them an interesting starting point for the development of new anti-chemoresistance treatment strategies.

Aims of the Review
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The aims of this review are to examine the various molecular pathways by which the NaK targeting can be more deleterious to biologically aggressive cancer cells than to normal cells. In order to achieve this goal, a computerized literature search (using Pub Med database, ASCO and AACR annual meetings' proceedings, and World Intellectual Property Organization database) identified relevant published/presented studies. References of papers thus obtained were studied, and most relevant papers included. In order to keep the number of cited papers on the reasonable level, for background parts, reviews from highly ranked Journals have been used/cited instead of original papers. For the discussion of CS-mediated chemoresistance, all available publications have been used in order to present the most complete overview.

Fighting Resistant Cancer Cells through Na^+/K^+-ATPase Targeting
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Migrating cells are particularly resistant to cytotoxic agents: involvement of the NaK β subunit in pro-cell attachment strategies

Resistance to chemotherapy is believed to cause treatment failure in more than 90% of patients with metastatic cancer. Because metastatic cancers originate from migrating cells, specific anti-migratory strategies should be added to conventional radio- and/or chemotherapy.

The Na^+/K^+-ATPase associates with a number of signalling molecules and with the actin cytoskeleton, forming a multiprotein complex (recently reviewed in [17, 18]). The effect of CS, and particularly ouabain, on the adhesive state of the cell was studied extensively, and signalling cascades involved in the so-called P → A mechanism (pump → attachment) were deciphered by Contreras et al. [12–16]. Contreras' group demonstrated that ouabain affects cell attachment through a complex signalling cascade and by sending β-catenin to the nucleus, where it is known to act as a transcriptional cofactor [12–16]. These reports further emphasise that the interactions of CS with NaK could markedly affect cell migration features. Furthermore, Rajasekaran et al. [10] presented evidence that NaK plays a crucial role in E-cadherin-mediated development of epithelial polarity and suppression of invasiveness and motility of carcinoma cells. Their results suggest that E-cadherin-mediated cell-cell adhesion requires the function of the NaK β subunit to induce epithelial polarisation and suppress the invasiveness and motility of carcinoma cells. Tummala et al. [55] revealed that reduced expression of the NaK β1 protein is associated with oxaliplatin resistance in cancer cells and demonstrated a novel role for this protein in sensitising the cells to oxaliplatin. Although the mechanism by which NaK β1 increases sensitivity to oxaliplatin is not known, it is tempting to speculate that the cell–cell adhesion function of NaK β1 might be involved in this process. Importantly, it has been widely reported that NaK β1 subunits are very frequently downregulated in human epithelial cancer cells [10, 11, 28–30, 56]. The Rajasekaran group [10, 11, 28, 33, 56, 57] noted that when these cells downregulate β1, they detach from each other as a result of a marked reduction in cadherin expression, a process in which the Snail transcription factor plays a major role [10, 29, 56]. Thus, downregulation of β1 subunits seems essential for epithelial cancer cells to become individually invasive and chemoresistant. The NaK β1 downregulation might result from its rapid degradation in cancer cells. Yoshimura et al. [58] recently demonstrated that the α and β subunits of NaK are assembled in the endoplasmic reticulum but are disassembled in the plasma membrane and undergo different degradation processes, leading to over-expression of the α subunits and faster degradation of the β subunit. Thus, restoration of NaK β1 expression might contribute to preventing cancer cell migration and the resulting invasion, metastasis, and chemoresistance. Alternatively, compounds inducing NaK β1 expression might provide an interesting complement to the

Table 1 Potential of CS to (i) act as apoptosis sensitizers, (ii) act as anoïkis sensitizers, and (iii) be potent inducers of autophagy-like cell death.

