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Evaluation of Cardiotonic Steroid Modulation of Cellular Cholesterol and Phospholipid

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Abstract

We have previously shown that 21-benzylidene digoxin (21-BD) increases the total cholesterol and phospholipid content on the membrane of HeLa cells. Lipid modulation caused by cardiotonic steroids (CTS) is still unexplored. Therefore, the aim of the present study was to evaluate the cholesterol and phospholipid modulation of the cell membrane caused by ouabain and 21-BD and the possible involvement of the caveolae on this modulation. For this, one cell line containing caveolae (HeLa) and other not containing (Caco-2) were used. The modulation of the lipid profile was evaluated by total cholesterol and phospholipids measurements, and identification of membrane phospholipids by HPTLC. The cholesterol distribution was evaluated by filipin staining. The caveolin-1 expression was evaluated by Western Blotting. Ouabain had no effect on the total membrane lipid content in both cell lines. However, 21-BD increased total membrane phospholipid content and had no effect on the membrane cholesterol content in Caco-2 cells. CTS were not able to alter the specific phospholipids content. In the filipin experiments, 21-BD provoked a remarkable redistribution of cholesterol to the perinuclear region of HeLa cells. In Caco-2 cells, it was observed only a slight increase in cholesterol, especially as intracellular vesicles. The caveolin-1 expression was not altered by any of the compounds. Our data mainly show different effects of two cardiotonic steroids. Ouabain had no effect on the lipid profile of cells, whereas 21-BD causes important changes in cholesterol and phospholipid content. Therefore, the modulation of cholesterol content in the plasma membrane of HeLa cells is not correlated with the expression of caveolin-1.

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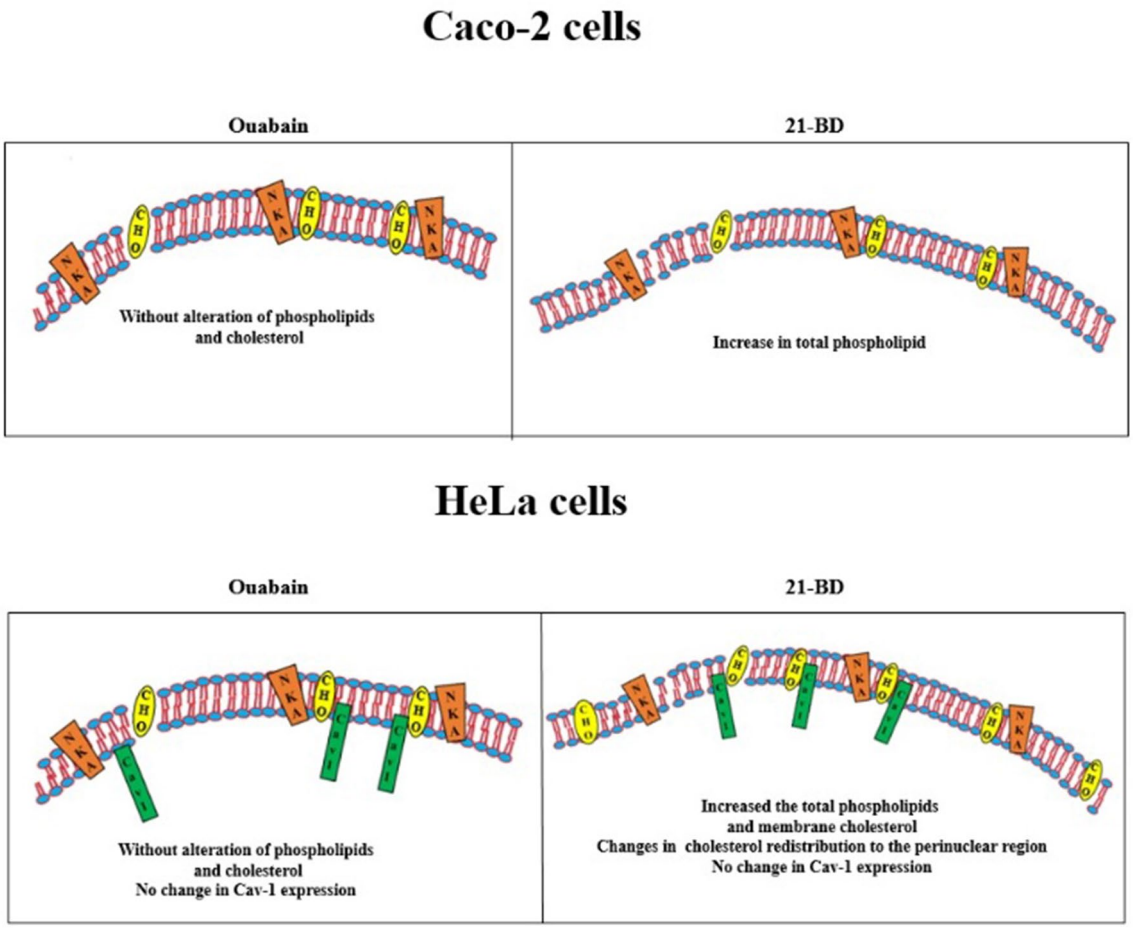
Graphic Abstract



Keywords Cardiotonic steroids · Caveolae · Na,K-ATPase · Lipid modulation

Introduction

Cell structure and function depend on the integrity of the plasma membrane and organelles that are essential in many biochemical processes. In 1972, Singer and Nicolson developed a model to characterize biological membranes in a unifying manner, since the knowledge about the plasma membrane composition and organization was very limited. This model, also known as the fluid mosaic model, states that membranes are asymmetric and composed of a phospholipid bilayer with several protein molecules floating around and embedded within it (Singer and Nicolson 1972).

Subsequently, a model was postulated demonstrating the existence of lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids. Caveolin-1 (Cav-1) is present in some of these microdomains, which results in the formation of membrane invaginations producing specific structures called caveolae (Simons and Ikonen 1997; Simons and van Meer 1988). Caveolin-1 is essential for the formation of caveolae in cells since the loss of this important protein results in cells with no production of this structure. Moreover, exogenous expression of Cav-1 in cells with apparent no expression of this protein, and consequently, absence of caveolae structures, results in caveolae formation (Drab et al. 2001; Fra et al. 1995). Caveolae regions of the plasma membrane have specialized functions in cells, such as signal transduction due to the interaction of Cav-1 with cellular signaling proteins (Couet et al. 1997; Okamoto et al. 1998). In addition, Cav-1 takes part on the transport of cholesterol to the membrane, and its interaction with cholesterol is necessary to form and stabilize the caveolae structure (Murata et al. 1995; Parton and del Pozo 2013).

Lipid composition of the cell membrane is asymmetric and heterogeneous. Usually, the inner monolayer contains a higher concentration of phosphatidylserine (PS) and phosphatidylethanolamine (PE), whereas the outer monolayer is

enriched in phosphatidylcholine (PC) and sphingomyelin (SM), which allows lipophilic or electrostatic interactions among different phospholipids permanently interchanging their partners through lateral diffusion along the cell membrane. Alterations in the distribution of phospholipids from the lipid bilayer may signal several cell processes, such as aggregation, adhesion, and apoptosis (Engelman 2005; Marquardt et al. 2015; Nicolson 2014).

