High-Throughput Screening Identifies Cardiac Glycosides as Potent Inhibitors of Human Tissue Kallikrein Expression: Implications for Cancer Therapies

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Abstract

Purpose: Human tissue kallikreins (KLK) comprise a subgroup of 15 homologous secreted serine proteases. Primarily known for their clinical use as cancer biomarkers (e.g., PSA), KLKs have recently been directly implicated in cancer-related processes, including invasion, angiogenesis, and tumor growth regulation. Therefore, the identification of compounds that would modulate expression of KLKs might be of considerable therapeutic value.

Experimental Design: A cell-based high-throughput screening (HTS) of three small molecule libraries (~4,500 compounds) was undertaken; KLK expression in the breast cancer cell line MDA-MB-468 was assessed with sensitive ELISAs.

Results: The initial screening resulted in 66 "putative hits" that decreased KLK5 expression by at least 50% over control. Secondary screening and mini-dose-response assays resulted in 21 "validated hits." These 21 compounds were clustered in only three distinct functional families and were further analyzed *in vitro* to determine their effectiveness (IC_{50}s). Hits that failed to show dose-responsiveness or interfered with the viability of the cells were excluded. Multiple members of the cardiac glycoside family were found to be novel inhibitors of KLK expression, acting at low concentrations (10-50 nmol/L). Furthermore, members of the same family induced marked decreases in c-MYC and c-FOS expression, in a dose-dependent manner that correlated the KLK inhibition, suggesting a transcriptional mechanism of regulation of KLK expression.

Conclusions: We conclude that cardiac glycosides can dramatically suppress the transcription of KLKs and that these effects may be linked to proto-oncogene (c-myc/fos) expression. These findings may partially explain the recently realized antineoplastic actions of cardiac glycosides.

New technologies are helping to uncover the molecules and pathways that are directly involved in cancer pathogenesis. Targeting the proteins involved in neoplastic initiation and progression seems to be one of the most promising strategies for cancer therapy (1). Thus, as a first step, identification of the key players associated with cancer progression is critical. Among a plethora of potential targets, secreted proteases are considered very promising targets for cancer therapeutics (2). Indeed, protease activity plays a fundamental role in the dynamic regulation of the cellular, tissue, and systemic microenvironment. Aberrations in protease expression or activity are associated with many pathologic states, including cancer. Diverse proteases are directly involved in the regulation of tumor-related processes, such as invasion, metastasis, angiogenesis, and growth (3). In fact, proteases represent one of the six most important classes of current drug targets (5-10% of total). In the last 50 years, many small molecule–based drugs that specifically target proteases entered the clinic or are undergoing clinical trials. Matrix metalloproteinases, proteasome proteases (cancer), HIV protease (AIDS), factor X, and thrombin (thrombosis) are already targets of small molecule inhibitors approved for clinical use (4).

In the last decade, accumulating evidence implicates human tissue kallikreins (KLK) in cancer initiation and progression. Human KLKs and related peptidases emerged as promising players in the cancer proteolysis scene. Human KLK comprise a family of 15 homologous secreted serine peptidases encoded by a large gene cluster located on chromosome 19q13.4 (5). KLKs are expressed mainly by secretory epithelial cells and found in the majority of human fluids and tissues, including skin, prostate, breast, and pancreas (6). Despite the fact that their physiologic roles are not as yet fully elucidated, recent findings show that KLKs are directly involved in a wide range of physiologic processes, including skin desquamation (7), semen liquefaction (8), neural plasticity (9), regulation of blood pressure (10), and antimicrobial defense (11).

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Translational Relevance

Cardiac glycosides are widely known for their ability to bind to and inhibit the sodium pump. Members of this family (digoxin, digitoxin) have been used for many years for the treatment of heart failure and atrial arrhythmia. The mechanism of their positive inotropic effect is one of the best described among all known drugs. Recent findings implicate cardiac glycosides in the regulation of several important cellular processes and highlight their potential new anticancer therapeutic roles. Here, we show that inhibition of human tissue KLK expression might partially account for the anticancer effects of these drugs. We have also identified a significant down-regulation of c-MYC after treatment of breast cancer cell lines with cardiac glycosides. We conclude that cardiac glycosides represent novel anticancer agents whose action may be linked to KLK and c-MYC down-regulation.

