21-Benzylidene Digoxin: A Proapoptotic Cardenolide of Cancer Cells That Up-Regulates Na,K-ATPase and Epithelial Tight Junctions

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Abstract
Cardiotonic steroids are used to treat heart failure and arrhythmia and have promising anticancer effects. The prototypic cardiotonic steroid ouabain may also be a hormone that modulates epithelial cell adhesion. Cardiotonic steroids consist of a steroid nucleus and a lactone ring, and their biological effects depend on the binding to their receptor, Na,K-ATPase, through which, they inhibit Na+ and K+ ion transport and activate of several intracellular signaling pathways. In this study, we added a styrene group to the lactone ring of the cardiotonic steroid digoxin, to obtain 21-benzylidene digoxin (21-BD), and investigated the effects of this synthetic cardiotonic steroid in different cell models. Molecular modeling indicates that 21-BD binds to its target Na,K-ATPase with low affinity, adopting a different pharmacophoric conformation when bound to its receptor than digoxin. Accordingly, 21-DB, at relatively high µM amounts inhibits the activity of Na,K-ATPase α1, but not α2 and α3 isoforms. In addition, 21-BD targets other proteins outside the Na,K-ATPase, inhibiting the multidrug exporter Pdr5p. When used on whole cells at low µM concentrations, 21-BD produces several effects, including: 1) up-regulation of Na,K-ATPase expression and activity in HeLa and RKO cancer cells, which is not found for digoxin, 2) cell specific changes in cell viability, reducing it in HeLa and RKO cancer cells, but increasing it in normal epithelial MDCK cells, which is different from the response to digoxin, and 3) changes in cell-cell interaction, altering the molecular composition of tight junctions and elevating transepithelial electrical resistance of MDCK monolayers, an effect previously found for ouabain. These results indicate that modification of the lactone ring of digoxin provides new properties to the compound, and shows that the structural change introduced could be used for the design of cardiotonic steroid with novel functions.


Introduction
Cardiotonic steroids are common substances in the plant kingdom that confer a competitive advantage against depredation, either to the plant that synthesizes them, or to the animals that accumulate them from the diet [1]. Some experimental evidence shows that animals can endogenously synthesize cardiac steroids and that these substances play an important role in the regulation of blood pressure, cell proliferation and cell death [2,3]. The basic structure of cardiotonic steroids consists of a steroid backbone with...
a cis/trans/cis configuration and a lactone moiety at position 17β, also known as the aglycone. In addition, these compounds frequently have a sugar attached at position 3β of the steroidal nucleus, for which they are commonly known as cardiac glycosides. The nature of the lactone ring distinguishes the cardenolides, that have an unsaturated butyro lactone ring, from the bufadienolides, which present an 2-pyrene ring. The only structural difference between the two main cardenolides that are used therapeutically, digoxin and digitoxin, is a single extra hydroxyl group (–OH) on digoxin, which considerably alters its pharmacokinetics. Digitoxin, is more lipophilic, shows strong binding to plasma proteins, is almost entirely metabolized in the liver and exhibits a very long half-life. In contrast, digoxin displays weak binding to plasma proteins, is not extensively metabolized and is excreted in a primarily unaltered form by the kidneys [2,3].

The main pharmacological effect of therapeutic doses of digoxin and digitoxin is their positive inotropic effect. This is mediated through the inhibition of the myocardial cell Na,K-ATPase, which results in an increase in the cytosolic levels of Na+ and secondarily Ca2+, due to reduction in transport of the Na+ dependent Na+/Ca2+ exchanger. The increased Ca2+ in the myocardial cell cytosol leads to further filling of the sarcoplasmic reticulum with Ca2+, which will be readily available for its release upon stimulation to produce enhanced muscle contraction [3–5].

In the context of cell biology, there are two areas in which cardiac steroids are of potential interest: cancer and cell adhesion. Both these phenomena are closely related, as shown by the loss of cell adhesion that takes place during cancer proliferation and metastasis. The effect of cardiac steroids on cell adhesion is poorly understood and is the focus of intense research [6–8]. Since the 1960s, several interesting antitumor effects have been observed for cardiac steroids, digoxin and digitoxin, is a single extra styrene group in the C21 carbon of the lactone ring, in cancer and normal cells.

Materials and Methods

General procedure for the synthesis of 21-benzylidene digoxin

Benzaldehyde (0.18 ml, 1.8 mmol), digoxin (0.469 g, 0.6 mmol), anhydrous KO3CO3 (0.249 g, 1.8 mmol) and 60 ml of methanol were added into a round bottom flask. After stirring for 6 h at 70°C, the solvent was evaporated in a rotary evaporator. The crude product was diluted with 20 ml of water and extracted with hot ethyl acetate (3×30 ml). The organic layer was washed with brine, dried over anhydrous Na2SO4 and concentrated under vacuum. The crude product was then purified via silica column chromatography (CH2Cl2/MEOH 11:1). After purification, the pure product was diluted in tetrahydrofuran (THF), precipitated with hexane and concentrated under reduced pressure to give 21-BD (0.325 g, 0.37 mmol, 62%) as a white solid.

