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Effect of the digitoxigenin derivative, INCICH-D7, on Na^+, K^+-ATPase

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Abstract

Compound 14β,17β-cycloketoester-3β-OH androstane (INCICH-D7) is a semisynthetic product of a structural modification of the digitoxigenin molecule. INCICH-D7 has a heterocyclic ketoester type fusion between positions C14 and C17 of the steroid nucleus, which confers this molecule stronger electronegativity than that of digitoxigenin. INCICH-D7 retained positive inotropic effect, with a greater safety margin, when compared to digitoxigenin and ouabain. In this study we have examinated the INCICH-D7 effect on Na^+, K^+-dependent adenosinetriphosphatase (Na^+, K^+-ATPase) and compared these results with the ones observed with digitoxigenin and ouabain. The inhibitory effect of INCICH-D7 on Na^+, K^+-ATPase was five times lower (IC_{50} = 4 μM) than that of ouabain (IC_{50} = 0.8 μM) and 70 times lower than that of digitoxigenin (IC_{50} = 0.06 μM). The inhibitory effect of INCICH-D7 and ouabain on the enzyme was irreversible while digitoxigenin's one was reversible in up to an 80%. Our results indicate that inclusion of the heterocycle between positions C14 and C17 in the digitoxigenin molecule lowers significantly the inhibitory effect on Na^+, K^+-ATPase and renders the interaction between INCICH-D7 and enzyme irreversible under the studied reaction conditions.

Keywords: Na^+, K^+-ATPase; Digitoxigenin derivates; Digitalis; Cardioactive steroid; Ouabain; Cardiac glycosides

1. Introduction

Therapeutic treatment of cardiac failure includes, among other measures, the use of cardiac glycosides or digitalis compounds [1–4]. The narrow safety margin of digitalis compounds has represented up to now a serious problem due to the high frequency and severity of digitalis intoxication. Studies searching for a cardioactive steroid, with selective inotropic activity, have taken scientists to modify the structure of the existing digitalis compounds [4–9], stimulating the research on the design of new digitalis compounds.

The digitalis compound 14β,17β-cycloketoester-3β-OH androstane (INCICH-D7), has been synthesized from digitoxigenin in the Department of Pharmacology of the National Institute of Cardiology "Ignacio Chávez" in Mexico City. The compound lacks the lactone ring at position 17 and the OH in position 14, having instead a ketoester type cyclic fusion at position β giving rise to a new chemical heterocycle within the steroid structure (Fig. 1). Structural modifications carried out on digitoxigenin didn't modify positive inotropic effect, but conferred to the molecule a greater safety margin, in comparison with digitoxigenin [10].

Cardiac glycosides bind specifically to the Na^+, K^+-dependent adenosine triphosphatase (Na^+, K^+-ATPase) [E.C.3.6.1.3]. This enzyme catalyzes the active transport of Na^+ and K^+ ions through the plasmatic membrane of most animal cells [11–13], utilizing the chemical energy produced by the hydrolysis of ATP [12,13]. The most accepted hypothesis for the inotropic effect mechanism of action of these pharmacological agents on the myocardium involves a partial inhibition of the Na^+, K^+-ATPase. This inhibition raises the intracellular concentration of Na^+ affecting the Na^+/Ca^{2+} exchange in the sarcolemma, leading to an increase in intracellular Ca^{2+} and in cardiac contraction force [14,15]. Therefore, the participation of Na^+, K^+-ATPase is a key factor for the therapeutical benefits achieved with digitalis compounds.