Uncontrolled proteolytic activity of KLKs is also associated with a wide range of neoplastic and nonneoplastic pathologies. Briefly, KLKs seem to be dysregulated in tumors, mainly adenocarcinomas, and their expression is often associated with patient prognosis. Accumulating evidence implicates this family of proteases in the regulation of many cancer-related processes, including invasion, metastasis, angiogenesis, and tumor growth regulation (12). Furthermore, increased KLK activity is associated with skin diseases, such as Netherton syndrome (13), psoriasis (14), atopic dermatitis (15), and rosacea (16). In light of these new findings, KLKs are proposed to represent attractive targets for therapeutic interventions.

In this study, we applied a cell-based high-throughput screening (HTS) approach in search of potential small molecule inhibitors of KLK expression.

Materials and Methods

Cell lines. The breast cancer cell line MDA-MB-468 was obtained from American Type Culture Collection.

Small molecule libraries. The following compound libraries were used for the screening campaign: LOPAC (1,280 bioactive compounds), Sigma-Aldrich Corp., PRESTWICK Chemical Library (composed of 1,120 samples, of which 85% are marketed drugs), Prestwick Chemical, Inc., and the SPECTRUM collection (2,000 compounds, including many natural product). Samples for follow-up studies were obtained from Sigma Chemical Co. The chemicals used for the screening campaign were stored as stock solutions in 100% DMSO at a concentration of 10 mmol/L for both LOPAC and Spectrum collections, and 2 mg/mL (~5 mmol/L) for the Prestwick Chemical library. Daughter plates were prepared by diluting stock solutions with water to obtain 100 μmol/L samples, which were dispensed (10 μL) in the 190-μL cell culture to obtain 5 μmol/L final drug concentrations. DMSO in the cell-based assay was either 0.05% (v/v) in the presence of LOPAC and Spectrum samples or 0.1% (v/v) with Prestwick drugs. Preliminary tests revealed that these levels of DMSO were irrelevant to morphology, viability, and proliferation of MDA-MB-468 cells.

HTS assay. The screens were done using a Biomek FX liquid handler (Beckman-Coulter) to dispense reagents. MDA-MB-468 cells were cultured in phenol red–free RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Before screening, assay conditions were optimized with respect to cell density, culture media selection, and incubation time. Thus, MDA-MB-468 cells were cultured to 75% confluency in RPMI supplemented with fetal bovine serum, trypsinized and seeded by the liquid handler into 96-well plates at a density of 50,000 cells per well in a total volume of 200 μL. Cells were then allowed to incubate and adhere to the wells for 48 h at 37°C in 95% humidity, after which wells were washed twice with 1× PBS via an EMBLA microplate washer (Molecular Devices) and the culture media replaced by serum-free CDCHO media to prevent any interference of the serum with drug activity. Small molecules were added to the wells in a final concentration of 5 μmol/L and incubated for 30 h. Supernatants were then collected, and KLK5 expression was measured by a sensitive KLK5 ELISA. Cells treated with DMSO were used as a negative control. In parallel, an Alamar Blue (Biosource International) viability test was also done to discriminate compounds that caused cytotoxic effects. Alamar Blue is a stable, soluble, and nontoxic agent that monitors innate metabolic activity of the cells and was used according to the manufacturer's instructions.

Statistical analysis. All experiments were done in triplicate, and data are reported as the mean score ± SD. To evaluate the dynamic range of the HTS assay, we estimated the *Z*-factor for each of the validated hits, as previously described (17).

