Cytotoxic Effects of Cardiac Glycosides in Colon Cancer Cells, Alone and in Combination with Standard Chemotherapeutic Drugs

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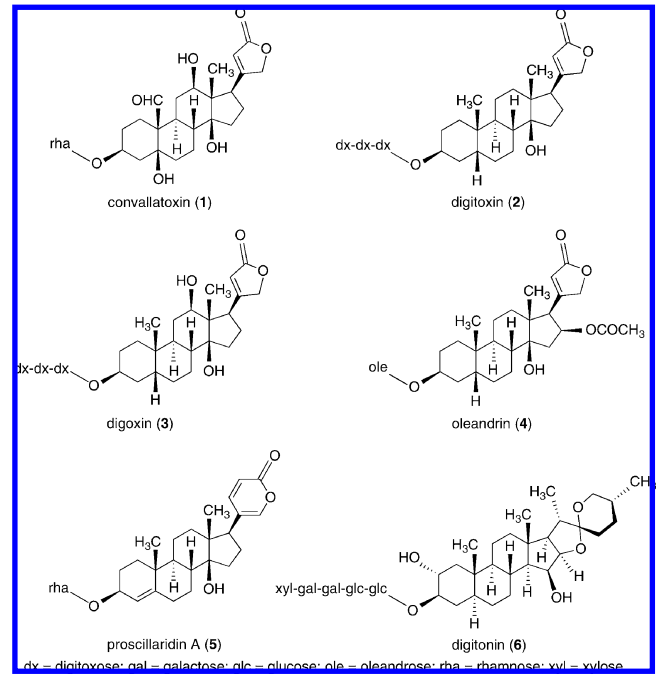
Cardiac glycosides have been reported to exhibit cytotoxic activity against several different cancer types, but studies against colorectal cancer are lacking. In a screening procedure aimed at identifying natural products with activity against colon cancer, several cardiac glycosides were shown to be of interest, and five of these were further evaluated in different colorectal cancer cell lines and primary cells from patients. Convallatoxin (**1**), oleandrin (**4**), and proscillaridin A (**5**) were identified as the most potent compounds (submicromolar IC_{50} values), and digitoxin (**2**) and digoxin (**3**), which are used in cardiac disease, exhibited somewhat lower activity (IC_{50} values 0.27−4.1 μM). Selected cardiac glycosides were tested in combination with four clinically relevant cytotoxic drugs (5-fluorouracil, oxaliplatin, cisplatin, irinotecan). The combination of **2** and oxaliplatin exhibited synergism including the otherwise highly drug-resistant HT29 cell line. A ChemGPS-NP application comparing modes of action of anticancer drugs identified cardiac glycosides as a separate cluster. These findings demonstrate that such substances may exhibit significant activity against colorectal cancer cell lines, by mechanisms disparate from currently used anticancer drugs, but at concentrations generally considered not achievable in patient plasma.

Cardiac glycosides are a group of natural products occurring in a limited number of plant families and are characterized by their ability to inhibit membrane-bound sodium−potassium-activated ATPase (Na^+/K^+-ATPase), causing a rise in intracellular calcium. Some of these, mainly digoxin and digitoxin, have clinical use in cardiology, in treatment of congestive heart failure and in atrial arrhythmias.

There are several reports on the cytotoxic activity of cardiac glycosides, and a potential anticancer effect has been suggested. From an ethnopharmacological perspective, it may be noted that leaves of *Digitalis purpurea* (containing digitoxin) and extracts of *Nerium oleander* (containing oleandrin) have been used traditionally to treat tumors in different parts of the world.[1−3] One epidemiological report demonstrated that tumor cell populations from breast cancer patients on digitalis medication had a lower proliferative capacity than tumor cells from control patients.[4] Furthermore, the tumor size was smaller and the death rate lower in the patients on digitalis.[4,5] Another study showed fewer cases of leukemia in a group of patients treated with digitoxin compared to the control group.[6] Cytotoxic effects of these glycosides against a number of types of cancer cell lines have also been demonstrated in vitro.[4,7−9] Interestingly, several reports indicate that malignant cells are more susceptible to the effects of cardiac glycosides compared to normal cells.[3,8] At the mechanistic level, many different pathways have been suggested as being responsible for mediating these cytotoxic effects. Calcium-dependent activation of caspases and other hydrolytic enzymes,[9,10] generation of reactive oxygen species (ROS),[11] topoisomerase inhibition,[12] interference with signal transduction pathways, e.g., Src, the epidermal growth factor receptor (EGFR), induction of the cell cycle inhibitor $p21^{Cip1}$,[13] and inhibition of hypoxia-inducible factor 1 alpha (HIF-1α) synthesis[14] have all been associated with cardiac glycosides. Consequently, these compounds may produce a spectrum of cellular effects eventually leading to cell death, and despite their narrow therapeutic indices, cardiac glycosides have been postulated for years as potential anticancer agents.[15]