Cell culture

HeLa (human cervix carcinoma; ATCC CCL2) and RK-OAS45-1 (colon carcinoma; ATCC CRL-2579) cells were cultured in RPMI or DMEM/HAM F-10 (1:1) medium (Sigma, St. Louis, MO, USA). CHO-K1 (ATCC CCL61) and MDCK-II cells (canine renal; ATCC CCL-34) were grown in DMEM. All media were supplemented with 10% fetal bovine serum (FBS; Hyclone) (CDMEM), 60 mg/ml streptomycin and 100 mg/ml penicillin. Cells were seeded at a density of 5×103 cells/cm2 and incubated in a humidified atmosphere with 5% CO2. The culture medium was changed every 48 h to avoid nutrient depletion.

Insect cell culture and viral infections

Sf-9 insect cells were grown in Grace’s medium with 3.3 g/l lactalbumin hydrolysate, 3.3 g/l yeastolate, and supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone. Cells were grown in suspension cultures and were transferred to 150 mm tissue culture plates before infection. Infections were performed as previously described [51]. After 72 h at 27°C, cells were scraped from the culture plates, centrifuged at 1,500×g for 10 min and suspended in 10 mM imidazole hydrochloride (pH 7.5) and 1 mM EGTA. Cells were homogenized on ice using a Potter-Elvehjem homogenizer and the lysate was centrifuged for 10 min at 1,000×g. The supernatant was removed, centrifuged for an additional 10 min at 12,000×g, and the final pellet was suspended in 250 mM sucrose, 0.1 mM EGTA, and 25 mM imidazole HCl, pH 7.4.
Cytotoxicity assay

Compound cytotoxicity effect was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetra-Zolium salt colorimetric method (Sigma, St. Louis, MO, USA). Briefly, the cells were plated in 96-well plates (1 x 10^4 cells/well) and incubated for 24 h at 37°C to confluence. Then, the wells were washed with culture medium and digoxin or 21-BD was added at different concentrations (0.05–500 μM). After incubation for 24 or 48 h, the plates were treated with MTT. Readings were performed in a Spectramax M5e microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 550 nm. Cytotoxicity was scored as the percentage of reduction of absorbance, relative to untreated control cultures [52]. All experiments were performed in triplicate. The results were expressed as the mean of LC50 (the drug concentration that reduced cell viability to 50%).

Apoptosis Assay

Comet assay. The single-cell gel electrophoresis assay (comet assay) was performed according to previously published protocols [55,56]. For this, CHO-K1 cells were treated with 20, 35 or 50 μM digoxin or 21-DB, which are concentrations that do not affect cell viability according to the MTT assay. The cells were seeded in 24-well plates. The next day cells were washed twice with PBS, and incubated 3 h with the corresponding compounds in culture media without serum. The negative and positive control groups were treated with PBS, and methyl methanesulfonate (400 μM), respectively. After 24 h, the cells were washed twice with PBS and detached using a trypsin-EDTA solution. Trypsin was inactivated with 3.0 ml of complete medium, followed by centrifugation (5 min, 150 x g). The pellet was then resuspended in 500 μl of PBS, and 30 μl aliquots of the cell suspensions were mixed with 70 μl of low melting point agarose (0.5%). These mixtures were placed on slides pre-coated with normal melting point agarose (1.5%) and covered with coverslips. The coverslips were removed after five minutes, and the slides were immersed overnight in lysis solution (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, pH 10; Triton X-100 1% and DMSO 10%). Next, slides were washed with PBS and maintained for 40 min in a horizontal electrophoresis box filled with cold alkaline buffer (EDTA 1 mM, NaOH 300 mM, pH >13). Electrophoresis was conducted at 0.86 V/cm and 300 mA (20 minutes) and the slides were subsequently neutralized (0.4 M de Tris, pH 7.5), fixed with methanol and stained with ethidium bromide. Visual analyses were performed under fluorescence [55,56].

Phosphatidylserine translocation. HeLa cells were plated in 60 mm diameter Petri dishes at confluence and incubated overnight in CDMEM. They were then treated with 2 μM digoxin or 50 μM 21-BD in CDMEM for 6, 12 or 24 h. After incubation, suspended cells were recovered from the media by centrifugation at 150 x g, 20°C for 5 minutes. Attached cells were obtained by a mild protease treatment 1 ml Accutase plus 3 ml PBS), centrifuged as indicated above and added to the cells obtained from the media. Annexin-V bound to the outer leaflet of the plasma membrane was measured with a commercial kit (Annexin-V-Fluos Staining kit, Roche, Germany) following manufacturer instructions. Briefly, cell suspension was incubated with a mixture of Annexin-F-FITC and propidium iodide in saline buffer for 15 min. Cell suspension was analyzed by flow cytometry (FACS Calibur flow cytometer, Fow Jo software).

Transepithelial electrical resistance (TER)

MDCK cells were cultured on Transwell permeable supports (3415, Corning Inc., NY, USA) The transepithelial electrical resistance was measured with an EVOM and the EndOhm-6 system (World Precision Instruments, Sarasota, FL, USA). Final values were obtained by subtracting the resistance of the bathing solution and the empty insert. The results are expressed in ohm-cm² (Ω-cm²) as a percentage of the control.

