Anti-Cancer Drugs 2001, **12,** pp. 475–483

Report

Cytotoxicity of digitoxin and related cardiac glycosides in human tumor cells

Senia Johansson,[1] Petra Lindholm,[1] Joachim Gullbo,[2] Rolf Larsson,[2] Lars Bohlin[1] and Per Claeson[1]

[1]Division of Pharmacognosy, Department of Medicinal Chemistry, Biomedical Centre, Uppsala University, PO Box 574, 751 23 Uppsala, Sweden. [2]Division of Clinical Pharmacology, University Hospital, Uppsala University, 751 85 Uppsala, Sweden.

The saponin digitonin, the aglycone digitoxigenin and five cardiac glycosides were evaluated for cytotoxicity using primary cultures of tumor cells from patients and a human cell line panel (representing different cytotoxic drug-resistance patterns). Of these seven compounds, proscillaridin A was the most potent (IC_{50}: 6.4–76 nM), followed by digitoxin, and then ouabain, digoxin, lanatoside C, digitoxigenin and digitonin. Correlation analysis of the log IC_{50} values for the cell lines in the panel showed that compound cytotoxicity was only slightly influenced by resistance mechanisms that involved P-glycoprotein, topoisomerase II, multidrug resistance-associated protein and glutathione-mediated drug resistance. Digitoxin and digoxin expressed selective toxicity against solid tumor cells from patients, while proscillaridin A expressed no selective toxicity against either solid or hematological tumor cells. The results revealed marked differences in cytotoxicity between the cardiac glycosides, both in potency and selectivity, and modes of action for cytotoxicity that differ from that of commonly used anticancer drugs. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cytotoxicity drug screening, digitalis glycosides, digitonin, digitoxigenin, digitoxin, digoxin, lanatoside C, ouabain, proscillaridin A, tumor cell lines.

Introduction

During a cytotoxicity screening of plant extracts, aimed at finding lead compounds for anticancer drug development, we observed that a moderately high molecular weight fraction of the ethanolic extract[1] of the foxglove (*Digitalis purpurea* Ehrl.) exhibited potent antitumor activity in a fluorometric microculture cytotoxicity assay (FMCA).[2] Using a bioassay-guided fractionation procedure, the cytotoxic substance in the foxglove was isolated and identified as the clinically well-known cardiac glycoside digitoxin.

Chemically, digitoxin belongs to the group of cardiac glycosides called cardenolides, which are steroidal compounds, characterized by the presence of a five-membered unsaturated lactone ring. Cardiac glycosides of the bufadienolides type are, on the other hand, characterized by the presence of a six-membered unsaturated lactone ring. The therapeutically most important bufadienolide is proscillaridin A, which in nature is a constituent of squill, *Drimia maritima* (L) Stearn. Cardenolides and bufadienolides are regarded as cardiac glycosides because both increase the contractile force of the heart by inhibiting the enzyme Na^+,K^+-ATPase. The chemical structures of digitoxin, proscillaridin A and the other substances included in this study are illustrated in Figure 1. Of these substances, cardenolides and bufadienolides have previously been tested for cytotoxicity in a variety of tumor models.[3–9]

The US National Cancer Institute (NCI) has performed large-scale screenings of substances for anticancer activity. Since 1990, tens of thousands of substances have been tested in the NCI cell line panel. This panel consists of 60 different human tumor cell lines, and has been shown to generate remarkably reproducible and characteristic profiles of differential *in vitro* sensitivity for cytotoxic agents with different mechanisms of actions.[10] Using the same general principles for data treatment, Dhar *et al.*[11] have shown that, by using a panel of only 10 human cell lines representing defined types of cytotoxic drug resis-

This work was supported by grants from the Swedish Cancer Foundation.

