

## Digitoxin Inhibits the Growth of Cancer Cell Lines at Concentrations Commonly Found in Cardiac Patients

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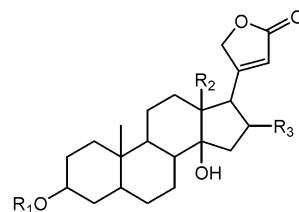
The cardiac glycosides digitoxin (**1**) and digoxin (**3**) have been used in cardiac diseases for many years. During this time several reports have suggested the possible use of digitalis in medical oncology. Several analogues of digitoxin (**1**) were evaluated for growth inhibition activity in three human cancer cell lines; this study showed that digitoxin (**1**) was the most active compound and revealed some structural features that may play a role in the growth inhibition activity of these drugs. The IC<sub>50</sub> values for **1** (3–33 nM) were within or below the concentration range seen in the plasma of patients with cardiac disease receiving this glycoside (20–33 nM). A renal adenocarcinoma cancer cell line (TK-10) was hypersensitive to this drug, and digitoxin toxicity on these cells was mediated by apoptosis. In vitro experiments showed that **1** at 30 nM induced levels of DNA–topoisomerase II cleavable complexes similar to etoposide, a topoisomerase II poison widely used in cancer chemotherapy. Using the individual cell assay TARDIS, cells exposed to **1** for 30 min showed low but statistically significant levels of DNA–topoisomerase II cleavable complexes; however these complexes disappeared after 24 h exposure.

Digitalis (the leaves of species of the genus *Digitalis* or steroidal glycosides isolated from them) has been used for many years mainly for the treatment of cardiac congestion and some types of cardiac arrhythmias. Over the years there have been various reports suggesting that digitalis may have an anticancer utilization. In the 1960s inhibition of malignant cells by cardiac glycosides in vitro was reported.<sup>1</sup> Short-term animal experiments have suggested that toxic doses would probably be needed to observe an anticancer effect in humans.<sup>2</sup> However, different species show a wide variability in susceptibility to these cardiac glycosides, making extrapolation from animal models into humans problematic.<sup>3</sup> In 1979, it was observed that breast cancer cells from women on digitalis had more benign characteristics than cancer cells from control patients not on these drugs<sup>4,5</sup> and that five years after a mastectomy, the recurrence among patients not taking digitalis was 9.6 times higher than in patients on these drugs.<sup>6</sup> Surprisingly, these results did not trigger much research activity at that time to further evaluate a possible benefit for cardiac glycosides in cancer treatment.

Cardiac glycosides have more recently been shown to induce apoptosis in different malignant cell lines.<sup>7–9</sup> For instance, oleandrin, ouabain, and digoxin induced apoptosis in human prostate cancer cell lines at 10–100 nM.<sup>8</sup> An interesting feature seems to be that malignant cells in general are more susceptible to the effects of digitalis than normal cells.<sup>7</sup> In 2001, a study with 9271 patients showed a relationship between high plasma concentration of digitoxin and a lower risk for leukemia/lymphoma as well as lower incidence of cancer of the kidney/urinary tract.<sup>10</sup> These interesting findings by Haux et al. could be related to the ability of digitoxin to inhibit cell growth at concentrations found in cardiac patients. We have investigated

this and the possible role of topoisomerase II in digitoxin activity, and report these results in the present article.

The growth inhibitory effects of six structurally related compounds derived from *Digitalis* species (**1–6**) were investigated. Initially, the cancer cell lines TK-10 (renal), MCF-7 (breast), and UACC-62 (melanoma) were used. These cell lines are derived from tumors that have different sensitivities to chemotherapeutic drugs and have been used routinely at the U.S. National Cancer Institute for screening for new anticancer agents.<sup>11</sup> Using the SRB assay the growth inhibitory activity of these drugs was assessed (Table 1). Table 1 reveals some structural features that may play a role in the growth inhibition activity of these drugs. If digitoxin (**1**), digoxin (**3**), and gitoxin (**5**) are compared with their corresponding aglycons (**2**, **4**, **6**, respectively), it is clear that the sugar moieties at position C-3 are fundamental for a high growth inhibition activity. By comparing digitoxin, digoxin, and their aglycons with gitoxin and its aglycon it appears that the lack of OH at position C-16 is also very important for growth inhibitory activity.



