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γ-Benzylidene digoxin derivatives synthesis and molecular modeling: Evaluation of anticancer and the Na,K-ATPase activity effect

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ABSTRACT

Cardiotonic steroids (CS), natural compounds with traditional use in cardiology, have been recently suggested to exert potent anticancer effects. However, the repertoire of molecules with Na,K-ATPase activity and anticancer properties is limited. This paper describes the synthesis of 6 new digoxin derivatives substituted (on the C17-butenolide) with γ-benzylidene group and their cytotoxic effect on human fibroblast (WI-26 VA4) and cancer (HeLa and RKO) cell lines as well as their effect on Na,K-ATPase activity and expression. As digoxin, compound BD-4 was almost 100-fold more potent than the other derivatives for cytotoxicity with the three types of cells used and was also the only one able to fully inhibit the Na,K-ATPase of HeLa cells after 24 h treatment. No change in the Na,K-ATPase α1 isoform protein expression was detected. On the other hand it was 30–40 fold less potent for direct Na,K-ATPase inhibition, when compared to the most potent derivatives, BD-1 and BD-3, and digoxin. The data presented here demonstrated that the anticancer effect of digoxin derivatives substituted with γ-benzylidene were not related with their inhibition of Na,K-ATPase activity or alteration of its expression, suggesting that this classical molecular mechanism of CS is not involved in the cytotoxic effect of our derivatives.

1. Introduction

The Na,K-ATPase is an enzyme that regulates the cellular homeostasis of Na^+ and K^+, through the transport of 3 Na^+ ions out and 2 K^+ ions into the cell. The enzyme is ubiquitously expressed in mammals and it has 3 subunits: α, β, and FXYD.[1] Four α, three β and seven FXYD isoforms have been described.[2] The α subunit contains the binding sites for ATP, Na^+, K^+ and specific ligands known as cardiotonic steroids (CS) and is responsible for the catalytic and ion transport activities of the enzyme. The β subunit is necessary for the activity of the enzyme complex and the assembling of the pump to the cell membrane and FXYD family members may affect the ionic transport properties of the Na,K-ATPase.[1,2]

CS are an important class of drugs classically known to inhibit the ion transport of the Na,K-ATPase. This inhibition leads to the positive inotropic effect derived from the increase of intracellular calcium in the cardiac muscle, and can be also responsible for cell death in toxic concentrations.[3,4] Besides, the activation of intracellular signaling pathways induced by CS using the Na,K-ATPase as a

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hormone receptor has been extensively investigated in recent years.[5]

Digoxin and digitoxin are naturally occurring CS used for congestive heart failure and arrhythmia and recently have been suggested to exert potent anticancer effects. Cancer cells have been shown to be highly sensitive to the toxic effects of CS and digoxin presented anticancer activity with induction of cell death through apoptosis[6] and involvement of established cell signaling pathways related to cancer cell proliferation.[7,8] Therefore, CS emerge as a promising class of molecules for cancer therapy.[2]

However, there are few works describing digoxin derivatives with modifications at the butenolide moiety. Wiesner et al.[9,10] described the synthesis of some cardenolides with important pharmacological properties related with modifications at C17β of steroidal skeleton keeping there a fractional positive charge, aiming at the conservation of the positive inotropic effect with reduced cardiotoxicity.

Xu et al.[11,12] described the synthesis and preliminary results of γ-alkylidenebutenolide derivatives, showing that some of them could increase myocardial contractility without cardiac toxicity, which is a significant drawback against the anticancer utilization of digoxin in humans. Huh et al.[13] synthesized non-toxic derivatives 20,22-dihydrodigoxin-21,23-diol and digoxin-21-salicylidene and observed that they blocked TH17 cells differentiation and function, which can be important for the development of Crohn's disease, rheumatoid arthritis and psoriasis. These compounds inhibited IL-17 production in human CD41 T cells. Recently, we have described the proapoptotic properties of 21-benzylidene digoxin (21-BD) in cancer cells and its effects on Na,K-ATPase, tight junctions and cell proliferation.[14]

Here we describe the synthesis, molecular modeling, Na,K-ATPase inhibition and cytotoxicity in human cancer cell lines of a series of γ-benzylidene digoxin derivatives.

2. Material and methods

2.1. Chemistry

Reagents and solvents were purchased as reagent grade and used without further purification. All NMR data were acquired in $CDCl_3$ or DMSO-d_6 on a Bruker AVANCE 400 NMR spectrometer, observing ^1H and ^{13}C at 400.13 and 100.61 MHz, respectively or Bruker ARX 200 NMR spectrometer, observing ^1H and ^{13}C at 200.13 and 50.3 MHz, respectively. The spectrometers were equipped with a 5-mm multinuclear direct detection probe with z-gradient. All ^1H and ^{13}C NMR chemical shifts are given in ppm (δ) relative to the TMS signal at 0.00 ppm as internal reference and the coupling constants (J) are in Hz. Preparative chromatography was performed using silica gel (230–400 mesh) following the methods described by Still et al.[15] Thin layer chromatography (TLC) was performed using silica gel GF254, 0.25 mm thickness. For visualization, TLC plates were either placed under ultraviolet light, stained with iodine vapor or acidic vanillin. HPLC analysis was performed in a Shimadzu Prominence liquid chromatography, equipped with a LC-20AT pump, SPD-M20A Photodiode Array detector, SIL-20A automatic injector and a Shim-pack C-18 VP-ODS 4.6 × 250 mm 5 μm reverse phase column. HRMS analysis was recorded on a micrOTOF-QII Bruker. IR spectra were recorded on a Shimadzu IRAffinity-1 spectrophotometer.