Correspondence to P Claeson, Division of Pharmacognosy, Department of Medicinal Chemistry, Biomedical Centre, Uppsala University, PO Box 574, 751 23 Uppsala, Sweden.
Tel: (+46) 18 4714479; Fax: (+46) 18 509101;
E-mail: per.claeson@fkog.uu.se

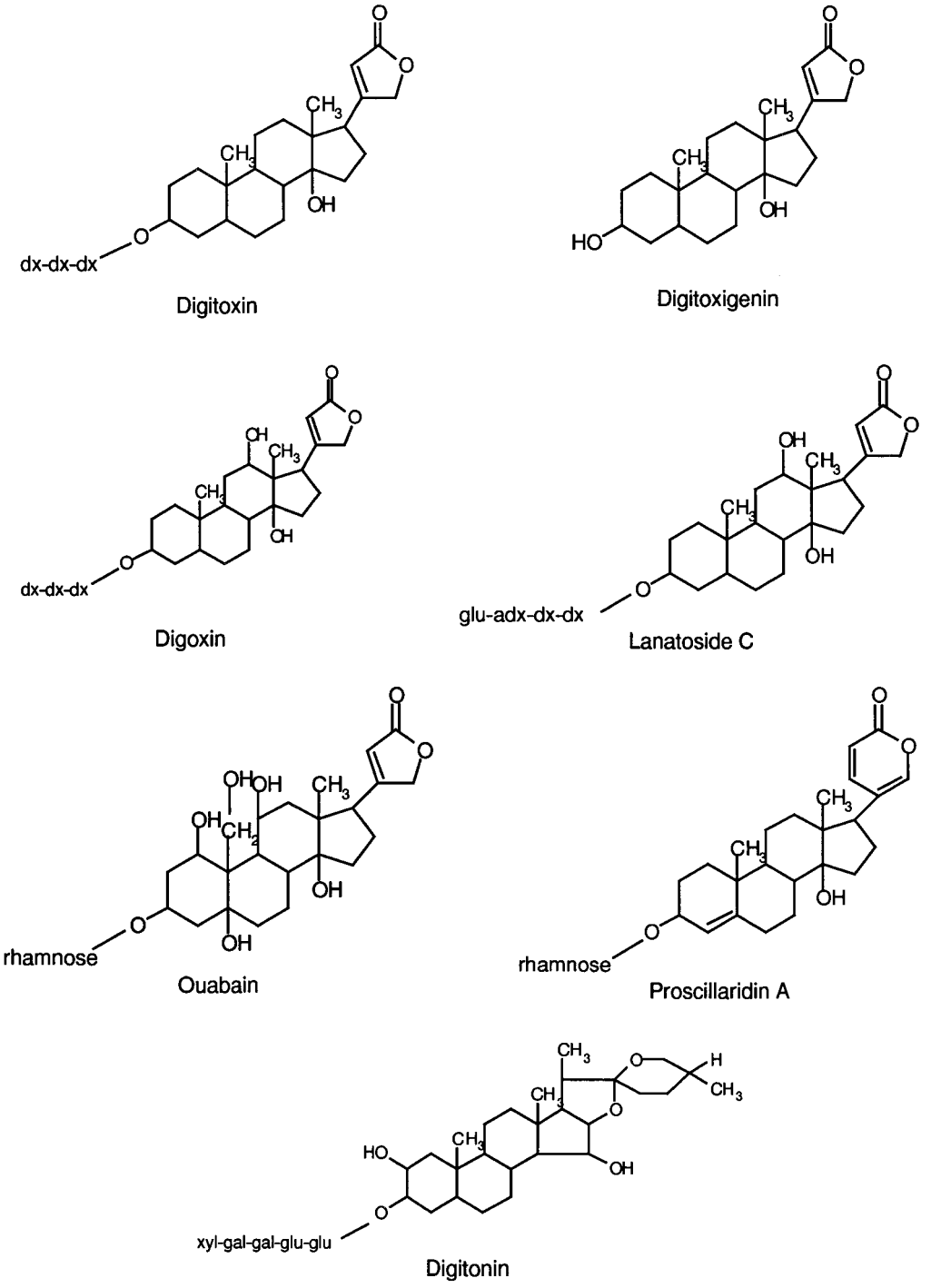

Figure 1. Chemical structures of the cardenolides digitoxin, digitoxigenin, digoxin, lanatoside C, ouabain, the bufadienolide proscillaridin A and the saponin digitonin. Abbreviations: dx (digitoxose), glu (glucose), adx (acetyldigitoxose), xyl (xylose) and gal (galactose).

tance, it is possible to predict mechanisms of action of anticancer drugs.

Applying this simplified approach to digitoxin and some related cardiac glycosides, we also investigated their structure–activity relationships for cytotoxic effects. For comparison, we included digitonin, the triterpene saponin detergent from foxglove and the aglycone of digitoxin (digitoxigenin). In addition, we

characterized the cytotoxic activities of the substances in different primary human tumor cells.