Cardiotonic steroids (CTS) are well-known compounds used in the treatment of congestive heart diseases for centuries. The cellular target for CTS is the membrane enzyme Na,K-ATPase, responsible for maintaining the cellular osmotic gradient and controlling the intracellular concentrations of sodium and potassium (Stucky and Goldberger 2015; Thomas et al. 1990). CTS are able to bind to the α-subunit of the Na,K-ATPase causing its inhibition and leading to an increase of intracellular Na^+, and consequently, an increase of intracellular Ca^{2+}. This effect promotes an increase of the cardiac contraction force In addition, CTS interaction with the Na,K-ATPase may trigger intracellular signaling pathways that are involved with the regulation of gene expression, protein synthesis, and cell proliferation (Chen et al. 2006; Xie and Askari 2002).

21-Benzylidene digoxin (21-BD), a semi-synthetic CTS derivative of digoxin, consists of an additional styrene group on the lactone ring, specifically at C21 position. This change provides different characteristics compared to other CTS, probably because the additional aromatic group might promote a steric hindrance at the Na,K-ATPase catalytic site. The Na,K-ATPase demonstrates a lower affinity for 21-BD, compared to digoxin and ouabain, and its activity when isolated from cell membrane preparations is not affected by 21-BD. Interestingly, cells treated with 10 μM 21-BD have an increase of Na,K-ATPase activity. Moreover, 21-BD induces apoptosis of cancer cells, and increases the sealing degree of tight junctions (Rocha et al. 2014). More recently, it has been demonstrated in HeLa cells that 21-BD decreases cell proliferation by reducing epidermal growth factor receptor (EGFR) and extracellularly regulated kinase (ERK) phosphorylation and promoted apoptosis by activating intrinsic and extrinsic pathways (Pessôa et al. 2018). Thus, the biological effects of 21-BD are different from those observed for classical CTS, such as ouabain and digoxin, thus being an interesting compound to study the diversity of biological effects modulated by CTS.

There are few studies in the literature concerning the effect of CTS on modulation of cellular lipids. CTS are able to modify the lipid metabolism of cells (Campia et al. 2009, 2012), altering the cholesterol and phospholipid content (Garcia et al. 2015, 2018, 2019; Silva et al. 2017), as well as the plasma membrane fluidity (Manna et al. 2006).

We have demonstrated that HeLa cells treated with 21-BD undergo lipid alterations. An increase in the total phospholipid and cholesterol content of the plasma membrane was observed after treatment of the cells with 50 μM of 21-BD. This treatment also caused a decrease in the Na,K-ATPase activity, possibly due to modulations of the lipid microenvironment around the enzyme, since Na,K-ATPase has low affinity for 21-BD and no changes in the enzyme expression have been observed. Lipid modulation could lead to alterations in the membrane fluidity resulting in changes of the enzyme conformation that could inhibit the catalytic activity of Na,K-ATPase by exposing its binding site to 21-BD (Silva et al. 2017).

It has been described that caveolae could be important for the modulation of CTS in activating signaling pathways (de Souza et al. 2014; Liang et al. 2007; Liu et al. 2011). Although several reports have shown the association of the Na,K-ATPase with the caveolae for signaling events, there are still some debate if the presence of caveolae is crucial for the Na,K-ATPase effects. We have previously demonstrated that ouabain treatment in Caco-2 cells was able to activate ERK1/2 signaling pathway (de Souza et al. 2014). Since caveolae is a cellular microdomain of the plasma membrane enriched in cholesterol, it is of great importance to the maintenance of lipid homeostasis in the cell. Moreover, Cav-1 is able to regulate the Na,K-ATPase endocytosis, which can result in changes of its activity and signaling properties (Chen et al. 2009). Therefore, the expression pattern of Cav-1 can modulate several cellular responses, such as cell proliferation (Quintas et al. 2010), migration (Grande-Garcia et al. 2007; Nunez-Wehinger et al. 2014), apoptosis and cell survival (Gargalovic and Dory 2003; Han et al. 2015; Torres et al. 2006).

Through all those important effects, we can assume that the presence of caveolae could be crucial for the cellular effects of CTS, especially concerning the regulation of cholesterol and phospholipids. The objective of this study was to evaluate the effect of 21-BD and ouabain in the lipid modulation and to analyze if caveolae is involved in the CTS modulation of the lipid profile from HeLa (a caveolae-containing cell line) (Hirama et al. 2017) and Caco-2 (cells with no caveolae) cell lines (Breuza et al. 2002; Vogel et al. 1998).

Methods

Cell Culture

HeLa (cervix adenocarcinoma—ATCC CCL-2) and Caco-2 (colorectal adenocarcinoma—ATCC HTB-37) cancer cells were kindly provided by Fundação Ezequiel Dias (FUNED) from Belo Horizonte, MG, Brazil. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. DMEM cell culture medium was used with fetal bovine serum (FBS) 10% and penicillin/streptomycin 0.1%. Medium was changed

every 48 h and cells were rinsed with sterile phosphate buffered saline (PBS—[pH 7.4], NaCl 137 mM, KCl 2.7 mM, and PO_4^{3-} 10 mM). For each experiment, cells were seeded in a density specified in each section.

Treatment

Ouabain was obtained from Sigma-Aldrich and 21-benzylidene digoxin (21-BD) was synthesized by the Laboratory of Organic Synthesis and Nanostructures of the Federal University of São João del-Rei, Minas Gerais, Brazil (Rocha et al. 2014). After 80% of confluence, cells were treated with the compounds for 48 h. Stock solutions were prepared by dilution of the compounds in DMSO and working concentrations were obtained by dilution in DMEM on the day of each experiment. Maximum concentration of DMSO on cells was 1%.

Cell Viability Assay

MTT assay was used to evaluate the cell viability. Viable cells can metabolize a yellow tetrazolium salt into a water-insoluble purple formazan crystal. The cytotoxic effect of CTS on the cells was observed to determine specific concentrations for the next assays. HeLa and Caco-2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and after 24 h they were treated with increasing concentrations (2 nM to 500 µM) of ouabain or 21-BD. After 48 h of treatment, the culture medium was discarded and 100 µL of MTT salt (0.5 mg/mL) was added to each well. Formazan crystals formed after 3 h were diluted with 50 µL of DMSO for 15 min. Finally, a microplate reader Biotech Instruments, Inc. Winooski, VT, USA was used to record the absorbance at 550 nm.