2.2. General procedure for the synthesis of 21-benzylidenes digoxin (BD-1,2,3,5 and 6)

Aldehyde (1.8 mmol), digoxin (0.469 g, 0.6 mmol), anhydrous K_2CO_3 (0.249 g, 1.8 mmol) were added to 60 mL of methanol in 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 Na_2SO_4 and concentrated under vacuum. The crude product was purified by silica column chromatography (CH_2Cl_2/MeOH 11:1). After purification, the pure product was diluted in THF, precipitated with hexane and concentrated under reduced pressure to give benzylidene digoxin derivative.

2.3. Procedure for the synthesis of 21-benzylidene digoxin (BD-4)

4-(Dimethylamino)benzaldehyde (0.298 g, 2 mmol), digoxin (0.781 g, 1.0 mmol), anhydrous K_2CO_3 (1.1 g, 0.152 mmol), were added to 4 mL of methanol and 2 mL THF in a round bottom flask. After stirring for 6 h at room temperature, 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 Na_2SO_4 and concentrated under vacuum. The crude product was purified by silica column chromatography (CH_2Cl_2/MeOH 11:1). After purification, the pure product was diluted in THF, precipitated with hexane and concentrated under reduced pressure to give benzylidene digoxin derivative (0.572 g, 0.63 mmol, 63%) as a yellow solid.

2.4. Cell culture

HeLa (human cervix carcinoma; ATCC CCL2), RKO-AS45-1 (human colon carcinoma; ATCC CRL-2579) and WI-26 VA4 (lung fibroblast; ATCC CCL-95.1) cells were cultured in RPMI medium (Sigma®, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 60 mg/mL streptomycin and 100 mg/mL penicillin in a humidified atmosphere of 5% CO_2. Cells were seeded at 5 × 10^5 cells/cm^2. The culture medium was changed every 48 h to avoid nutrient depletion.

2.5. Cytotoxicity assay

The cytotoxicity effect was assessed using a tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma®, St. Louis, MO, USA) colorimetric method. Briefly, the cells were plated in 96-well plates (1 × 10^5 cells/well) and incubated for 24 h at 37 °C in humid atmosphere with 5% CO_2 until adhesion. After this period, the wells were washed with culture medium and incubated for 48 h with the compounds at different concentrations (0.05–500 μm). After the incubation, the plates were treated with MTT. The reading was performed in a microplate reader Spectramax M5e (Molecular Devices, Sunnyvale, CA, USA) at 550 nm. Cytotoxicity was scored as the percentage of absorbance reduction when compared to untreated control cultures. All experiments were performed in triplicate. The results were expressed as the means of IC_{50} (the drug concentration that reduced cell viability to 50%). A selectivity index (SI), corresponding to the ratio between the cytotoxic activity of each compound against the fibroblast cell and the carcinoma cells was calculated as follows: SI = IC_{50} WI-26 VA4/IC_{50} HeLa or RKO-AS45-1.

2.6. Molecular modeling studies

Initially, digoxin and six steroids (*E* and *Z* isomers) were generated and refined by Universal Force Field (UFF)[16] implemented in Gaussian 09 W software.[17] Following, a model of the human Na,K-ATPase was built using the isoform ATP1A1 located at chromosome 1 with accession P05023[18] in the Uniprot database.[19] The search for suitable templates was carried out by the Template Identification Tool in the Swiss-Model Workspace.[20]

The model was evaluated using the Ramachandran plot generated using the Procheck software.[21] Once the model built and validated, a rigid re-dock of ouabain was carried out in order to validate the methodology. A grid box was constructed exploring all active sites, which was defined as a cube with the geometric centred in the ligand, with dimensions of 18 × 10 × 24 Å, spaced points of 1 Å and *X*, *Y* and *Z* coordinates of 147.345, 15.455 and −6.352. Following, digoxin and others compounds were docked against our ATPase human model using the AutoDockVina 1.0.2 software.[22] The search algorithm used was Iterated Local Search Global Optimizer for global optimisation. In this process, a succession of steps with a mutation and local optimisation (Broyden–Fletcher–Goldfarb–Shanno [BFGS] method) were conducted. Each step was followed by the Metropolis criterion.[23] All molecular modeling figures were constructed by DS Visualizer 3.1.[24]

2.7. Na,K-ATPase preparation from human kidney and rat brain hemispheres

Normal human renal samples were obtained and prepared as previously described.[25] All procedures for the use of discarded organ portions were done in accordance to the Institutional Ethical Committee from Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Brazil (process number 038/08). Briefly, samples were homogenized with motor-driven teflon Potter homogenizer in ice cold 0.25 M buffered sucrose (pH 7.4), containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and ultracentrifuged at 100,000*g* for 1 h. The final pellets were resuspended in the same buffer without PMSF and stored in liquid N_2.

