SHORT COMMUNICATION

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

Naira F. Z. Schneidera, Fabiana C. Gellera, Lara Persicha, Lucas L. Marosticaa, Rodrigo M. Páduab, Wolfgang Kreisc, Fernão C. Braga and Cláudia M. O. Simões

aDepartamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil; bFaculdade de Farmácia, Departamento de Produtos Farmacêuticos, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil; cDepartment of Biology, Friedrich-Alexander Universität, Erlangen-Nürnberg, Germany

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 tumour cell lines. Both compounds showed anti-proliferative effects in all tumour 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.

ARTICLE HISTORY
Received 17 March 2015
Accepted 20 May 2015

KEYWORDS
Convallatoxin; digitoxigenin monodigitoxoside; proliferation; migration; invasion; A549 cell line; lung cancer

CONTACT
Cláudia M.O. Simões claudia.simoes@ufsc.br
Supplementary material relating to this article is available online, http://dx.doi.org/10.1080/14786419.2015.1055265 together with Tables S1–S3 and Figures S1–S3.

© 2015 Taylor & Francis
1. Introduction

Natural compounds play a dominant role in the development of anticancer and anti-infectious agents, and several plant-derived compounds are currently employed in cancer therapy (Unnati et al. 2013). Cardiac glycosides belong to a class of natural products that includes cardenolides and bufadenolides, which are clinically used for the treatment of congestive heart failure and atrial arrhythmia (Rahimtoola & Tak 1996). In the last few decades, several studies have demonstrated other biological activities for cardenolides, including anticancer (Wang et al. 2012; Cerella et al. 2013) and antiviral effects (Su et al. 2008; Bertol et al. 2011). Based on these findings, extensive experiments have been conducted to determine the action mechanism of cardenolides, demonstrating the ability of these compounds to inhibit cell growth, arrest cell cycle, and induce apoptosis and/or autophagy in different cancer cells (Juncker et al. 2011; Wang et al. 2012; Cerella et al. 2013). Additionally, cardenolides such as ouabain have been reported to reduce the migration and invasive characteristics of some cancer cell lines (Pongrakhananon et al. 2013).

However, only a few studies have evaluated the migration and invasion potential of cardenolides by in vitro assays (Klausmeyer et al. 2009; Pongrakhananon et al. 2013; Yang et al. 2014). Thus, further studies are needed, to elucidate their metastatic potential. In this study, we evaluated digitoxigenin monodigitoxoside (DGX) and convallatoxin (CON) (Figure S1A and S1B, respectively) for their cytotoxic activity, and the anti-migratory and anti-invasive effects on non-small cell lung cancer (NSCLC, A549 cells).

2. Results and discussion

2.1. DGX and CON reduced cancer cells proliferation

In a preliminary screening, DGX and CON showed more effectiveness in reducing the proliferation of two cancer cell lines amongst 64 cardenolides, especially in A549 cells (Carvalho 2012). DGX, found in Digitalis lanata Ehrh. (Braga et al. 1996) was evaluated for the first time in this study. CON, isolated from Convallaria majalis L., has been reported to possess cytotoxic effects against several cancer cells (Juncker et al. 2011; Yang et al. 2014). In order to investigate the cytotoxic effects of these compounds in other cancer cells, human non-small cell lung cancer (NSCLC, A549), human rhabdomyosarcoma RD, human colon carcinoma HCT-8 and human prostate carcinoma LNCaP cell lines were selected. The anti-proliferative/cytotoxic activity of these compounds on normal human gingival fibroblasts (HGF) was also tested. Cancer cells were treated with various concentrations (160, 80, 40, 20, 10, 5, 2.5 and 1.25 nM) of DGX and CON for 48 h, and stained with SRB (Tables S1 and S2). The results revealed that these compounds and the positive control paclitaxel inhibited the proliferation of these four cell lines at nanomolar concentrations. In A549 cell line, the most sensitive cell line for both compounds, the IC_{50} values were 12.34 and 11.03 nM, respectively, after 48 h. CON was identified as a potent cardenolide in different reports, with IC_{50} values at nM concentrations, as herein observed. Its anti-proliferative effects were evaluated in colorectal cancer (HT29, HCT116 and CC20) (Felth et al. 2009), non-small cell lung cancer (NIH-H460 and A549) (Liu, Tang, et al. 2013; Yang et al. 2014), glioblastoma (U87MG), cervical cancer (HeLa), liver carcinoma (HepG2) and fibrosarcoma (HT1080) (Yang et al. 2014) human cell lines. It was also shown that CON caused a dual induction of apoptosis and autophagy in HeLa cells (Yang et al. 2014). The anti-cancer agents used as positive controls (paclitaxel, cisplatin and irinotecan) showed a high impact on A549 cell proliferation, with IC_{50} values ranging from
0.194 to 8.77 μM after 48 h of treatment (Table S1). Nevertheless, CON and DGX showed the strongest effects compared to the positive controls, with IC_{50} values at nM concentrations.