Plasma Membrane Preparation

An amount of 2.25×10^6 cells were cultured in a 75 cm^2 culture flask up to 80% confluence. Then, cells were treated for 48 h with ouabain (10 nM or 100 nM) or 21-BD (5 µM or 50 µM). Afterward, cells were rinsed with PBS and harvested in 3 mL of preparation buffer (Tris–HCl 6 mM [pH 6.8], imidazole 20 mM, sucrose 250 mM, sodium dodecyl sulfate 0.01%, EDTA 3 mM, and protease inhibitor cocktail 1:100). Samples were submitted to a Potter tissue homogenizer for 20 times, and subsequently, centrifuged at 10,000×*g* for 20 min. The pellet was discarded, and the supernatant was centrifuged at 70,000×*g* for 1 h using a WTi 45 ultracentrifuge rotor. The pellet was suspended in 300 µL of preparation buffer. Samples were stored at − 20 °C.

Protein Measurement

Protein measurement was evaluated using the Bradford method (Bradford 1976) in 96-well plates. Samples were diluted in ultrapure water and an aliquot of 40 µL/well was used. Afterward, 200 µL of Bradford reagent was added, and 15 min later a microplate reader BioTek Instruments, Inc. Winooski, VT, USA was used to record the absorbance at 595 nm. Albumin was used to create a standard curve.

Lipid Extraction

Membrane fractions from HeLa and Caco-2 cell lines were submitted to a solvent-based extraction using an adaption of the Folch method (Folch et al. 1957). After protein measurement, samples were evenly diluted to the same protein concentration. Five milliliters of chloroform/methanol (2:1) were added to the samples and they were kept under agitation for 1 h at room temperature. Afterward, 1 mL of NaCl 0.9% was added, samples were mixed using a vortex, and centrifuged at 670×*g* for 20 min. To improve phase separation, samples were kept resting for 30 min at room temperature. The organic phase was separated from the water-soluble phase, followed by the addition of methanol/water (1:1) (v/v) to the organic phase and chloroform (v/v) to the water-soluble phase. Samples were mixed again in a vortex and kept at 4 °C overnight. The water-soluble phase was discarded, and the organic phase was concentrated in rotary evaporator, followed by suspension in 300 µL of chloroform. Lipid extracts were stored at − 20 °C.

Cholesterol Measurement

Cholesterol content was determined using the Higgins method (1987) based on cholesterol complexation with ferric chloride ($FeCl_3 \cdot 6H_2O$) (Higgins 1987). Forty microliters of lipid extract were dried under a nitrogen stream, followed by addition of 750 µL of acetic acid and 500 µL of reagent B composed of reagent A (2.5 g of $FeCl_3 \cdot 6H_2O$ in 100 mL of orthophosphoric acid 85%) in 46 mL of sulfuric acid. Samples were mixed in a vortex and kept at room temperature for 10 min. Finally, the absorbance was recorded in a spectrophotometer at 550 nm. The cholesterol content was calculated based on a cholesterol standard curve.

Phospholipid Measurement

Phospholipid content was determined using the Chen method (Chen et al. 1956). Forty microliters of lipid extract were dried under a nitrogen stream, followed by digestion with 500 µL of nitric acid 65% (v/v) in an incubator at 120 °C. Then, 500 µL of distilled water and 1 mL of Chen reagent were added to the samples. Afterward,

samples were kept in a water bath at 45 °C for 20 min, followed by the spectrophotometry reading at 820 nm. A standard curve was created using sodium dihydrogen phosphate for calculating phospholipid content. Chen reagent was prepared by adding ascorbic acid to the solution B (1:6). Solution B was prepared diluting 1.25 g of ammonium molybdate in 30 mL of ultrapure water, followed by addition of 7.3 mL of sulfuric acid, and finally the volume was completed to 300 mL with ultrapure water.

Identification of Membrane Phospholipids

After the solvent-based lipid extraction from plasma membrane, samples were dried under a nitrogen stream, and suspended to 20 µL of chloroform/methanol (1:1). Phospholipid identification was performed using a one-dimensional high performance thin-layer chromatography (HPTLC) in silica gel 60 plates. Samples and phospholipid standards were applied to the plates at 1 cm from the margin (origin) and 0.5 cm from each other. The solvent system was composed of acetone:methanol:acetic acid:chloroform:water (15:13:12:40:8) and the plates were kept in a chamber for approximately 30 to 40 min (Ruiz and Ochoa 1997). The run was performed up to 1 cm of the superior margin (ending). The plates were developed using the Charring reagent ($CuSO_4$ 10% in H_3PO_4 8%) for 10 min at 200 °C. Then, plates were digitalized in a scanner and submitted to a densitometry analysis using the software Image Master Total Lab version 1.11 (Amersham Pharmacia Biotech). Phospholipids were analyzed according to the migration pattern of their corresponding standards (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, sphingomyelin).

Filipin Staining

Cholesterol localization was evaluated by filipin staining, a cholesterol probe (Carozzi et al. 2000). An amount of 2×10^5 cells was cultured in coverslips up to 80% confluence, followed by treatment with ouabain 10 nM or 21-BD 50 µM. Afterward, cells were fixed with 4% paraformaldehyde in PBS—Ca^{2+}/Mg^{2+} (Ca^{2+} 0.1 mM and Mg^{2+} 1 mM) for 30 min at room temperature and the aldehyde groups quenched with NH_4Cl 50 mM in PBS for 10 min. Then, cells were permeabilized with saponin 0.1% in PBS for 10 min and incubated in the dark with the filipin probe 0.05 mg/mL at room temperature for 1 h. Finally, coverslips were mounted onto slides, rinsed with PBS to remove filipin probe excess, and analyzed using a fluorescence microscope (Axio Vert. A1—Zen Imaging Software—ZEISS, Oberkochen, Germany).

Caveolin-1 Expression

Samples of membrane preparation, total extract, and cell fractionation were diluted in sample buffer (120 mM Tris–HCl [pH 6.8], 0.02% Bromophenol Blue, 10% 2-Mercaptoethanol, 20% Glycerol, and SDS 4%) for the same protein concentration. Subsequently, the samples were applied and separated by polyacrylamide gel electrophoresis. After running the samples, the proteins were transferred from the polyacrylamide gel to the nitrocellulose membrane in transfer buffer. The transfer efficiency was evaluated by staining the nitrocellulose membrane with Ponceau-Xylidine red solution. To block unspecific binding of the antibodies used, the membrane was blocked for 1 h with 5% BSA diluted in T-TBS (Composition of T-TBS-100 mM Tris-Base, 0.9% NaCl, and 0.1% Tween). Caveolin-1 was detected with primary anti-caveolin-1 antibody (ab 2910, Abcam) diluted in T-TBS (1:1000), and tubulin (T5168, Sigma Aldrich) diluted in T-TBS (1:3000). Then the membrane was incubated with secondary antibody and developed by chemiluminescence method. The bands corresponding to each sample were quantified by densitometry using the program Image J 1.47.

Statistical Analysis

One-way ANOVA followed by Tukey's post hoc test was used to compare the sample means. The significance was set to $p < 0.05$. All analyses and graphs were performed using the software GraphPad Prism 5.