Function	CS	Mechanism	Reference
Apoptosis sensitizer	oleandrin	Apo2L/TRAIL-induced apoptosis via upregulation of death receptors 4 and 5 in non-small cell lung cancer cells	[60]
Apoptosis sensitizer	oleandrin, ouabain, digoxin	stimulate Ca2+ increases and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells	[61]
Apoptosis sensitizer	oleandrin	oleandrin-mediated expression of Fas that potentiates apoptosis	[62]
Apoptosis sensitizer	bufalin, bufotalin, gamabufotalin	TRAIL-sensitising agents, especially for the triple negative breast cancer	[63]
Anoïkis sensitizers	ouabain, peruvoside, digoxin, digitoxin, strophanthidin	anoïkis sensitisation in anoïkis-resistant PPC-1 prostate adenocarcinoma cells through the mitochondrial pathway of caspase activation and by inducing hypoosmotic stress	[64]
Inducers of autophagy-like cell death	oleandrin	authophagic cell death of pancreatic cancer cells	[65]
Inducers of autophagy-like cell death	19-hydroxy-2′′-oxovoruscharin	disorganisation of the actin cytoskeleton and induction of severe autophagic process	[34]
Inducers of autophagy-like cell death	19-hydroxy-2′′-oxovoruscharin	decrease of Hsp70 expression and induction of the lysosomal membrane permeabilisation	[59]

standard anti-metastatic therapy. To the best of our knowledge, no such compound has been reported.

A number of cancers display intrinsic resistance to pro-apoptotic stimuli: targeting of NaK α subunit by CS

The malignant transformation of cells is associated with a constellation of pro-survival mutations that increase the cells' resistance to apoptosis. Because most of the agents used in current anti-cancer therapies are pro-apoptotic agents, agents that induce other types of cell death or act as apoptosis sensitizers might offer better therapeutic results. Consistent with this idea, as summarised in **Table 1**, several reports [34, 59–65] have documented the potential of CS, at least *in vitro*, to (i) act as apoptosis sensitizers, (ii) act as anoïkis sensitizers, and (iii) be potent inducers of autophagy-like cell death.

Multidrug resistance as one of the major reasons for the failure of anti-cancer therapy: targeting of the NaK α subunit by anti-MDR CS

Available research data point to the divergent behaviour of CS with respect to the induction and repression of MDR. Most known cardenolides have been reported to antagonise the activity of several chemotherapeutic agents. Digoxin was shown to up-regulate MDR1 mRNA, [66] and Huang et al. [67] reported that ouabain and digitoxin induced resistance to tubulin-dependent anti-cancer drugs such as paclitaxel, colchicine, vincristine, and vinblastine in androgen-independent human prostate cancer. It was suggested that these cardenolides inhibit the G2/M arrest induced by tubulin-binding anti-cancer drugs via an indirect blockage of microtubule function. Furthermore, a decline in the transport of these tubulin-dependent anti-cancer drugs into the nucleus may explain the antagonistic action of these cardenolides. Ouabain provokes reduced doxorubicin-mediated cytotoxicity in human A549 non-small cell lung cancer (NSCLC), HT29 colon cancer, and U1 melanoma by decreasing doxorubicin-induced topoisomerase-mediated DNA strand breakage [68]. This response indicates that altered ionic gradients are a potential cause of resistance to drugs that use topoisomerase II as a target [68]. Additionally, Ahmed et al. [69] reported that cisplatin accumulation in oral squamous carcinoma cells is regulated by NaK and thus, its inhibition markedly reduced intra-cellular cisplatin accumulation. In contrast, the reports on less thoroughly investigated CS indicate the potential usefulness of these CS to combat chemoresistant cancers [52–54, 70]. Bufalin has been reported to reverse multi-drug resistance in some human leukemia MDR cells. Indeed, Efferth et al. [70] reported that bufalin caused a significant increase in the accumulation of daunorubicin in CEM/VLB100 and CEM/E1000 cells. Moreover, some cardenolides from *Calotropis procera, Pergularia tomentosa*, and *Nerium oleander* can overcome MDR [52–54]. Interestingly, some of these compounds can overcome MDR from multiple origins. Indeed, we previously reported that 19-hydroxy-2″oxovoruscharin-mediated potent anti-cancer activity is not limited by the intrinsic MDR conferred by the over-expression of key drug-transporter proteins acquired as a result of exposure to a range of chemotherapeutic agents or loss of wild-type p53 [52]. This was confirmed in human cancer cell lines of different origin including HeLa-derived KB carcinoma, MDA-MB-231 breast cancer, GLC4 small cell lung cancer, SW-1573 and A549 NSCLC, S1 and HCT116 colon cancer, HL-60 leukaemia, and adenovirus transformed HEK293 cells; these were selected given their resistance to various chemotherapeutic agents (adriamycin, vincristine, cisplatin, oxaliplatin, mitoxantrone, hydroxyurea) and/or their over-expression of different MDR-related proteins (ABCB1, ABCC1 (MRP1), ABCC2, ABCC10, ABCG2 (BCRP), and MVP). In general, the sensitivity of all tested cell lines to 19-hydroxy-2″-oxovoruscharin was in the low nM range (IC_{50} range for both sensitive and resistant cells: 7–32 nM). It must be emphasised that in cardenolides from the *Digitalis* and *Strophantus* plant species (such as digoxin and digitoxin), steroidal rings A/B and C/D are *cis* fused, while rings B/C are *trans* fused. Such ring fusion gives the aglycone nucleus of these cardiac glycosides a characteristic "U" shape. In contrast, in cardenolides produced by plants from the milkweed family *Asclepiadaceae* (such as calactin uscharin and 2″-oxovoruscharin) A/B rings are *trans* fused resulting in rather flat structures. Whereas the cardiac glycosides from *Digitalis* and *Strophantus* species carry sugar units linked through the 3β-OH of the steroid aglycone (single link), some of those produced by plants from the milkweed family *Asclepiadaceae* contain a single sugar in a unique "dioxanoid" attachment (double link; [27, 34, 35, 49, 71–73]). The consequences of these structural differences on the NaK binding of these compounds have been reported previously [34, 35, 44] and indicate the markedly more potent binding (particularly to NaK α1 subunits) of the *trans-trans-cis* cardenolides.