Immunofluorescence

After TER measurements, MDCK monolayers were washed three times with ice-cold PBS/Ca²⁺, fixed with 4% paraformaldehyde for 30 min at 4°C, permeabilized with 0.1% Triton X-100 for 5 min, blocked for 30 min with 3% BSA and treated for 1 h at 37°C with a specific primary antibody. The monolayers were then rinsed 3 times with PBS/Ca²⁺, incubated with an appropriate FITC or TRITC-labeled antibodies for 30 min at room temperature and rinsed as indicated above. Filters with the cells were excised with a scalpel and mounted in Vectashield (Vector Labs, Burlingame, CA, USA). The preparations were examined with a Leica confocal SP5 microscope (Leica Microsystems, Wetzlar, Germany). The captured images were imported into FIJI, version 2.8 (National Institutes of Health, Baltimore, USA), to obtain maximum projections and into the GNU Image Manipulation Program (GIMP) to normalize brightness and contrast in all images and construct figures.

Immunoblotting

Western blot analysis of whole-cell protein extracts was performed as described previously [57]. Briefly, MDCK and HeLa cells were washed with PBS and solubilized with radioimmunoprecipitation assay (RIPA) buffer (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 7.4, 150 mM NaCl, 2 mM ethylendiamine-tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). The protein content of the cell lysate was measured (BCA protein assay reagent; Pierce Chemical, Rockford, IL) and prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by boiling in sample buffer. The resolved proteins were electrotransferred to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The proteins of interest were then detected with the specific polyclonal or monoclonal antibodies indicated in each case, followed by species-specific peroxidase-conjugated antibodies (Zymed Laboratories, South San Francisco, CA) and chemiluminescent detection (ECL PLUS; GE Healthcare).

Detection of Na,K-ATPase and claudin expression via real-time quantitative RT-PCR

For real-time quantitative RT-PCR (RT-qPCR), total RNA was extracted from the cell samples using TRizol (Life technologies, USA). The concentration was estimated via spectrophotometry with a Nanodrop ND2000 (Thermo Scientific, USA). All reverse transcriptase reactions were performed using 5 μg of RNA with the Superscript III kit (Invitrogen, USA) according to the manufacturer’s instructions. All RNA samples were reverse transcribed simultaneously to minimize the inter assay variation associated with the reverse transcription reaction. Real-time quantitative PCR was performed on an ABI Prism 7500 fast sequence detection system (Applied Biosystems) using Go Taq qPCR master mix (Promega, USA). The following primers and concentrations were employed (Murphy et Al, 2004): Na,K-ATPase δ subunit (GenBank accession number NM_000701): Fw (300 nM): 5'-TGTCCAGAATTGCAGGTCTTTG-3', Rv (300 nM): 5'-TGCCCGTCTTAAAGAATAGGTAGGT-3'; Na,K-ATPase β subunit (GenBank accession number NM_001677): Fw

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Biological Effect of 21-Benzylidene Digoxin
Molecular modeling analyses

Initially, digoxin, ouabain, and 21-BD (Figure 1A) were generated and refined via the semi-empirical PM6 method [60] implemented in Gaussian 09 W software [61]. The crystallographic structure of Na,K-ATPase (target protein) complexed with ouabain was obtained from the Protein Data Bank (PDB ID: 4HYT [62] with a 3.40 Å resolution, using the αs subunit of Na,K-ATPase). The magnesium ion and three interstitial water molecules were kept in the binding site. Next, a rigid re-dock of ouabain was carried out to validate our system. Subsequently, digoxin and 21-BD were docked against the ATPase. The docking analyses were conducted using AutoDock Vina 1.0.2 [63]. The applied search algorithm was Iterated Local Search Global Optimizer for global optimization. In this process, a succession of steps with mutation and local optimization (the Broyden-Fletcher-Goldfarb-Shanno [BFGS] method) were conducted, and each step followed the Metropolis criterion [64]. In this study, a grid box was constructed exploring all active sites, which was designed as a cube with the geometric center in ouabain, with dimensions of 20 Å × 20 Å × 20 Å. In each step, the Metropolis criterion was followed by the Metropolis criterion [64]. In this study, a grid box was constructed exploring all active sites, which was designed as a cube with the geometric center in ouabain, with dimensions of 20 Å × 20 Å × 20 Å. In each step, the Metropolis criterion was followed by the Metropolis criterion [64].

Preparation of Pdr5p plasma membranes

Plasma membranes containing the overexpressed Pdr5p protein were prepared from the Saccharomyces cerevisiae mutant strain AD124567 as previously reported [66].

Fractionation of cell lysates and preparation of membrane fractions

A total of 2.5 × 10^7 cells were grown in 75 cm² culture bottles and treated with digoxin and 21-BD. After 48 hours of treatment, cells were washed three times with cold PBS and scrapped from the culture bottle with a rubber policeman in a membrane preparation buffer (6 mM Tris pH 7.5, 4 mM MgCl₂, 75 mM KNO₃, 7.5 mM NaN₃, and 0.3 mM ammonium molybdate in the presence of 3 mM ATP). The reaction was initiated by the addition of 13% DMSO, 21-BD and digoxin were added up to a 5% v/v final concentration. The released inorganic phosphate (Pi) was measured as described elsewhere [69]. Using stock solutions in DMSO, 21-BD and digoxin were added up to a 5% v/v final concentration. The difference in ATPase activity in the presence of 3 μM oligomycin corresponded to a Pdr5p-mediated ATPase activity.