Results

Cell Viability After Ouabain and 21-BD Treatment

In previous studies, we have determined the IC_{50} of 21-BD for HeLa cells as 50 µM (Rocha et al. 2014). To define the concentrations to be used in the next experiments, we performed a cell viability assay after treatment of HeLa cells with increasing concentrations of ouabain, and after treatment of Caco-2 cells with increasing concentrations of ouabain or 21-BD. Ouabain showed a higher cytotoxicity on both cells when compared to 21-BD (Fig. 1), demonstrating a lower IC_{50} (50 nM in HeLa cells and 227 nM in the Caco-2 cells). For Caco-2 cells 21-BD was cytotoxic only with 500 µM.

Based on these results and considering that most studies involving signaling triggered by binding of CTS to Na,K-ATPase use concentrations of 10 and 100 nM ouabain, we have defined to use these concentrations in this study. Our previous studies, in different cell lines, have demonstrate that the significant effects caused by 21-BD occur at a concentration of 50 µM, even though each cell line has a different IC_{50}

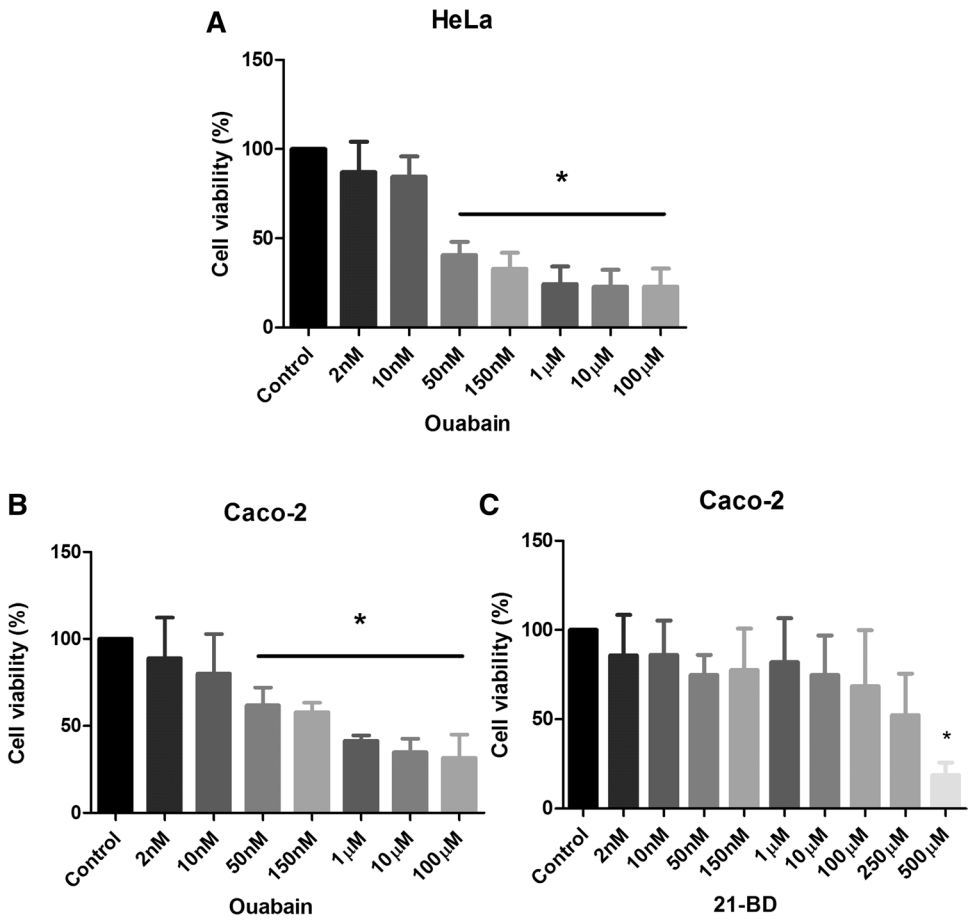


Fig. 1 HeLa and Caco-2 cell viability (%). HeLa (**A**) and Caco-2 (**B** and **C**) cells were treated for 48 h with increasing concentrations of ouabain or 21-BD. $*p < 0.05$ $n = 3$

for 21-BD (Rocha et al. 2014; Silva et al. 2017). Therefore, we decided to continue the work using 50 μM of 21-BD for both cells.

Evaluation of Lipid Modulation on the Plasma Membrane

In vitro and in vivo experiments have shown that CTS may alter the lipid composition of plasma membrane (Garcia et al. 2015, 2018, 2019; Silva et al. 2017). We have demonstrated that treatment of HeLa cells with 21-BD caused a significant increase in the total phospholipid and cholesterol content of plasma membrane (Silva et al. 2017). For this reason, we investigated here whether treatment with ouabain or 21-BD for 48 h would have the same effect in Caco-2 cells. No significant alterations were observed in the total content of phospholipids and cholesterol in plasma membrane after treatment with ouabain (Fig. 2A and B).

On the other hand, 21-BD (5 μM) treatment showed a significant increase in total phospholipid content in Caco-2 cell membrane compared to control group. The membrane cholesterol content was not altered after treatment with 21-BD (Fig. 2C and D). Moreover, ouabain treatment on HeLa cells did not cause changes in the lipid profile of plasma membrane (Fig. 3).

Thus, our data show different effects for two CTS on the membrane lipid profile of human cells. Only 21-BD demonstrated significant changes in total cellular lipid content of the plasma membrane. Interestingly, the cholesterol content was altered only in the caveolae-containing HeLa cell line.

Even though ouabain did not provoke an alteration in total membrane phospholipid, changes of specific phospholipids could occur. Therefore, considering changes in phospholipids caused by the treatment with 21-BD on both cell lines, we assessed the contents of specific phospholipids, such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin (Figs. 4 and 5). We did not identify any significant differences in plasma membrane phospholipids from both Caco-2 and HeLa cell lines after treatment with CTS.

Cholesterol Distribution

One important effect to consider is whether an increment or redistribution of cholesterol would induce caveolae formation and, therefore, a stronger signaling. We