Materials and methods

Isolation and identification of digitoxin

A specific fraction (fraction P; substances with molecular weights over >700 Da) from the 50% ethanolic extract of the leaves of *D. purpurea* Ehrl. was isolated according to a previously described protocol.[1] The fraction P was dissolved in a mobile phase of aqueous 25% acetonitrile (CH_3CN) and 0.1% trifluoroacetic acid (TFA), and then fractionated on a semipreparative high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10Avp photodiode array detector and a 250×10 (i.d.) mm Dynamax column (C_{18}, 5 μm, pore size 300 Å). The column was eluted with a linear gradient from 25% CH_3CN:0.1% TFA to 75% CH_3CN:0.1% TFA for 30 min. Fractions were collected and tested for cytotoxicity in a cell line panel. The most potent fraction was further purified by HPLC to yield a homogenous component, which was unambiguously identified as the cardiac glycoside digitoxin by spectroscopic methods. The ^{1}H-NMR spectrum (recorded at 600 MHz) and mass spectrum (obtained by the electrospray ionization technique) were in agreement with those of an authentic sample of digitoxin (Sigma, St Louis, MO).

Cell line panel

The compounds were tested for growth inhibitory response at concentrations ranging from 6.4 nM to 20 μM in a standard panel consisting of 10 cell lines. After 72 h of treatment, the percentage of living cells was determined. The cell line panel, maintained as described earlier,[11] represents a set of defined mechanisms of resistance. Table 1 lists the cell lines, origin and mechanism of resistance.

Patient samples

Seven 'patient samples' (from tumors of seven untreated patients) comprising two ovarian carcinoma (Ovcas), three chronic lymphocytic leukemia (CLLs) and two breast carcinoma (BCs) were obtained from routine peripheral blood or surgical procedure (Ovca), with sampling approved by the local ethics committee at the Uppsala University Hospital. These seven patient samples (Ovcas, CLLs and BCs) were compared to three samples of normal peripheral blood mononuclear cells (PBMCs) from healthy blood donors.

Table 1. The cell lines, their origins and their mechanisms of resistance[11]

Cell line	Origin	Mechanism of resistance
8226-S	myeloma	parental
8226-LR5	myeloma	GSH-associated
8226-Dox40	myeloma	P-gp-associated
U-937 GTB	histiocytic lymphoma	parental
U-937 Vcr	histiocytic lymphoma	tubulin-associated
NCI-H69	small cell lung cancer	parental
H69 AR	small cell lung cancer	MRP-associated
CCRF-CEM	leukemia	parental
CEM-VM-1	leukemia	Topo II-associated
ACHN	renal	primary resistant

Abbreviations: Topo II (topoisomerase II), MRP (multidrug-associated protein), P-gp (P-glycoprotein) and GSH (glutathione).

The leukemic cells and PBMCs were isolated from peripheral blood by 1.077 g/ml Ficoll-Paque (Amersham Pharmacia-Biotech, Uppsala, Sweden) density-gradient centrifugation.[2] Tissue from each solid tumor sample was minced into small pieces, from which tumor cells were then isolated by collagenase dispersion followed by Percoll (Amersham-Pharmacia Biotech) density-gradient centrifugation.[12] Cell viability was determined by the Trypan blue exclusion test.

Reagents and drugs

Seven pure compounds of natural origin, digitoxin, digoxin, lanatoside C, digitoxigenin, ouabain, proscillaridin A and digitonin (all from Sigma), were tested for cytotoxicity in the cell lines, using the FMCA.[13,14] For each compound, six different concentrations, obtained by 5-fold serial dilution from a maximum concentration of 20 μM, were tested in the cell line panel and against patient samples.