Brain hemispheres from adult male Wistar rats were rapidly collected after decapitation (protocol approved by the Institutional Commission for Ethics in the Use of Animals, process code DFBCICB011, 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 organ, a source of ouabain-sensitive (α_2/α_3) Na,K-ATPase isoforms was homogenized in 250 mM sucrose, 2 mM dithiothreitol, 0.1 mM PMSF, 5 mM Tris/HCl (pH 7.4) with a motor-driven teflon Potter homogenizer. Subsequently, chaotropic treatment with 2 M KI for 1 h under constant stirring and centrifugation for three times at 100,000*g* for 1 h were performed. The final pellet was resuspended in 250 mM sucrose, 0.1% sodium deoxycholate, 20 mM maleate/Tris (pH 7.4), stored overnight at −20 °C and submitted to differential centrifugation after thawing.[26] The final pellets were resuspended in the same buffer without PMSF and stored in liquid N_2.

2.8. Fractionation of cell lysate and preparation of membrane homogenate

2.5×10^6 cells were grown in 75 cm^2 culture bottles and treated with the compounds. After 24 h 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 6.8), 20 mM imidazole, 0.25 M sucrose, 0.01% SDS, 3 mM EDTA and 2 mM PMSF). The cells were homogenized ten times in a potter in ice and sonicated in an ultrasonic cell disruptor on ice for 10 s at 45% power with resting periods of 5 s. The homogenate was subjected to centrifugation at 20,000*g* for 1 h and 30 min at 4 °C. The supernatant was discarded and the pellet resuspended with 250 µl of membrane preparation buffer. Finally, the final content was sonicated for 10 s at 25% power until complete dispersion of the homogenate.

2.9. Na,K-ATPase activity

Human kidney or rat brain hemispheres preparations were incubated at 37 °C for 2 h in the presence of 87.6 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 3 mM $ATPNa_2$, 1 mM EGTA, 10 mM sodium azide and 20 mM maleic acid/Tris (pH 7.4). The cell membrane preparations were incubated at 37 °C for 1 h in the presence of 120 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, 3 mM $ATPNa_2$ and 50 mM HEPES, (pH 7.5), both in the absence and presence of 1 mM ouabain or increasing concentrations of the compounds. Na,K-ATPase activity was determined by measuring the Pi released according to a colorimetric method described previously.[27,28]

2.10. Immunoblotting

The cell membrane homogenate was used for immunoblotting experiments. The protein content of the cell lysate was measured by Hartree method and prepared for SDS–polyacrylamide gel electrophoresis (PAGE) by boiling in sample buffer. The resolved proteins were electrotransferred to a nitrocellulose membrane (Ultracruz, Santa Cruz Biotechnology, CA). The proteins of interest were then detected with the specific polyclonal or monoclonal antibodies indicated in each case, followed by species-appropriate peroxidase-conjugated antibodies (Santa Cruz Biotechnology, CA) and chemiluminescent detection. Following detection of bands by chemiluminescence, images were scanned and were quantified for band intensity using ImageJ software[29] and values were expressed as density units relative to the untreated controls.

2.11. Statistical analysis

The parameter IC_{50} (the concentration of an inhibitory drug that reduces a response by 50% of the maximal inhibition) was estimated by nonlinear regression analysis of the data (Prism®, GraphPad Software Inc., version 5), assuming a sigmoidal dose–response curve model.[28] In order to evaluate a putative correlation between Na,K-ATPase inhibition and cytotoxicity, a Pearson correlation test was used considering the logIC_{50} values since the concentrations of drugs that produce half-maximal responses are only normally distributed when expressed as logarithms.[30]

3. Results and discussion

3.1. Chemistry

CS have arisen as an important pharmacological class with anticancer properties[31] and the synthesis of new compounds is a growing matter of interest.[14,32] The requirement of a lactone ring for inhibition of Na,K-ATPase is a strategy for the development of new CS.[33,34] However, a critical disadvantage of CS is the narrow therapeutic index and the resultant risk of toxicity that can lead to life-threatening heart arrhythmias and is a drawback for the development of new anticancer compounds.[34] Therefore, the goal of our chemical modifications was to introduce an aromatic group in the lactone ring of digoxin to promote a steric hindrance in the binding site of the Na,K-ATPase (Fig. 1A). Moreover, this chemical modification in the lactone ring of digoxin has been performed to be easy and with high yield.

Preparation of digoxin derivatives was carried out by a stereoselective vinylogous aldol reaction described by Xu et al.[11] (Fig. 1B). All compounds were characterized on the basis of extensive analyses of ^1H, $^{13}C\{^1H\}$[34] and DEPT-135 NMR, HR-MS and IR data (Supplementary data).