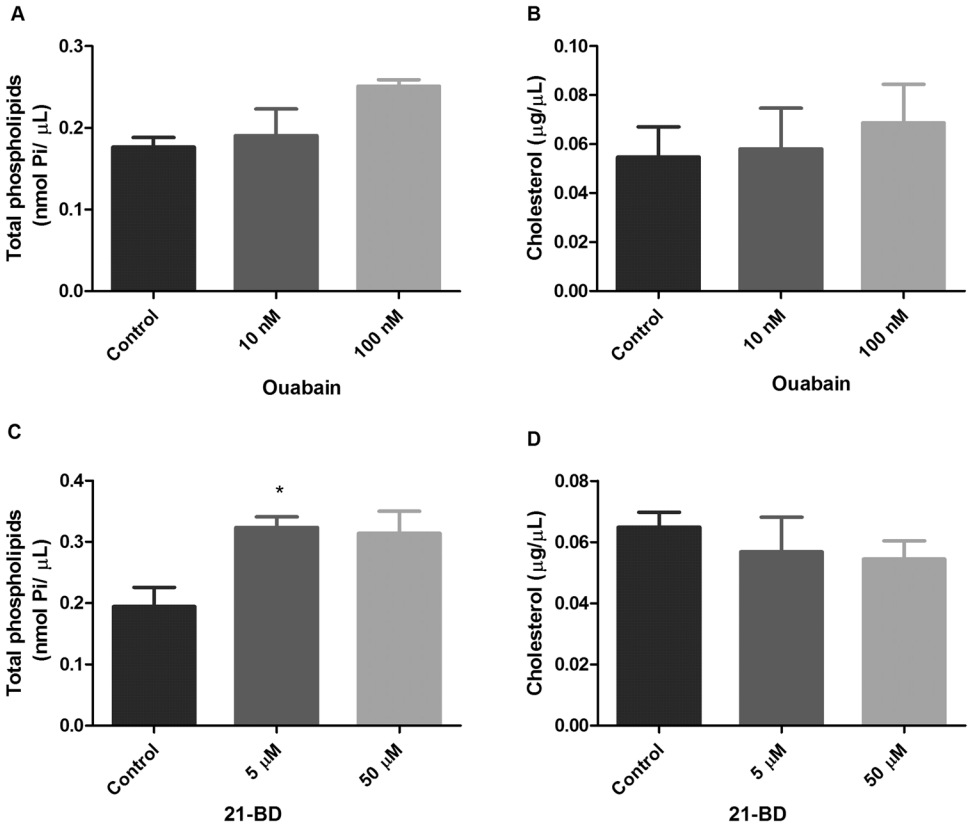


Fig. 2 Total lipid content of the cell membrane from Caco-2 cells. Total phospholipid (**a**) and cholesterol content (**b**) after treatment with different concentrations of ouabain for 48 h. Total phospholipid (**c**) and cholesterol content (**d**) after treatment with different concentrations of 21-BD for 48 h. *$p < 0.05$ $n = 3$

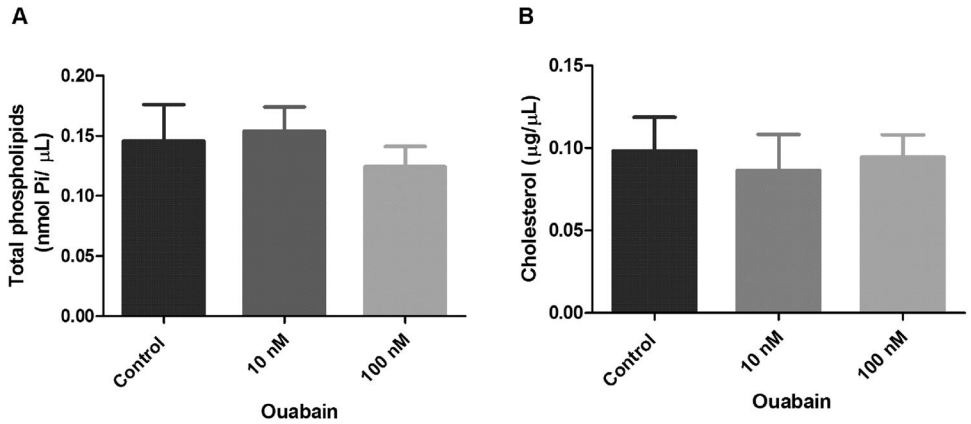


Fig. 3 Total lipid content of the cell membrane from HeLa cells. Total phospholipid (**A**) and cholesterol content (**B**) after treatment with different concentrations of ouabain for 48 h. $p > 0.05$ $n = 3$

investigated the cellular distribution of cholesterol using filipin, a fluorescent antimycotic that binds to membrane domains rich in cholesterol, in HeLa and Caco-2 cells, after treatment with ouabain or 21-BD for 48 h. In non-treated cells we observed a high fluorescent signal on the plasma membrane, reflecting the normal subcellular localization of cholesterol. Treatment with ouabain 10 nM affected neither the intensity nor the distribution of cholesterol in both cell lines. On the other hand, treatment with 50 µM of 21-BD on HeLa cells markedly increased the fluorescence intensity in the perinuclear region. In Caco-2 cells, fluorescence was also detected in the intracellular vesicles displaying a characteristic ‘‘chicken fence’’-like pattern, while fluorescence on the plasma membrane was similar to that in non-treated cells (Fig. 6).

Caveolin-1 Expression

21-BD increases the plasma membrane cholesterol of the caveolae-containing HeLa cells. Therefore, Cav-1 may be involved in regulating metabolism and cholesterol trafficking

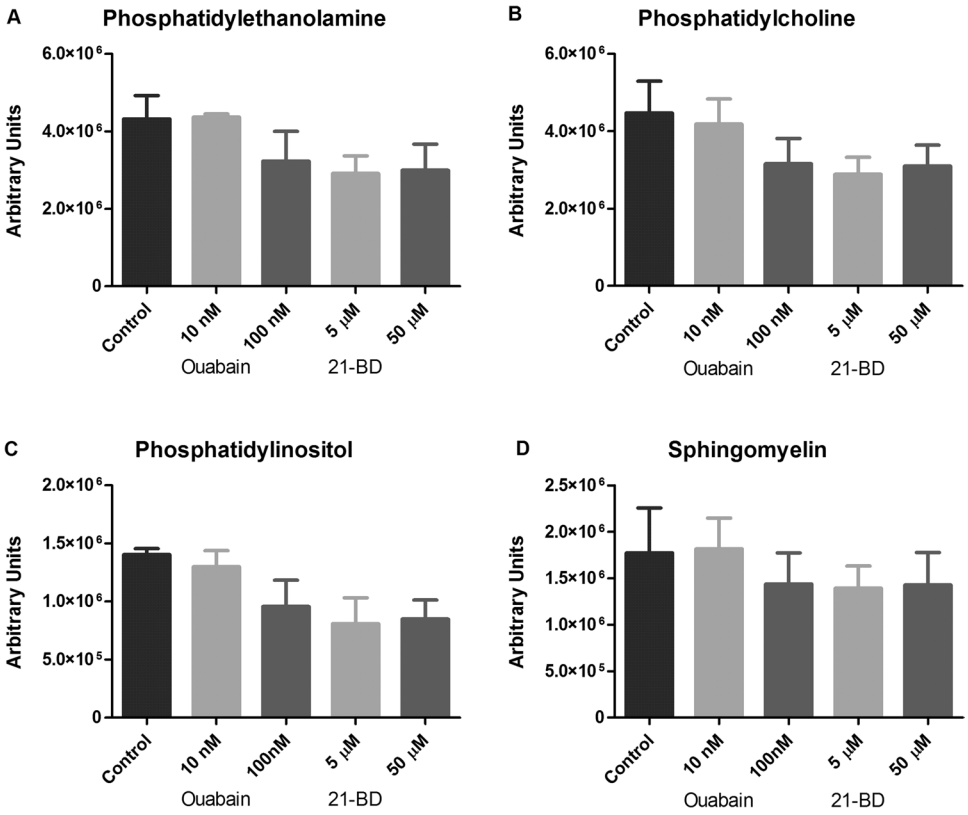


Fig. 4 Specific phospholipid composition of cell membrane from Caco-2 cells treated with different concentrations of ouabain or 21-BD for 48 h. Membrane fractions were submitted to a lipid extraction and analyzed using HPTLC. Phosphatidylethanolamine (**A**), phosphatidylcholine (**B**), phosphatidylinositol (**C**), sphingomyelin (**D**). $p > 0.05$ $n = 3$