In an attempt to identify novel compounds, in particular molecules of natural origin, with activity against colorectal cancer, the Spectrum Collection compound library, which contains 2000 compounds (of which 624 are natural products), was screened. By screening for cytotoxicity in human colon cancer cell lines several "hits" among the cardiac glycosides were found. Little or nothing has been published on the cytotoxic effects of the cardiac glycosides against colorectal cancer cells.

The present study was undertaken to investigate the cytotoxic effects of five cardiac glycosides [convallatoxin (**1**), digitoxin (**2**), digoxin (**3**), oleandrin (**4**), proscillaridin A (**5**)] and the saponin digitonin (**6**) alone and in combination with four standard chemotherapeutic drugs against colorectal cancer cell lines. Additionally, the activity was confirmed in primary cells from colon cancer patients. The rationale for evaluating cardiac glycosides in this context was based on the results of high-throughput screening (HTS) of the Spectrum Collection compound library and on the lack of data for natural products of this type against colon cancer.



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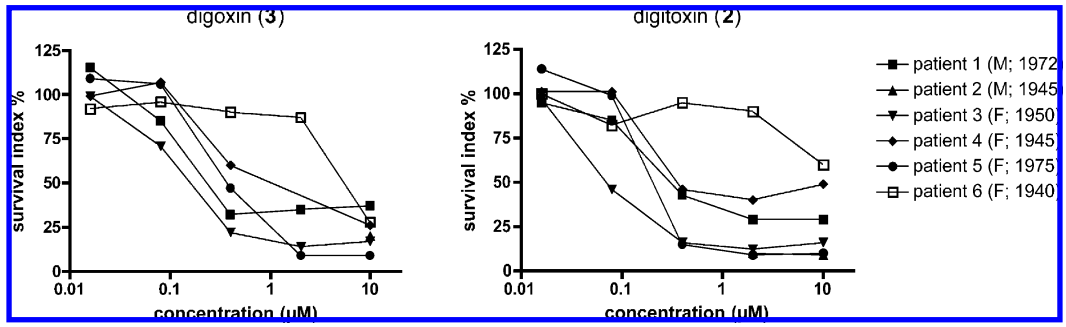

Figure 1. Concentration-dependent cytotoxic activity of digitoxin (**2**) and digoxin (**3**) in primary cultures of colorectal adenocarcinoma from two male (M) and four female (F) patients.

Results and Discussion

Cytotoxic Activity and Patient Sample Validation of Selected Cardiac Glycosides. The cytotoxic activities of convallatoxin (**1**), digitoxin (**2**), digoxin (**3**), oleandrin (**4**), proscillaridin A (**5**), and digitonin (**6**) against three colon cancer cell lines (HT29, HCT116, and CC20) with different inherent sensitivity to standard cytotoxic drugs, were studied using a fluorometric microculture cytotoxicity assay (FMCA). The cells were susceptible to cytotoxic effects of the cardiac glycosides, with IC_{50} values typically in the low micromolar range. The HT29 cell line was significantly more resistant than the CC20 and HCT116 cell lines to cardiac glycosides as well as standard drugs. Convallatoxin (**1**), oleandrin (**4**), and proscillaridin A (**5**) were identified as the most potent test compounds, with IC_{50} values ranging from 0.007 to 0.55 μM (Figure S1, Supporting Information). Digitoxin (**2**) and digoxin (**3**) were less potent although still active (IC_{50} 0.27−4.1 μM). The saponin digitonin (**6**) showed low activity for all three cell lines (IC_{50} approximately 10 μM), consistent with its suggested permeating mechanism (as discussed below). It should be noted that the validity of results for these cell lines may be hampered due to genetical drift and altered phenotypes over many years. Importantly, however, in addition to their effects on these cell lines, digitoxin (**2**) and digoxin (**3**) were found to inhibit the survival of primary cultures of tumor cells from surgical specimens obtained from patients diagnosed with colon cancer ($n = 6$), with IC_{50} values in the range 0.1−1.9 μM (Figure 1).