Measurement of the effect of 21-BD on Na,K-ATPase activity

Brain hemispheres from adult male Wistar rats were rapidly collected after diethyl ether anesthesia and decapitation. The protocols used for the use of rats were approved by the Institutional Commission for Ethics in the Use of Animals, process code DBFCICB011 and conformed to the Guide for the Care and Use of Laboratory Animals, published by US National Institute of Health (NIH publication No. 85-23, revised in 1996). The brain tissues, used as a source of ouabain-sensitive (αs/αs) Na,K-ATPase isoforms, were homogenized in 250 mM buffered sucrose, 2 mM diithiothreitol, 0.1 mM PMSF and 5 mM Tris/HCl (pH 7.4) with a motor-driven Teflon Potter-Elvehjem homogenizer. Subsequently, chaotropic treatment with 2 M KI was performed for 1 h under constant stirring, followed by centrifugation three times at 100,000 × g for 1 h. The final pellet was resuspended in 250 mM sucrose, 0.1% sodium deoxycholate and 20 mM maleate/Tris (pH 7.4), then stored overnight at −20°C and subjected to differential centrifugation after thawing [67]. The pellets were resuspended in the same buffer without PMSF and stored in liquid N₂.

Na,K-ATPase preparation from mouse kidney

Kidney tissue was isolated from adult mice and was homogenized in 250 mM sucrose, 0.1 mM EGTA, and 25 mM imidazole HCl, pH 7.4, using a motor-driven Teflon Potter-Elvehjem homogenizer. The sample was then subjected to centrifugation at 4,500 × g for 10 min. The resulting supernatant was centrifuged at 70,000 × g for 1 h. The final pellet was resuspended in the homogenization solution and used for Na,K-ATPase activity assays. All experimental protocols involving mice were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

NTPase Assays

Enzymatic activities were assayed using ATP as a substrate in standard medium (50 μl final volume) containing 100 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 75 mM KNO₃, 7.5 mM NaN₃, and 0.3 mM ammonium molybdate in the presence of 3 mM ATP. The reaction was initiated by the addition of 13 μg/ml of the plasma membrane preparations, then maintained at 37°C for 60 min and stopped by the addition of 1% SDS, as previously described [68]. The released inorganic phosphate (Pi) was measured as described elsewhere [69]. Using stock solutions in DMSO, 21-BD and digoxin were added up to a 5% v/v final concentration. The difference in ATPase activity in the presence or absence of 3 μM oligomycin corresponded to a Pdr5p-mediated ATPase activity.

Measurement of the effect of 21-BD on Na,K-ATPase activity

The brain hemisphere preparations were incubated at 37°C for 2 h in medium containing 87.6 mM NaCl, 3 mM KCl, 3 mM MgCl₂, 3 mM ATPNa₂, 1 mM EGTA, 10 mM sodium azide and 20 mM maleic acid/Tris (pH 7.4), and the cell membrane preparations were incubated at 37°C for 1 h in 120 mM NaCl, 20 mM KCl, 2 mM MgCl₂, 3 mM ATPNa₂ and 50 mM Hepes (pH 7.5), both in the absence and presence of 1 mM ouabain or increasing concentrations of 21-BD and digoxin. Na,K-ATPase activity was determined by measuring the Pi released according to a colorimetric method described previously [69], and specific
activity was considered as the difference between the total and ouabain-resistant ATPase activities [70].

Ouabain binding

HeLa cells were plated on 24 multi well plates at confluence and cultured overnight. Monolayers were then rapidly washed three times with potassium free saline buffer (140 mM NaCl, 1.8 mM CaCl2, 5 mM sucrose, 10 mM Tris-HCl pH 7.4 at room temperature) and incubated 30 min with total binding solution (0.1×10⁻⁶ ³H-ouabain plus 0.9×10⁻⁶ cold ouabain in potassium free saline buffer) under gentle agitation and at room temperature. Then monolayers were washed four times, 1 min each, with ice cold 0.1 M MgCl₂ and dissolved with 400 μl of SDS 1%. ³H activity was measured in samples of 350 μl by scintillation counting. Total ³H-ouabain binding was competed by adding to the total binding solution the necessary amount of 21-BD to reach the concentrations indicated in results. A subset of monolayers was exposed to competed binding solution (total binding solution plus 0.5×10⁻⁴ M cold ouabain) to measure the unspecific binding and subtract it to the total binding values.

Statistical analyses

Statistical analyses were performed with GraphPad Prism software, version 5. The results were expressed as mean ± error standard of the mean. Statistical significance in a one-way analysis of variance (ANOVA), followed by a Bonferroni’s selected pairs comparison test, was set at P < 0.05 (*), P < 0.01 (**), or P < 0.001 (***) vs. the control condition, and “n” represents the number of independent experiments.

Results

We synthesized 21-BD via a simple stereo selective vinylogous aldol reaction, according to Xu et al. [50], and characterized the final product through conventional NMR, HRMS and IR analysis (Figure 1A; Figure S1 and Data S1).

To perform the molecular modeling analyses we started with the geometric optimization of ligands, through a semi-empirical approach, to correct geometric parameters such as bond lengths and refine the structure. The re-dock structure obtained with the software Autodock Vina, shows that the refined and the crystallographic ligand share the same conformation at the active site, with a root mean square deviation value of 2.24 Å for the best
solution, which indicated the accuracy of the methodology used (see Figure S2A). Figure 1B shows a simulation of the docking of ouabain to its binding site in the Na,K-ATPase α1 isoform. As shown, the enzyme preserves the secondary structural elements of the ATPase family (Figure 1B, left) and that the active site is composed of Glu111, Glu117, Pro110, Asn112, Leu125, Glu312, Ile315, Gly319, Val322, Ala323, Phe703, Phe706, Leu793, Ile800, Arg880 (Figure 1B, right), in agreement with known structures [71].