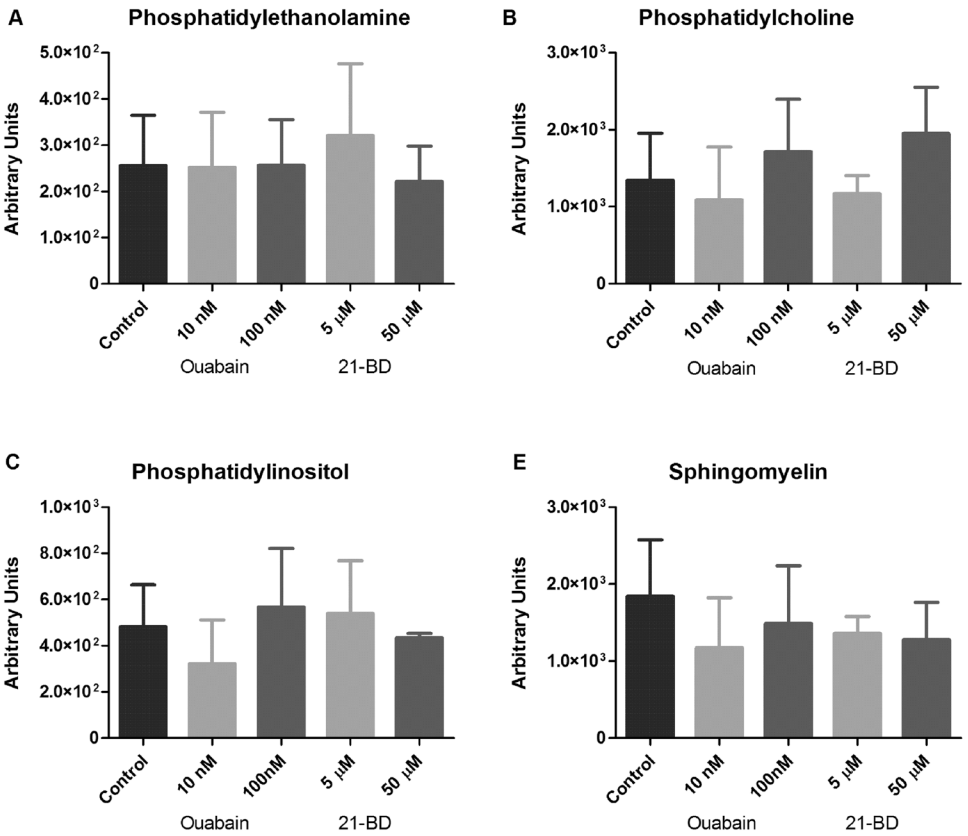


Fig. 5 Specific phospholipid composition of cell membrane from HeLa cells treated with different concentrations of ouabain or 21-BD for 48 h. Membrane fractions were submitted to a lipid extraction and analyzed using HPTLC. Phosphatidylethanolamine (**A**), phosphatidylcholine (**B**), phosphatidylinositol (**C**), sphingomyelin (**D**). $p > 0.05$ $n = 3$

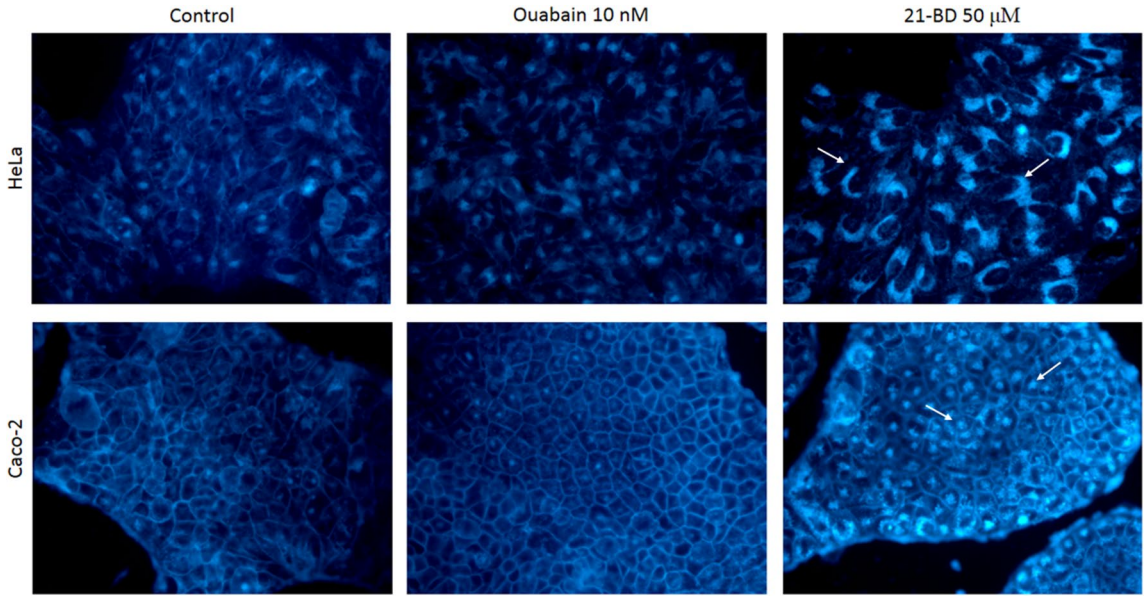


Fig. 6 CTS effect on cholesterol distribution. HeLa and Caco-2 cells were treated with ouabain 10 nM or 21-BD 50 μM for 48 h. Images were obtained at ×40 magnification by fluorescence microscopy after incubation with filipin. Arrows point to intracellular filipin signals. Images were edited using the software Image J 1.51 k. $n=3$

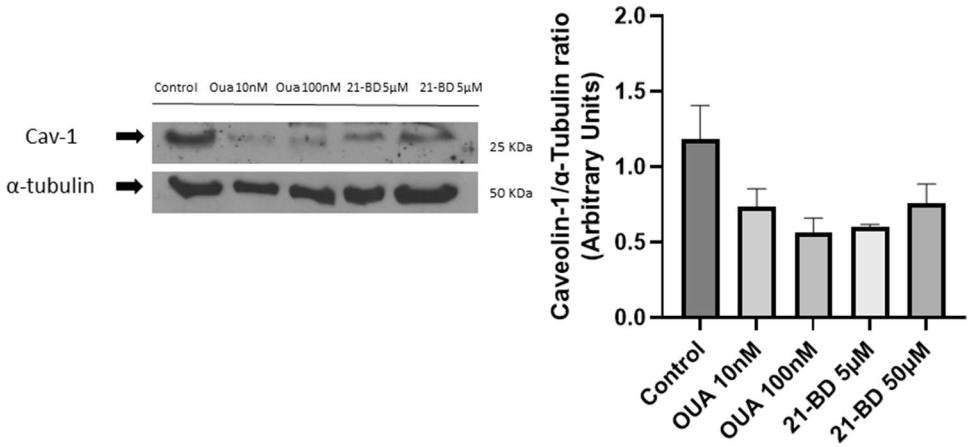


Fig. 7 CTS do not alter the expression of caveolin-1. The analysis of the expression of caveolin-1 was performed in total cell extract after treatment with ouabain and 21-BD for 48 h. $p>0.05$ $n=3$

to the plasma membrane and caveolae in these cells. Thus, we evaluated the expression of this protein in HeLa cells treated with 21-BD (Fig. 7). Our data show that neither 21-BD nor ouabain alter the expression of Cav-1. It is important to note that this data were obtained through the analysis of the total extract of these cells and not membrane preparations.