The IC_{50} values in vitro were higher than achievable plasma concentrations in vivo; that is, the plasma concentrations in the treatment of cardiac disease are about 1.5 nM for digoxin (**3**) and 30 nM for digitoxin (**2**),[16] which suggests their limited therapeutic potential as anticancer agents. However, direct comparison with concentrations in plasma may be misleading, as the volume of distribution for cardiac glycosides generally is high, being up to 780 L for digoxin,[17] and furthermore, the distribution in different organs varies considerably.[18] Certainly, following oral intake, high local concentrations in the intestine and portal circulation may be achieved, which may be sufficient for effects in the intestine or on early metastases in the liver. Digitoxin (**2**) and its metabolites are known to be eliminated very slowly from the human body, due to biliary excretion and enterohepatic circulation, suggesting relatively high concentrations in this compartment, while digoxin (**3**) is more rapidly eliminated through glomerular filtration.[16]

The cardiac glycosides have previously been reported to be generally more toxic against cancer cells, as compared to normal peripheral blood mononuclear cells (PBMCs), suggesting an in vitro therapeutic index and possible selectivity.[8,19,20] However, previous results from FMCA analyses on PBMCs yielded slightly lower IC_{50} values than for the colorectal cancer cell lines studied here.[7] Stenkvist observed that breast cancer patients treated with digoxin or digitoxin for cardiac disease had lower death rates and less aggressive tumors than control patients.[4,5] To the best of our knowledge, no such study has been performed regarding the connection between colon cancer and digitalis glycoside therapy.

Combination of Cardiac Glycosides with Standard Chemotherapeutic Agents. The effects of cardiac glycosides were evaluated in combination with cisplatin, 5-fluorouracil, irinotecan, and oxaliplatin, all drugs with a demonstrated effect against colon cancer and therefore used clinically as standard agents.[21] The results from the combination analysis are summarized in Table 1, and selected graphs/isobolograms are shown in Figure 2, and Figures S2 and S3 (Supporting Information). Using the median effect method of Chou and Talalay,[22] with a fixed ratio design (i.e., varying the concentration of both drugs), all compounds tested showed additive activity in combination with 5-fluorouracil, while they were synergistic [digoxin (**3**), digitonin (**6**), convallatoxin (**1**; HT29)] or additive [oleandrin (**4**), digitoxin (**2**), convallatoxin (**1**; HCT116)] in combination with oxaliplatin. Interestingly, the combination of the clinically used digoxin (**3**) with oxaliplatin retained synergistic cytoxicity also for the otherwise highly drug-resistant HT29 cell line (Table 1). In the case of irinotecan, commonly used as a second-line therapy for patients with advanced colon cancer, opposing effects were found when combined with cardiac glycosides, depending on the cell line. Concurrent exposure of colon cancer cell lines to irinotecan and the test compounds demonstrated cell-specific activities ranging from synergistic cytotoxicity in HCT116 cells to antagonism against HT29 cells (Table 1). The cellular events underlying this intriguing observation are unknown, but may be associated with multifactorial mechanisms contributing to multidrug resistance in the HT29 cell line. Interestingly, an interaction between topoisomerase II inhibition and cardiac glycosides has been previously noted. Digitoxin (**2**) significantly reduces the etoposide- and idarubicin-induced topoisomerase II cleavable complexes in K562 leukemia cells,[23] and blockade of the Na^+/K^+ pump by ouabain caused doxorubicin resistance and decreased topoisomerase II-mediated DNA strand breakage in hamster cells as well as in three human tumor cell lines, including HT29 colon carcinoma cells.[24]

Additional experiments with fixed concentrations of **1**−**3** were also performed. At clinically achievable concentrations (in plasma) effects were marginal, but at cytotoxic concentrations, interaction effects could be detected (Figures S4 and S5, Supporting Information).

The saponin digitonin (**6**) differs structurally and pharmacologically (Table S1, Supporting Information) from the cardiac glycosides and has been shown to increase cell membrane permeability and enhance the cytotoxic effects of cisplatin in experimental cancer models in vitro and in vivo.[25,26] Digitonin (**6**) was found to act synergistically or additively in combination with the chemotherapeutic drugs tested, consistent with its known ability to increase cell membrane permeability.

