SUPPLEMENTARY MATERIAL

Inhibition of cell proliferation, invasion and migration by the cardenolides digitoxigenin monodigitoxoside and convallatoxin in human lung cancer cell line

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ABSTRACT

Cardiac glycosides consist of a large family of naturally derived compounds that are clinically used to treat congestive heart failure, and also present anticancer properties. In this study, the cytotoxic effects of two cardenolides, digitoxigenin monodigitoxoside (DGX) and convallatoxin (CON) were screened in four human tumor cell lines. Both compounds showed anti-proliferative effects in all tumor cells, at nanomolar concentrations. Since the human lung cancer cell line A549 was the most sensitive, we investigated the anti-proliferative, anti-migratory and anti-invasive effects of these cardenolides. DGX and CON reduced A549 cell migration, being able to reduce more than 90% of cell invasion. Their effects on the expression of key regulators of metastatic mechanism showed decreased levels of MMP-2, MMP-9 and p-FAK. Both compounds also presented low toxicity for healthy cells. Finally, this work provides the first insights into the effects of these cardenolides on key steps of lung cancer metastasis.

Keywords: convallatoxin, digitoxigenin monodigitoxoside, proliferation, migration, invasion, A549 cell line, lung cancer.

Experimental

Cardenolides, anticancer drugs and cell lines

Digitoxigenin monodigitoxoside (DGX) was isolated from *Digitalis lanata* Ehrh. leaves. In summary, a methanolic extract from *D. lanata* leaves was pre-purified by filtration over activated charcoal. The obtained residue was dissolved in dichloromethane and filtered over a silica gel column. Further elution of this column with a gradient of chloroform and ethyl acetate led to the isolation of DGX (yield = 1.5 % w/w), recovered in the fraction eluted with chloroform / ethyl acetate (3:7). The identification of DGX was accomplished by comparison with an authentic compound, employing TLC and UPLC-MS analyses. The purity of the isolated compound (> 98%) was checked by HPLC-DAD and UPLC-MS data (Braga et al., 1996). Convallatoxin (CON) and the anticancer drugs used as positive controls (irinotecan, cisplatin and paclitaxel) were purchased from Sigma-Aldrich, USA. Human non-small cell lung cancer (NSCLC, A549 cells, ATCC: CCL185) and human rhabdomyosarcoma RD (Adolfo Lutz Institute, SP, Brazil) were grown in Minimal Essential Medium (MEM; Cultilab, Brazil). Human colon carcinoma cells HCT-8 (Texas A&M University System, USA) and human prostate carcinoma cells LNCaP (ATCC: CRL-1740) were grown in RPMI 1640 medium (Gibco, USA). All the cell lines were maintained in medium supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified incubator under 5% CO₂/95% air.

Human gingival fibroblasts (HGF), obtained from human gingival primary cell culture (process approved by the Ethics Committee on Human Research, UFSC, authorization number 062, process number 021/09), were grown in Dulbecco's Modified Eagle Medium (DMEM; Cultilab) supplemented with 10% FBS, 2 mM glutamine (Cultilab) and 1% nonessential amino acids (Gibco).

Screening for cytotoxic effects

The screening of cell viability was performed by sulforhodamine B assay (SRB) (Vichai and Kirtikara, 2006). Briefly, A549, LNCaP, HCT-8, RD, and HGF cells were cultured in 96-well plates (1×10⁴ cells/well), and after 24 h the cells were exposed to DGX and CON at various concentrations and experimental times. A549 and HGF cells were exposed to paclitaxel, irinotecan and cisplatin as positive controls. After incubation, 10% trichloroacetic acid (TCA) was added to each well to fix the cells and then washed and stained with SRB. The protein-bound dye was dissolved in 10 mM Tris-Base [(tris(hydroxymethyl) aminomethane] solution and optical densities (OD) were read at 510 nm on a spectrophotometer Spectra Max M2 (Molecular Devices, USA). The inhibitory concentration (IC₅₀) of each compound was calculated as the concentration that inhibited cell proliferation of cancerous cells (A549, LNCaP, HCT-8, RD) by 50%, when compared to untreated controls. In order to differentiate the cytotoxic effects for cancerous and non-cancerous cells, the cytotoxic concentration (CC₅₀) was used to calculate the concentration that inhibited cell proliferation of non-cancerous cells (HGF).