Discussion

Changes in the composition of phospholipids and cholesterol can lead to alterations in plasma membrane fluidity and may impair crucial cellular functions. There are few studies about the lipid modulation caused by CTS. Oleandrin has been shown to induce changes in membrane fluidity of different cell lines (Manna et al. 2006; Raghavendra et al. 2007). In vivo studies with male Wistar rats have demonstrated that ouabain was able to cause an increase in the total phospholipid content from the hippocampus (Garcia et al. 2015). Interestingly, the ouabain effect appears to be different depending on the region of the brain, since it caused a decrease in the levels of total membrane phospholipids in the cerebellum (Garcia et al. 2018). In these neuronal tissues, no changes in membrane cholesterol levels were found (Garcia et al. 2015, 2018). In a previous study by our research group, we have demonstrated that 21-BD significantly increased the total phospholipids and membrane cholesterol contents

in HeLa cells. As a consequence of this modulation, 21-BD decreased the Na,K-ATPase activity most likely due to alterations of the membrane fluidity of HeLa cells (Silva et al. 2017).

The present data demonstrated that ouabain treatment had no effect on the phospholipid content of plasma membrane from HeLa and Caco-2 cells. However, 21-BD caused a significant increase in total phospholipid content of Caco-2 cells (Figs. 2 and 3) and the same effect has also been previously demonstrated in HeLa cells (Silva et al. 2017).

We identified the main classes of plasma membrane phospholipids in HeLa and Caco-2 cells, such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin. Surprisingly, we did not find any significant changes in the content of these specific phospholipids (Figs. 4 and 5). Reports in the literature have demonstrated that CTS can alter the content of specific phospholipids. For instance, an ouabain treatment caused alterations of the phosphatidylinositol and sphingomyelin content in prostate cancer cells (PC-3) (Gasper et al. 2011). In vivo experiments have demonstrated that ouabain increased the turnover of phosphatidic acid in the brain from guinea pigs (Yoshida et al. 1961) and rabbits (Nicholls et al. 1962). Moreover, ouabain was able to increase the contents of phosphatidylinositol, phosphatidylethanolamine, and phosphatidylcholine in rabbit cerebral cortex slices (Nicholls et al. 1962). Another study has described that an ouabain injection increased the content of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol in the hippocampus of Wistar rats (Garcia et al. 2019).

In our study, the treatment with ouabain caused no changes in the total or specific phospholipid contents. On the other hand, 21-BD caused an increase of total membrane phospholipid content of Caco-2 cells in the same extent as reported in our previous work for HeLa cells (Silva et al. 2017). We expected to find differences in the content of specific phospholipids after treatment with 21-BD; however, no significant alterations were observed. In addition to the phospholipids that we have analyzed, there are other subtypes present in the cell membrane, such as phosphatidylserine, lysophosphatidylcholine, phosphatidylglycerol, and phosphatidic acid that are important for cellular functions (Zegarlinska et al. 2018). We believe that the high phospholipid content induced by 21-BD described in the present study could be the effect of those other phospholipids. For example, 21-BD treatment could be causing an increase of activity of enzymes involved in the hydrolysis of membrane phospholipids, such as phospholipase D and lysophospholipases, leading to an increase of lysophospholipid levels that we were unable to identify in this study.

Ouabain is the most used CTS in kinetic studies and cell signaling involving the Na,K-ATPase. Moreover, it has been described that Na,K-ATPase function depends on "annular lipids", and also, the presence of sites of interaction between the membrane lipids and the enzyme (Contreras et al. 2011; Cornelius 2001; Cornelius et al. 2003). There are specific binding sites within the enzyme able to interact with phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol, all of them required to maintain the enzyme stability, and to stimulate and inhibit its catalytic activity (Cornelius et al. 2015; Habeck et al. 2015, 2017). Thus, alterations in the lipid environment of the enzyme could modify its function (Garcia et al. 2015, 2018, 2019; Silva et al. 2017). Therefore, ouabain at the concentrations used in this study does not cause lipid changes in the plasma membrane that could impair kinetic and signaling studies.

By using a cholesterol probe (filipin), we demonstrated here that ouabain does not alter the cholesterol composition of the plasma membrane of HeLa and Caco-2 cells. However, in both cell lines treated with 21-BD, we could observe changes in cholesterol distribution compared to non-treated cells (Fig. 6). In Caco-2 cells, intracellular vesicles containing cholesterol were observed, whereas in HeLa cells, an increase of the cholesterol in the perinuclear region could be observed, and perhaps in the total cellular cholesterol content. However, in both of the cells, the cholesterol content of the plasma membrane seems to be similar to that in non-treated cells.

The differences in the effect of ouabain and 21-BD in HeLa and Caco-2 cells, in relation to the changes caused in the content of cholesterol and phospholipids, are probably due to differences in the interaction of the molecules with the Na,K-ATPase. In molecular docking studies it was shown that 21-BD interacts at sites other than ouabain in the α1 subunit of Na,K-ATPase. The aromatic ring attached to the lactone ring in 21-BD helps the molecule to interact with a hydrophobic pocket formed by Ala330, Glu786, Ph790, Leu800, Ile807. The lactone ring of ouabain interacts with Val329 and Gly803 by electrostatic interactions. Hydrogen bonds are formed between C12 of the molecule and residue Thr804, while ouabain interacts through hydrogen bonds between C11α, C19α, and C14β with residues Arg118, Asp128, and Thr804, respectively. The presence of 2 extra distal sugar units in the molecule causes it to establish new polar interactions with Asp892 and Arg893, as well as hydrogen bonds with Trp984. Thus, all these different features observed at the molecular level about the interaction of 21-BD with Na,K-ATPase, compared to ouabain, could be the reason why the effects observed with 21-BD are different from those observed with ouabain. Furthermore, these differences are evidenced by the absence of any modulatory effect of 21-BD, at nanomolar concentrations, on the Na,K-ATPase activity (Pessôa et al. 2018).