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
digitoxin ( <b>1</b> )	(digitoxose) <sub>3</sub>	H	H
digitoxigenin ( <b>2</b> )	H	H	H
digoxin ( <b>3</b> )	(digitoxose) <sub>3</sub>	OH	H
digoxigenin ( <b>4</b> )	H	OH	H
gitoxin ( <b>5</b> )	(digitoxose) <sub>3</sub>	H	OH
gitoxigenin ( <b>6</b> )	H	H	OH

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**Table 1.** IC<sub>50</sub> Values (nM) of Several Structurally Related Cardiac Glycosides and Aglycons against the Human Cancer Cell Lines TK-10, MCF-7, and UACC-62

compound	TK-10	MCF-7	UACC-62	plasma
digitoxin ( <b>1</b> )	3.2 ± 0.1	10.2 ± 0.3	33.5 ± 0.9	19.6–32.6
digitoxigenin ( <b>2</b> )	76.5 ± 5.8	194.4 ± 2.4	348.8 ± 2.1	
digoxin ( <b>3</b> )	14.6 ± 2.2	24.1 ± 2.1	29.5 ± 4.8	0.6–2.0
digoxigenin ( <b>4</b> )	317.5 ± 5.0	507.0 ± 55.0	416.1 ± 9.5	
gitoxin <sup>a</sup> ( <b>5</b> )	131.2 ± 24.6	251.9 ± 30.2	369.4 ± 12.9	
gitoxigenin <sup>a</sup> ( <b>6</b> )	415.6 ± 30.4	1805.6 ± 324.1	2838.9 ± 170.6	
etoposide <sup>b</sup>	9952.5 ± 88.0	877.5 ± 213.3	1130.0 ± 214.3	

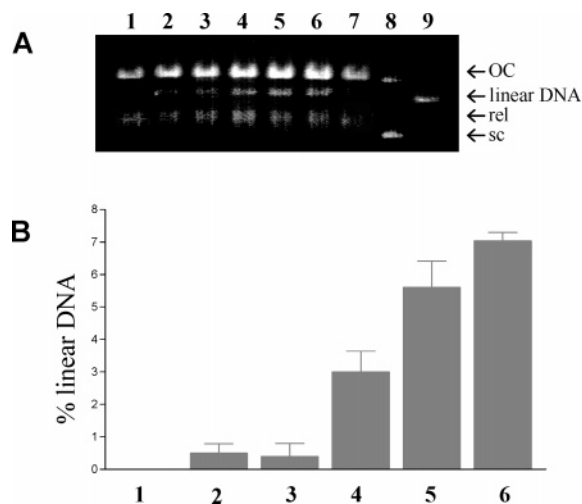
<sup>a</sup> Data taken from ref 9 for the structure–activity relationship analysis. <sup>b</sup> Positive control.

The two drugs currently used in cardiac disease, digitoxin (**1**) and digoxin (**3**), showed the highest level of growth inhibition in the three cell lines investigated (Table 1). Since **1** has been reported to lower the risk of leukemia/lymphoma, we looked at these two compounds in the K-562 leukemia cell line; the IC<sub>50</sub> values were 6.4 ± 0.4 nM for **1** and 28.2 ± 2.9 nM for **3**. In all four cell lines studied the IC<sub>50</sub> values for **1** were within or below the concentration range seen in the plasma of patients receiving digitoxin for cardiac treatment, while for **3** they were around 10-fold higher than its normal plasma concentrations<sup>7,12</sup> (Table 1).