Following these molecular modeling analyses we docked ouabain, digoxin and 21-BD to Na,K-ATPase (Figure S2B and Figure 1C and D). Simulations resulted in binding energies for ouabain, digoxin, and 21-BD of −9.8, −1.9, and −10.0 kcal/mol, respectively. These data suggest that 21-BD may have a similar binding affinity to the Na,K-ATPase than ouabain and digoxin. However, both these last compounds exhibit different pharmacophoric conformations in the binding site, principally at the level of the lactone ring. Figures 1C and D highlight the most important intermolecular interactions between the ligands and the target protein. Digoxin binds to the enzyme via hydrogen bonding with Thr797, Asp834 and Lys905 aminoacids. The electrostatic and hydrophobic interaction occur with Glu111, Asn120, Asp121, Asn122, Ile315, Gly319, Val322, Ala323, Arg880, Asp884, Tyr901, Glu908 and Glu111, Leu125, Phe316, Phe783, Phe786, Leu793, Arg886, Phe909, Arg972, respectively (Figure 1C). In contrast to natural cardiac glycosides, 21-BD complexes to the Na,K-ATPase through Glu111, Glu312, and Thr797 hydrogen bonding. In addition, the aromatic ring reaches a hydrophobic pocket formed principally by Cys104, Val322, Ala323, Glu327, Ile800 (Figure 1D). Moreover, the pharmacophoric conformation is completely different from that of the natural glycosides, whose aromatic moiety binds to water molecules and magnesium ion.

To directly determine binding of 21-BD to the ouabain site of the Na,K-ATPase, we first tested if the synthetic steroid could compete with ³H-ouabain binding in HeLa cells, which express the α1 isoform of the Na,K-ATPase [72]. As shown in Figure 2A, 21-BD significantly competed with ouabain binding at concentrations in the μM range, indicating that the affinity of the Na,K-ATPase for this ligand is low. We next studied if 21-BD affects Na,K-ATPase activity of a membrane preparation from rat cerebral hemispheres. This tissue is mainly composed of the ouabain sensitive α2 and α3 isoforms of the enzyme, which comprise approximately 80% of the total Na,K-ATPase activity, with the remaining 20% corresponding to the α1 isoform [73]. Digoxin inhibited most of the Na,K-ATPase activity, with an IC50 of 219 ± 40 nM, corresponding to the high-affinity sites of the α2 and α3 isoforms, and at higher concentrations it further inhibited the α1 isoform. In contrast, 21-BD had no effect on rat brain Na,K-ATPase, even at the highest concentration tested (100 μM) (Figure 2B).

To further determine if 21-BD had any effect on the α1 isoform of the Na,K-ATPase, we tested its activity of two different sources of Na,K-ATPase rich in α1. We used membrane fractions from Sf9 insect cells exogenously expressing the rat Na,K-ATPase α1 and β1 subunits and membrane fractions from mouse kidney, which primarily contains the Na,K-ATPase α2 isoform. The expression in Sf9 cells provides a useful system to specifically investigate effects on the exogenously expressed Na,K-ATPase, since these cells virtually lack expression of an endogenous Na,K-ATPase. As shown in Figure 2C–D, 21-BD inhibited activity of α1/β1 from Sf9 cells, but had little effect on parallel preparations expressing only the β1 subunit of the enzyme, which by lacking the catalytic α1 subunit serve as a control. The effect of 21-BD only took place at high concentrations (100 μM) of the compound. Similarly, 21-BD inhibited the mouse kidney Na,K-ATPase with similar kinetics. These results agree with the ³H-ouabain binding experiments in HeLa cells, suggesting that 21-BD interacts with Na,K-ATPase with low affinity.

Although the results described above suggest that Na,K-ATPase α1 is a receptor for 21-BD, the possibility exists that 21-BD could be also targeting another protein/s in the membrane of the cells. Steroids have been shown to inhibit the activity of yeast and mammalian ATP-dependent efflux pumps [74,75]. Therefore, we investigated the effect of 21-BD on Pdr5p, a member of the Saccharomyces cerevisiae ABC transporters family that shares many substrates and inhibitors with the mammalian P-glycoprotein [76]. Interestingly, after incubation with membrane preparations from Saccharomyces cerevisiae, 21-BD showed a concentration-dependent inhibitory effect on NTPane activity, with an IC50 of 1.25 ± 0.36 μM. In contrast, digoxin had no significant effect on Pdr5p (Figure 2E). This suggests that, while 21-BD has an effect on its Na,K-ATPase target, it can also affect other proteins, such as the ATP dependent transporter of the plasma membrane.

To further study the effects of 21-BD, its action was tested and compared to that of digoxin in whole cells. Thus, the compounds were directly applied to HeLa cervix cells, which express the Na,K-ATPase α1 isoform, and RKO colorectal cancer cells that, in addition to α1, express the α2 isoform [77]. After incubation with the compounds for 48 h, cells were harvested and Na,K-ATPase activity determined. As expected, treatment with 150 nM digoxin for 48 h inhibited Na,K-ATPase activity (Figure 3A, green columns). Surprisingly, 10 μM 21-BD increased Na,K-ATPase activity in both cell lines (Figure 3A, red columns). To explore whether the increase in Na,K-ATPase activity was caused by an increase in expression of the Na,K-ATPase, the levels of Na,K-ATPase subunits in the cells was determined by RT-PCR. As depicted if Figure 3B, incubation with 10 μM 21-BD for 48 h increased mRNA of the Na,K-ATPase α1 and β1 subunits in HeLa cells.