Ethanol was used to dissolve digitoxin, ouabain, lanatoside C, proscillaridin A and digitonin, and DMSO, to dissolve digoxin and digitoxigenin. The final concentrations of ethanol and DMSO were 1% or less, since cell viability has been shown to be unaffected by solvent concentrations up to 1%.[15]

V-shaped 96-well microtitre plates (Nunc, Roskilde, Denmark) were prepared with 20 μl/well of drug solution at 10 times the desired concentration, with the aid of a programmable pippeting robot (Pro/Pette; Perkin-Elmer, Norwalk, CT). Each plate contained three substances, using triplicate wells for each concentration and compound, six control wells, six blank wells and three wells for each positive (0.1% Triton X-100)

and negative (PBS) controls. For later use, the plates were stored frozen at −70°C for up to 3 months.[2]

Measurement of cytotoxic activity

The FMCA is based on measurement of fluorescence generated by hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes, as previously described.[13,14] Quality criteria for a successful analysis included a fluorescence signal in the control wells of more than 10 times mean blank value and a mean coefficient of variation (CV) in the control wells of less than 30%.

Data analysis and quantification

For each compound, the IC_{50} value was calculated (i.e. the concentration giving a survival index of 50%) and for each compound not providing an IC_{50} value the lowest tested concentration was used instead,[10] as in the case of proscillaridin A on the cell line CCRF-CEM. Also, the resistance factor (RF) was calculated for each compound, defined as the value obtained by dividing the IC_{50} value of the resistant subline with the IC_{50} value of its sensitive parental cell line. The pairs of resistant/parental cell lines used for RF calculations of P-gp, GSH, MRP, Topo II and tubulin resistance were RPMI 8226-Dox40/RPMI 8226-S, RPMI 8226-LR5/RPMI 8226-S, H69AR/NCI-H69, CEM-VM-1/CCRF-CEM and U-937-Vcr /U-937-GTB, respectively.[11]

A procedure similar to the COMPARE analysis described by Paull *et al.*[16] (using Pearson's correlation coefficients) was used for comparing compounds and rank-ordering them for their similarity to a 'mean' profile (see Appendix for further detail). Also, the correlation of log IC_{50} values for two compounds x and y was determined using Excel (Microsoft) and the formula $R=(\Sigma_i \{(\log x_i-\log x_{mean})(\log y_i-\log y_{mean})\}/\{[\Sigma_i (\log x_i-\log x_{mean})^2][\Sigma_i (\log y_i-\log y_{mean})^2]\}^{1/2}$, where R is the correlation coefficient, x_i is the log IC_{50} value of compound x for cell line i (i=1, 2, ..., 10); x_{mean} is the mean log IC_{50} value for compound x, y_i is the log IC_{50} value of compound y for cell line i and y_{mean} is the mean log IC_{50} value for compound y. When there is no correlation, R=0, and when $R>0.9$, the correlation is considered high.[17]

Results

Cytotoxic activity in the cell line panel

Seven related compounds (Figure 1)—five cardenolides (digoxin, digitoxin, lanatoside C, ouabain and digitoxigenin), one bufadienolide (proscillaridin A) and one saponin (digitonin)—induced a dose-dependent decrease in cell viability. The IC_{50} values for these compounds (Table 2) ranged from 6.4 to 7000 nM, with digitonin (IC_{50} ranging from 1070 to 7000 nM; Table 2) being the least potent, and proscillaridin A (IC_{50} values ranging from 6.4 to 76 nM), the most potent. The order of potency (high to low) of these substances on the cell line panel was in most cases proscillaridin A, digitoxin, ouabain, digoxin, lanatoside C, digitoxigenin and digitonin. An exception to this was observed in the cell line NCI-H69, where digitoxin was more potent than proscillaridin A. In the renal adenocarcinoma cell line ACHN, digitoxin was equipotent with proscillaridin A. Also, in the cell lines NCI-

Table 2. IC_{50} values (nM) of cardiac glycosides and digitonin for the human tumor cell line panel

Cell line	Compounds							Mean IC_{50} cell line
	Digitoxin	Digitoxigenin	Digoxin	Lanatoside C	Ouabain	Proscillaridin A	Digitonin	
8226-S	34	242	84	220	73	13	1280	278
8226-LR5	25	201	64	150	57	15	1310	260
8226-Dox40	59	395	172	339	148	20	1070	315
U-937-GTB	32	251	68	142	66	6.4	1880	349
GTB-Vcr	36	344	74	133	63	6.4	1100	251
NCI-H69	12	635	56	137	70	66	2600	511
H69AR	31	200	41	154	45	10	4730	744
CCRF-CEM	25	283	49	127	57	<6.4[a]	2650	457
CCRF-VM-1	40	441	63	190	74	9	4430	750
ACHN	76	658	125	602	126	76	7000	1237
Mean IC_{50} compounds	37	365	80	219	78	23	2805	

[a]Since the IC_{50} value is below the tested concentrations, the lowest tested concentration is used.[10]
Each IC_{50} value is calculated from the mean of two or three experiments, all performed in triplicate.