The finding that cardiac glycosides additively (cardiac glycoside plus 5-fluorouracil) or synergistically (cardiac glycoside plus

Table 1. Mean Combination Indices (CI) at IC_{70}, with 95% Confidence Intervals for Cardiac Glycosides and Selected Anticancer Drugs Using Two Colon Cancer Cell Lines

	HT29		HCT116	
compound combination	CI	effect[a]	CI	effect[a]
convallatoxin (**1**) + 5-fluorouracil	ND[b]		1.41 (0.65−2.16)	additive
convallatoxin (**1**) + cisplatin	ND		1.47 (0.77−1.47)	additive
convallatoxin (**1**) + irinotecan	ND		ND	
convallatoxin (**1**) + oxaliplatin	0.51 (0.41−0.60)	synergistic	0.94 (0.70−1.18)	additive
digitoxin (**2**) + 5-fluorouracil	1.27 (0.44−2.10)	additive	ND	
digitoxin (**2**) + cisplatin	0.95 (0.57−1.34)	additive	0.90 (0.70−1.11)	additive
digitoxin (**2**) + irinotecan	4.02 (2.07−5.97)	antagonistic	1.06 (0.83−1.29)	additive
digitoxin (**2**) + oxaliplatin	1.37 (0.48−2.27)	additive	1.08 (0.73−1.44)	additive
digoxin (**3**) + 5-fluorouracil	1.02 (0.60−1.43)	additive	1.04 (0.72−1.36)	additive
digoxin (**3**) + cisplatin	0.94 (0.85−1.02)	additive	0.87 (0.73−1.01)	additive
digoxin (**3**) + irinotecan	3.49 (3.11−3.87)	antagonistic	0.62 (0.51−0.73)	synergistic
digoxin (**3**) + oxaliplatin	0.86 (0.78−0.94)	synergistic	0.77 (0.57−0.73)	synergistic
oleandrin (**4**) + 5-fluorouracil	1.20 (0.81−1.59)	additive	ND	
oleandrin (**4**) + cisplatin	1.54 (0.52−2.56)	additive	1.12 (0.63−1.60)	additive
oleandrin (**4**) + irinotecan	3.79 (2.75−4.83)	antagonistic	0.31 (0.24−0.39)	synergistic
oleandrin (**4**) + oxaliplatin	1.20 (0.75−1.65)	additive	0.72 (0.60−0.84)	synergistic
proscillaridin A (**5**) + 5-fluorouracil	ND		ND	
proscillaridin A (**5**) + cisplatin	ND		ND	
proscillaridin A (**5**) + irinotecan	ND		ND	
proscillaridin A (**5**) + oxaliplatin	ND		ND	
digitonin (**6**) + 5-fluorouracil	ND		0.90 (0.80−1.01)	additive
digitonin (**6**) + cisplatin	1.07 (0.73−1.40)	additive	0.86 (0.84−0.89)	synergistic
digitonin (**6**) + irinotecan	0.98 (0.97−1.00)	additive	1.15 (0.94−1.36)	additive
digitonin (**6**) + oxaliplatin	0.84 (0.78−0.90)	synergistic	0.61 (0.41−0.80)	synergistic

[a] Synergism is defined as a CI statistically significantly lower than 1; antagonism, as a CI statistically significantly higher than 1, tested with one-sample *t* test ($p > 0.05$). [b] ND, not determined, analysis did not fulfill the quality criteria: $r > 0.85$ (see Experimental Section).