To test the possibility that 21-BD could exhibit anticancer effects, such as those described for digoxin, we used HeLa, RKO cancer cells, and normal epithelial MDCK cells. Both digoxin and 21-BD showed a cytotoxic effect on HeLa cells after treatment for 24 and 48 h. Digoxin produced the classical time- and dose-dependent decrease in viability (IC50 of 2.2 ± 0.8 μM), being approximately 25 times more potent that 21-BD (IC50 of 56.16 ± 8.12 μM) (Figure 4A). In RKO cells, digoxin decreased viability with higher potency, with an IC50 of 0.42 ± 0.1 μM, compared to that of 21-BD, which had a IC50 of 55.81 ± 15.15 μM. Thus, while RKO cells showed a higher sensitivity to digoxin than HeLa cells (Figure 4B), they exhibited the same sensitivity to 21-BD than HeLa cells. Surprisingly, different from digoxin, 21-BD increased the viability of MDCK cells (Figure 4C). This unexpected result may depend on the fact that 21-BD is not toxic to MDCK cells and that it may induce a higher mitochondrial activity to metabolize the substrate in the MTT assay.

Digoxin is known to induce apoptosis in several cell types [78,79]. Therefore, the effect of 21-BD in reducing HeLa and RKO cell viability could be due to induction of cell apoptosis. To assess this possibility, we followed two crucial events of the apoptotic process after 21-BD treatment: DNA fragmentation and the translocation of phosphatidylserine from the inner towards the outer leaflet of the plasma membrane of CHO-K1 cells. As shown in Figures 5A and B, 21-BD causes primary DNA fragmentation as determined by comet assay. The amount of phosphatidylserine translocated was measured through the binding of fluoresceinated annexin-V and the amount of necrotic cells was estimated by the
incorporation of propidium iodide into the cell nucleus (Figure 5C). We found that 21-BD increases phosphatidylserine translocation (red bar, Apoptotic) but not necrosis (Necrotic, red bar). Altogether, these results show that 21-BD is able to induce cell apoptosis.

The integrity of cell junctions is a key factor in cancer biology, as several authors have demonstrated that alterations of tight junctions are associated with cell transformation and metastasis [6,23,41,80,81]. We tested whether 21-BD treatment modifies transepithelial electrical resistance (TER) or the distribution of the tight junction proteins Cldn-2 and -4 and ZO-1. For this purpose, we used MDCK cells, which are a well-known model for tight junction studies [82]. We first examined whether 21-BD altered TER. As shown in Figure 6A, 50 μM 21-BD induces a sustained increase in TER for at least 87 h of treatment and in a dose-dependent manner. This change in TER is associated with an increase in the expression of tight junction proteins. Figure 6B shows that 21-BD increases the cellular content of claudin-4 mRNA at all concentrations tested (Figure 6B, red circles) and claudin-2 mRNA only at the lowest concentration (Figure 6B, red triangles). Conversely, digoxin increases claudin-4 (Figure 6B, green circles) but not claudin-2 mRNA (Figure 6B, green triangles). The increase in claudin-4 mRNA results in a corresponding increment of the protein at high 21-BD concen-
trations (Figure 6C) while claudin-2 protein strongly decreases (Figure 6C, Cldn-2, red bars). 21-BD also induces the increment of ZO-1, an important peripheral membrane protein of tight junctions (Figure 6C).

Figure 7 illustrates that 21-BD increases the expression of claudin-4 and ZO-1 at the tight junction (arrows) and the cytoplasm of MDCK cells (arrowheads D and F vs A and C), displaying their characteristic “chicken fence” like pattern of expression of these junctional proteins. Conversely, 21-BD reduces the expression of claudin-2 (Figure 7E vs B). The coordinated antagonistic variation of claudins -4 and -2 has also been shown in epithelial cells treated with other factors, e.g. EGF [83,84].

21-BD also increases the cellular content of the α1 Na,K-ATPase at the highest concentration tested (Figure 8A) and its localization in the plasma membrane (Figure 8C, arrow) and in the cytoplasm (Figure 8C, arrowheads) of MDCK cells.

Discussion

We have synthesized a digoxin derivative, 21-BD, that reduces cell viability by inducing apoptosis, and increases the hermeticity of tight junctions through the up-regulation of claudin-4 and ZO-1 and the down-regulation of claudin-2 junctional proteins. These effects take place after 12 to 48 h incubation with 21-BD. These delayed responses most probably result from the activation of signaling cascades that result in changes in expression of different genes, as has been reported for other cardenolides [85–87]. An important question is whether 21-BD triggers those effects as a result of its binding to the Na,K-ATPase or to another membrane protein. 21-BD is able to displace 3H-ouabain from the Na,K-ATPase (Figure 2A), demonstrating that 21-BD has the capacity to bind to the Na,K-ATPase with a low affinity and that 21-BD occupancy of the sites in the pump may trigger cellular responses via the classical Na,K-ATPase mediated cascade of intracellular mediators. Molecular modeling also indicates the binding of 21-BD to the α1-Na,K-ATPase (Figure 1D), although with a pharmacophoric conformation very different from that of digoxin. Also, supporting the role of the Na,K-ATPase as a 21-BD receptor is the regulation that this cardiotonic steroid exerts on the tight junctions in MDCK cells, which is similar to that caused by ouabain [35]. In addition, 21-BD induces apoptosis like other cardiotonic steroids do. Nevertheless, it is known that adrenocortical bovine cells express high affinity binding sites for ouabain that are distinct from the Na,K-ATPase [88]. Here we show that 21-BD, but not digoxin, is able to inhibit the activity of the yeast ABC ATPase Pdr5p (Figure 2E), suggesting the possibility that 21-BD might, in addition to the Na,K-ATPase, act through a different receptor.