H69, CCRF-CEM, and its sublines H69AR and CEM-VM-1, digoxin showed a lower IC_{50} value than that of ouabain.

Correlation analysis of the cardiac glycosides

Testing on the cell line panel for correlation of the seven natural compounds with one another (Table 3a), we found that the correlation of digitoxin with digoxin, ouabain and lanatoside C was moderately high, whereas correlation with digitoxigenin, proscillaridin A and digitonin was much lower. The highest correlation was between digoxin and ouabain (0.946). The correlation of those natural compounds with standard drugs (Table 3b) was very low to moderately high (−0.3 to 0.7). Digitoxin and digoxin were less correlated with standard drugs than proscillaridin A.

Resistance factors of the cardiac glycosides in the mechanism-based cell line evaluation

In Table 4, the RFs have been calculated for the P-gp, Topo II, MRP, GSH and tubulin resistance mechanisms. The overall low resistance factors for the substances (<3) indicate minimal dependence on the resistance mechanisms examined. Proscillaridin A was more active against the MRP-expressing resistant subline NCI-H69AR than to its parental cell line H69 (RF=0.15).

The activity of the cardiac glycosides in primary tumor cells from patients

The IC_{50} values obtained in primary tumor cells ranged from 17 to 7050 nM (Table 5). As in the cell line panel, proscillaridin A was the most potent compound, and digitonin, the least potent. The ratios between the hematological and the solid tumor samples (CLL/Ovca; CLL/BC) were calculated (Table 5). In most cases, the solid tumor samples (Ovca, BC) were more sensitive than the hematological (CLL) sample, which in turn was more sensitive than its normal counterpart (PBMC), in several cases.

The solid tumor toxicity was most pronounced for digitoxin and digoxin, while proscillaridin A, lanatoside C and digitoxigenin showed no selectivity for hematological or the solid tumor samples.

Discussion

The molecular structures of the cardiac glycosides can be divided into three parts: a sugar moiety, an unsaturated lactone ring (five or six membered) and a steroidal skeleton. Digitoxigenin has no sugar moiety and is therefore an aglycone, by definition. The triterpene saponin digitonin does not have a lactone ring (Figure 1), and thus no cardiac activity. However, known to lyse plasma membranes,[18] digitonin was included to evaluate the importance of the lactone ring on cytotoxicity.

Table 3. For the cell line panel, correlation coefficients (*R*) for analyzing relationships of the log IC_{50} values of the compounds: (a) among themselves and (b) with standard drugs

	R[a]						
	Digitoxin	Digitoxigenin	Digoxin	Lanatoside C	Ouabain	Proscillaridin A	Digitonin
(a)							
Digitoxin	1.000	0.146	0.713	0.796	0.652	0.033	0.160
Digitoxigenin	0.146	1.000	0.417	0.502	0.634	0.716	0.381
Digoxin	0.713	0.417	1.000	0.803	0.946	0.398	−0.256
Lanatoside C	0.796	0.502	0.803	1.000	0.849	0.608	0.317
Ouabain	0.652	0.634	0.946	0.849	1.000	0.548	−0.021
Proscillaridin A	0.033	0.716	0.398	0.608	0.548	1.000	0.344
Digitonin	0.160	0.381	−0.256	0.317	−0.021	0.344	1.000
(b)							
Doxorubicin	0.38	0.43	0.26	0.59	0.39	0.59	0.57
Vincristine	−0.02	0.43	0.00	0.34	0.15	0.70	0.54
Cytarabine	0.02	0.05	0.32	0.44	0.28	0.68	−0.04
Melphalan	−0.03	−0.30	0.09	0.20	0.00	0.39	−0.06
Topotecan	0.12	0.07	0.05	0.43	0.09	0.58	0.43

[a]Pearson's correlation coefficient calculated as [$\log_{10}$ (IC_{50}) for the compound]/[$\log_{10}$ (IC_{50})] for other tested compounds on the tumor cell line panel.