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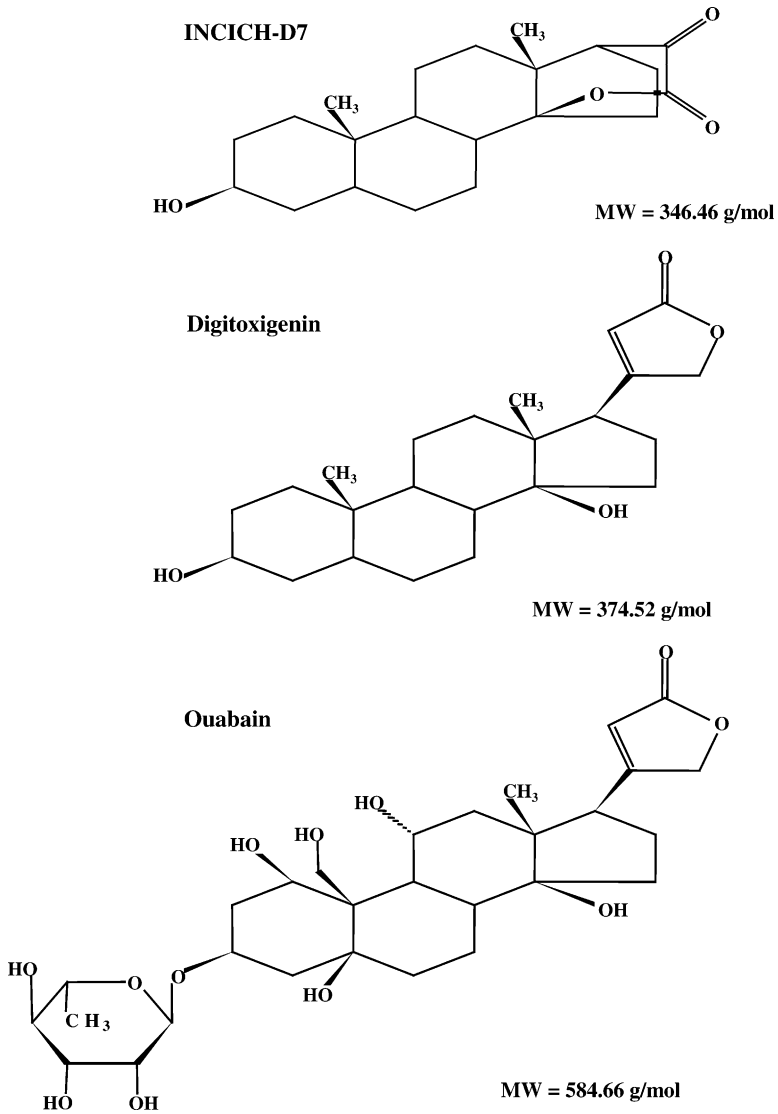

Fig. 1. Chemical structures of the digitalis compounds used in the study.

The aim of this study was to assess the effect of this new digitalis compound (INCICH-D7) on the hydrolytic activity of Na^+, K^+-ATPase, and compare these results with digitoxigenin and ouabain.

2. Material and methods

2.1. Materials

Ouabain and digitoxigenin were purchased from Sigma Chemical Co., St. Louis, MO, USA. The INCICH-D7 (Fig. 1) was synthesized from digitoxigenin [10] and its structure was determined through selective chemical reactivity tests [16], infrared spectroscopy (Duhamm's method) [17], and X-ray diffraction crystallography [18].

Ouabain was dissolved in dimethyl sulfoxide (DMSO), digitoxigenin and INCICH-D7 were dissolved in 7% ethanol–water. Concentration of the solvent in the reaction medium was less than 1% and did not affect the activity of the enzyme.

2.2. Purification of Na^+, K^+-ATPase

External medulla of Mongrel dog's kidneys was used as a source of Na^+, K^+-ATPase. Purification was accomplished following the method described by Jorgensen [19]. Preparations with an average specific activity of 6 μmol Pi/min per milligram of protein were obtained and more than 95% was ouabain-sensitive. Protein concentration of each preparation was determined by the Lowry method [20].

2.3. Na^+, K^+-ATPase activity

Na^+, K^+-dependent hydrolysis of ATP was assayed at 37 °C in a medium containing: 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 5 mM NaN_3, 50 mM TEA–HCl, pH 7.4, 2.5 mM ATP–Tris, in a final volume of

1 ml. The enzyme (7 μg/ml) was preincubated for 5 min, in the reaction medium without ATP, reaction was started by adding ATP–Tris. Reaction time lasted 10 min. ATP hydrolysis was linear with time (ATP hydrolysis was 10–20%). Reaction was stopped by adding an equal volume of ice-cold 10% trichloroacetic acid (TCA). The tubes were then centrifuged at 3000 rpm for 5 min. The hydrolized ATP was measured by determining inorganic phosphate (Pi) released according to the method of Taussky and Shorr [21]. The activity inhibitable by 50 μM ouabain corresponded to the Na^+, K^+-dependent ATPase. Control reactions were assayed simultaneously in the absence of the enzyme to measure ATP hydrolysis at times 0 and 10 min. TCA was added before ATP–Tris at time 0. The hydrolysis obtained under these conditions was subtracted from the ATP hydrolysis in the presence of the enzyme.