Hypoxia-mediated drug resistance: targeting of the NaK *α* subunit by CS

For decades, tumour hypoxia has been known to have a negative effect on therapy outcomes (recently reviewed in [74]). Hypoxia inhibits tumour cell proliferation and induces cell cycle arrest, ultimately conferring chemoresistance because anti-cancer drugs preferentially target rapidly proliferating cells. However, this knowledge has been largely neglected during screening for antiproliferative substances *in vitro*, resulting in hypoxia-mediated failure of most newly identified substances *in vivo*. The hypoxia-inducible factor (HIF) family of hypoxia-inducible transcription factors represents the main mediator of the hypoxic response and is often upregulated in human cancers. The oxygen-regulated HIF isoforms, HIF-1*α* and to a less extent HIF-2*α*, have been associated with chemotherapy failure, and interference with HIF function holds great promise for improving future anti-cancer therapy (recently reviewed in [74]). Accordingly, Zhang et al. [75] screened a library of drugs that are in clinical trials or in use for inhibitors of HIF-1. Twenty drugs inhibited HIF-1-dependent gene transcription by > 88% at a concentration of 0.4 µM. Eleven of these drugs were cardiac glycosides, including digoxin, ouabain, and proscillaridin A, which inhibited HIF-1*α* protein synthesis and the expression of HIF-1 target genes in cancer cells [75]. Digoxin administration increased the latency and decreased the growth of tumour xenografts, whereas treatment of established tumours resulted in growth arrest within one week. Enforced expression of HIF-1*α* by transfection was not inhibited by digoxin, and xenografts derived from transfected cells were resistant to the anti-tumour effects of digoxin [75], demonstrating that HIF-1 is a critical target of CS for cancer therapy.