Table 4. RFs for the different cardiac glycosides and digitonin

Resistance mechanism	RF[a]						
	Digitoxin	Digitoxigenin	Digoxin	Lanatoside C	Ouabain	Proscillaridin A	Digitonin
P-gp associated MDR	1.74	1.63	2.05	1.54	2.03	1.54	0.84
GSH-associated MDR	0.74	0.83	0.76	0.68	0.78	1.15	1.02
Topo II-associated MDR	1.60	1.56	1.29	1.50	1.30	1.4	1.67
Tubulin-associated MDR	1.13	1.37	1.09	0.94	0.95	1.00	0.59
MRP-associated MDR	2.58	0.31	0.73	1.12	0.64	0.15	1.82
Primary MDR[b]	2.95	1.87	1.95	3.85	1.89	3.31	3.33

[a]RF=(IC_{50} resistant cell line)/(IC_{50} parental cell line).
[b]RF defined as (IC_{50} ACHN)/(mean panel IC_{50} for parental cell lines).

Table 5. Comparison of the IC_{50} values (nM) of cardiac glycosides and digitonin for tumor cells from patients

	Compounds						
	Digitoxin	Digitoxigenin	Digoxin	Lanatoside C	Ouabain	Proscillaridin A	Digitonin
Patient samples							
PBMC (*n*=3)	106	1000	327	740	277	26	4640
Ovca (*n*=2)	55	339	232	335	229	17	2637
BC (*n*=2)	65	489	127	316	147	21	4786
CLL (*n*=3)	150	527	520	424	358	18	7050
Ratio							
PBMC/CLL	0.71	1.90	0.63	1.75	0.77	1.44	0.66
CLL/Ovca	2.73	1.55	2.24	1.27	1.56	1.06	2.67
CLL/BC	2.31	1.08	4.09	1.34	2.44	0.86	1.47

Each IC_{50} value is calculated from the mean of two or three experiments, all performed in triplicate.

In this study we found that the bufadienolide proscillaridin A in nine out of 10 human tumor cell lines was the most potent substance (Table 2). These results agree with literature data showing that the cytotoxic activity of cardenolides is generally weaker on primary liver carcinoma cells PLC/PRF/5 than the corresponding bufadienolides having the same steroidal skeletons.[19] The order of cytotoxic potency of the cardiac glycosides found in this study virtually parallels the inhibitory potency of the cardiac glycosides on the Na^+/K^+-transporting ATPase from human cardiac muscle from the literature data.[20] The cytotoxic concentrations are higher than the concentrations required to inhibit Na/K-ATPase activity from human cardiac muscle, but for digitoxin the average IC_{50} (37 nM; Table 2) is still within the therapeutic concentration used for cardiac congestion (13–45 nM).[21]

Digitoxigenin is the aglycone of digitoxin. This structural difference caused marked differences in the cytotoxicity patterns of digitoxin and digitoxigenin (Tables 2 and 3a), and virtually no significant correlations were found between the activity profiles of these two compounds. Moreover, for the cell line panel tests, digitoxin's additional three digitoxose sugar units increased its potency 6.5–50 times compared to that of digitoxigenin, suggesting that an intact glycoside is essential for potent cytotoxic activity (Table 2). The correlation of digitoxin with other intact cardenolides (i.e. digoxin, ouabain and lanatoside C) was moderately high (Table 3a), which may indicate (as might be expected) a similar mode of action for these compounds. The highest correlation occurred for digoxin and ouabain (Table 3a). Only low correlation of the activity patterns of digitoxin with that of proscillaridin A, digitoxigenin and digitonin was found.

Lanatoside C and digoxin are structurally closely related in that digoxin can be obtained from lanatoside C by hydrolytic removal of the acetyl and glucose moieties (Figure 1). This structural difference in the sugar part of the compounds was manifested in an approximately 3 times higher mean potency of digoxin compared to lanatoside C (Table 2).

For digitonin, which was less cytotoxic than all tested cardiac glycosides (Table 2), the differential cytotoxicity patterns were different, and correlation with glycosides and standard drugs was low (Table 3), suggesting that cardiac glycosides possess a mechanism of action other than just the cell membrane lysing

effect of digitonin.