Digitoxin (**1**) has been reported to induce apoptosis in several human cancer cell lines.<sup>7</sup> To confirm if digitoxin was acting via apoptosis in the TK-10 cell line, which displayed the highest growth inhibition with this compound, we used a three-fluorescent-dye method<sup>13</sup> to clearly distinguish between live, apoptotic, or necrotic cells. Digitoxin was assayed at its IC<sub>50</sub> concentration of 3 nM. Following 48 h drug exposure, drug removal, and a period of 3 days in fresh medium, 52.1 ± 4.4% of cells were alive, 41.3 ± 7.3% of the cells were apoptotic, while necrotic cells accounted for only 6.6 ± 4.6%. Compared to the control cells, 72.8 ± 2.8% were alive, 22.4 ± 4.5% were apoptotic, and 4.8 ± 2.9% were necrotic. This showed that digitoxin is acting via apoptosis in the TK-10 cell line and confirms that cardiac glycosides can induce apoptosis in cancer cells.

As discussed in a previous report, several lines of evidence have been used to suggest that digitoxin (**1**) might affect topoisomerase II (topo II).<sup>9</sup> For instance, like digitoxin, several topoisomerase poisons currently used in the clinic (e.g., etoposide and camptothecin derivatives) have a lactone moiety, which seems to be crucial for their activity. In addition, topo II has been proposed to be involved in radiosensitization processes, and digitoxin has shown a radiosensitizing effect on malignant cell lines. Indeed, it has recently been observed that bufalin, a cardiotonic drug chemically related to digitoxin, affects topo II and delays the repair of X-ray-induced DNA breaks.<sup>9</sup> Therefore, we investigated the possibility of digitoxin acting on topo II both in vitro and using a cell-based assay. Figure 1A shows in vitro cleavage experiments with digitoxin and purified topo II and DNA. Data from three experiments are shown in the histogram in Figure 1B. At 30 and 100 nM digitoxin strongly stimulated in vitro topo II cleavage of plasmid DNA. However, at higher concentrations the cleavage stimulation was reduced, so that at 1 and 10 μM low levels of cleavage were seen and at 100 μM no cleavage was observed. It is not unusual for high concentrations of topo II poisons to produce less cleavable complex formation than lower concentrations.<sup>14</sup>

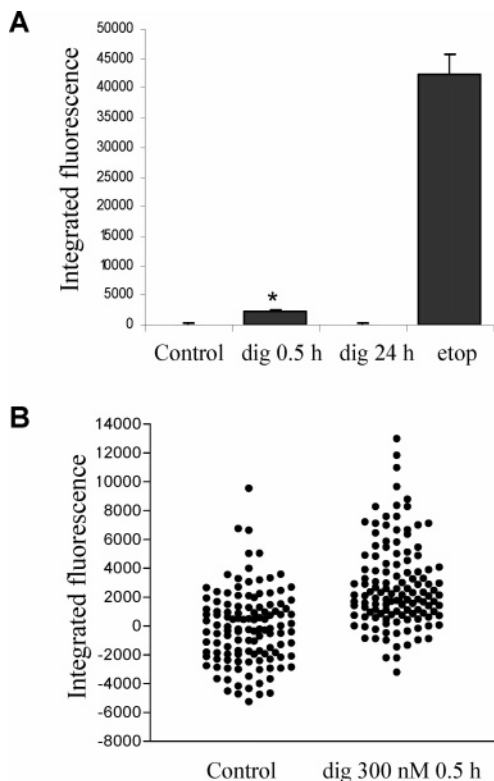
The high cleavage activity seen in vitro was not found in vivo when the topo II cleavage complexes were analyzed using the TARDIS assay. This assay allows the detection of topo II cleavable complexes in individual cells. Initially, K-562 leukemia cells were treated for 0.5 h with digitoxin



**Figure 1.** Evaluation of the in vitro DNA–topo II cleavable complexes induced by digitoxin (**1**). (A, B) 1. Topo II + DNA (pRYG) + 100 μM digitoxin. 2. Topo II + DNA + 10 μM digitoxin. 3. Topo II + DNA + 1 μM digitoxin. 4. Topo II + DNA + 100 nM digitoxin. 5. Topo II + DNA + 30 nM digitoxin. 6. Topo II + DNA + 100 μM etoposide. 7. Topo II + DNA. 8. DNA. 9. Linear DNA. (OC: open circular DNA, rel: relaxed DNA, sc: supercoiled DNA.) (B) Percentage of linear DNA form, indicating topo II poison activity, induced by digitoxin and etoposide. Data are given as the mean of three independent experiments ± SEM.