Quantification of KLKs in cell culture supernatants. The concentration of each KLK was measured with specific and quantitative immunofluorometric ELISA assays developed in our laboratory. In brief, 96-well polystyrene plates were first coated with 500 ng/well of a KLK-specific capture antibody. After overnight incubation (~12 h), the plates were washed, 50 μL of culture supernatant or standards and an equal volume of assay buffer were added and incubated at room temperature for 2 h. Plates were washed, and biotinylated antibodies were subsequently added. After incubation and wash, alkaline phosphatase–conjugated streptavidin was added. Finally, diflunisal phosphate and terbium-based detection solutions were added, and fluorescence was measured with the Envision time-resolved fluorometer (Perkin-Elmer). The calibration and data reduction were done automatically. More details for the ELISA assays used have been described elsewhere (6).

RNA extractions and reverse transcription–PCRs. MDA-MB-468 cells were seeded in six-well plates and inhibitor treatments at various doses were carried out using RPMI 1640 supplemented with 10% charcoal-dextran stripped fetal bovine serum. After 8-h inhibitor treatment, total RNA was extracted from the breast cancer cells using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Total RNA concentration was determined spectrophotometrically, and 3 μg of total RNA were reverse-transcribed into first strand cDNA using the Superscript First Strand Synthesis kit (Invitrogen) using an Oligo(dT) primer. PCRs were carried out using Qiagen Hotstar Taq Polymerase (Qiagen) on first strand cDNA for several KLKs. The primers used for the PCRs are shown in Supplementary Table S1. An equal amount of each PCR product was run on 0.9% agarose gels and visualized by ethidium bromide staining.

Western blot analysis. MDA-MB-468 cells were treated with inhibitors overnight, then washed twice with 1× PBS and lysed using 1× cell lysis buffer, supplemented with 1× complete protease inhibitor cocktail (Roche Diagnostics). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology). Ten micrograms of cleared cell lysates were used for Western blot analysis. Antibodies for Western blot analysis using manufacturer's protocols (and product codes) included β-actin (C4), c-JUN (D), c-FOS (6-2H-2F), NF-κB [p65 (A), p50 (E10)], ATF2 (N-96; Santa Cruz Biotechnology), and c-MYC (Upstate).

Results

Initial HTS. In pursuit of novel small molecule transcriptional inhibitors of human KLKs, three small molecule