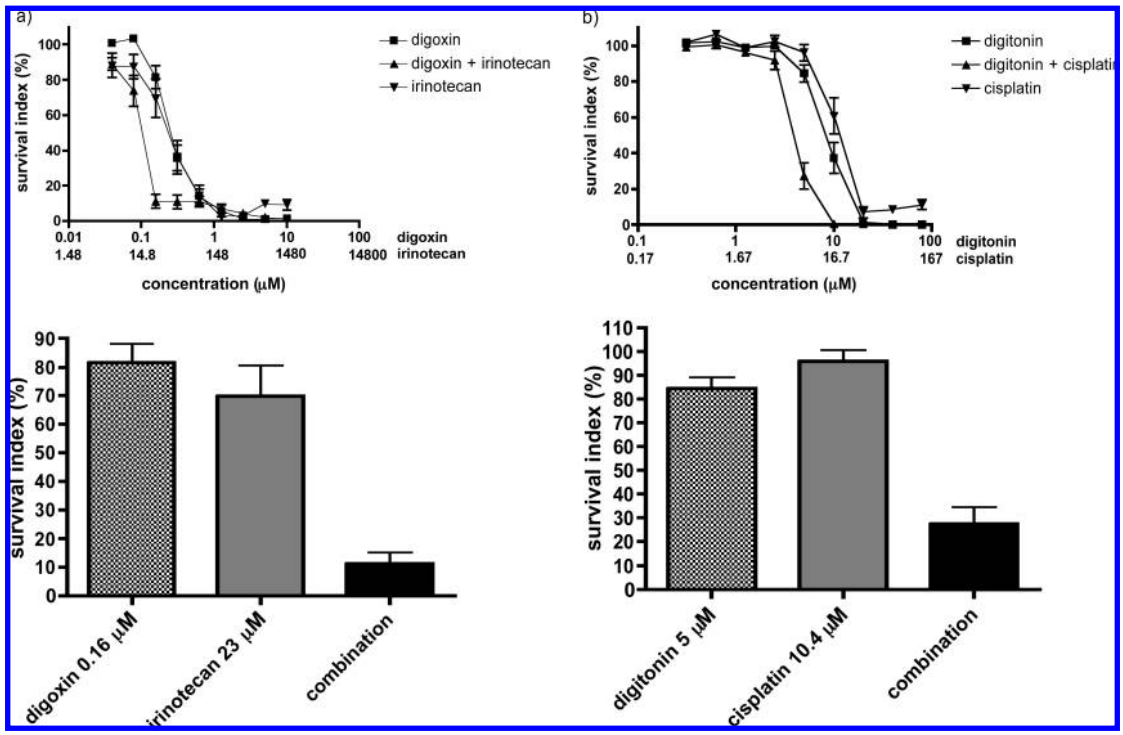

Figure 2. Synergistic combinations of (a) digoxin (**2**) and irinotecan and (b) digitonin (**6**) and cisplatin in the HCT116 colon cancer cell line. The first row displays the concentration effect curves from FMCA analysis, and the second row shows a bar graph of a selected concentration for each combination. Data are presented as means ± standard errors from three independent experiments.

oxaliplatin) kill colon cancer cells when combined with standard chemotherapeutic drugs (Table 1) is novel and of potential clinical relevance. In this context, particularly, the present results for digitoxin (**2**) and digoxin (**3**) in combination with standard cytotoxic drugs may be significant.

Activity on Na^+/K^+-ATPase. The mechanism of the cytotoxic action of cardiac glycosides and their potential use as anticancer agents has been discussed in recent years.[15,27] It is known that these compounds interact with the cardiac glycoside Na^+/K^+-ATPase receptor and have the potential to influence proliferation, differentiation, and cell death.[27] Alterations in the expression of the α isoforms of the catalytic subunit of Na^+/K^+-ATPase have been observed in tumor cells and have been suggested to be potential targets for anticancer drugs.[28,29] The relative cytotoxic potencies in colon cancer cell lines compared to the relative Na^+/K^+-ATPase inhibitory potencies, as reported by Paula et al.,[30] are given in Table 2. From this comparison, no strong correlation between the inhibitory effects of ion transport and cytotoxic activity was

Table 2. Relative Colon Cancer Cytotoxic Potencies of Cardiac Glycosides Compared to Their Relative ATPase Inhibitory Potencies

compound	relative cytotoxic potency in colon cancer cell line[a]			relative ATPase inhibitory potency[b]
	HT29	HCT116	CC20	
convallatoxin (**1**)	0.39	0.04	0.03	n/a
digitoxin (**2**)	2.91	2.74	1.71	0.30
digoxin (**3**)	1.00	1.00	1.00	1.00
oleandrin (**4**)	0.31	0.30	0.38	0.42
proscillaridin A (**5**)	0.39	0.22	0.21	0.054

[a] All values are calculated on the basis of IC_{50} values and presented in relation to the potency of digoxin (1.00). Compounds with low values have a higher inhibitory potency. [b] As previously reported.[30] Values were originally reported relative to ouabain. Here, the values have been recalculated and are presented relative to digoxin.