2.2. DGX- and CON-reduced migration of A549 cells

The treatment of tumour metastasis is one of the aims of cancer therapy, particularly in lung cancer, due to the high degree of metastasis, resulting in high mortality rates (Ferlay et al. 2010; Perlikos et al. 2013). This hallmarks of cancer involve complex processes; two of them, cell migration and invasion, require various coordinated cellular activities. To mimic this complex phenomenon, several assays have been developed. One of these, the scratch assay – performed in this study – is used to access directional cell migration or proliferation in vitro (Menon et al. 2009).

In order to exclude the growth inhibitory effect of DGX and CON on cell migration, A549 cells were exposed to 0, 10, and 50 nM of these compounds. The highest tested concentration of both compounds (50 nM) did not affect the growth of cancer cells for 16 h (data not shown). As shown in Figure S2A and B, DGX and CON at 10 nM reduced the migration and/or proliferation of A549 cells by 65 ± 6.26% and 63.40 ± 7.35%, respectively, after 16 h. The cardenolide percentages of inhibition were similar to that of paclitaxel at 100 nM, a drug commonly used for the chemotherapy of NSCLC, but no significant statistical differences were detected. Paclitaxel at 10 nM was not able to reduce cell migration.

2.3. DGX- and CON-reduced invasion of A549 cells

In cell invasion, tumour cells must cross the extracellular matrix (ECM) layer and penetrate the underlying stroma in order to invade the tissue and generate distant metastasis. For this approach, the invasion Transwell® assay contains the Matrigel® layer to mimic the ECM layer, thereby allowing the cell invasive matrix degradation, similarly to what occurs in vivo (Valster et al. 2005). As shown in Figure S2C, DGX and CON demonstrated anti-invasive potential. After 48 h, the highest concentration tested (50 nM) of DGX and CON were able to reduce more than 90% of cell invasion. Interestingly, CON showed similar results at the two tested concentrations, being able to reduce cell invasion by approximately 97% at 10 nM. These results are similar to that of paclitaxel, which showed 85.4 ± 8.6% of inhibition at 100 nM. At the lowest concentration, DGX showed no anti-invasive profile. A recent study revealed that CON was able to inhibit invasion in HeLa cells (Yang et al. 2014). CON also reduced vascular endothelial growth factor (VEGF) levels in human glioma cells (Klausmeyer et al. 2009) and inhibited VEGF in healthy cells (HUVEC) (Yang et al. 2014).

