Biomedicine & Pharmacotherapy 97 (2018) 684–696

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Cytotoxic and cytostatic effects of digitoxigenin monodigitoxoside (DGX) in human lung cancer cells and its link to Na,K-ATPase

Naira Fernanda Zanchett Schneider[a], Lara Persich[a], Sayonarah C. Rocha[b], Ana Carolina Pacheco Ramos[b], Vanessa Faria Cortes[b], Izabella Thaís Silva[d], Jennifer Munkert[e], Rodrigo M. Pádua[d], Wolfgang Kreis[e], Alex G. Taranto[c], Leandro A. Barbosa[b], Fernão C. Braga[d], Cláudia M.O. Simões[a,*]

[a] Programa de Pós-Graduação em Farmácia, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[b] Laboratório de Bioquímica Celular, Universidade Federal de São João del Rei, Campus Centro-Oeste Dona Lindu, Divinópolis, MG, Brazil
[c] Laboratório de Química Farmacêutica Medicinal, Universidade Federal de São João del Rei, Campus Centro-Oeste Dona Lindu, Divinópolis, MG, Brazil
[d] Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[e] Department of Biology, Friedrich-Alexander Universität, Erlangen-Nürnberg, Germany

ARTICLE INFO

Keywords:
Cardenolides
Digitoxigenin monodigitoxoside
Short and long-term analyses
Non-small cell lung cancer
Cell death
Na,K-ATPase

ABSTRACT

Cardiac glycosides (CGs) are natural compounds widely used to treat several cardiac conditions and more recently have been recognized as potential antitumor agents. They are known as Na,K-ATPases ligands, which is a promising drug target in cancer. In this study, the short and long-lasting cytotoxic effects of the natural cardenolide digitoxigenin monodigitoxoside (DGX) were evaluated against two non-small cell lung cancer lines (A549 and H460 cells). It was found that DGX induced cytotoxic effects in both cells and the apoptotic effects were more pronounced on H460 cells. In long-term analysis, using the clonogenic and the cumulative population doubling (CPD) assays, DGX showed a reduction of cell survival, after 15 days without re-treatment. To better understand DGX effects in A549 cells, several assays were conducted. In cell cycle analysis, DGX caused an arrest in S and G2/M phases. This compound also increased the number of cells in subG1 phase in a concentration- and time-dependent manner. The presence of β-galactosidase positive cells, large nucleus and flattened cells indicated senescence. Additionally, DGX inhibited Na,K-ATPase activity in A549 cells, as well as in purified pig kidney and in human red blood cell membrane preparations, at nanomolar range. Moreover, results of molecular docking showed that DGX binds with high efficiency (−11.4 Kcal/mol) to the Na,K-ATPase (PDB:4HYT). Taken together, our results highlight the potent effects of DGX both in A549 and H460 cells, and disclose its link with Na,K-ATPase inhibition.

1. Introduction

Cardiac glycosides (CGs) are natural compounds with positive inotropic effects that lead to an increase of myocardial contractile force. They present a steroid skeleton with an unsaturated lactone moiety at C17. The nature of this lactone moiety characterizes two subgroups: cardenolides (five-membered α,β-unsaturated butyrolactone ring) and bufadienolides (2-pyrone ring). The main pharmacological effect of this group of compounds results from the inhibition of Na,K-ATPase leading to an increased intracellular Na^+ and Ca^{2+} ions, as well as a decreased intracellular K^+ ions. They are used to enhance cardiac contractile force in patients with congestive heart failure and arrhythmias [1].

In the last years, several studies have highlighted potent antineoplastic effects of CGs by *in vitro* studies and clinical trials, as recently reviewed by [2–4]. They can act as apoptotic, antiproliferative and antimigratory agents against a variety of cancer cells [5]. The antiproliferative effects of CGs occurs by the inhibition of several signal transduction cascades, including the inhibition of antiapoptotic proteins, such as Bcl-xL and Bcl-2, transcription factors, such as NF-kB and activator protein 1 (AP-1), and modulation of cell cycle [6,7].

Indeed, the cytotoxic effect of CGs in cancer cells have also been correlated with the inhibition of Na,K-ATPase. More recently, a new hypothesis has been raised that the sodium pump is associated with other proteins in caveolae, and modulates several downstream signaling pathways, such as elevation of intracellular Ca^{2+}, activation of Src and ERK1/2 kinase pathways, inactivation of the PI3K/Akt

* Corresponding author at: LVA, CIF, CCS, Universidade Federal de Santa Catarina (UFSC), Florianópolis, 88040-900, SC, Brazil.
E-mail address: claudia.simoes@ufsc.br (C.M.O. Simões).

http://dx.doi.org/10.1016/j.biopha.2017.10.128
Received 11 September 2017; Received in revised form 11 October 2017; Accepted 23 October 2017

pathways, production of reactive oxygen species (ROS) and mitochondria injury. These effects vary depending on the cell type as well as the chemical structures and concentrations of CGs [8,9].

Na,K-ATPase is, thus, a vital enzyme, ubiquitously present in every mammalian cell. It is a transmembrane heterodimeric protein, containing a catalytic α-subunit, a glycosylated β-subunit, responsible for the assembly of the enzyme to the plasma membrane, and a tissue specific γ-subunit member of the FXYD family [10]. CGs bind to the α-subunit and four different isoforms were identified in mammalian cells (α1, α2, α3 and α4). The expression pattern of the α-subunit-isoforms is tissue-specific and is markedly altered in cancer [11].

Several changes in the expression of Na,K-ATPase have been observed in cancer cells, including enhanced activity during the transformation to malignant cells. In addition, roles in cell survival, proliferation, adhesion and migration have been also described [12].