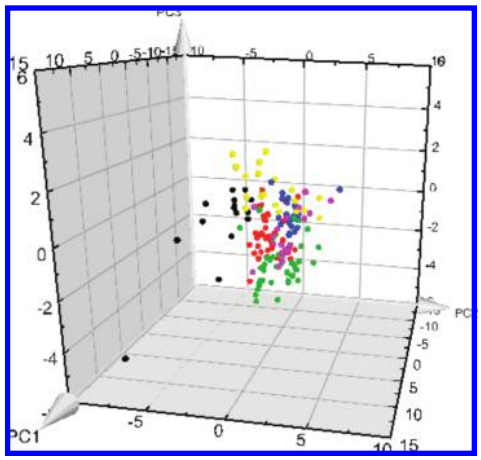

Figure 3. Score plot of the three first dimensions (principal components 1−3), from analysis of cardiac glycoside compounds in the ChemGPS-NP model for prediction of mode of action. The cardiac glycosides (black) are forming their own cluster, separated from the standard anticancer agents in the model: topoisomerase I inhibitors (blue), topoisomerase II inhibitors (pink), antimetabolites (green), alkylating agents (red), and tubulin inhibitors (yellow).

observed, but there are several confounders in the analysis, such as the different isoforms of Na^+/K^+-ATPase, which may have different relevance in the two events.

ChemGPS-NP Analysis Indicating a Potential New Mode of Action for Cardiac Glycosides. ChemGPS-NP is a principal component analysis (PCA)-based model for navigation in biologically relevant chemical space[31] and has proven suitable for the differentiation of biological activities, in a study reporting how anticancer drugs acting by several different cytotoxic mechanisms cluster in accordance with their respective mode of action.[32] When applying cardiac glycosides to this model, they did not coincide with any of the predefined mode of action clusters representing antimetabolites, alkylating agents, topoisomerase I and II inhibitors, and tubulin-active agents (Figure 3). Instead, the cardiac glycosides formed their own cluster, both in the activity profile-based analysis and in an analysis based on molecular descriptors calculated from simplified molecular input line entry specifications (SMILES). Hence, the results of the ChemGPS-NP analysis indicate a possible new common mode of action for the cardiac glycosides. However, this mode of action remains to be further elucidated. As expected, the results from the ChemGPS-NP analysis also showed that the saponin digitonin (**6**), which is a larger molecule than **1**−**5**, does not cluster with the cardiac glycosides, on the basis of its molecular properties (Figure 3).

Inhibition of Nuclear Factor Kappa B (NF-κB) Translocation. From the ChemGPS-NP analysis, it seems that the mode of action for cardiac glycoside cytotoxicity is mediated through another pathway than those of some other anticancer drugs. The transcription factor NF-κB has been proposed as being involved in processes of importance for carcinogenesis and is one of the targets suggested to be affected by cardiac glycosides.[33] However, as analyzed here, tumor necrosis factor alpha (TNF-α)-stimulated NF-κB translocation was unaffected by digitoxin (**2**), digoxin (**3**), and oleandrin (**4**) in HT29 colon cancer cells, as well as in breast (MCF-7) and cervical (HeLa) cancer cells (data not shown), thus not confirming actions via this pathway, as has been suggested by others.[8,33,34] Future studies should clarify the role of NF-κB in the context of cardiac glycoside-mediated cytotoxicity for cancer cell lines.

In conclusion, the tested cardiac glycosides are cytotoxic to human colon cancer cells, but at concentrations generally not achievable in human plasma. In vitro interactions with some standard chemotherapeutic drugs can be detected. The effects of the cardiac glycosides appear as class effects, and the molecular mechanism is not obviously linked to inhibitory effects on the Na^+/K^+-ATPase.

Experimental Section

Chemicals and Reagents. The Spectrum Collection (MicroSource Discovery Systems Inc., Gaylordsville, CT) was screened for cytotoxic effects against six human colon cancer cell lines. The library contains 2000 compounds (with 624 being natural products). The compounds were supplied as 10 mM solutions in DMSO and were further diluted with phosphate-buffered saline (PBS) and transferred to 384-well microplates (NUNC Brand Products, Roskilde, Denmark), using a Biomek 2000 pipetting station (Beckman Coulter Inc., Fullerton, CA). All compounds were screened at a final concentration of 10 μM.

Six pure compounds of natural origin were further evaluated for concentration-dependent cytotoxic activity against human colorectal cancer cells. Convallatoxin (**1**), digitoxin (**2**), digoxin (**3**), proscillaridin A (**5**), and digitonin (**6**) were obtained from Sigma (Sigma-Aldrich, Stockholm, Sweden), while oleandrin (**4**) was purchased from MicroSource Discovery Systems. The compounds were dissolved in DMSO and further diluted with PBS. Four conventional drugs used clinically to treat colon cancer were selected: cisplatin, 5-fluorouracil, irinotecan, and oxaliplatin (Apoteket AB, Uppsala, Sweden). Drugs were diluted and stored as instructed from the manufacturer; dilutions from stock were made with PBS. All compounds and drugs were tested in duplicate, serially diluted, and transferred to 384-well microplates using the Biomek 2000 pipetting station. Test compounds were evaluated at nine different concentrations, achieved by 1:2 serial dilutions in PBS.