Cytoprotective effects caused by constitutively activated NF-*κ*B: targeting of the NaK *α* subunit by CS

Constitutive or drug-induced activation of the NF-*κ*B signalling cascade represents one of the major pathways by which tumour cells avoid cytotoxicity [76–78]. Many tumour cells display constitutively high levels of nuclear NF-*κ*B activity due to the hyperactivation of the NF-*κ*B signalling pathways or to inactivating mutations in the regulatory I*κ*-B subunits [76–78]. Several CS have already been shown to interfere with the NF-*κ*B pathway [51, 79–81]. We previously reported that 19-hydroxy-2″-oxovoruscharin (UNBS1450) is able to sensitise chemoresistant, highly aggressive, and naturally therapy-resistant A549 NSCLC cancer cells by deactivating the cytoprotective effects caused by constitutively activated NF-*κ*B [51]. This UNBS1450-induced deactivation of the NF-*κ*B pathways occurs at several levels, including both the inhibitory I-*κ*B portion of the NF-*κ*B signalling pathway and its stimulatory p65/Rel-A NF-*κ*B portion. With respect to the I-*κ*B portion of the NF-*κ*B signalling pathway, the compound acts at the levels of i) the upregulation of inhibitory protein expression (as observed for I-*κ*B*β*), ii) the downregulation of the phosphorylation levels of I-*κ*B*α*, and iii) the downregulation of the expression of CDC34. With respect to the stimulatory p65/Rel-A NF-*κ*B portion, the compound induces i) the downregulation of the expression levels of p65, ii) the downregulation of the DNA binding capacity of the p65 subunit, and iii) the downregulation of the NF-*κ*B transcriptional activity [51].

How might CS overcome cancer cells' chemoresistance?

We were able to show that NaK *α*1 targeting by siRNA induced the death of resistant cancer cells with the same morphologic features as those induced by 19-hydroxy-2″-oxovoruscharin [35]. Thus, cancer cells need abundantly expressed NaK for their survival, which seems not to be the case for normal, non-tumour cells [35].

The observed hypersensitivity of some MDR cells to CS [52] suggests a rather specific MDR targeting. The multifactorial nature of MDR indicates that it may be important to develop modulators that can simultaneously inhibit the expression of the drug transporters and the key signalling pathways, which are responsible for this phenomenon [8, 82]. The available, yet scarce, data argue in favour of this double mechanism: (a) the inability of tumour cells to acquire resistance to 19-hydroxy-2″-oxovoruscharin, (b) genome-wide microarray analyses performed after 19-hydroxy-2″-oxovoruscharin treatment of cancer cells revealed downregulation of different MDR-related mRNAs (our unpublished data), and (c) by binding to the sodium pump, CS affect multiple signalling pathways [27, 37, 45, 48, 50]. Furthermore, post-translational modifications seem to play major roles in the MDR-related regulation of protein expression. N-glycosylation was shown to contribute to the stability of P-gp [83], and inhibiting glycosylation reduced membrane-associated P-gp and altered the MDR phenotype [84]. Consistent with this observation, Beheshti Zavareh et al. [85] identified CS as the most potent inhibitors of the N-glycosylation pathway. Zhang et al. [86] demonstrated that the stability and function of P-glycoprotein can be regulated by the ubiquitin-proteasome pathway and suggested that modulating the ubiquitination of P-glycoprotein might be a novel approach to the reversal of drug resistance. Consistent with this suggestion, we demonstrated that 19-hydroxy-2″-oxovoruscharin induced an increase in the accumulation of ubiquitinylated proteins in the MDR A549 tumour cells and that some other ubiquitinylation-related enzymes are also affected by this CS [51].