The HPLC and NMR analysis indicated the presence of stereoselectivity in the synthesis of the compounds BD-1, -4 and -5,

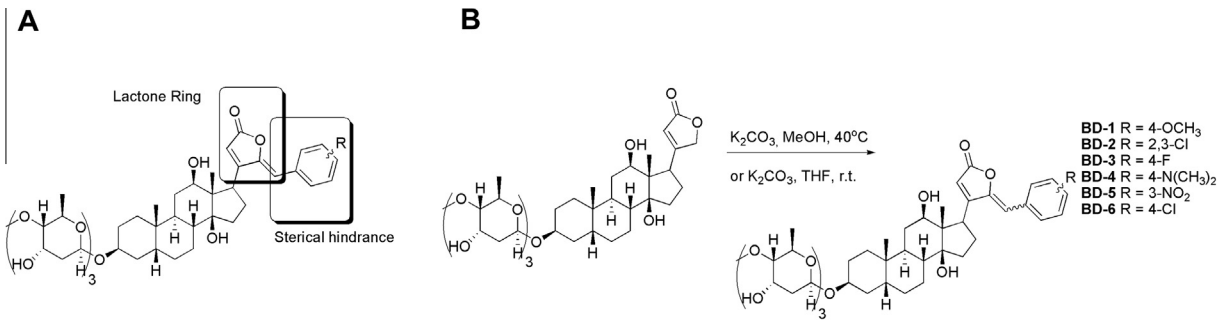

Figure 1. Synthesis of digoxin derivatives. (A) Digoxin derivatives proposed in this work. (B) Synthesis of BD-1 to -6.

Table 1
In vitro cytotoxic potency of digoxin and its derivatives BD-1 to -6 against HeLa, RKO and WI-38 cell lines

Compound	R		IC_{50} ± SD (μM)			Selectivity index	
			HeLa	RKO	WI-26 VA4	HeLa	RKO
BD-1	4-OCH_3	*Z*	61.4 ± 16.8	79.0 ± 15.1	78.3 ± 8.1	1.27	0.99
BD-2	2,3-Cl	Z/E	42.5 ± 15.1	19.0 ± 8.1	15.5 ± 1.7	0.36	0.81
BD-3	4-F	Z/E	60.9 ± 18.5	41.4 ± 9.9	54.1 ± 6.1	0.89	1.31
BD-4	4-$N(CH_3)_2$	Z	0.26 ± 0.06	0.48 ± 0.16	0.65 ± 0.11	2.5	1.35
BD-5	3-NO_2	Z	65.2 ± 8.5	41.3 ± 20.0	44.1 ± 2.6	0.68	1.07
BD-6	4-Cl	Z/E	40.1 ± 5.5	23.8 ± 1.1	28.7 ± 3.4	0.72	1.21
Digoxin	—	—	2.2 ± 0.8	0.42 ± 0.1	ND	—	—

Table 2
Inhibitory concentration of digoxin and its derivatives BD-1 to -6 for Na,K-ATPase inhibition of human kidney or rat brain preparation

	Na,K-ATPase inhibition IC_{50} ± SEM (μM)						
	Digoxin	BD-1	BD-2	BD-3	BD-4	BD-5	BD-6
Human kidney (α1)	0.29 ± 0.02	0.57 ± 0.10	N.D.	0.34 ± 0.09	9.8 ± 2.4	11 ± 3	N.I.
Rat brain (α2α3)	0.22 ± 0.04	0.46 ± 0.10	18 ± 10	0.66 ± 0.20	20 ± 7	4 ± 1	N.I.

IC_{50} were obtained by non-linear regression analysis of average curves from 3 to 9 independent experiments performed in triplicate. SEM represents here the goodness of fit for such parameter.
N.I., no inhibition (less than 20% inhibition at 10 μM).
N.D. not determined.

whereas a mixture of geometric isomers (*E* and *Z* compounds) were detected with BD-2, -3 and -6. For the synthesis of compound BD-4 we used THF as solvent at room temperature to afford a stereoselective product. According to Xu et al.[11] the thermodynamic preference for the double bond configuration is the Z configuration.

3.2. Biological activity

3.2.1. Cytotoxic effect

MTT assay in cancer cell lines gave IC_{50} values between 0.26 and 65 μM for HeLa cells, 0.48–79 μM for RKO cells and 0.65–78 μM for WI-26 VA4 cells (Table 1). As a whole, the BD compounds presented similar IC_{50} values, in the micromolar range, with the remarkable exception of BD-4 that was about 100 times more potent with IC_{50} values ranging from 260 to 480 nM. The compounds have no significant selectivity for the cancer cells, as indicated by the selective index (SI) ranging from 0.36 to 2.5 (Table 1).

3.2.2. Effect on Na,K-ATPase activity and expression

Table 2 and Figures 2 and 3 show that our digoxin derivatives have different capacities to inhibit the Na,K-ATPase activity of human kidney and rat brain Na,K-ATPase. Indeed, BD-1 and -3 have similar potencies as digoxin whereas BD-2, -4 and -5 are less potent (higher IC_{50} values) and BD-6 has no significant effect (less than 20% inhibition at 10 μM).