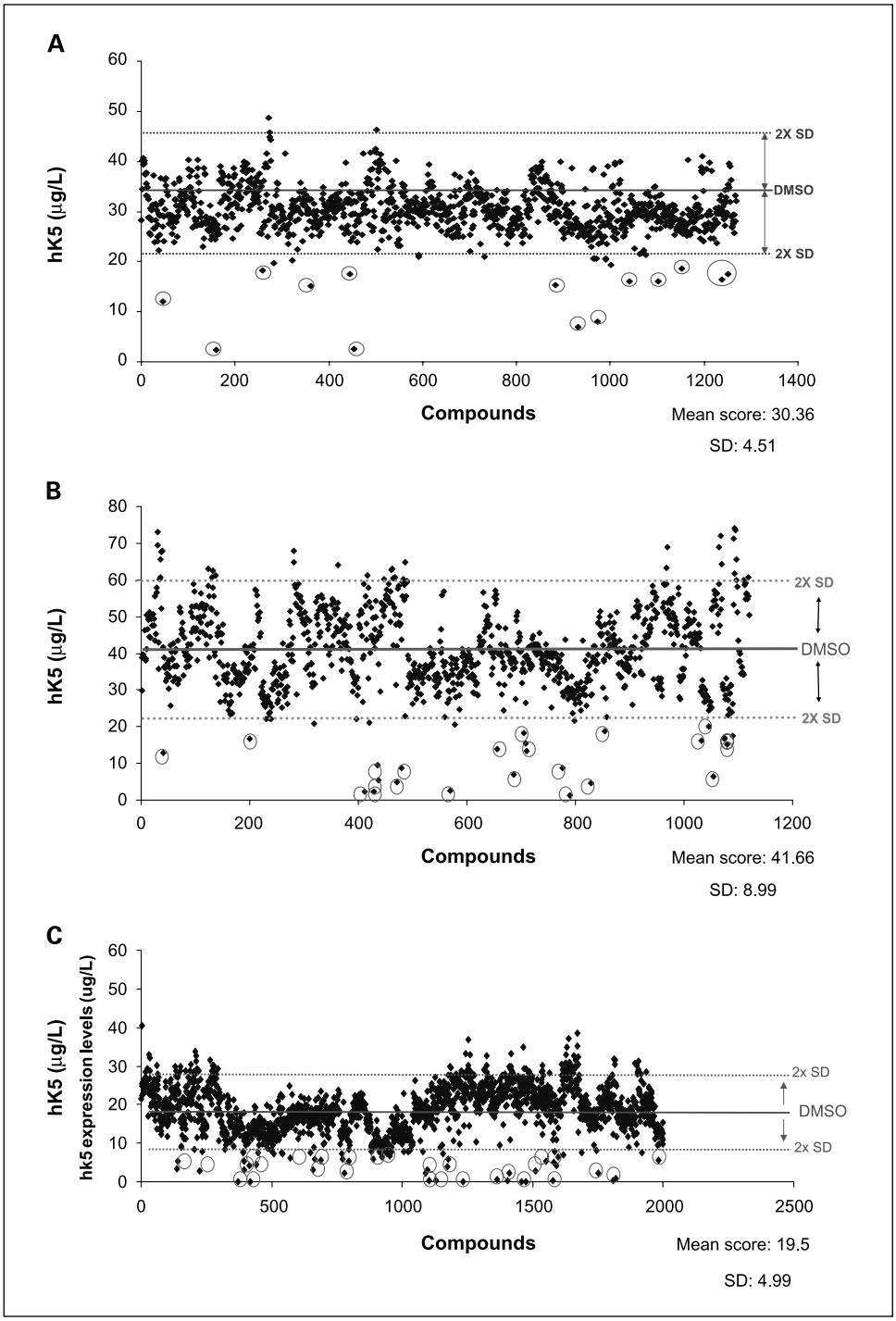
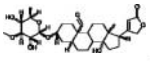
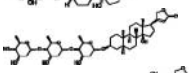
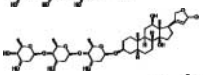
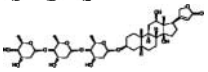
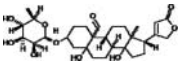
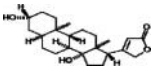


Fig. 1. Graphical representation of KLK5 protein expression of HTS of small molecule compound libraries. SD for each graph was determined from the average KLK5 expression levels from the DMSO-vehicle controls. Circled points represent candidate hits.

libraries (LOPAC, PRESTWICK, SPECTRUM; a total of ~4,500 compounds) were screened for their ability to inhibit KLK5 expression in MDA-MB-468 breast cancer cells. MDA-MB-468 is a well-characterized metastatic breast cancer cell line that expresses relatively high levels of several KLKs, including KLK5, KLK6, and KLK10. KLK5 is expressed at levels several hundred-fold greater than the other KLKs, and it was selected as for monitoring during our initial HTS. Furthermore, by scaling-up the same protocol, we were also able to assess the modulation of other KLKs (KLK6 and KLK10) and, therefore, investigate any potential differential effects of the compounds on KLK expression. The selection of the cell line, as well as the specific variables of the HTS assay (number of cells, type of media, time course of the assay), were optimized before the actual screening (data not shown).

The initial screening strategy of the three libraries is illustrated as a flow chart in Supplementary Fig. S2. The compounds that qualified as initial "hits" were those that exceeded an arbitrarily selected threshold of KLK5 expression (KLK levels lower than the mean of the controls minus 2 SDs).