Two major mechanisms might be responsible for CS-induced effects on chemoresistant cancer cells. The first mechanism relates to the inhibition of the glycolytic pathway and reduction of intracellular ATP levels [87–89] because these cancer cells have increased metabolic requirements for ATP [87–89]. This hypothesis is also supported by our data on the 19-hydroxy-2″oxovoruscharin-induced drop in intra-cellular ATP concentrations in cancer, but not in normal, cell lines [34, 35, 90]. It is interesting that aerobic glycolysis is linked to the activity of Na^+/K^+-ATPase and that CS can inhibit aerobic glycolysis (reviewed in [91]). The mechanism by which a decrease in the activity of the Na^+/K^+-ATPase produces glycolysis inhibition is not completely understood. However, it has been reported that glycolysis is inhibited by ATP via an allosteric inhibition of phosphofructokinase (PFK), a key enzyme in the control of glycolysis. Thus, cells need to hydrolyse ATP in order to release PFK inhibition and activate glycolysis. One of the major ATPases involved in the hydrolysis of ATP is indeed Na^+/K^+-ATPase [91]. Thus, Na^+/K^+-ATPase inhibition by CS could prevent the hydrolysis of ATP, which in turn may inhibit PFK and glycolysis, leading ultimately to cancer cell death. In addition, glucose transport into cells is mediated by facilitative glucose transporters (GLUTs) and in some cell types (such as small intestine and renal epithelial cells) by sodium glucose transporters (SGLT), the activity of which depends on Na^+/K^+-ATPase [91]. Therefore, Na^+/K^+-ATPase inhibition by CS may also reduce glucose transport into these cells resulting in further inhibition of glycolysis [91].

The second mechanism relates to CS-induced changes in cell ion concentrations, with an increase of Ca^{2+}_i following the Na_i increase due to NaK blockage contributing to the increase of MDR-1 mRNA [92]. In contrast, CS do not affect cell ion concentrations when used at their IC_{50} or concentrations that decrease MDR [52]. Additional data are, however, needed to decipher the details of the mechanism(s) by which CS circumvent cancer cell chemoresistance.

In summary, the multiplicity of potential targets might underlie the ability of CS to overcome the multiple anti-cell death mechanisms established in cancer.

Which signalling pathways are affected by NaK targeting in resistant cancer cells?

Although CS-mediated signalling has been investigated in normal cells (indicating the involvement of ERK, MAPK, PLC, PKC, and Ras-Raf), only a few studies of NaK-mediated signalling in cancer cells in general and in chemoresistant cancer cells in particular, have been reported.

By binding to the sodium pump, CS elicit several downstream signalling cascades affecting a number of different targets (reviewed in [27, 37, 45, 48, 50, 93]). Among the multiple targets are certain key markers. One pathway that might link NaK and MDR is the one related to c-Myc because c-Myc is involved in regulating the expression of MDR [94] and P-gp, the product of the MDR1 gene [95]; c-Myc activates MDR-1 transcription by binding the E-box motif (CACGTG) in the MDR1 gene promoter [96]. Our data indicate that (i) CS anti-tumour efficiency is correlated with the ability to down-regulate c-Myc [97] and (ii) 19-hydroxy-2″-oxovoruscharin impairs the expression of five Myc-related genes [90], suggesting a broad effect on the c-Myc pathway. As a reminder, the c-Myc oncoprotein regulates transcription of genes associated with cell growth, proliferation, and apoptosis [98]. The c-Myc protein is required for activating ribosomal DNA transcription in response to mitogenic signals, and it coordinates the activity of all three nuclear RNA polymerases, thereby playing a key role in regulating ribosome biogenesis and cell growth [99, 100]. Stimulation of ribosomal RNA synthesis by c-Myc is a key pathway driving cell growth and tumourigenesis [99]. Furthermore, oncogenic signalling through the Myc pathways directly controls glutamine uptake, which is of vital importance in cancer cells that must satisfy the metabolic requirements associated with anabolism and rapid growth rates [99]. Experimental evidence shows that inhibiting c-Myc significantly halts tumour cell growth and proliferation [101].

The way cardiotonic steroids down-regulate c-Myc expression has not been deciphered. Among the possible mechanisms are: (i) rapid compound-induced increases in ROS (as we previously reported [90]), which can inhibit gene expression partly by the oxidation of Sp1, which decreases its DNA-binding activity and contributes to the suppression of a number of genes, including c-Myc [102]; and (ii) compound-induced STAT3 downregulation (as we previously reported [90]).