When HeLa cells were treated for 24 h, 150 nM digoxin caused a ~60% decrease of Na,K-ATPase activity as also observed with 150 nM BD-4. At 10 μM, BD-4 caused a total loss of Na,K-ATPase activity (Fig. 4A) and the other compounds failed to produce any significant inhibition of the activity, even at 10 μM. Moreover, the levels of α1-Na,K-ATPase were not changed on membrane preparations (Fig. 4B) and this open the possibility of the modulation of Na,K-ATPase activity instead of changes of Na^+ pump expression on the cell membrane by activation of signaling pathways.

CS are toxic to the cells due to inhibition of the Na-pump, considered as the main mechanism for digoxin toxicity.[4] The cytotoxic effect of digoxin in RKO cells (0.42 μM, Table 1) occurred at concentrations similar to the ones inhibiting the Na,K-ATPase activity of human kidney and rat brain preparations (IC_{50} = 0.22 and 0.37 μM, Table 2). Surprisingly, BD-4 was the most cytotoxic compound among our derivatives, being almost 100-fold more potent than the other derivatives with the three types of cells used (Table 1), despite its 30–40 fold lower potency for Na,K-ATPase inhibition, when compared to the most potent derivatives, BD-1 and BD-3 (Table 2). Moreover, the compounds demonstrated similar affinity for all isoforms of Na,K-ATPase.

Such lack of relation between these two effects in the present study was further supported by the absence of significant correlation between the log IC_{50} for cytotoxicity and Na,K-ATPase

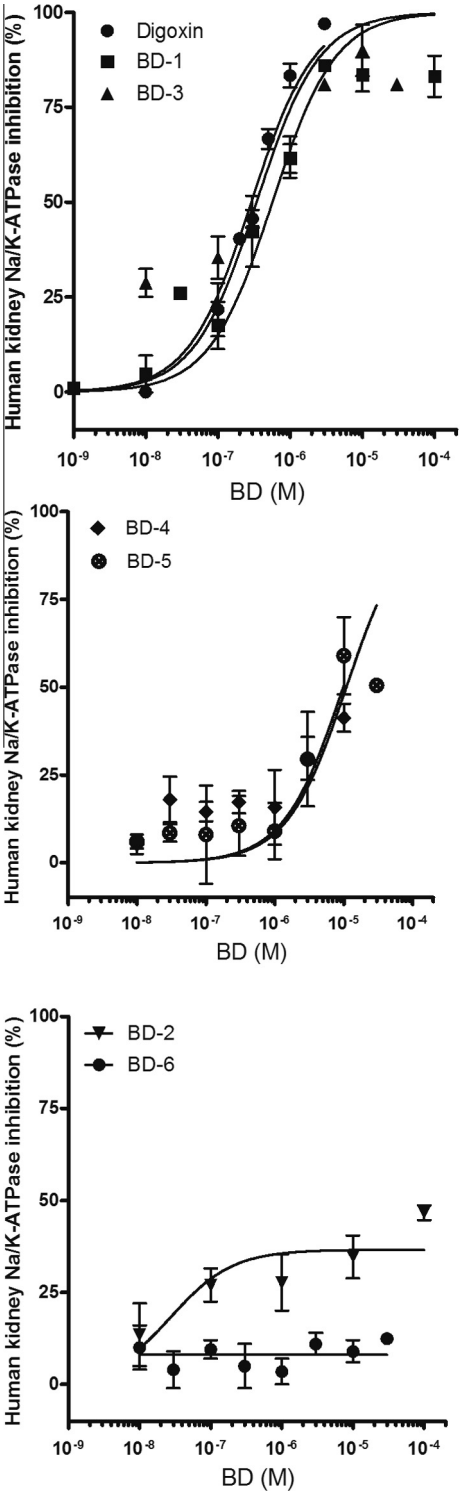

Figure 2. Inhibition of Na,K-ATPase from human kidney preparation. Average curves were obtained by non-linear regression analysis. Each point represents the IC_{50} ± SEM of 2–8 independent experiments performed in triplicate.