(**1**) at increasing concentrations (30–1000 nM); this showed that the highest levels of topo II cleavable complexes appeared at 300 nM (results not shown). Next, cells were treated with 300 nM digitoxin for 0.5, 2, 6, and 24 h; this experiment revealed that digitoxin induced a decrease in the levels of topo II cleavable complexes in a time-dependent fashion (results not shown). Figure 2A represents the levels of cleavable complexes in K-562 cells exposed to digitoxin (300 nM) for 0.5 and 24 h, and they are compared with etoposide. After K-562 cells were exposed to 300 nM digitoxin for 0.5 h, low levels of topo II cleavable complexes were seen, which were significantly above background ( $p < 0.05$ , two-tailed paired  $t$ -test). The levels of cleavable complexes induced by digitoxin (300 nM, 0.5 h) in relation to untreated cells can be appreciated better in Figure 2B. These levels, however, were 20 times lower than that observed in cells treated with the strong topo II poison etoposide (2 h, 10 μM). After exposure to 300 nM digitoxin for 24 h, no signal was seen (Figure 2A). Under these conditions (300 nM, 24 h) preliminary experiments carried out in our laboratory suggest that digitoxin may behave as a catalytic inhibitor of topo II in intact cells (our unpublished data).

Johansson et al. showed that digitoxin was 1.6 times less cytotoxic on the topo II-deficient CCRF-VM-1 leukemia cell line than on the parental cell line CCRF-CEM.<sup>15</sup> This suggests that topo II poisoning contributes to digitoxin-induced cytotoxicity, but only slightly. This seems to agree with the results represented in Figure 2 and suggests that



**Figure 2.** Evaluation of digitoxin (**1**) top II poison activity in K-562 leukemia cells using the TARDIS assay. (A) Plot showing mean immunofluorescence values (with standard error bars, three independent experiments) induced by 300 nM digitoxin (0.5 and 24 h) and the etoposide (10  $\mu$ M, 2 h) in relation to untreated cells (control). (B) Representative individual experiment that shows the distribution of cleavable complexes in individual cells treated with digitoxin in relation to untreated cells.

other mechanisms of action are involved in the cytotoxic effect of digitoxin.

The growth inhibition of cancer cell lines shown by digitoxin (**1**) is worth noting. The  $IC_{50}$  value against K-562 was 3–5 times lower than the concentration commonly found in cardiac patients treated with this drug,<sup>7,12</sup> which may explain the lower risk of leukemia/lymphoma reported in patients taking digitoxin.<sup>10</sup> The activity of digitoxin against the MCF-7 breast adenocarcinoma cell line (Table 1) might also explain the anticancer effects reported in breast cancer patients taking digitalis cardiac glycosides.<sup>4–6</sup> The TK-10 renal adenocarcinoma cell line is generally considered as one of the most resistant to cancer chemotherapeutic drugs, and, accordingly, it was approximately 10 times more resistant to etoposide than the MCF-7 and UACC-62 cell lines (Table 1). However, Table 1 shows that the TK-10 cell line was hypersensitive to digitoxin when compared with the other cell lines. The high sensitivity of this cancer cell line to digitoxin supports data from a recent study carried out by Haux and co-workers<sup>10</sup> in which the incidence of different cancers over nine thousand patients taking digitoxin was evaluated. An internal dose–response analysis revealed a relationship between high plasma concentration of digitoxin and a lower risk for cancer of the kidney/urinary tract. In fact, patients with plasma concentrations of **1** within the 21–29 nM range showed 3.3 times less incidence of these cancers of the kidney/urinary tract than patients with plasma concentrations lower than 21 nM. The present article shows that digitoxin induces apoptosis in TK-10 cells at 3 nM, a concentration almost 10 times lower than that commonly found in cardiac patients. The fact that the plasma half-life of digitoxin in

humans is several days and that 30% of untransformed digitoxin is excreted via the renal route<sup>12</sup> suggest that the concentration of digitoxin in the kidneys is prolonged and high enough to display an anticancer effect. Interestingly, no important toxicity has been reported in cardiac patients on digitoxin with plasma concentrations of 30 nM;<sup>12,16</sup> this sustains experimental data showing that the most interesting feature about digitalis is that malignant cells, in general, seem more susceptible to its effects than normal ones.<sup>7</sup> All these data suggest that **1** has the potential to be tested in the treatment of cancers of the kidney/urinary tract.