It is important to consider Na^+/K^+-ATPase as a signal transducer able to mediate CS-induced effects in a compound, concentration, and cell type-specific manner [27, 37, 45, 93]. Thus, while binding to the same receptor, CS display different spectra of signatures indicating the differences in their modes of action and subsequent effects on cell behaviour. Indeed, using Fourier Transform Infrared (FTIR) analyses on the prostate cancer PC-3 cell line treated with four different CS (two cardenolides and two bufadienolides), we demonstrated the differences in the signatures of the metabolic changes induced by these four compounds [103]. This could explain, at least partly, the differences in CS behaviours toward the MDR of cancer cells.

Finally, a question remains about the possible intracellular roles of NaK and CS. Several studies showed NaK internalisation upon CS binding, and some of them demonstrated NaK accumulation in the nuclei, suggesting a direct role of NaK in gene expression [104, 105]. In contrast, the internalisation of CS together with NaK has still not been demonstrated. If some CS could undergo internalisation, this might explain, at least partly, why certain CS are substrates of P-gp and others are not.

Potential NaK isoform-related specificities in overcoming cancer cells' resistance

Using a baculovirus expression system for studying Na^+/K^+-ATPase-mediated ouabain effects, Pierre et al. [106] showed that there were important isoform-specific differences in NaK signalling. It is important to remember that different CS display different NaK inhibitory properties and that most, if not all, of them display higher binding affinity for the $\alpha 2$ and $\alpha 3$ isoforms compared to the $\alpha 1$ isoform [107]. Furthermore, conspicuous kinetic differences exist among sodium pump isozymes from different species in their interaction with CS [107–110]. According to Crambert et al. [108], human α/β complexes formed with $\alpha 1$ and $\alpha 3$ subunits have slow dissociation rate constants corresponding to half-lives ($t_{1/2}$) between 30 and 80 min, whereas those formed with $\alpha 2$ have rapid dissociation kinetics with $t_{1/2}$ of about 4–5 min. Similarly, the association kinetics of ouabain with human Na^+/K^+-ATPase isozymes followed the order $\alpha 2 >> \alpha 3 = \alpha 1$, with the times required to reach equilibrium binding being approximately 10 min ($\alpha 2,\beta$) and 60 min ($\alpha 1,\beta$ and $\alpha 3,\beta$). The association rate of ouabain seems to depend on the steroid moiety, whereas the dissociation rate depends on both the steroid and the sugar moieties. Several amino acids are involved in the ouabain binding kinetics [108, 111]. Whether there is isoform-specific mediated sensitivity towards the CS that display anti-cancer effects remains an open question. Currently, most of the published data link the $\alpha 1$ NaK subunit over-expression with cancer progression [27, 31, 32, 34–38]. Newman et al. [45] suggested that rather than an increase or decrease in NaK α subunit expression, the ratio of $\alpha 3$ to $\alpha 1$ should be used as the prognostic indicator for candidate patients to be treated with CS. This proposal was based on their data obtained with pancreatic cancer cell lines. The data suggest that the higher the ratio of $\alpha 3$ to $\alpha 1$, the greater the sensitivity to oleandrin. Unfortunately, this type of investigation cannot be conclusively conducted with a large panel of human cancer cell lines because the NaK α subunit expression is significantly influenced by culture conditions *in vitro* [112, 113], which generally lead to the sole expression of $\alpha 1$.

CS-mediated NaK targeting: from bench to bedside – how far are we?