2.4. Na^+, K^+-ATPase inhibition by digitalis compounds

The effects of ouabain, digitoxigenin, and INCICH-D7 were determined in the standard reaction medium at different concentrations (0.1 nM–1 mM). The enzyme (7 μg/ml) was preincubated for 10 min at 37 °C in the reaction medium with ouabain, digitoxigenin or INCICH-D7. The reaction was started by adding ATP (2.5 mM), lasted for 10 min, and stopped by adding an equal volume of 10% iced-cold TCA. Pi released was measured as mentioned above.

The data are presented as the percentage activity remaining at a particular digitalis concentration relative to the total Na^+, K^+-ATPase activity in the absence of the inhibitor. Percentage values were plotted as a function of the logarithm of the concentration of the digitalis compounds, and the IC_{50} values were obtained from these curves. IC_{50} is the inhibitor's concentration that inhibits 50% of the Na^+, K^+-ATPase activity.

To obtain the K_i of the INCICH-D7's inhibitory effect, two fixed concentrations were used, 2.25 and 6.3 μM which inhibited, respectively, 30 and 70% the Na^+, K^+-ATPase activity in the presence of Na^+, K^+, Mg^{2+}, and increasing ATP concentration (0–4 mM).

The K_i value was obtained substituting the V_{max} values obtained from the double reciprocals graph in the equation:

$$\frac{1}{V_{max_{ap}}} = \frac{(1 + [I]/K_i)}{V_{max}}$$

where V_{max} is the maximal hydrolysis velocity in the absence of INCICH-D7 and $V_{max_{ap}}$ is the maximal hydrolysis velocity obtained in the presence of INCICH-D7.

2.5. Reversibility study

The reversibility of the inhibitory action was determined by the method described by Ross and Pessah [22]. Briefly, the enzymatic preparation (60 μg protein) was incubated with INCICH-D7, ouabain or digitoxigenin (0.1 mM) in 1.0 ml of a solution containing: 80 mM NaCl, 2 mM ATP–Na_2, 2 mM $MgCl_2$, 5 mM NaN_3, 1 mM EDTA, 50 mM TEA, pH 7.5 for 20 min at 37 °C. At the end of the incubation time, the mixture was diluted 10 times in a solution containing: 1 mM EDTA, 10 mM TEA pH 7.5 (TEA–EDTA) and immediately placed on ice. Before dilution, a 100 μl aliquot was removed to determine Na^+, K^+-ATPase activity in the standard reaction medium. The diluted mixture was centrifuged at 100,000 × *g* at 4 °C for 45 min in a Sorval Ultra Pro 80 (T-880 rotor) ultracentrifuge. The pellet was resuspended in 5 ml TEA–EDTA and centrifuged again at 100,000 × *g* for 45 min, the supernatant was discarded and the pellet was resuspended in 0.15 ml of a solution of 25 mM Imidazol–1.0 mM EDTA, pH 7.5 to measure Na^+, K^+-ATPase activity and determine proteins. Controls of enzymatic activity before and after washing were simultaneously performed in the absence of digitalis compounds.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.) of three different experiments. Statistical evaluation of the data was performed using ANOVA followed by Tukey's test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Effect of INCICH-D7 on the hydrolytic activity of Na^+, K^+-ATPase

The incubation of purified Na^+, K^+-ATPase with Na^+, K^+, Mg^{2+} and ATP showed a mean basal hydrolitic activity 6 μmol/Pi/min/mg Prot (100% activity). The incubation of Na^+, K^+-ATPase with INCICH-D7 (0.1 nM–1.0 mM) produced an inhibition of the hydrolitic activity starting at a concentration as low as 0.1 μM. The concentration-response curve followed a sigmoidal path and reached maximal inhibition (88%) at 0.1 mM concentration (Fig. 2).