2.4. DGX and CON downregulated FAK, MMP-2 and MMP-9 in A549 cells

Western blotting was used to verify the expression of key regulators that play an important role in cell migration, invasion and angiogenesis. Both cardenolides decreased the levels of matrix metalloproteinases (MMP-2 and MMP-9) and p-FAK in A549 cells, especially at 50 nM after 48 h of treatment (Figure S2D). In addition, CON elicited a strong downregulation of MMP-2 expression. These findings suggest that the inhibition of cell invasion and migration by these cardenolides could be mediated by a reduction of the proteolytic action of MMPs. In addition, MMP-2 has been reported to be a more sensitive marker of progression, metastasis and survival of non-small cell lung cancer, than MMP-9 (Gupta & Massague 2006; Duffy et al. 2008). Nevertheless, p-FAK was also downregulated by CON and DGX. In line with these results, ouabain, another well-known cardiac glycoside, inhibited cancer cell migratory
behavior by suppression of FAK activation (Pongrakhananon et al. 2013) and decreased MMP-2 and MMP-9 expression (Liu, Li, et al. 2013) on lung cancer cells.

2.5. **DGX and CON induced migration in HGF cells**

Concerning the specificity of these compounds for cancer cells, their effects on HGF cells were further investigated. The SRB assay was previously performed in order to assess the concentrations of these compounds that were not cytotoxic for HGF. The IC_{50} values obtained after 16 h of treatment with DGX and CON were higher than 250 μM. After 48 h, it was possible to calculate the IC_{50} for both compounds, at a range of μM (Table S3). In this experiment, the selective indices (SI), which correspond to the ratio of CC_{50} and IC_{50} obtained with HGF and A549 cells, respectively, were also calculated for both compounds. It was found that DGX is 2,325 times more selective to A549 cells than to HGF, whereas CON was less selective than DGX to cancer cells with a SI of 442. According to the obtained IC_{50} values, the non-cytotoxic concentrations of both compounds were used for wound healing migration assay. Figure S3A and B shows that CON and DGX were able to induce cell migration in HGF at 10 and 50 nM. At the highest tested concentration (50 nM), DGX and CON induced 26 and 44% of cell migration and/or proliferation, respectively. CON showed pro-migration effects at a concentration-dependent manner (p < 0.05). Furthermore, platelet-derived growth factor (PDGF) was used as positive control, and the number of cells in the wound area increased by 55% after 16 h of incubation (Figure S3B). PDGF is characterized as a stimulating agent of cell proliferation at different concentrations, as described by Fronza et al. (2009).

3. **Conclusion**

In summary, these cardenolides showed anti-proliferative activity, decreasing migration and invasion of lung cancer cells by suppressing MMP-2, MMP-9 and p-FAK expression. Taken together, our findings suggest that DGX and CON might work as anticancer agents to decrease metastasis in lung cancer. Further in vivo studies of cancer metastasis are required, to confirm these effects and to clarify the mechanism of action, as well as to define the doses required to reduce lung cancer progression.

**Acknowledgements**

The authors would like to thank Dr Ariadne Cristiane Cabral da Cruz for providing the human gingival fibroblasts. The Brazilian authors thank CNPq (MCTI) and CAPES (MEC) for their research fellowships.

**Disclosure statement**

The authors declare that there are no actual or potential conflicts of interest in this work.

**Funding**

This work was supported by the Brazilian agency CNPq/MCTI [grant number 472544/2013-6], [grant number 490057/2011-0], Marie Curie Foundation IRSES and the European Community Marie Curie Foundation by IRSES, European Community [grant number 2011-IRSES-295251].

**References**

Bertol JW, Rigotto C, de Pádua RM, Kreis W, Barardi CR, Braga FC, Simões CM. 2011. Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of *Digitalis lanata*. Antiviral Res. 92:73–80.
Braga FC, Kreis W, Braga de Oliveira A. 1996. Isolation of cardenolides from a Brazilian cultivar of Digitalis lanata by rotation locular counter-current chromatography. J Chromatogr A. 756:287–291.

Carvalho A. 2012. Avaliação dos efeitos citotóxicos de cardenolídeos em células tumorais [Evaluation of cytotoxic effects of cardenolides in tumor cells] [dissertation]. Florianópolis (BR): Federal University of Santa Catarina. p. 126.