As already emphasized above, interest in developing the CS as anti-cancer agents has grown progressively in the last two decades despite their potential cardiotoxic effects and very narrow therapeutic index. Within the past 15 years, there has been a marked increase in the number of reports of CS-induced anti-cancer effects (recently reviewed in [26, 27, 37, 39, 45, 47, 48]). While *in vitro* anti-cancer properties of CS have been widely studied, few publications have demonstrated their *in vivo* activity in animal models or in clinical studies. Either these compounds demonstrated appreciable *in vivo* anti-tumour activity but were quite toxic (e.g., ouabain) or they were found to be relatively devoid of anti-tumour activity at the tolerated dose levels (e.g., digoxin). The studies published by Perne et al. and Hallböök et al. [114, 115] raised the concern about the potential use of CS in therapy since their results demonstrated that CS (digoxin and digitoxin) induced cell death in human cells by inhibiting general protein synthesis, pointing to the need of very detailed assessment of mechanism of action of potential therapeutic CS. Despite, recently, Platz et al. [116] reported on a novel two-stage, transdisciplinary study identifying digoxin as a possible drug for prostate cancer treatment. They investigated whether any clinically-used drugs might have utility for treating prostate cancer by coupling a high-throughput laboratory-based screen and a large, prospective cohort study. Stage 1 was based on an in vitro prostate cancer cell cytotoxicity screen of 3,187 compounds in which digoxin emerged as the leading candidate given its potency in inhibiting proliferation *in vitro* (mean IC_{50} = 163 nM) and common use. Stage 2 was based on evaluating the association between the leading candidate drug from stage 1 and prostate cancer risk in 47 884 men followed 1986–2006 and uncovered that regular digoxin users had a ~ 25 % lower prostate cancer risk. Thus this transdisciplinary approach for drug repositioning provides compelling justification for further mechanistic and possibly clinical testing of this class of compounds as drugs for cancer treatment. As a reminder, retrospective epidemiological studies conducted by Stenkvist revealed some intriguing results: very few patients that underwent CS treatment for heart problems died from cancer [41]. In a 20-year follow-up [117], Stenkvist has reported that the death rate from breast carcinoma (excluding other causes of death and confounding factors) was 6 % (two out of 32) among patients who were treated with digitalis, compared with 34 % (48 of 143) among patients who were not treated with digitalis (p = 0.002). On the other hand, a very recent report from Biggar et al. [118] reported an increasing risk of breast cancer in women taking digoxin for cardiac conditions: 2.05 % (2144 out of 104 648) of women using digoxin developed breast cancer. Two oncology clinical trials involving digoxin have recently been completed: i) a Phase I clinical trial (ClinicalTrials.gov Identifier NCT00650910) combining digoxin with Lapatinib (an oral receptor tyrosine kinase inhibitor that targets HER2 and the EGFR) in treatment for metastatic ErbB2 breast cancer and ii) a Phase II clinical trial (ClinicalTrials.gov Identifier NCT00281021) combining daily digoxin with Erlotinib, an EGFR inhibitor, in treatment for NSCLC. Unfortunately, a remarkable digoxin-mediated anti-tumour effect was not observed in any of these trials, emphasising the need for more clinically efficient anti-tumour CS. Interestingly, it is somewhat perplexing to observe the large number of patents filed (see [39]) for novel anti-cancer CS and the very limited number of these compounds being further assessed in pre-clinical investigations and clinical trials. Indeed, a very limited number of new CS are presently being evaluated in clinical trials: (i) *Nerium oleander* extract (PBI-05240) is in Phase I clinical trials (ClinicalTrials.gov Identifier NCT00554268) at the MD Anderson Cancer Center and an interim analysis presented at 2009 ASCO Conference reported that 20 % of evaluable patients achieved stable disease for more than 4 months [119]; (ii) one modified cardenolide, UNBS1450, selected to minimize cardiotoxicity while preserving potent anti-proliferative properties [49], is also currently in Phase I clinical trials in Europe (Belgium and The Netherlands); and (iii) a traditional Chinese medicine Huachansu (containing mainly bufadienolides) is currently being evaluated in a Phase II clinical trial along with gemcitabine in pancreatic cancer patients (ClinicalTrials.gov Identifier NCT00837239). Negative perceptions of CS toxicity and reticence of medical community might be part of the explanation for the observed discrepancy. Furthermore, elevated costs of pre-clinical investigations might be one of the major reasons for the lack of translational research, knowing that large number of the patent applications for novel anti-cancer CS came from academic investigators. On the other hand, the lack of available clinical data evidencing safety margin and therapeutic window of assessed new anti-cancer CS prevent pharmaceutical industry to consider large investments in order to investigate CS as potential new anti-cancer compounds.