To obtain the K_i of the INCICH-D7's inhibitory effect, two fixed concentrations were used, 2.25 and 6.3 μM which inhibited, respectively, 30 and 70% the Na^+, K^+-ATPase activity in the presence of Na^+, K^+, Mg^{2+}, and increasing ATP concentrations. Fig. 3 depicts the velocity values of ATP hydrolysis (in μmol Pi/min/mg Prot.) obtained for the control reaction (absence of INCICH-D7) and in its presence at 2.25 and 6.3 μM concentrations in relation to ATP concentration. ATP hydrolysis velocity is lower at greater digitalis compound concentration. Values of hydrolysis velocity were plotted as double reciprocal (Fig. 4a) to obtain V_{max} and K_s of the ATP

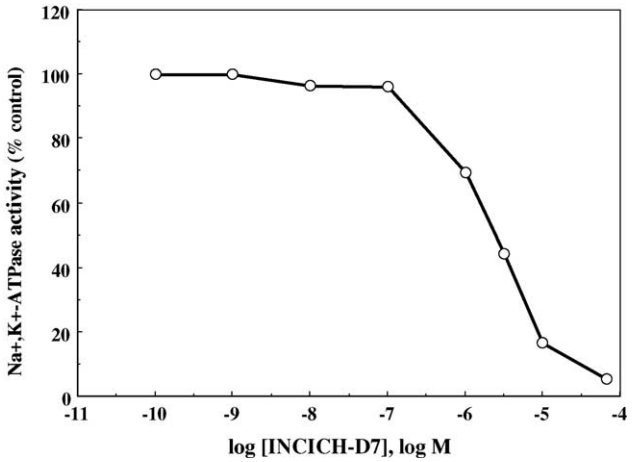

Fig. 2. Concentration-response curve for the INCICH-D7-induced inhibition of Na^+, K^+-ATPase activity. Data represent the percentage of Na^+, K^+-ATPase activity remaining at the indicated INCICH-D7 concentrations relative to total activity in the absence of the compound. Values represent the average of three assays ±S.D.

activator effect in the presence and absence of INCICH-D7. The V_{max} and K_s values are presented in Table 1, which shows that the maximal hydrolysis velocity decreases with increasing concentrations of INCICH-D7, whereas K_s remains practically constant.

K_i value was obtained by substituting the V_{max} values as indicated under Material and Methods. The K_i value obtained was 4 μM. Plotting the ATP hydrolysis velocity values in the presence and absence of INCICH-D7 in the Hanes-Woolf graph (Fig. 4b) revealed that the INCICH-D7 interaction does not affect the affinity of the enzyme for the substrate (ATP) since the three straight lines intercept at the "*X*" axis yielding the same K_m value for ATP (K_m = 0.4 mM).

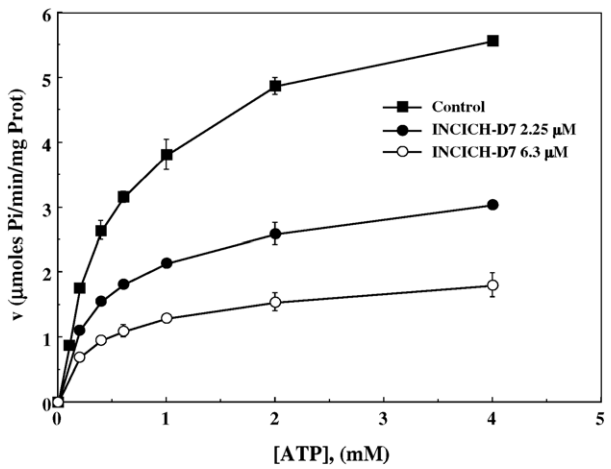

Fig. 3. Effect of INCICH-D7 on Na^+, K^+-ATPase activity. Hydrolysis velocity (v) of ATP in the presence and absence of INCICH-D7. Reaction conditions are as described under Section 2. Significance: control vs. INCICH-D7 6.3 μM $p < 0.001$, control vs. INCICH-D7 2.25 μM $p < 0.01$, INCICH-D7 2.25 vs. 6.3 μM $p < 0.05$.