Table 1. IC_{50} of the six most potent cardiac glycosides as inhibitors of KLK5 and KLK6 expression

Compound	Structure	KLK5 IC_{50} (nmol/L)	KLK6 IC_{50} (nmol/L)
Peruvoside		8	6
Digitoxin		42	50
Digoxin		90	100
Ouabain		120	65
Convallatoxin		150	130
Digitoxigenin		210	380

This threshold selection assumes a similar magnitude of random errors for all measurements, and it could lead to inferential errors in hit detection (18). To avoid position-specific effects, the DMSO-vehicle controls were scattered in different positions in all plates. Figure 1 depicts KLK5 expression upon completion of the initial screening of the three small molecule compound libraries. Initial "hits" were carried forward for further analysis.

Secondary screening. The potential hits from the primary HTS were subjected to filtering to eliminate false positives. Firstly, to check the reproducibility of the initial results, each compound was tested in triplicate, at the same concentration as the initial screening (5 μmol/L). Then, promising hits were tested across a small concentration gradient (0, 1, 2, 5 μmol/L) to select only dose-responsive hits. An additional cell viability test (measuring lactate dehydrogenase in supernatants) was done to exclude any cell death effects that may have escaped the Alamar Blue cell viability test (data not shown). A total of 21 compounds were carried over as "validated hits" for further cell-based *in vitro* studies.

We identified a highly restricted functional classification of the 21 validated compounds. Based on the Chembank database (19), we classified the compounds in the following classes: ion pump inhibitors (cardiac glycosides), translation inhibitors, and trafficking inhibitors. For further studies, we focused primarily on the ion pump inhibitors (cardiac glycosides), because they represent the largest (14 of 21) and most potent group of the validated compounds. Also, recently accumulated evidence suggests novel anticancer properties for these drugs (see also later sections). The well-established druggability of some of these compounds, such as digoxin and digitoxin (Food and Drug Administration–approved drugs), could potentially catalyze a shorter pathway toward future clinical trials.

Biological potency. To further characterize the effects of cardiac glycosides on KLK expression, we determined the IC_{50} for each identified member of this family. We retained the same protocol as for the initial HTS, but scaled-up the experiment to 12-well plates and ~500,000 cells per well. This allowed us to also assay for KLK6 and KLK10 and investigate possible differential effects of each compound on KLK expression. We found that KLK5, KLK6, and KLK10 showed a similar pattern of inhibition by cardiac glycosides (Table 1). Striking differences in the potency of structurally similar cardiac glycosides were identified (Fig. 2).

Changes in KLK expression seem to be a transcriptional event. We did reverse transcription–PCR analysis for KLK5, KLK6, and KLK10 in MDA-MB-468 cells treated with the most potent inhibitor of the cardiac glycoside family, peruvoside, and with emetine (a commonly studied translation inhibitor). Given that various members of the KLK family have similar functions, it was important to check for specificity of these compounds within the KLK family. Because our initial screening was carried out in CDCHO serum-free media, we modified our protocol to better mimic the tumor microenvironment with the addition of charcoal-dextran stripped fetal bovine serum. KLK5, KLK6, and KLK10 mRNA expression was reduced in a dose-dependent manner upon peruvoside treatment compared with the DMSO vehicle control. However, emetine mediated different effects. Although KLK5 and KLK6 transcript levels were reduced, they did not show dose dependence and KLK10 mRNA was not affected (Fig. 3). The differences between peruvoside and emetine on KLK transcription would suggest that their mechanisms of action are different. Indeed, emetine is a translation inhibitor; therefore, changes in KLK expression may not represent transcriptional events but alterations in protein synthesis.