Several CGs have been extensively investigated, but not digitoxigenin monodigitoxoside (DGX), also known as evatromonoside. This compound showed potent effects against HSV-1 and HSV-2 viruses [13] and cytotoxic effects for several cancer cell lines in screenings conducted by Wang et al. [14] and Schneider et al. [5].

Even though previous works have addressed the cytotoxic effects of the CGs, the investigation of their distinct mechanisms of cytostasis and their effects in long-term treatments has not been explored so far. We describe herein, for the first time, the long-term effects prompted by DGX in A549 and H460 cells, more specifically its cytostatic and cytotoxic effects against the less sensitive NSCLC A549, including cell cycle modulation and the effect of DGX on Na,K-ATPase enzyme activity, the main target of CGs.

2. Material and methods

2.1. Cardenolide, chemicals and cells

Digitoxigenin monodigitoxoside (DGX) was obtained by chemical degradation of digitoxin [15]. X-gal was purchased from Sigma-Aldrich (St. Louis, MO, USA). Prior to dilution in culture medium, samples were dissolved in dimethyl sulfoxide (DMSO, Merck Millipore, Billerica, MA, USA). Caspase inhibitor (z-VAD-FMK) was also purchased from Merck. Human non-small cell lung cancer (NSCLC) A549 cells (ECACC: 86012804) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco® Carlsbad, CA, USA). H460 cells, another NSCLC (ATCC: HTB-177) were grown in RPMI 1640 medium (Gibco). To verify selectivity on non-tumoral cells, human fetal lung fibroblast-like cells (MRC-5, ECACC: 05090501) were grown in DMEM and supplemented with 1% glutamine (Cultilab, Campinas, Brazil) and 1% non-essential amino acids (Gibco). All cells lines were supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C and 5% CO_2 in a humidified atmosphere. Cell lines were maintained free of antibiotics, and were routinely screened for microorganisms including mycoplasma.

2.2. Sulforhodamine B assay

Briefly, A549, H460 and MRC-5 cells were cultured in 96-well plates (1×10^4 cells/well), and after 24 h exposed to different concentrations of DGX for 24 and 48 h. After incubation, 10% trichloroacetic acid (TCA) was added to each well to fix cells for one hour. Plates were then washed with water to remove TCA and stained with SRB for 30 min. Afterwards, the plate was washed with 1% acetic acid to remove the unbound SRB [16]. Then, 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 microplate spectrophotometer Spectra Max M2 (Molecular Devices, Sunnyvale, CA, USA).

2.3. Cell morphology evaluation

To evaluate morphological alterations, A549 and H460 cells were seeded in 12-wells plates and treated with DGX (10 and 100 nM) for 24 and 48 h. After treatment, brightness-contrast images were obtained using an inverted microscope Olympus BX-41 (Olympus Corporation, Tokyo, Japan).

2.4. Flow cytometry for apoptosis/necrosis analyses

A549 and H460 cells were treated with DGX (10 and 100 nM) for 48 h and then subjected to the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) [17]. Data were obtained with a FACS Canto II cytometer (Becton Dickinson (BD) GmbH, Heildelberg, Germany), and analyses were performed using Flowing 2.5.0 software (University of Turku, Finland). Camptothecin (5 μM) (Sigma-Aldrich) was used as a positive control for apoptosis, and a pellet of cells freezing (−80 °C)/defrosting (56 °C) was used as necrosis control. Apoptotic cells were identified by annexin V − propidium iodide double staining.

2.5. Clonogenic assay

Clonogenic assay was performed according to Franken et al. [18]. A549 and H460 cells (2.5×10^5 cells/well in 6-well plates were incubated at 37 °C for 24 h, and treated for 48 h with DGX (10 and 100 nM). Then, cells were harvested and plated at 2×10^2 cells/well in 6-well plates to evaluate the ability of the remaining cells to form colonies after 10 days of DGX treatment, when they received fresh medium supplemented with 10% FBS. Medium was replaced every three days. On day 10, colonies were fixed with paraformaldehyde, stained with 0.1% of crystal violet, photographed and counted using a stereomicroscope (Olympus, Center Valley, PA, USA).

2.6. Determination of cumulative population doublings (CPD)

A549 and H460 cells were seeded in 12-well plates at a density of 5×10^4 cells/well and exposed to DGX (10 and 100 nM) for 48 h. This treatment was followed by 15 days analysis without treatment or 30 days analysis within two re-treatments. Well plates with a minimum 20% and a maximum 80% of cells confluence were trypsinized, and population doublings (PD) were determined according to the formula $PD = [\log N(t)^*\log N(to)]/\log 2$, where $N(t)$ is the number of cells per well at time of passage, and $N(to)$ is the number of cells seeded in the previous passage. The sum of PDs was then plotted against the culture time (CPDs). CPDs counting in both cells were used to calculate four parameters: relative end CPD (RendCPD), to identify the end point analysis of proliferation; relative area under curve (rAUC), to quantify the global chronic effect of DGX treatment; relative time to cross a threshold (RTCT), to indicate the delay in cell population recovery produced by DGX treatment; and relative proliferation rate (RPR), to describe the relative regrowth velocity of the cells that survived after DGX treatment in both cell lines. All formulas are described by Silva et al. [19].

2.7. Flow cytometry for cell cycle analyses

To verify the pronounced cytostatic effects on A549 cells, they were treated with DGX for 24, 48 and 72 h, and the effects on cell cycle were evaluated by propidium iodide staining (PI − Sigma-Aldrich) according to a standard protocol [20]. Flow cytometry analyses were performed on a FACS Canto II cytometer (BD) and 20,000 events were acquired for each group. The distribution of cells in each phase of the cell cycle was determined using the Flowing 2.5.1 free software.