Human Cancer Cell Lines. The colorectal adenocarcinoma cell line, HT29, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the HCT116 cell line was kindly provided by Professor Stig Linder of the Karolinska Institute, Solna, Sweden. The CC20 cell line was a gift from Dr. Christian Sundberg (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden). HT29 and CC20 cells were cultured in monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich). HCT116 was cultured in monolayer in McCoy's 5A medium (Sigma-Aldrich). All media used were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (all purchased from Sigma-Aldrich). The breast cancer cell line, MCF7, and the cervical adenocarcinoma cell line, HeLa (ATCC, Manassas, VA), were grown in minimum essential medium Eagle (MEME; Sigma-Aldrich), supplemented as above and with 1 mM sodium pyruvate. All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Primary Cultures of Human Colorectal Carcinoma. Tumor tissue samples from six patients with colorectal adenocarcinoma were obtained after a standard surgical procedure. The sampling for drug sensitivity testing (approval number Dnr 21/93−930125) was approved by the ethics committee at Uppsala University Hospital. The specimens were minced into small pieces, and tumor cells were isolated by collagenase dispersion followed by Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation.[35] Cell viability (>90%) was determined by the Trypan blue dye exclusion test, and the proportion of tumor cells in the preparation was estimated by the microscopic

examination of May−Grünwald−Giemsa-stained cytospin preparations (>70% tumor cells). Concentration-dependent cytotoxic effects of digitoxin (**2**) and digoxin (**3**) were studied at concentrations ranging from 0.016 to 10 μM.

Measurement of Cytotoxic Activity. To measure cytotoxic activity of the cardiac glycosides, a fluorometric microculture cytotoxicity assay (FMCA) was used, as previously described.[36,37] The method is based on measurement of fluorescence generated by hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes, i.e., viable cells. In brief, the cell suspension was seeded into test compound-containing 384-well microplates at a density of 5000 cells per well. The plates were incubated at 37 °C for 72 h and were then transferred to an automated HTS system controlled by an Optimized Robot for Chemical Analysis (ORCA; Beckman Coulter Inc., Fullerton, CA), programmed through the software SAMI (Beckman Coulter). The plates were washed and FDA was added to the wells followed by 50 min of incubation (37 °C). The fluorescence generated was measured at 485/520 nm using a FLUOstar Optima microplate reader (BMG Technologies, Offenburg, Germany). The fluorescence measured is proportional to the number of living cells in each well. Cell survival is presented as the Survival Index,[34] defined as the fluorescence value in the wells analyzed in percent of the value in the control wells, with blank values subtracted. Quality criteria included a signal/blank ratio of >10 and a coefficient of variation in control and blank wells of <30%. The experiments (with duplicates) were performed three times for cell lines and once for patient samples.

Identification of Cardiac Glycosides as Candidate Drugs for Colon Cancer Therapy. The Spectrum Collection compound library was screened for cytotoxicity, by means of FMCA, against three different human colon cancer cell lines (HT29, HCT116, and CC20). These were selected from a larger panel of six colon cancer cell lines, due to their differential sensitivity to 14 cytotoxic drugs, with HT29 being generally drug-resistant, HCT116 intermediately sensitive, and CC20 the most chemosensitive. From the initial high-throughput screening, cardiac glycosides were identified as a group of compounds with cytotoxic activity and were consequently selected for further evaluation. On the basis of previously published data,[7] the bufadienolide proscillaridin A (**5**; which is not part of the Spectrum Collection compound library) was also included in the present investigation.

Combination Analysis. The cytotoxic effects of five glycosides [convallatoxin (**1**), digitoxin (**2**), digoxin (**3**), oleandrin (**4**), and proscillaridin A (**5**)] and the saponin digitonin (**6**) were investigated in combination with four standard chemotherapeutic drugs used in colorectal cancer (5-fluorouracil, cisplatin, oxaliplatin, and irinotecan) in three cell lines (HT29, HCT116, and CC20). The studies were designed using a fixed ratio of the drugs across a concentration gradient. Single drug activity in the cell lines was estimated, and microplates for combination analysis were prepared by 2-fold serial dilutions in nine steps for all test compounds. Combinations were tested using a fixed concentration ratio. All compound concentrations and combinations were tested in duplicate, and the experiments were repeated three times.