The mechanisms of cardiac glycoside regulation of gene transcription are not clear. Accumulating evidence now supports

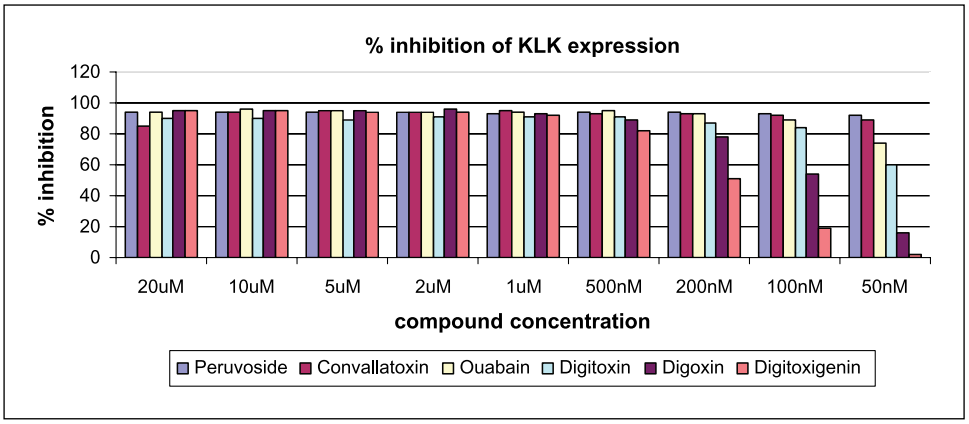


Fig. 2. Potency of six cardiac glycosides in inhibiting KLK5 expression. Each experiment was done in triplicate. The IC_{50}s are shown in Table 1.

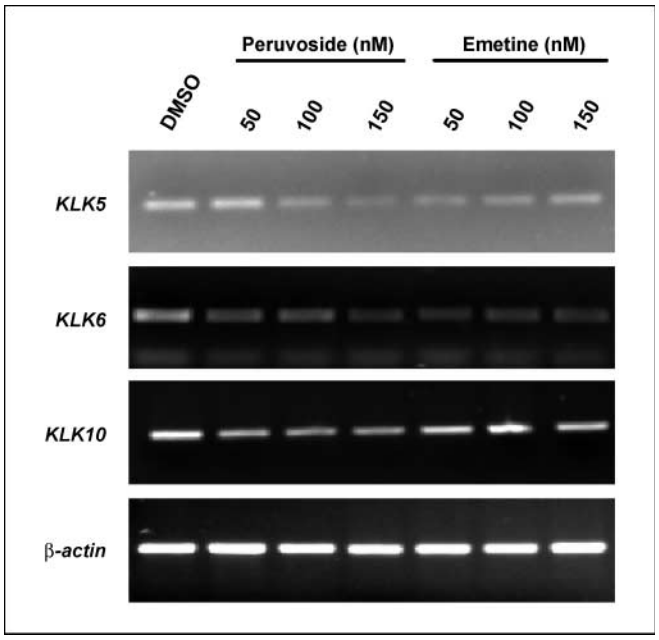


Fig. 3. Reverse transcription – PCR analysis of KLK5, KLK6, and KLK10 of MDA-MB-468 breast cancer cells treated with either peruvoside or emetine at three different concentrations. β-Actin was used as a control. For discussion, see text.

a pivotal role of Na^+-K^+-ATPase as both an ion pump and a versatile signal transducer; therefore, certain signaling pathways may be associated with their action, as exemplified below.

Cardiac glycosides affect transcription factor expression. To gain more insights into the transcriptional changes of KLK expression upon treatment with cardiac glycosides, we examined changes in the expression of certain transcription factors. We studied two compounds of the cardiac glycoside family, peruvoside and ouabain, which exhibit different potencies, based on our IC_{50} results. MDA-MB-468 cells were then treated with the same concentrations of peruvoside and ouabain and changes in a panel of transcription factors (c-MYC, c-JUN, c-FOS, NF-κB/p65, NF-κB/p50, ATF2) were analyzed by Western blotting (Fig. 4).

Peruvoside and ouabain at 150 nmol/L induced significant decreases in c-MYC expression. Reduced c-MYC expression was also observed at even lower concentrations (100 and 50 nmol/L) of peruvoside treatments. Peruvoside also reduced c-FOS expression at higher concentrations. The differences between peruvoside and ouabain, in their ability to repress transcription factor expression, correlated to their overall potency in suppressing KLK expression (Fig. 2).