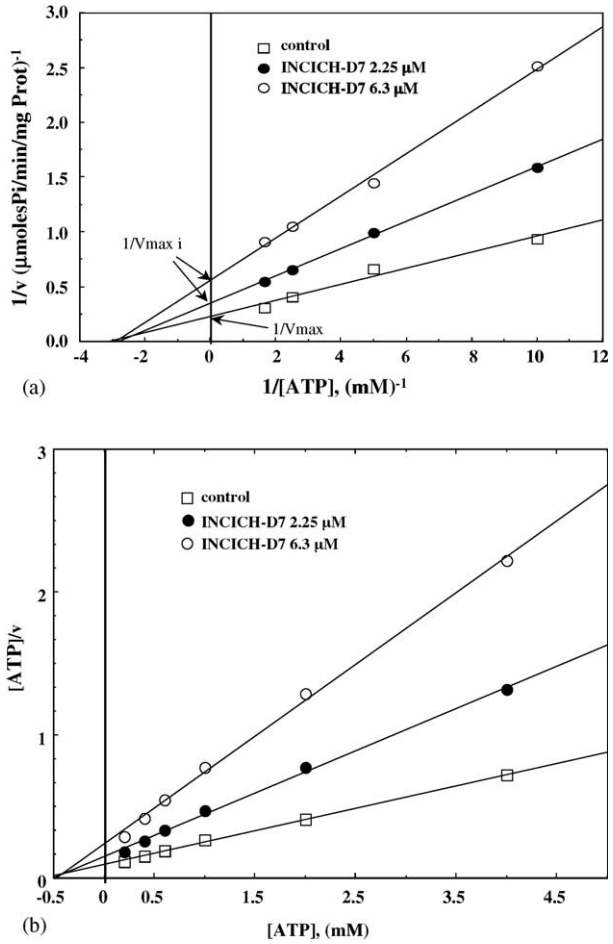

Fig. 4. Effect of INCICH-D7 on ATP hydrolysis. (a) Double reciprocal (1/v vs. 1/[ATP]) Lineweaver-Burk plot. (b) Hanes-Woolf representation of the ATP hydrolysis velocity values. The ATP hydrolysis velocity values were measured in the absence (control) and presence of fixed concentrations of INCICH-D7 (2.25 and 6.63 μM).

3.2. *Effects of INCICH-D7, digitoxigenin, and ouabain on Na^+, K^+-ATPase*

The inhibitory effect of INCICH-D7 was compared with that of digitoxigenin, from which it had been synthesized, and with the digitalis glycoside ouabain. Fig. 5 shows the percentage values of Na^+, K^+-ATPase activity in relation to the concentration of the studied

Table 1
Effect of INCICH-D7 on the activation of Na^+, K^+-ATPase by ATP

INCICH-D7 (μM)	K_s (mM)[a]	V_{max}[b]
0	0.33	4.459
2.25	0.36	2.884
6.30	0.35	1.795

Kinetic constants obtained for the ATP activating effect in the presence of INCICH-D7. Reaction conditions were standard for Na^+, K^+-ATPase. The ATP concentrations used are indicated in Fig. 3.

[a] K_m for ATP.

[b] Maximal hydrolysis velocity in μmol Pi/min/mg of protein.