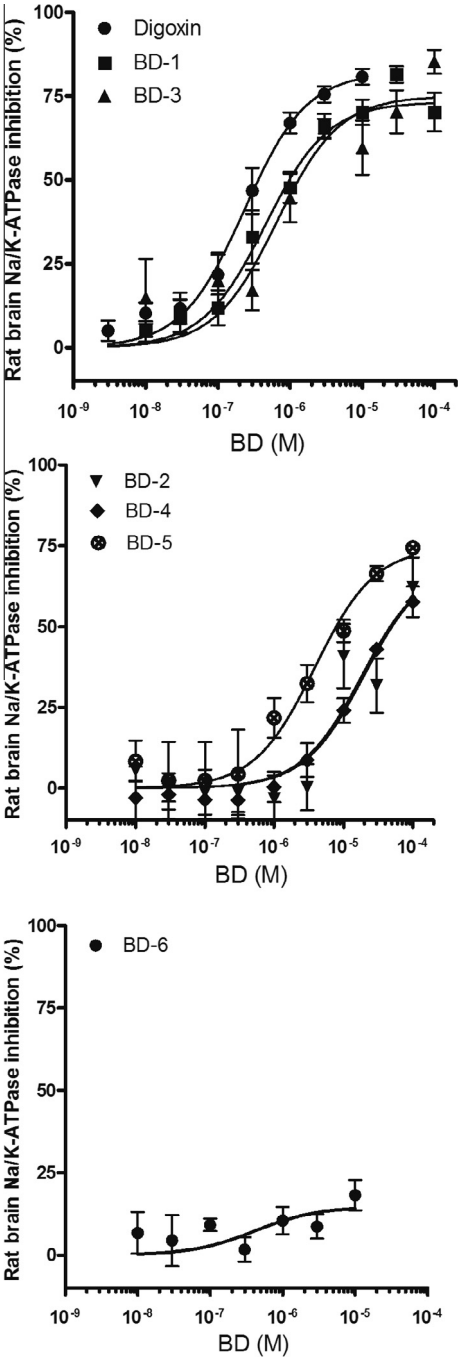

Figure 3. Inhibition of Na,K-ATPase from rat brain preparation. Average curves were obtained by non-linear regression analysis. Each point represents the IC_{50} ± SEM of 3–9 independent experiments performed in triplicate.

inhibition observed when testing digoxin and BD-1 to -6, for the three kinds of cells (HeLa, correlation coefficient $r^2 = 0.363$, $p = 0.282$; RKO, correlation coefficient $r^2 = 0.528$, $p = 0.164$; and WI-26 VA4 cells, correlation coefficient $r^2 = 0.602$, $p = 0.123$). These results suggest that cytotoxicity in these cells is not dependent on inhibition of the Na^+ pump and can be modulated by other mechanisms, such as the signaling pathways that were described to be mediated by Na,K-ATPase. In fact, different CS have been shown to induce Na,K-ATPase endocytosis at concentrations that do not inhibit the enzyme activity[35–37] and permanent depletion of plasma membrane Na,K-ATPase stimulates the cell cycle inhibitor $p21^{cyp}$ and triggers cell arrest and death.[37]

3.3. Molecular modeling

The search for a suitable template showed that the crystallographic structure of Na,K-ATPase of *Squalus acanthias* complexed with ouabain, PDB ID: 3A3Y with 2.80 Å of resolution, shared 89% of identity with human ATPase.[38] This high value of identity

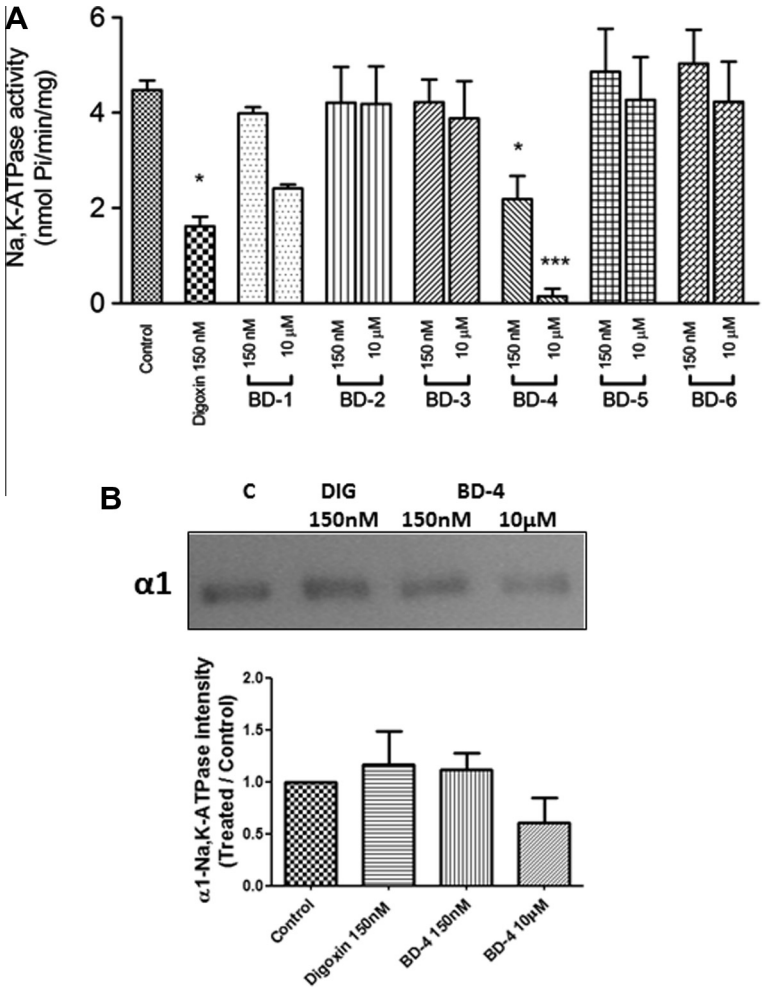

Figure 4. Na,K-ATPase activity in cancer cells. HeLa cells were incubated with digoxin and its derivatives for 24 h with 150 nM and 10 µM and membrane preparation was performed. Na,K-ATPase activity (A) or α1-Na,K-ATPase expression (B) were obtained. Each bar represents the mean ± SEM of 3–5 independent experiments (* = $p < 0.05$; *** = $p < 0.001$).