For the transcription factors c-Jun, NF-κB, and ATF2, as well as for β-actin (control), we could not discern any changes in expression upon cardiac glycoside treatments. Because the amount of total protein loaded in Western blots was the same, the fact that the expression levels of these transcription factors did not change upon inhibitor treatment is another indication (together with Alamar Blue and lactate dehydrogenase data) that the effects of cardiac glycosides are pathway-specific and not due to cell death. These results suggest that cardiac glycosides may regulate the KLK genes through the activity of specific transcription factors, such as c-MYC and c-FOS.

Discussion

The objective of this study was to identify novel inhibitors of tissue KLK expression. The cell-based HTS approach identified <2% of ~4,500 compounds as potential hits after the first screen. The overlap of the compounds in the three libraries confirmed the excellent reproducibility of the HTS assay. For example, emetine and ouabain were identified as hits from more than one library. Furthermore, the identification of previously known inhibitors of KLK expression, such as Wortmannin (20), represented another independent positive control. These data confirm the sensitivity and reproducibility of the proposed assay and suggest that it can be effectively used in similar cell-based HTS experiments.

Most of the validated hits belong to the cardiac glycoside family. Cardiac glycosides are common compounds in the plant kingdom. Their ability to bind to and inhibit the Na^+-K^+-ATPase has been extensively studied over the last 50 years (21). The resulting increase in intracellular Ca^{2+} is responsible for their positive iontropic effects, and members of this family (digoxin, digitoxin) are still in clinical use for cardiac disorders (22). More recently, the increasing use of unbiased and hypothesis-neutral HTS of large compound libraries revealed new roles for these compounds and highlighted novel therapeutic aspects for the treatment of noncardiac diseases, including cancer (23, 24). Strong evidence now supports the anticancer properties of these drugs. Early epidemiologic studies by Stenkvist et al. reported significantly lower mortality rates of cancer patients under cardiac glycoside treatment (25, 26). Because then, numerous *in vitro* studies have reported potent antiproliferative effects

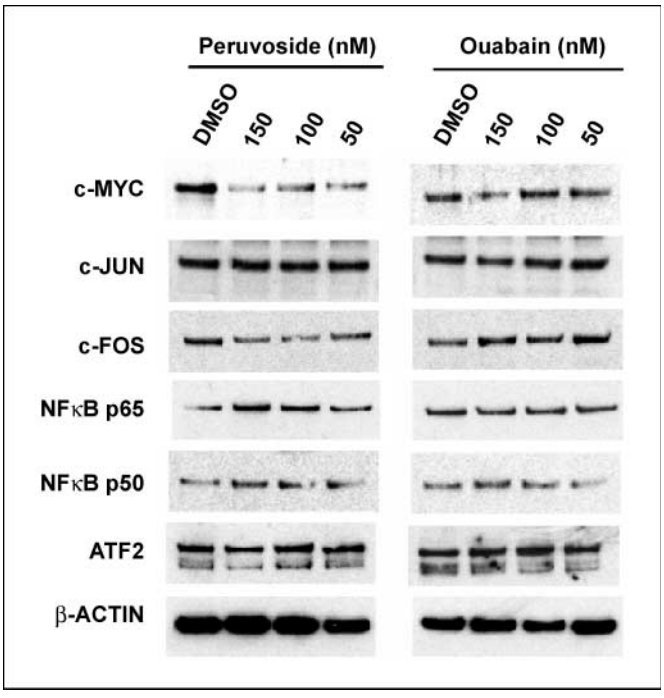


Fig. 4. Western blot analysis of a group of transcription factors in MDA-MB-468 cells, treated with either peruvoside or ouabain at three different concentrations. β-Actin was used as a control. For discussion, see text.