The promising anticancer effects shown by digitoxin (**1**) in human studies, along with the ability of this drug to inhibit the growth of cancer cell lines at concentrations commonly found in cardiac patients, deserve further research. Future clinical studies should be done to try to answer whether this natural product has a place in medical oncology. An advantage in such studies is that the safety profile of this drug is well-known due to its wide use in medical cardiology; this should facilitate any investigation carried out on cancer patients.

## Experimental Section

**General Experimental Procedures.** The TK-10 renal adenocarcinoma, MCF-7 breast adenocarcinoma, UACC-62 malignant melanoma, and K-562 chronic myelogenous leukemia cell lines were used. All cell lines were maintained in RPMI 1640 medium containing 20% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin, at 37 °C in a 5% CO<sub>2</sub> atmosphere with 95% humidity. Cell culture reagents were obtained from Life Technologies. Digitoxin (**1**), digitoxigenin (**2**), digoxin (**3**), digoxigenin (**4**), gitoxin (**5**), gitoxigenin (**6**), etoposide, and proteinase K were purchased from Sigma Chemical Co. Purified human top II and supercoiled pRYG DNA were purchased from TopoGen, Inc. (Columbus, OH). Anti-topo II polyclonal antibody ( $\alpha$ CT) was raised in rabbits to recombinant human top II  $\alpha$  C-terminal fragment that detected both isoforms of top II ( $\alpha$  and  $\beta$ ). This antibody was diluted in PBS containing 0.1% Tween 20 and 1% BSA at a 1:50 dilution. The anti-rabbit FITC-conjugated second antibody (1262), from Sigma, was used at 1:200 dilution.

**Growth Inhibition Assays.** For the human cancer cell lines UACC-62, TK-10, and MCF-7, the SRB assay was used following protocols established by the National Cancer Institute, National Institutes of Health, Bethesda, MD.<sup>11</sup> This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. The cancer cells were seeded in 96-well plates. According to their growth profiles, the optimal plating densities of each cell line were determined (15  $\times$  10<sup>3</sup>, 5  $\times$  10<sup>3</sup>, and 100  $\times$  10<sup>3</sup> cells/well for TK-10, MCF-7, and UACC-62, respectively). After 24 h the cells were treated for 48 h with the drugs and the SRB assay was carried out before reading the plates at 492 nm, as described previously.<sup>9,11</sup> For the K-562 cell line, the XTT assay was employed. Exponentially growing cells were seeded (2  $\times$  10<sup>3</sup>/well in 100  $\mu$ L) into 96-well plates. Drugs were added to plates 24 h later. Following the incubation period indicated in figure or table legends, cell viability was estimated using an XTT cell proliferation kit assay (Roche, Mannheim, Germany). This colorimetric assay is based on the capability of viable cells to transform the tetrazolium salt XTT into a formazan dye. After drug exposure, plates were incubated for 4 h with XTT, before being read on a Bio-Rad 550 plate reader at 450 nm. In both assays (SRB and XTT) cell viability was expressed as percentage in relation to controls. Data were averaged from at least three independent experiments  $\pm$  SEM.