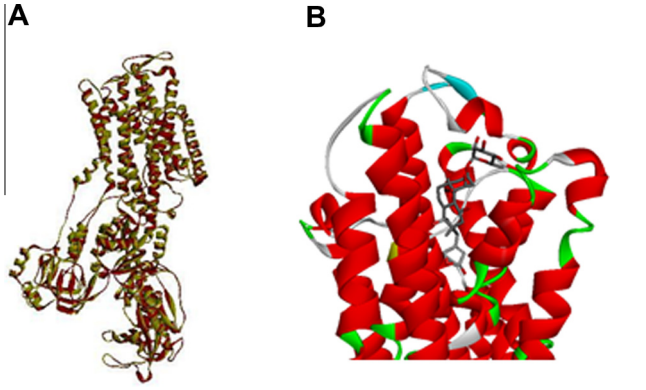

Figure 5. Crystallographic structure of Na,K-ATPase. (A) The superposition between the crystallographic structure 3A3Y (red) and the human model (yellow). (B) A close view of active site of human model (ribbons representation) complexed with ouabain (tube representation).

implies in low root-mean-square deviation (RMSD) value, which was 0.06 Å for α-carbons (Fig. 5). The model and 3A3Y has the same active site as well, which is composed for Glu319, Ile322, Phe323, Gly326, Ile327, Ile328, Val329, Ala330, Phe790, Phe793, Leu800, Thr804, Arg887 (Fig. 6).

Finally, the Ramachandran plot was used to check stereochemical accuracy of the model (Fig. 6). As can be seen, both model and template (Fig. 7A and B, respectively) had a similar plot, in which 88% and 12% of residues are in the most favorable region and in additional allowed region, respectively. Only Glu314 and Pro501 residues of the model are in generously allowed region and disallowed region, respectively. However, these residues are not present in the active site. In other words, the model was evaluated in relation to identity, RMSD value and the stereochemical quality, which the limits values are 30%, 1.2 Å and 90%. These results motivated to subsequent docking studies.

Initially, a re-dock was carried out in order to validate the method. As a result, the RMSD value for the superposition of crystallographic and docked ouabain was 1.59 Å (Fig. 8) in good accordance with values considered as satisfactory for re-docking (around 2.0). Next, all compounds were docked in the same conformation in the active site (Fig. 9). However, the lactone moiety of compound BD-3 changes the conformation in relation to the others. Table 3 shows the binding energy of all compounds studied. As can be seen, in general, all BD's can bind to the active site with a favorable binding energy and all of them have more affinity than ouabain and digoxin. Figure 9 illustrates the main intermolecular interaction between compounds BD-4 and BD-5 against human model, chosen as example due to experimental results. Docking results show that the aromatic moiety of all compounds reaches

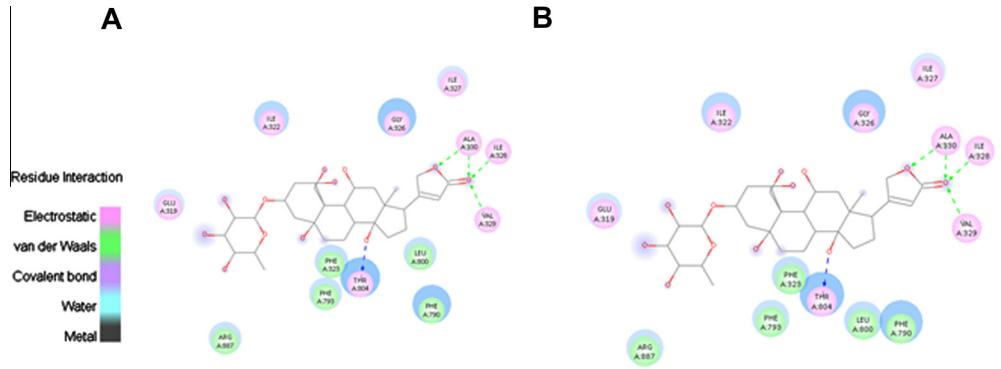

Figure 6. Active site of ATPases complexed with ouabain. (A) Human model; (B) 3A3Y. The dashed lines represent hydrogen bonds.

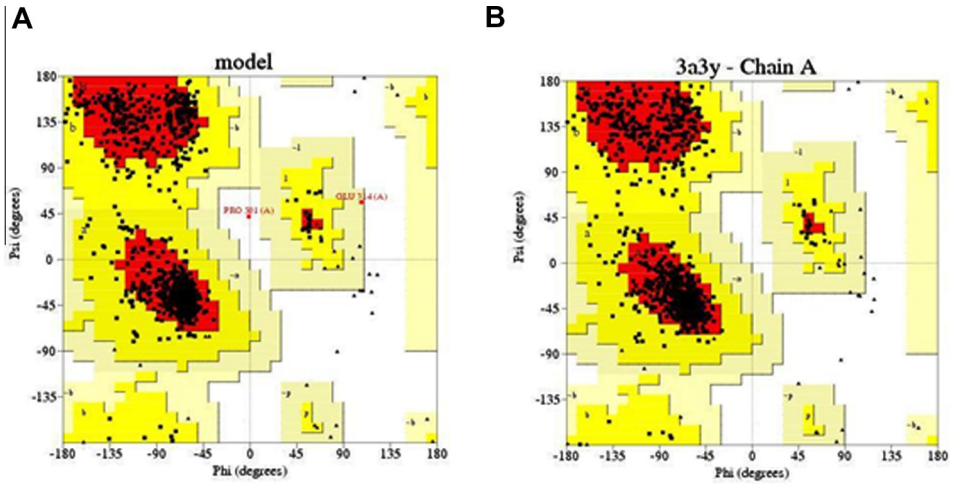

Figure 7. Ramachandran plot of Na,K-ATPase. (A) ATPase human model; (B) Crystallographic structure PDB code 3A3Y.