A549 cell migration assay

Cell migration was assessed by examining the ability of the cells to move into a cellular space in an *in vitro* wound healing assay known as scratch assay (Valster et al., 2005). Briefly, A549 cells were

grown to confluence in 12-well plates with MEM supplemented with 10% Fetal Bovine Serum (FBS) and incubated for 24 h and an artificial wound was generated by scratching the cell monolayer. Cells were washed and then incubated in medium, in the absence or presence of DGX and CON (10 and 50 nM) or 0.5% DMSO and paclitaxel (PAC) (10, 100 and 1000 nM) as controls for 16 h. The cells were fixed with paraformaldehyde, stained with 4',6-diamidino-2-phenylindole (DAPI), and subsequently photographed in an inverted fluorescence microscope BX-41 (Olympus, USA). Six representative images from each well of the scratched areas were photographed in order to determine the width of the scratch. The zero scratch well was fixed immediately after the generation of the artificial wound. The quantification of the migration percentages was performed using the free CellC[®] Software (Selinummi et al., 2005).

Healthy human gingival fibroblasts migration assay

This assay was carried out as described above (Section experiments: A549 cell migration assay) to evaluate the selectivity of both cardenolides in healthy cell lines. The fibroblasts were seeded into 12-well plates with DMEM supplemented with 10% FBS and incubated for 24 h. Further, a wound was created by scratching a line and the treatments were added: DGX and CON (10 and 50 nM), 0.5% DMSO and 2 ng/ml of platelet-derived growth factor (PDGF) (Gibco) as controls. The plates were incubated for 16 h, and the cells were fixed, stained, photographed and counted as described above.

A549 cell invasion assay

The assay was conducted using Transwell[®] inserts (8 µm pore size polycarbonate membrane, Millipore Corporation, Germany) coated with Matrigel[®]. The cells placed in the invasion chambers were rehydrated with MEM, and were seeded in 24-well plates. The lower chamber received MEM without serum, and they were incubated for 24 h to achieve cell confluence. After, in the upper chambers, both cardenolides and DMSO (control) diluted in serum-free medium were added. The basolateral compartments received MEM with 10% FBS (used here as a chemoattractant) and were incubated for 48 h. After this time, non-invading cells were removed and cells that invaded the membrane but do not arrived in the lower compartments were trypsinized and fixed with paraformaldehyde and stained with DAPI. Images were obtained with a fluorescence microscope BX-41 (Olympus) and the quantification was performed using the free CellC[®] Software (Selinummi et al., 2005).

Western blotting analysis

A549 cells were treated with both cardenolides at different concentrations or the vehicle (DMSO) for 24 h and 48 h. Whole cells were lysated and centrifuged at $10,000 \times g$ for 15 min, at 4 °C. Protein concentration was determined by using Bradford assay (kit from Bio-Rad, Hercules, USA). Equivalent amounts of protein were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electron transfer to PVDF membranes (Millipore). After blocking with 5% nonfat milk in PBS, membranes were incubated overnight at 4 °C with the appropriate primary antibodies. After incubation with the corresponding secondary antibodies

conjugated to horseradish peroxidase, protein bands were revealed by using Pierce Enhanced Chemiluminescence ECL substrate (Thermo Scientific, USA). Protein bands were quantified using the ChemiDoc MP equipment (Bio-Rad, Hercules, USA). The following antibodies were used: MMP-2, MMP-9, p-FAK and FAK (Cell Signaling Technology, USA). An antibody against β -actin (Sigma-Aldrich) was used as control for equal loading.

Statistical analysis

The results were expressed as mean $\pm$ SD of three or two independent experiments. Statistical analyses were performed by one-way ANOVA followed by Dunnet's post test ($p < 0.05$). GraphPad Prism 5 Software (GraphPad, USA) was used to calculate IC₅₀ values and the confidence intervals.

Table S1: Values of growth inhibition of human non-small cell lung cancer A549 by digitoxigenin monodigitoxoside, convallatoxin and chemotherapy drugs after 24 and 48 h of treatment obtained by sulforhodamine B assay.

Cardenolides	24h	Confidence Interval (95%)	48h	Confidence Interval (95%)
IC₅₀ (nM)				
DGX	67.16	45.04 to 90.1	12.34	9.10 to 16.74
CON	76.23	30.01 to 113.7	11.03	4.80 to 23.44
Positive controls				
IC₅₀ (μM)				
CIS	94.76	43.31 to 207.30	8.77	6.47 to 11.90
IRI	45.89	34.61 to 60.86	3.33	2.49 to 4.47
PAC	0.266	0.097 to 0.320	0.194	0.083 to 0.286

The data represent the mean of three independent experiments and 95% confidence interval. IC₅₀: concentration that inhibits 50% of cell growth of cancerous cells. DGX: digitoxigenin monodigitoxoside; CON: convallatoxin; CIS: cisplatin; IRI: irinotecan; PAC: paclitaxel

Table S2: Values of growth inhibition of different human cancer cell lines by digitoxigenin monodigitoxoside, convallatoxin and paclitaxel after 48 h of treatment obtained by sulforhodamine B assay.