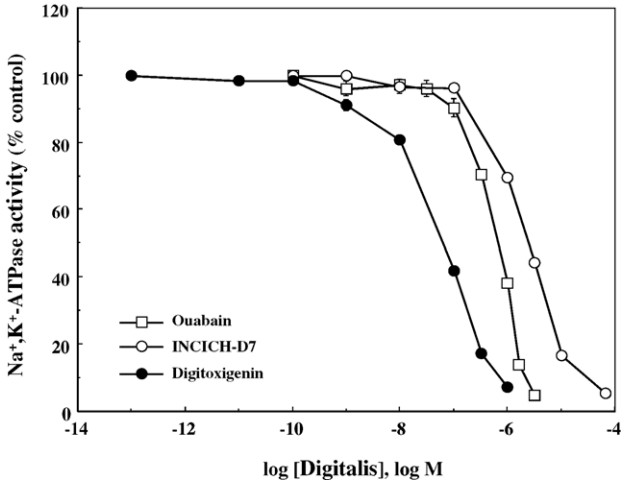

Fig. 5. Effects of digitalis compounds on Na^+, K^+-ATPase activity. Data represent the percentage of Na^+, K^+-ATPase activity remaining by the effect of the indicated digitalis concentrations; 100% corresponds to the total activity in the absence of the inhibitor. Plotted values represent the mean of three assays ±S.D. Reaction conditions are described under Section 2. Significance: digitoxigenin vs. INCICH-D7 $p < 0.001$, ouabain vs. INCICH-D7 $p < 0.01$.

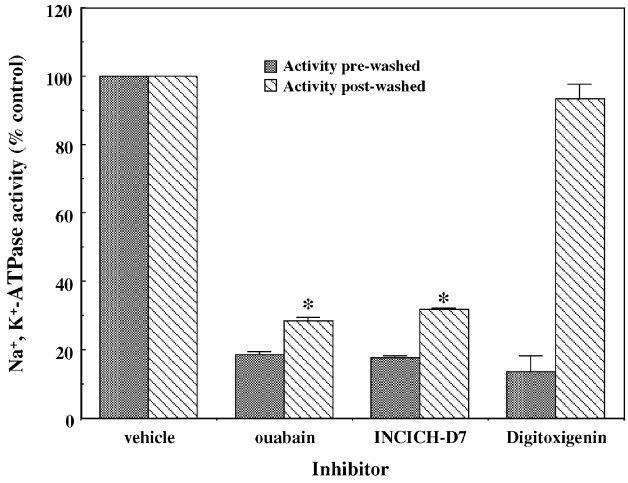

Fig. 6. Reversibility of the inhibitory effect of digitalis on Na^+, K^+-ATPase activity. Stability of the binding between the enzyme and the digitalis compounds was measured under conditions that favor the formation of the E_2P enzymatic complex. The reaction was considered reversible when the activity was recovered after dilution of the reaction medium and washing. Details and conditions are described under Section 2. *Significance: ouabain vs. digitoxigenin $p < 0.001$, INCICH-D7 vs. digitoxigenin $p < 0.001$.

digitalis compounds. The concentration-response curve obtained for INCICH-D7 is displaced to the right compared to the inhibition curves of ouabain and digitoxigenin. Differences between the obtained curves with INCICH-D7 and digitoxigenin ($p < 0.001$) are greater to the observed with INCICH-D7 and ouabain ($p < 0.01$). The IC_{50} for ouabain was 0.8 μM while digitoxigenin was 0.06 μM. Comparing these values with the K_i (4 μM) for INCICH-D7, it is evident that the concentration of INCICH-D7 needed to achieve a 50% inhibition is five times higher than that of ouabain and 70 times higher than that of digitoxigenin.

3.3. Study on the reversibility of the inhibitory action

The presence of Na^+, Mg^{2+}, and ATP in the reaction medium facilitates the formation of the E_2P enzymatic conformation of Na^+, K^+-ATPase, which has great affinity for digitalis compounds. Fig. 6 depicts the effect of INCICH-D7, ouabain, and digitoxigenin on the enzymatic activity under these conditions, before and after dilution and washings; the enzymatic activity in the absence of the corresponding digitalis compound was taken as 100%, which did not change in response to dilution or washing. The three compounds inhibited the enzymatic activity in approximately the same proportion: ouabain, 81%; INCICH-D7, 82.34%, and digitoxigenin, 86.34%. Dilution of the mixture and washing induced an 80% recovery of the activity in the enzyme incubated with digitoxigenin, whereas the enzyme incubated with either ouabain or INCICH-D7 recovered only 10 and 14% of the activity, respectively (observed differences had significance, $p < 0.001$).

4. Discussion

Synthesis of new digitalis compounds is aimed mainly at obtaining a pharmacological compound with a wide safety margin.