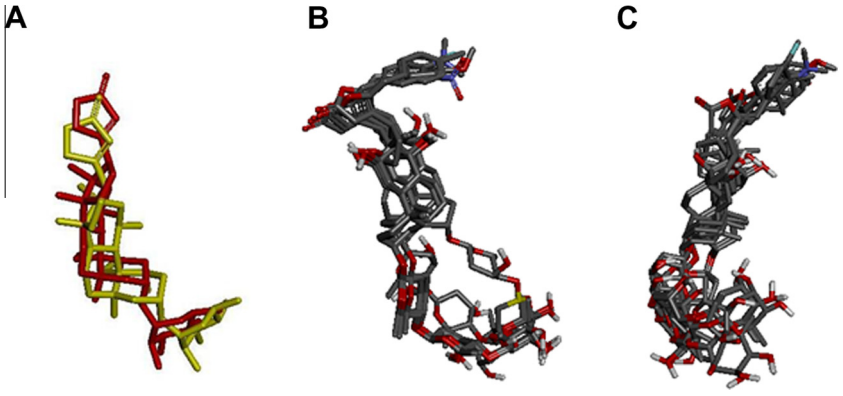

Figure 8. Re-docking of BD-1 to 6 on Na,K-ATPase. (A) Re-docking of ouabain in the human ATPase model. Red: crystallographic; Yellow: docked structure. (B) Cluster of *E*-BD's in the active site. (C) Cluster of *Z*-BD's in the active site.

a hydrophobic pocket of Na,K-ATPase carrying out a strong van der Waals interaction with Phe790. Note that the nitro polar moiety of BD-5 decreases the number of hydrophobic interactions so that it cannot assume a suitable fit in the active site.

4. Conclusion

In conclusion, we performed the synthesis of 6 butenolide derivatives of digoxin and showed that the dimethylamine benzylidene butenolide derivative (BD-4) is as potent as digoxin against cancer cells. In contrast to digoxin, however, BD-4 is a poor Na,K-ATPase inhibitor but after 24-h incubation downregulates Na,K-ATPase activity without altering its cellular expression, suggesting that the classical molecular mechanism of CS is not involved in the cytotoxic effect of this compound. The recently discovered Na,K-ATPase-mediated mechanisms, such as signal transduction by protein-protein interactions, is a matter that deserves further investigation.

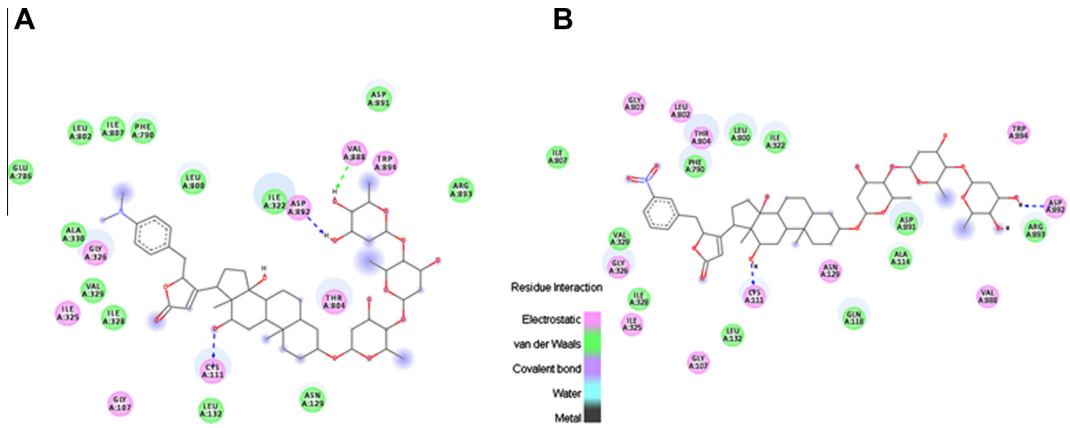

Figure 9. Pharmacophoric conformations of selected BD's. (A) BD-4; (B) BD-5. The dashed lines represent hydrogen bonds.

Table 3
Binding energy of BD's compounds on Na,K-ATPase

Compound	Binding energy (kcal/mol)
BD-1 (E/Z)	−8.6/−10.2
BD-2 (E/Z)	−9.6/−9.6
BD-3 (E/Z)	−9.0/−9.8
BD-4 (E/Z)	−9.5/−9.5
BD-5 (E/Z)	−9.3/−9.5
BD-6 (E/Z)	−8.8/−8.9
Ouabain	−8.5
Digoxin	−9.0

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.06.028.

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