Cell lines	Samples					
	DGX		CON		PAC	
	IC ₅₀ (nM)	Confidence Interval (95%)	IC ₅₀ (nM)	Confidence Interval (95%)	IC ₅₀ (nM)	Confidence Interval (95%)
HCT-8	71.62	59.7 to 85.9	13.71	12.4 to 15.2	20.8	13.5 to 31.9
RD	22.20	14.3 to 34.3	15.83	9.4 to 26.3	46.1	28.3 to 55.4
LNCaP	20.80	17.9 to 24.2	16.84	9.7 to 29.3	10.5	6.3 to 14.7

The data represent the mean of three independent experiments and 95% confidence interval. IC₅₀: concentration that inhibits 50% of cell growth of cancerous cells.
DGX: digitoxigenin monodigitoxoside; CON: convallatoxin; PAC: paclitaxel (used as positive control)
HCT-8: human colon carcinoma cell line; RD: human rhabdomyosarcoma cell line; LNCaP: human prostate carcinoma cell line.

Table S3: Values of growth inhibition of healthy human gingival fibroblasts by digitoxigenin monodigitoxoside, convallatoxin and paclitaxel obtained by sulforhodamine B assay.

Samples	CC ₅₀ (μ M) 16 h	CC ₅₀ (μ M) 48 h	95% Confidence Interval 48 h	SI (CC ₅₀ /IC ₅₀) 48 h
DGX	>250	28.70	16.92 to 48.66	2,325x
CON	>250	4.879	2.381 to 10.23	442x
PAC	>250	>250	-	>2500x

Values represent the mean of three independent experiments; CC₅₀ is the concentration that showed cytotoxic effects in 50% non-malignant HGF cells after 16 and 48 h of treatment; IC₅₀: concentration that inhibits 50% of cell growth in cancerous cells; SI=Selectivity Index: calculated as CC₅₀ HGF cells/IC₅₀ A549 cells, after 48 h of treatment. DGX: digitoxigenin monodigitoxoside; CON: convallatoxin; PAC: paclitaxel (used as positive control).

Figure S1. Chemical structures of **(A)** digitoxigenin monodigitoxoside (DGX) and **(B)** convallatoxin (CON).

Figure S2. Digitoxigenin monodigitoxoside (DGX) and convallatoxin (CON) inhibited A549 cell migration and invasion. **(A)** Fluorescent microscope images employed for the evaluation of wound healing activity by the scratch assay. Cell migration into the wound was observed in response to an artificial injury. **(B)** Effects of DGX and CON on A549 cell migration after 16 h of incubation. Data are expressed as percentages of cells in the wounded area, when compared to the control DMSO (0.5%). Data represent the mean $\pm$ SD of two independent experiments. * $p < 0.05$ (ANOVA followed by Dunnett's test) indicate statistically significant differences when compared to paclitaxel 100 nM. **(C)** Inhibitory effects of both cardenolides and paclitaxel on A549 cell invasion using Transwell® assay after 48 h of incubation. A549 cells that penetrated through the Matrigel® and went to the lower chambers were stained and photographed under a fluorescent microscope. Data represent the percentages of cells that have invaded the inserts, when compared to the control DMSO (0.5%). Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$ indicates statistically significant differences between DGX/CON and paclitaxel treatments (ANOVA followed by Dunnett's test). **(D)** Effects of DGX and CON treatments on MMP-2, MMP-9 and FAK proteins expression. A549 cells were treated with the compounds for 24 and 48 h and the samples were assayed by Western blotting. Expression levels of MMP-2, MMP-9 and p-FAK were quantified by using ImageJ software (National Institutes of Health, Bethesda, USA). Numbers below western blot signal represent quantification of proteins signals using β -actin for normalization of the sample input.

A

B

Figure S3. Comparative effects of digitoxigenin monodigitoxoside (DGX) and convallatoxin (CON) in healthy cells (human gingival fibroblasts – HGF) in order to demonstrate selectivity. **(A)** Fluorescent microscope images employed for the evaluation of wound healing activity by the scratch assay, using a confluent monolayer of HGF. Cell migration into the wound was observed in response to an artificial injury. **(B)** Effects of DGX and CON on HGF migration and proliferation using the scratch assay after 16 h of incubation. Data are expressed as percentages of cells in the wounded area when compared to the control DMSO (0.5%). Data represent the mean±SD of two independent experiments. * $p<0.05$ (ANOVA followed by Dunnett's post-test) indicate statistically significant differences when compared to PDGF positive control.

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