**Apoptotic Index.** For morphological evaluation, to discriminate between cells in the process of apoptosis, living, and dead (either necrotic or apoptotic), a previously reported

fluorescence microscopy assay was employed.<sup>13</sup> The procedure consisted of the use of a mixture of three fluorescent dyes in phosphate-buffered salt solution (PBS) at pH 7.4: 5  $\mu\text{g}/\text{mL}$  of the nonpermeant dye propidium iodide (PI), 15  $\mu\text{g}/\text{mL}$  of fluorescein diacetate (FDA), and 2  $\mu\text{g}/\text{mL}$  of Hoechst 33342. After drug exposure 2 mL aliquots from each sample were used from the cell cultures and 40  $\mu\text{L}$  of the fluorescent mix was added. After 5 min incubation in a  $\text{CO}_2$  atmosphere at 37  $^\circ\text{C}$ , the cell suspension was transferred to centrifugation tubes, washed with PBS, and resuspended in 1 mL of medium. Samples were dropped onto slides; for observation and quantification a fluorescent microscope (Olympus, Vanox AHB73) equipped with ultraviolet light filters was used. At least 500 cells were counted for each compound and concentration, and three independent experiments were carried out. Data are given as the mean  $\pm$  SEM.

**In Vitro DNA Cleavage with Topoisomerase II.** The assay is based upon the ability of topo II to break and rejoin double-stranded pRYG DNA (supercoiled). An inhibitory effect on the enzyme, through poisoning and stabilization of the ternary structure DNA–topo II–drug, the so-called “cleavable complex”, leads to an increased yield of the linear form as a consequence of a lack of rejoining of the double-strand breaks. These structures are evidenced by a proteolysis of the DNA-linked topo II carried out by proteinase K. The protocol has been described in detail before.<sup>17</sup> Briefly, the cleavage reaction (20  $\mu\text{L}$ ) contained water, cleavage buffer, the test drugs dissolved in 2  $\mu\text{L}$  of dimethyl sulfoxide/ $\text{H}_2\text{O}$  (2.5%), pRYG DNA (0.25  $\mu\text{g}$  in 1  $\mu\text{L}$  of buffer), and 2  $\mu\text{L}$  of topo II $\alpha$  (4 units), which were mixed in this order in ice/water. Reactions were carried out by incubation at 37  $^\circ\text{C}$  for 30 min and terminated by additional 15 min incubation at 37  $^\circ\text{C}$  after the addition of 2  $\mu\text{L}$  of SDS 10% and 1  $\mu\text{L}$  of proteinase K (stock solution 20  $\mu\text{g}/\text{mL}$ ). Afterward, the samples were extracted with chloroform–isoamyl alcohol (24:1), and 2  $\mu\text{L}$  of bromophenol blue was added. Samples were loaded onto 1% agarose gels and electrophoresed at 6 V/cm for 3 h in a Tris-acetate-EDTA buffer. Gels were then stained with ethidium bromide and washed in water. For quantitative determination, the software PCBAS was used. The presence of the linear DNA form, which indicates topo II poisoning, was expressed as percentage of total DNA. Data are given as the mean of three independent experiments  $\pm$  SEM.

**TARDIS Assay.** The TARDIS (trapped in agarose DNA immuno staining) assay is an immunofluorescence assay that allows measurement of drug-induced topo I and topo II ( $\alpha$  and  $\beta$ ) cleavable complexes that form in individual cells and has been described in detail previously.<sup>18</sup> Briefly, cells were treated with drugs at the concentrations and for the times specified in the figure legends, and embedded in agarose on microscope slides. After placing the slides in a lysis buffer (10% SDS, 80 mM phosphate, pH 6.8, 10 mM EDTA plus protease inhibitors), proteins that were not covalently bound to the DNA were removed by placing the slides in 1 M NaCl plus protease inhibitors. Slides were incubated with primary topo II antibody

and then a fluorescein isothiocyanate (FITC)-conjugated (secondary antibody) before being counterstained with Hoechst 33258. Images were captured using an epi-fluorescence microscope attached to a cooled slow scan charge-coupled device camera that separately visualizes blue (Hoescht-stained DNA) and green (FITC-stained topo) fluorescence. For each of the eight randomly chosen fields of view, images of blue and green fluorescence were captured to give a total of approximately 100 cells/dose. Imager 2 software (Astrocam, Cambridge, UK) was used to analyze and quantify levels of blue and green fluorescence. Figures show integrated green fluorescence values, which indicates drug-stabilized topo–DNA cleavable complexes. Data were averaged from at least three independent experiments  $\pm$  SEM.

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