In a previous study by Del Valle et al. [10] the chemical and pharmacologic properties of INCICH-D7 were described. The pharmacologic studies performed on isolated guinea pig hearts (Langendorff preparation) show that this new compound has a positive inotropic effect 2.8 times stronger than that of digitoxigenin. In this study it is also demonstrated that INCICH-D7 reverts completely the heart failure induced experimentally in vivo in dog hearts (Starling cardiopulmonary preparation) with a safety margin 2.58 times wider than that of digitoxigenin (safety margin = lethal dose/minimal therapeutic dose) [10].

The modification of the digitoxigenin molecule by replacing the lactone ring at position 17 and the OH at position 14 by the ketoester group and, thus, forming a new cycle between positions 17 and 14, as shown in Fig. 1, did not affect the inhibitory capacity of the steroid nucleus on the Na^+, K^+-ATPase activity, the enzyme identified as the pharmacological receptor of digitalis compounds [9,23]. Interaction of INCICH-D7 with the enzyme did not modify its affinity for the natural substrate (ATP), as observed in the double reciprocal and Hanes-Woolf graphs (Fig. 4a and b, respectively) in both, the K_m value for ATP remained the same, independently from the presence of the digitalis compound. This kinetic behavior corresponds to a non-competitive inhibitor [24], which has been reported for digitalis compounds [25]. Digitalis compounds do not interrupt the catalytic cycle of the enzyme by occupying

the site of the binding effectors of the enzyme, but rather by binding to a specific conformational state and, thus, avoiding a change in the conformation of the enzyme and continuation of the cycle [26].

The inactivation kinetics on Na^+, K^+-ATPase by INCICH-D7, although following a parallel path to that of digitoxigenin, reveals that its affinity for the enzyme is significantly lower since the concentration required for INCICH-D7 to inhibit 50% the hydrolytic activity is 70 times higher than that needed for digitoxigenin to obtain the same effect. The affinity of INCICH-D7 for the enzyme is closer to that shown by ouabain, but this does not mean that the interaction with the binding sites for ouabain is similar. Although, the steroid nucleus is the same, the substituents of the molecules are different since ouabain has more functional groups to interact with the enzyme (Fig. 1).

The changes made to the digitoxigenin molecule, i.e., substituting the lactone group and the OH at C14 by the cyclic ketoester group, provided larger electronegativity to the new compound [10]; this characteristic grants it less affinity for Na^+, K^+-ATPase, but probably with very slow liberation kinetics as shown by the reversibility studies, in which enzymatic activity was not recovered even after washing. The reaction conditions in the reversibility study used high concentrations of digitalis compounds with a reaction time of 20 min, favoring completely the enzymatic form E_2P (absence of K^+) which shows greater affinity for digitalis, and it is possible that in an ''in vivo'' situation this inhibitory effect could revert. Although the safety margin for INCICH-D7 is wider than the one of the original molecule, extensive studies are being conducted to evaluate the safety of this compound.

Studies on the pharmacological effects of this compound and digitoxigenin done in dog and guinea pig isolated hearts demonstrate that the inotropic effect and the safety margin for INCICH-D7 are superior to the ones of digitoxigenin [10] while the inhibitory action on the enzyme is weaker (70 times), and there is no proportional relationship between this inhibitory action and the inotropic effect. Consequently, we believe that this compound could be acting through two mechanisms: one is the inhibition of Na^+, K^+-ATPase, and a possible interaction with a Ca^{2+} releasing mechanism from the sarcoplasmic reticulum which produces the inotropic effect with a wider safety margin, as has been suggested by Wasserstrom et al. [27] to explain the mechanism of action of the semisynthetic cardiac steroid actodigine.

We conclude that the synthesized digitalis compound INCICH-D7 preserves the inhibitory capacity on Na^+, K^+-ATPase in a non-competitive way.

Substitution of the lactone ring at position 17 and the OH at position 14 by the heterocycle in the digitoxigenin molecule induces a marked decrease in its inhibitory capacity upon Na^+, K^+-ATPase.

The modifications made to the digitoxigenin molecule render irreversible the interaction between the compound and the enzyme under the reaction conditions studied.

INCICH-D7 may be showing a combined cellular mechanism related to the inotropic effect of digitalis.

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