Digitoxin has recently been shown to induce apoptosis,[22] and to inhibit prostate[23] and breast cancer cell lines in therapeutic concentrations used in the treatment of heart congestion.[22] In this study, cardiac glycosides and digitonin were tested against cells from patients, i.e. primary BC cells, Ovca cells and CLL cells. Comparing the cytotoxicity of the compounds to patient samples and to cell lines, the order of potency was similar (Table 5). For some of the compounds, such as digitoxin, digoxin and, to some extent, ouabain, a tendency of selective toxicity against the solid tumor cells of Ovca and BC was observed, when compared to the hematological samples of CLLs. This observation is encouraging, since drugs with high solid tumor activity are rare.[24–26] Proscillaridin A, lanatoside C and digitoxigenin exhibited less selective toxicity, as shown by low IC_{50} ratios (hematological/solid tumor cells), which were less than 2 (Table 5).

The low correlations to standard drugs in our database[11] (Table 3b) support the idea that the cardiac glycosides may act by cytotoxic mechanisms other than those of standard drugs. Furthermore, the glycosides overall yielded low resistance factors (Table 4), indicating little or no dependence on the common resistance mechanisms studied here.

Early epidemiological studies show that congestive heart-failure patients who receive cardiac glycosides have a tendency to develop breast tumors of lower growth potential than untreated patients.[27,28] The observed activity is proposed to be associated with an estrogen-like effect[27–30] of cardiac glycosides. This hypothesis conflicts with our result in which cardiac glycosides inhibited the growth not only of the cells of breast carcinoma, but of all tumor cells tested (Tables 2 and 5). Other papers also report[31,32] that it seems unlikely that estrogen-like properties of digoxin are of major importance in the growth suppression of breast tumors.

In a study where the post-operative fate of breast cancer was investigated, the risk of recurrence for patients that had not taken digitalis within 5 years after mastectomy was almost 10 times that of patients receiving digitalis medication.[29] Others have found no connection between digitalis medication and prevention of breast cancer.[33] Recent data on the potential of cardiac glycosides as anticancer agents[22,23,34,35] show that digitoxin inhibits proliferation and induces apoptosis in various cell lines. Also, reports show that the mortality of the patients on digitalis treatment was lower compared to patients without digitalis treatment.[30]

In our study, digitoxin and digoxin showed a tendency of selective toxicity towards the solid tumor patient samples of ovarian carcinoma and breast carcinoma compared to the hematological malignancy CLL (Table 5). The different cardiac glycosides varied considerably in their cytotoxic potency towards different cell lines and towards the primary cultures of tumor cells from patients. In agreement with the reports of Haux *et al.*,[22,23] digitoxin was shown to be more potent than digoxin. The bufadienolide proscillaridin A was found to be of even higher potency (average IC_{50} 23 nM, Table 2), but the cytotoxic concentration is not within the range of therapeutic plasma concentration (0.9–1.1 nM) used for this drug in the treatment of heart congestion.

Correlation analysis indicates that the different cardiac glycosides act by different modes of action than the mechanistically archetypal anticancer drugs doxorubicin, vincristine, cytarabine, melphalan and topotecan. These results provide new information for use in further research that may clarify a possible role of cardiac glycosides in chemotherapy of malignant diseases.

Acknowledgments

Mrs L Lenhammar's skilful technical assistance is gratefully acknowledged. We also thank Professor Karl-Erik Andersson Department of Clinical Pharmacology, University Hospital of Lund, Sweden, for valuable discussions about the therapeutic concentration of proscillaridin A.

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(Received 13 February 2001; accepted 10 March 2001)

Appendix: comparing compounds

Suppose n compounds are being evaluated and compared for toxicity using a panel of m cell lines. For each compound, the IC_{50} value for each cell line in the panel is converted to its log IC_{50} value and the mean panel $\log_{10}$ IC_{50} value is obtained by averaging the individual $\log_{10}$ IC_{50} values (one for each cell line). For the given compound, the $\log_{10}$ IC_{50} value of each cell line is then subtracted from the panel mean to

create a corresponding delta value for the cell line. These *m* delta values are the values for the 'mean graph' of the given compound.

A particular selected mean-graph profile (i.e. the set of n mean graphs, one for each compound) is used to probe a given database, as follows. Consider a given compound. For each cell line, that compound's delta value is compared to that of each of the $n-1$ remaining compounds. This procedure for comparing a compound's delta values with that of the remaining compounds is done for each of the *n* compounds. In this manner, the mean-graphs of all compounds in the specified database can be rank-ordered for similarity to the mean-graph profile.