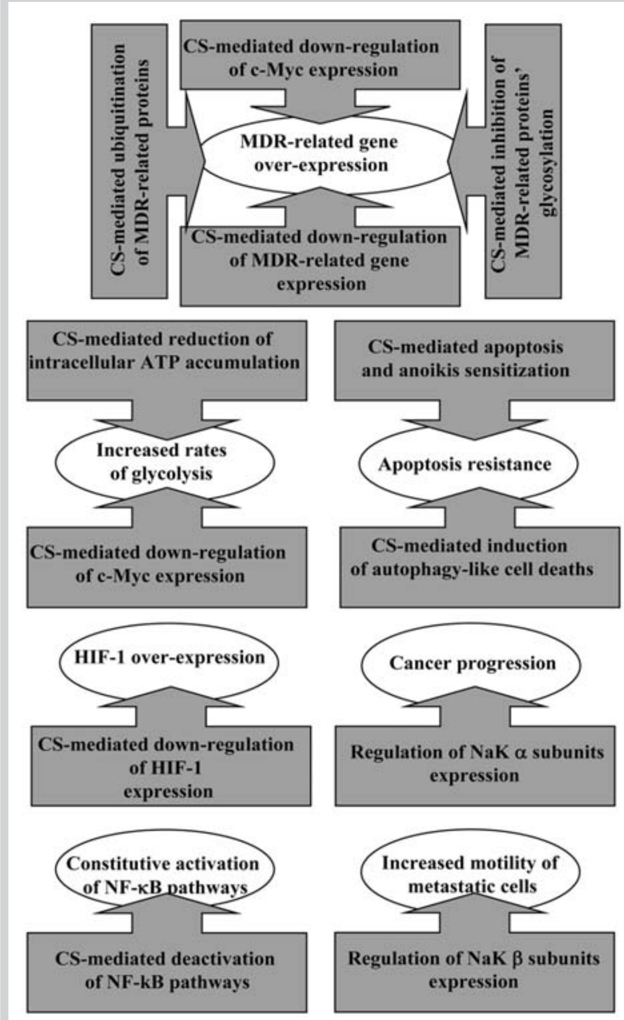

Fig. 3 Summary of postulated mechanisms resulting from Na^+/K^+-ATPase targeting and leading to overcoming of cancer cells chemoresistances. Pathways contributing to chemoresistance and MDR are represented in ellipses while their counteracting by Na^+/K^+-ATPase targeting is represented by block arrows.

Conclusions

Considering the severe limitations of current cancer chemotherapy, it is desirable to identify novel drugs that (i) are active against otherwise resistant tumour cells and (ii) modulate resistance to established drugs. An ideal compound would contain both features. Compelling evidence from published research data suggests that some cardiotonic steroids could act as such potential "two-in-one" drugs able to circumvent the chemoresistance of cancer cells. It is the multiplicity of potential targets rather than the specific action on one particular target that might enable certain cardiotonic steroids to overcome the multiple anti-cell death mechanisms established in cancer cells. While no precise mechanism has yet been deciphered for how NaK targeting might overcome cancer chemoresistance, several hypotheses converge to indicate the most probable pathways (summarized in ▶ **Fig. 3**). These pathways include the involvement of the NaK *β* subunit in invasion and the NaK *α* subunits in chemosensitisation by means of specific CS-mediated (a) apoptosis and anoïkis-sensitisation, (b) regulation of expression of MDR-related genes, (c) post-translational regulation, including glycosylation and ubiquitinylation, of MRD-related proteins, (d) c-Myc downregulation, (e) HIF downregulation, (f) downregulation and deactivation of NF-*κ*B pathways, (g) inhibition of the glycolytic pathway and reduction of intra-cellular ATP levels, and (h) induction of non-apoptotic cell death.

Thus, a completely new way of targeting chemoresistant cancer cells would rely on targeting the sodium/potassium pump, i.e., the Na^+/K^+-ATPase. This attractive hypothesis urgently needs medical validation, and it is expected that all the results originated from fundamental research would motivate further translational and clinical research aiming on use of specially designed CS for treatment of chemoresistant malignancies.

Conflict of Interest

The authors declare no conflict of interest.

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