ChemGPS-NP. The PCA-based model ChemGPS-NP (http://chemgps.bmc.uu.se) is a tool for navigation in biologically relevant chemical space.[31] It has eight principal components (dimensions), derived from 35 molecular descriptors describing physical-chemical properties such as size, shape, polarizability, lipophilicity, polarity, flexibility, rigidity, and hydrogen bond capacity for a reference set of compounds. New compounds are positioned onto this map using interpolation in terms of PCA score prediction. The properties of the compounds together with trends and clusters can be interpreted from the resulting projections.

It is a central principle of medicinal chemistry that structurally similar molecules often have similar biological activities.[38] In a recent study reporting how anticancer drugs that act by several different cytotoxic mechanisms cluster in accordance with their respective mode of action, ChemGPS-NP was demonstrated to be suitable for differentiation of biological activities.[32] Also, a procedure was proposed for prediction of the mode of action of cytotoxic compounds.[32]

In the present work, the cardiac glycosides were analyzed in the above-mentioned two-step model for prediction of mode of action. The ChemGPS-NP descriptors were calculated for the cardiac glycosides on the basis of their structure information as simplified molecular input line entry specification (SMILES) using the software DRAGON Professional 5.3 software (Talete srl, Milan, Italy). They were then mapped onto ChemGPS-NP together with a reference set of known anticancer agents with previously studied modes of action (Anticancer Agent Mechanism Database; http://dtp.nci.nih.gov/docs/cancer/searches/standard_mechanism.html). The compounds in the reference anticancer agent data set were classified with regard to the following mode of action classes: antimetabolites, alkylating agents, topoisomerase I and topoisomerase II inhibitors, and tubulin active agents. Principal component analysis and PCA score prediction were performed using the SIMCA P+ 11.5 software (Umetrics AB, Umeå, Sweden), with the training set ChemGPS-NP. Prior to PCA determination, all data were centered and scaled to unit variance.

Inhibition of NF-κB Translocation. The effects of digitoxin (**2**; 0.4, 2, 10 μM), digoxin (**3**; 0.4, 2, 10 μM), and oleandrin (**4**; 0.2, 1, 5 μM) on NF-κB translocation in HT29, MCF-7, and HeLa cells were studied using the NF-κB Activation HCS HitKit (Cellomics, Pittsburgh, PA). The NF-κB translocation assay was carried out according to the manufacturer's instructions.[39] Quantification of NF-κB activation was performed by measuring the spatial translocation of NF-κB from the cell cytoplasm to the nucleus, using the ArrayScan HCS reader (Cellomics), as previously described.[39] TNF-α and bortezomib were used as controls.

Data Analysis. The cytotoxic IC_{50} values (inhibitory concentration 50%) for the drugs were determined from log concentration−effect curves in GraphPad Prism (GraphPad Software Inc., La Jolla, CA), using nonlinear regression analysis. Three independent experiments were carried out. Data are presented as the means ± SEM. Outliers were identified and excluded using the GraphPad online outlier calculator based on Grubbs' test (www.graphpad.com).

To test combination effects, data were analyzed using the median-effect method of Chou and Talalay,[22] utilizing the CalcuSyn, Version 2, software (Biosoft, Cambridge, UK). Each concentration−response curve (individual agents as well as combinations) was fitted onto a linear model using the median effect equation, allowing calculation of a median effect value D (corresponding to the IC_{50}) and slope. Goodness of fit was assessed using the linear correlation coefficient, r, and $r > 0.85$ was required for a successful analysis. The extent of drug interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs: $CI = d_1/D_1 + d_2/D_2$ where D_1 and D_2 represent the concentration of drug 1 and 2 alone, required to produce a certain effect and d_1 and d_2 are the concentration of drugs 1 and 2 in combination required to produce the same effect. Different CI values are obtained when solving the equation for different effect levels, and the 70% effect was chosen for presentation. A CI equal to 1.0 indicates additivity; a significantly lower CI value was defined as synergy, while a significantly higher value was defined as antagonism. One-sample t-tests were used to determine if CIs differed from 1.0 ($p < 0.05$).

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Supporting Information Available: Survival indices, dose−response curves, and IC_{50} values for the compounds tested, isobolograms, and combination dose−response curves with fixed glycoside concentrations are available free of charge via the Internet at http://pubs.acs.org.

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