Table 2. *In vitro* antiproliferative and/or apoptotic effects of cardiac glycosides in cancer cell lines

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

NOTE: Specific references are listed in the text.

of these compounds in a panel of cancer cell lines, including breast (27), prostate (28), melanoma (29), pancreatic (30), lung (31), leukemia (32), neuroblastoma (33), and renal adenocarcinoma (ref. 34; Table 2). Moreover, the ability of nontoxic concentrations of cardiac glycosides to inhibit tumor formation was more recently shown in mouse models of induced carcinogenesis (23). The already well-established pharmacodynamics and pharmacokinetics of these compounds provided a shorter pathway to clinical trials, and as a result, the first cardiac glycoside-based anticancer drug (Anvirzel) is now undergoing phase II trials (35).

The molecular mechanisms underlying the anticancer effects of these drugs are not yet elucidated. It is now established that, in addition to pumping ions, Na^+-K^+-ATPase acts as a multisignaling molecule that transmits signals to various intracellular compartments and modulates several cellular processes, such as proliferation, cell-cell interaction, and apoptosis, mainly through altering the gene expression profile of the cell (36). A characteristic illustration of this is the study by Johnson et al., who screened 9,000 compounds for their ability to simultaneously inhibit expression of six commonly overexpressed genes in prostate cancer cells. Interestingly, members of the cardiac glycoside family (digitoxin and ouabain) were the only compounds found to confer significant inhibition in the expression of four of the target genes, including transcription factors Hoxb-13, hPSE/PDEF, hepatocyte NF-3α, and the apoptosis inhibitor survivin (37). Herein, our data suggest an additional role for the action of cardiac glycosides as potent inhibitors of tissue KLK expression. Follow-up robust studies (e.g., whole genome microarrays) are needed to elucidate all the genes that are downstream targets of these compounds.

The future therapeutic value of these compounds could not be clearly estimated without an understanding of their mode of action. To gain more insights, we examined the effects of two of these compounds (peruvoside, ouabain) on the intracellular levels of common transcription factors (Fig. 4). At the concentrations used (~50-150 nmol/L), these compounds are noncytotoxic and do not mediate any morphologic changes in the cancer cells (data not shown). However, further experiments are needed to exclude any long-term apoptotic effects of these compounds. Both compounds mediated significant decreases in the expression of c-MYC. To our knowledge, this is the first documented association between cardiac glycoside action and expression of c-MYC. This finding is further supported by the data of Mijatovic et al., who reported that low concentrations of UNBS1450 (a novel cardenolide, chemical derivative of 2′-oxovorusharin) provoke a dramatic down-regulation of c-MYC in a panel of different cancer cell lines (38). C-MYC is a transcription factor commonly dysregulated in many tumor types, and for many years, c-MYC–targeted therapeutics represent a main anticancer therapeutic avenue (39, 40). It is thought that c-MYC mediates its diverse cellular effects by altering the expression of specific target genes. Indeed, in the past 5 years, microarray experiments generated a wealth of information regarding c-MYC responsive and target genes (41). However, among this plethora of potential targets, only a few have been verified by chromatin immunoprecipitation experiments to directly interact with c-MYC. In an extensive chromatin immunoprecipitation and CpG island microarray analysis, Mao et al. reported that c-MYC binds directly to the promoter of KLK10 gene and regulates its expression (ref. 42; no evidence exist, thus far, for the direct interaction of c-MYC with promoters of other members of the KLK family).

In conclusion, our HTS revealed that cardiac glycosides represent a novel family of potent tissue KLK transcriptional inhibitors. C-MYC was also found to be markedly down-regulated upon treatment with these compounds. Emerging literature suggests a relationship between cardiac glycosides with c-MYC, and c-MYC and KLK expression. In light of this evidence, we postulate that cardiac glycosides might mediate down-regulation of tissue KLK gene expression through down-regulation of c-MYC. Down-regulation of c-MYC might also account for the recently identified anticancer effects of cardiac glycosides. Further experiments will be necessary to further elucidate the pathways associating cardiac glycoside action, c-MYC expression, and KLK regulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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