Cerella C, Dicato M, Diederich M. 2013. Assembling the puzzle of anti-cancer mechanisms triggered by cardiac glycosides. Mitochondrion. 13:225–234.

Duffy MJ, McGowan PM, Gallagher WM. 2008. Cancer invasion and metastasis: changing views. J Pathol. 214:283–293.

Felth J, Rickardson L, Rosén J, Wickström M, Fryknäs M, Lindskog M, Bohlin L, Gullbo J. 2009. Cytotoxic effects of cardiac glycosides in colon cancer cells, alone and in combination with standard chemotherapy drugs. J Nat Prod. 72:1969–1974.

Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 127:2893–2917.

Fronza M, Heinzmann B, Hamburger M, Laufer S, Merfort I. 2009. Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts. J Ethnopharmacol. 126:463–467.

Gupta GP, Massagué J. 2006. Cancer metastasis: building a framework. Cell. 127:679–695.

Juncker T, Cerella C, Teiten MH, Morceau F, Schumacher M, Ghelfi J, Gaascht F, Schnekenburger M, Henry E, Dicato M, Diederich M. 2011. UNBS1450, a steroid cardiac glycoside inducing apoptotic cell death in human leukemia cells. Biochem Pharmacol. 81:13–23.

Klausmeyer P, Zhou Q, Scudiero DA, Uranchimeg B, Melillo G, Cardellina JH, Shoemaker RH, Chang CJ, McColl TD. 2009. Cytotoxic and HIF-1α inhibitory compounds from Crossosoma bigelovii. J Nat Prod. 72:805–812.

Liu N, Li Y, Su S, Wang N, Wang H, Li J. 2013. Inhibition of cell migration by ouabain in the A549 human lung cancer cell line. Oncol Lett. 6:475–479.

Liu Q, Tang JS, Hu MJ, Liu J, Chen HF, Gao H, Wang GH, Li SL, Hao XJ, Zhang XS. 2013. Antiproliferative cardiac glycosides from the latex of Antiaris toxicaria. J Nat Prod. 76:1771–1780.

Menon MB, Ronkina N, Schwermann J, Kotlyarov A, Gaestel M. 2009. Fluorescence-based quantitative scratch wound healing assay demonstrating the role of MAPKAPK-2/3 in fibroblast migration. Cell Motil Cytoskeleton. 66:1041–1047.

Perlikos F, Harrington KJ, Syrigos KN. 2013. Key molecular mechanisms in lung cancer invasion and metastasis: a comprehensive review. Crit Rev Oncol Hematol. 87:1–11.

Pongtrakhananon V, Chunhacha P, Chanvorachote P. 2013. Ouabain suppresses the migratory behavior of lung cancer cells. PLoS ONE. 8:e68623.

Rahimtoola SH, Tak T. 1996. The use of digitalis in heart failure. Curr Probl Cardiol. 21:781–853.

Su CT, Hsu JT, Hsieh HR, Lin PH, Chen TC, Kao CL, Lee CN, Chang SY. 2008. Anti-HSV activity of digitoxin and its possible mechanisms. Antiviral Res. 79:62–70.

Unnati S, Ripal S, Sanjeev A, Niyati A. 2013. Novel anticancer agents from plant sources. Chin J Nat Med. 11:16–23.

Valser A, Tran NL, Nakada M, Berens ME, Chan AY, Symons M. 2005. Cell migration and invasion assays. Methods. 37:208–215.

Wang Y, Qiu Q, Shen JJ, Li DD, Jiang XJ, Si SY, Shao RG, Wang Z. 2012. Cardiac glycosides induce autophagy in human non-small cell lung cancer cells through regulation of dual signaling pathways. Int J Biochem Cell B. 44:1813–1824.

Yang SY, Kim NH, Cho YS, Lee H, Kwon HJ. 2014. Convallatoxin, a dual inducer of autophagy and apoptosis, inhibits angiogenesis in vitro and in vivo. PLoS ONE. 9:e91094.