Our docking results show that the aromatic ring of 21-BD may reach a hydrophobic pocket in the binding site (Figure 1),

**Figure 3. 21-BD increases Na,K-ATPase expression in cancer cells.** (A) Na,K-ATPase activity after incubation of HeLa cells with 21-BD or digoxin for 48 h with different concentrations of 21-BD. B) mRNA content of the Na,K-ATPase α1 and β1 subunits of HeLa cells after 48 h incubation with various concentrations of 21-BD.
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**Figure 4. High concentrations of 21-BD reduce cell viability of HeLa and RKO.** HeLa (A) or RKO (B) cells were treated with digoxin (green symbols) or 21-BD (red symbols) for 24 (circles) or 48 (squares) h. Viability was measured by MTT reduction assay. 100 and 25 μM 21-BD induced the statistically significant reduction of HeLa and RKO viability, respectively (p<0.0084). Digoxin reduces HeLa viability starting with 150 μM for 24 h and 50 μM for 48 h (p<0.001). RKO cells have a higher sensitivity to digoxin that induces statistically significant differences starting from 1.6 μM for 48 h (p<0.0001).
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suggesting that intermolecular hydrophobic interactions may be optimized by linking alkyl groups to the aromatic ring or by increasing the aliphatic chain between lactone and aromatic rings. In other words, extending the molecule in the direction of the hydrophobic pocket may improve the affinity of new compounds through extra van der Waals interactions with the enzyme. Additionally, the docking simulations revealed a large change in the pharmacophoric conformation of 21-DB at the lactone ring, which may explain the biological effect of this compound.

Retrospective clinical observations performed 40 years ago suggest that cardiac steroids have anticancer effects [10,89,90]; however, few studies have been performed to elucidate the
underlying mechanisms of these effects [3,4]. It has been proposed that cardiotonic steroids induce necrosis through the inhibition of the ion transport activity of the Na,K-ATPase, the resulting sustained increase of the cytosolic calcium concentration and the activation of cellular signaling cascades [16,91–94]. Nevertheless, low concentrations of ouabain (10 nM) do not induce apoptosis in MDCK cells [35], while high concentrations (300 nM - 3 μM) induce necrotic processes such as cell swelling independent of Na,K-ATPase pumping activity [95] and permeabilization of lysosomal membranes [42]. These findings indicate that cardiotonic steroids induce cell death though complex mechanisms named oncosis, a type of cell death that involves apoptotic (caspase activation) as well as necrotic (cell swelling) processes [95]. Our data indicate that 21-BD induces DNA damage, which is a signature characteristic of apoptosis but is also compatible with oncosis. In vitro studies show that other mechanisms may be related to cell responses triggered by cardiac glycosides. For instance, bufalin, a hydrophobic cardiotonic steroid, downregulates the expression of Cyclin A, Bcl-2 and Bcl-XL and upregulates the expression of p21 and Bax in ovarian endometrial cyst stromal cells, affecting cell cycle progression and inducing apoptosis [96]. Digoxin applied at low concentrations (<10 nM) prevents apoptosis in HeLa cells, whereas at higher concentrations induces the release of cytochrome-c, thereby triggering apoptosis [97]. UNBS1450 induces death in A549 lung cancer cells through the NFκB signaling pathway [42]. The diversity of reported effects indicates the need for further studies aimed to elucidate the possible mechanisms involved in cell death induced by digoxin and other types of cardiac steroids.

21-BD is moderately cytotoxic for HeLa and RKO cells (LC50 of approximately 50 μM). This cytotoxicity is correlated with the induction of DNA damage. Studies addressing the genotoxicity of cardiac glycosides are scarce [98,99]. Given that cardiac glycosides produce reactive oxygen species (ROS) [4,100] and that these substances are well-known genotoxic agents [101], it is possible that 21-BD could induce an increase in ROS. Alternatively, 21-BD may inhibit topoisomerase II, as is known for other cardiac glycosides, including digoxin [91].
When directly applied to HeLa and RKO cells and in relatively low concentrations, 21-BD induces the increase of Na,K-ATPase activity through up-regulation of the $\alpha_1$ and $\beta_1$ subunits of the enzyme (Figures 3A, B). In contrast, in relatively high concentrations, 21-BD inhibits the activity of the Na,K-ATPase $\alpha_2$ isofrom of cell homogenates (Fig. 2C, D). This effect appears to be isoform specific, since 21-DB has no effect on the $\alpha_2$ and $\alpha_3$ isoforms of brain that have a high affinity for for cardiac steroids. The requirement of high concentrations of 21-DB to inhibit $\alpha_2$ shows the low affinity of this Na,K-ATPase isoform for the compound. The effects that we observe with lower concentrations of 21-BD are consistent with the activation of the signaling functions of the Na,K-ATPase that are known to trigger a variety of cellular phenomena including induction of endocytosis of junctional proteins [34,83] and the Na,K-ATPase itself [102–104], the detachment of epithelial cells from the substrate and from themselves [24,105], cell proliferation [106], protection from cell death caused by the addition of serum [107], cell survival [108] and the sealing of tight junction through the upregulation of claudinins [35]. The effects produced by 21-BD depend on the cellular context. While this compound reduces cell viability and induces apoptosis in cells derived from tumors (HeLa and RKO), it does not affect the viability of normal kidney epithelial cells (MDCK) as indicated by the increase in the transepithelial electrical resistance. The overexpression of claudin and the strengthening of tight junctions is an important mechanism against the phenotypic characteristics of cancer, which can regulate events such cell migration, transformation, proliferation and invasiveness [109–113].