In HeLa cells, an important increase in fluorescence that would correspond to an increase in intracellular cholesterol

in the perinuclear region was observed. This finding confirms the increase of total cholesterol in cell lysates and the increase of the plasma membrane cholesterol in plasma membrane fractions, demonstrated previously by us in HeLa cells (Silva et al. 2017). These cells present caveolae, in contrast with Caco-2 cells that do not. Therefore, it is important to address whether caveolae could be involved in the CTS modulation of the lipid environment of the cells.

Caveolin-1 plays an important role in the cholesterol homeostasis, being involved in the trafficking of newly synthesized cholesterol to the membrane (Smart et al. 1996) and modulating the activity of other proteins that are involved in the regulation of intracellular cholesterol. Caveolin-1 binds directly to cholesterol and transports it to the membrane. This interaction, of caveolin-1 with cholesterol, is important to form the caveolae microdomains (Cai et al. 2008).

In fact, the formation and maintenance of caveolae depends on the membrane and intracellular cholesterol levels. The plasma membrane cholesterol levels can regulate Cav-1 expression, where the cholesterol depletion can disrupt the formation of caveolae and, consequently, reduce the number of these structures and the expression of caveolin-1 in cells (Hailstones et al. 1998). In addition, the inhibition of cholesterol biosynthesis leads to a marked reduction in cholesterol content causing disruption of the lipid rafts, with redistribution of caveolin-1 (Sánchez-Wandelmer et al. 2009). On the other hand, intracellular accumulation of cholesterol promotes Cav-1 expression and Cav-1 directs the trafficking of cholesterol to caveolae, in response to increased intracellular cholesterol uptake or synthesis (Frank et al. 2006). Therefore, Cav-1 expression can be regulated by cholesterol and this regulation is reciprocal (Fu et al. 2004; Hailstones et al. 1998). It has been observed an increase in cholesterol in cells overexpressed with exogenous Cav-1, whereas Cav-1 knockout mouse embryonic fibroblasts (MEFs) and mouse peritoneal macrophages (MPMs) was associated with reduced free cholesterol synthesis but increased acyl-CoA:cholesterol acyl-transferase (ACAT) activity (Frank et al. 2006). Thus, differences in the caveolar content in the two cells analyzed in this work could be one of the reasons why these cells present different responses in relation to cholesterol content after treatment with 21-BD.

Another relevant point is that the interaction between Na,K-ATPase and Cav-1 is important for the maintenance of the membrane cholesterol homeostasis (Cai et al. 2008; Chen et al. 2009, 2011). Downregulation of Na,K-ATPase redistributes the cholesterol from the plasma membrane to the cytosol, and decreased expression of caveolin-1 from LLC-PK1 cells also alters the cholesterol metabolism in mice $\alpha1^{+/-}$ (Chen et al. 2009). Cholesterol depletion stimulates the endocytosis and degradation of α1 through a Src-dependent pathway, and consequently decreases the expression of α1 Na,K-ATPase (Chen et al. 2011). This data shows that this regulation between α1 expression and the cholesterol content is mutual. The interaction of Na,K-ATPase and Src is important for the regulation of the trafficking of Cav-1 and cholesterol to the cell membrane. Cholesterol specifically regulates the expression of Na,K-ATPase α1 subunit and the complex Na,K-ATPase/Src/caveolin-1 is essential for this regulation (Zhang et al. 2020).

Our data show that Cav-1 expression was not altered after treating HeLa cells with 21-BD (Fig. 7); however, this was observed in total cell lysates and not membrane fractions. In a previous study, we have observed an increase in the levels of α1 subunit mRNA in HeLa cells treated with 50 μM of 21-BD (Rocha et al. 2014). However, we have demonstrated that the expression of the Na,K-ATPase in HeLa cells after treatment with 21-BD remained unchanged in the plasma membrane (Silva et al. 2017). Therefore, interestingly in our hands, the increasing cholesterol levels in HeLa cells did not alter the Na,K-ATPase/Cav-1 expression.

The mechanism by which 21-BD affects cell lipids is not well understood. We know that the lipid changes caused by 21-BD, is not due to a direct action of the compound on the membrane. This was demonstrated in a previous study where human erythrocyte membrane fractions were treated with 21-BD. In this case, the content of cholesterol and phospholipids was not changed (Silva et al. 2017). Perhaps, treatment of HeLa cells with 21-BD alters the cholesterol homeostasis by increasing the synthesis or uptake of this lipid. CTS, such as digoxin and ouabain, can modulate the cholesterol content by interfering with the lipid metabolism. Studies have shown that CTS stimulated the cholesterol synthesis in different cell types (liver cells—HepG2, colon cancer cells—HT29, leukemia cells—THP-1, and cardiomyoblasts—H9c2). Both CTS increased the cholesterol synthesis by increasing the activity and expression of HMG-CoA reductase (Campia et al. 2009, 2012).

Some studies have shown that endogenous and exogenous CTS can interact with nuclear receptors causing different effects on processes regulated by gene transcription (Karaś et al. 2020). For instance, RORγ has been found to be involved in the control of several metabolic pathways, including lipid metabolism by positively regulating the transcription of genes related to lipid metabolism (Takeda et al. 2014; Urlep et al. 2017). It has been shown that some CTS, such as digoxin and synthetic analogues derived from digoxin, can interact with RORγ (Karaś et al. 2018), but not ouabain. CTS can act as receptor antagonists (Huh et al. 2011; Wu et al. 2013) or agonists as demonstrated by other authors (Huh et al. 2011). In addition, intermediaries in the cholesterol biosynthesis pathway can bind to RORγ and increase its activity (Zou et al. 2021). Thus, we can also raise the possibility of involvement of the orphan receptor related to retinoid acid (RORγ) in the modulation of lipid content in HeLa cells, which would be interesting to investigate to

better understand the mechanisms of 21-BD to increase cholesterol levels.

Conclusion

21-BD caused important lipid changes in cells, although the major changes in the lipid content were observed predominantly in HeLa cells that produce caveolae, even though the Cav-1 expression was not increased in these cells. Therefore, 21-BD does not increase the cholesterol content by increasing the expression of Cav-1 and the increase in cholesterol does not cause an increase in the expression of Cav-1.

21-BD was able to increase phospholipids in Caco-2 cells. In addition, ouabain was not able to cause modification in the lipid content of any cell line in this study. We could observe the same effect in a previous study using digoxin in HeLa cells. Apparently, the additional styrene group on the lactone ring of 21-BD structure promotes its ability to affect the lipid content of these cells. This could be of relevance to develop new compounds focused on the modulation of cholesterol and phospholipids as a mechanism of action to prevent diseases.

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Author contributions LNDS, IJPG wrote the main manuscript text. LNDS and IJPG performed the lipids and filipin experiments, JMMV and MMT performed the caveolae blotting, MTCP performed MTT experiments, MVM performed the 21-BD synthesis, MSB and IR performed the quantification of phospholipids. JAFPV, GCA, VFC, HLS, and LAB reviewed all the experiments, mentored the students, and reviewed the manuscript. GCA and LAB supported the paper with grants.

Declarations

Conflict of interest No conflicts of interest.

Ethical Approval Not applicable.

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