Our results suggest that the modification of lactone ring of digoxin and maybe other cardiotonic steroids, could be an interesting alternative for chemical modification of this class of compounds. The effects presented by 21-BD appear to be unrelated with the classical effects that the cardiotonic steroids exert on intracellular ion changes through changes in Na,K-ATPase activity and raises the possibility that 21-BD (and maybe other cardiotonic steroids) may be involved with other proteins different from the Na,K-ATPase. Interestingly, digoxin has been shown to bind to other proteins besides Na,K-ATPase, such as the retinoic acid receptor-related orphan nuclear receptor RORγ. Digoxin-21-salicylidene is another digoxin derivative, with similar modification than 21-BD, that does not have high cytotoxic effect and selectively binds to RORγ, reducing progression of autoimmune encephalitis [49]. So, the decrease of cardiotonic steroid cytotoxicity opens the opportunity to use this class of compounds in other diseases.

Surprisingly, Na,K-ATPase is not the only enzyme that is responsive to 21-BD. Although Na,K-ATPase activity was not directly affected by 21-BD, the activity of the yeast multidrug exporter Pdr3p was largely inhibited by 21-BD. Cholesterol-derivated steroids can block the pumping function of this transporter, showing an IC$_{50}$ in the micromolar range, and some derivatives are considered interesting leads for use as drugs for the putative treatment of multidrug-resistant (MDR) tumors [74,75]. It is also known that several cardiotonic steroids exhibit antiancer activity in cells that express the chemotheraphy resistance phenotype [43,44,114–117]. This activity may be explained if as suggested by our 21-BD results, these cardiotonic steroids, have also the capacity to inhibit MDR proteins. Alternatively, anti MDR activity may be caused by downregulation of c-Myc or MDR-related genes, the inhibition of the glycosylation of MDR-related proteins or the inhibition of glycoslysis [43]. Our results show, for the first time, that a cis-trans-cis steroid is also able to cause direct inhibition of Pdr3p activity and opens the possibility that 21-BD may act as a drug to reverse the MDR phenotype.

Another important action of 21-BD is its interaction with junctional complexes. This increases TER as a result of the upregulation of the tight junction proteins Claudin-4 and ZO-1, which increase the sealing of tight junctions [118–120], and downregulates the levels of Claudin-2, which is expressed in epithelia with low TER [121,122]. Additionally, 21-BD induces an increase in the levels of Claudin-4 mRNA. This effect shows that ouabain is not the sole cardiotonic steroid that alters the molecular composition of tight junctions and TER [24,35], but that 21-BD can induce these effects as well.

**Conclusion**

The combination of an styrene group with the lactone moiety of digoxin produced a cardiotonic steroid, 21-BD, that enhances Na,K-ATPase catalytic activity in intact cells and stimulates Na,K-ATPase mediated signaling events. The later include reduction of cell viability via apoptosis in cancer, but not normal epithelial cells and increase in the closure of tight junctions of epithelial cells. The chemical modification of cardiotonic steroids with the styrene group has the potential to provide compounds with new functional properties that could results beneficial to treat cancer and perhaps other diseases in which cell proliferation occurs.

**Supporting Information**

**Figure S1** Synthesis of 21-BD. (TIF)

**Figure S2** Structure and pharmacophoric conformation of ouabain. (A) Crystallographic (green) and docked (red) structures of ouabain. (B) Pharmacophoric conformation of ouabain. Ouabain complexes with the receptor via hydrogen bonding between Gln111, Gln117, Asp121, Asn122, Gla312 and Thr797. In addition, ionic and hydrophobic interactions perform around the steroid core composed by Prol18, Leu125, Gly319, Phe783, Phe786, Leu793, Ile800, Arg880 and Asp121, Ile315, Val322, Ala323, respectively. (TIF)

**Data S1** Spectroscopic characterization of 21-BD. (DOC)

**Author Contributions**

Conceived and designed the experiments: FPV LMS AGT MC HLS FVS LEQ FGN AFP OCM GB JAFPV RGC LAB. Performed the experiments: SCR MTCP LDRN SLGA SMFO IVG AGCST NLSG RRH NP. Analyzed the data: FPV LMS AGT MC FVS LEQ FGN AFP OCM GB JAFPV RGC LAB. Contributed reagents/materials/analysis tools: FPV HLS MC FVS LEQ FGN AFP OCM GB JAFPV RGC LAB. Contributed to the writing of the manuscript: FPV LMS AGT MC HLS FVS LEQ FGN AFP OCM GB JAFPV RGC LAB.

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