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2-Methoxy-3,8,9-trihydroxy coumestan: a new synthetic inhibitor of Na^+,K^+-ATPase with an original mechanism of action

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Abstract

The aim of the present work was to analyse the interaction between Na^+,K^+-ATPase and one of our recent synthesized coumestans, namely the original molecule 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36). Rat brain (mainly α2 and α3 Na^+,K^+-ATPase isoforms) and kidney (α1 isoform) fractions enriched with Na^+,K^+-ATPase were utilized to compare the inhibition promoted by PCALC36 with that of classical inhibitors like ouabain and vanadate. Analysis of inhibition curves revealed that unlike ouabain, which was about a thousand times more potent to inhibit brain isoforms than kidney isoform, PCALC36 had a similar affinity for brain ($IC_{50} = 4.33 \pm 0.90$ μM) and kidney ($IC_{50} = 11.04 \pm 0.86$ μM) isoforms. The inhibitory effect of PCALC36 was not antagonized by 1–10 mM K^+, as observed with ouabain. Whereas vanadate was more potent in ionic conditions promoting the E2 conformation of the enzyme, the inhibitory effect of PCALC36 was equal in ionic conditions favouring either the E1 or E2 conformations. Equilibrium binding assays with [^{3}H]ouabain revealed that the addition of 2–10 μM PCALC36 did not change the K_d of ouabain but decreased its maximal binding (B_{max}) in a concentration-dependent manner (from 76.6 to 44.0 pmol/mg protein). This inhibitory effect of PCALC36 was not reverted after an extensive washing procedure indicating that it forms a very stable complex with Na^+,K^+-ATPase. We conclude that PCALC36, a new molecule with a non-steroidal skeleton, inhibits the Na^+,K^+-ATPase by a mechanism of action different from the cardiac glycosides and could thus serve as a structural paradigm to develop new inotropic drugs.

Keywords: ATPase-Na^+,K^+; Cardiac glycoside; Coumestan; Inotropic

1. Introduction

Since 1785, the cardiac glycosides have been widely used in the treatment of congestive heart failure because of their positive inotropic effect and beneficial effects on hemodynamics. The molecular mechanism of action of these drugs was suggested for the first time in 1963, when Repke described the Na^+,K^+-ATPase as the receptor for cardiac glycosides [1]. This enzyme is a membrane-bound protein that maintains the low Na^+/high K^+ cellular concentrations using the energy derived from the hydrolysis of ATP. Further studies indicated that the inhibition of Na^+,K^+-ATPase promotes an increase in intracellular Na^+ concentration, decreasing the driving force for the extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchanger. As a consequence, there is an increase of the intracellular Ca^{2+} pumped into the sarcoplasmic reticulum, increasing the intracellular stores which, when released, strengthen subsequent contractions [2].

Although new therapies improving morbidity and mortality from chronic heart failure have emerged (e.g. angiotensin-converting enzyme inhibitors), digoxin continues to be indicated, mainly in combined therapy [3], due to its positive effect on morbidity and its neutral effect on mortality [4]. These positive effects of digoxin, confirmed

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]-benzene; PPO, 2,5-diphenyloxazole; Tris, tris(hydroxymethyl)aminomethane; DMSO, dimethyl sulfoxide.

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in the withdrawal studies PROVED and RADIANCE [5,6] and the very large trial sponsored by the NIH [7], explain its widespread use in moderate and severe congestive heart failure [6]. However, the adverse effects and very low therapeutic index of cardiac glycosides difficult their use. The inadequacy of currently available drugs and the prevalence of congestive heart failure, that is high in the developed countries and increasing in developing countries, renewed the interest of searching new inotropic compounds acting through the inhibition of Na^+,K^+-ATPase [8].

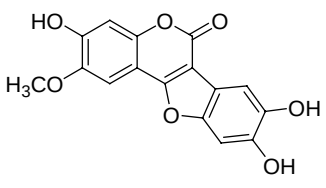
As the cause of failure to discover digoxin-like inotropes with improved therapeutic range has been attributed to the preservation of the C/D-*cis* junction of steroid backbone unique to digitalis steroid [8], different attempts are actually performed with altered steroid-like compounds [9–11]. Looking for non-steroidal structures potentially able to interact with Na^+,K^+-ATPase through a mechanism different from digoxin, we recently described that wedelolactone and its new synthetic analogues were able to inhibit Na^+,K^+-ATPase activity [12]. This was the first register of Na^+,K^+-ATPase inhibition by coumestans, a special class of isoflavonoids, of particular interest since they are non-steroidal molecules.

The aim of the present work was to analyse the interaction between Na^+,K^+-ATPase and one of our recently synthesized coumestans, namely the original molecule 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36). Present results indicate that PCALC36 inhibits the Na^+,K^+-ATPase by a mechanism different from the classical inhibitors as cardiac glycosides and vanadate, further demonstrating the interest of searching new structural pattern for inhibitors of this therapeutically relevant target.

2. Materials and methods

2.1. Synthesis of 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36)

The compound PCALC36 (Fig. 1) was prepared according to the methodology described previously for the synthesis of five coumestans with different patterns of oxygenation in rings A and D [12], using available benzaldehydes, isovanillin and piperonal, as starting materials. PCALC36 was dissolved in 100% DMSO to obtain a 30 mM stock solution. The final concentration of DMSO in the assay never exceeded 0.3% (v/v).



PCALC 36

Fig. 1. Structure of PCALC36.

2.2. Preparation of Na^+,K^+-ATPase from brain and kidney

Adult male Wistar rats were killed by decapitation and their brain hemispheres and kidneys were rapidly excised and stored at −80°. Preparations enriched in Na^+,K^+-ATPase were obtained by chaotropic treatment with 2 M KI for 1 hr and 0.1% DOC (sodium deoxicholate) overnight, followed by differential centrifugation, as earlier described [13]. The protein concentration was measured according to the method of Lowry *et al*. [14] using bovine serum albumin as the standard. The maximal Na^+,K^+-ATPase activity was about 75 μmol Pi mg/protein·hr^{-1}, corresponding to 84% of the total ATPase activity in brain preparation and 60 μmol Pi mg/protein·hr^{-1}, corresponding to 70% of the total ATPase activity, in kidney.

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

The Na^+,K^+-ATPase activity was determined by the Fiske and Subbarow method [15] with slight modifications, as described by Noël and Pardon [16]. The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain (ouabain-resistant activity). The quantity of protein was adjusted in order to hydrolyse no more than 10–15% of the substrate during the incubation period. The reaction was started by addition of the brain or kidney preparation, incubated at 37° for 2 hr, in a total volume of 0.5 mL. Unless otherwise stated, the incubation was performed in the presence of 84 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1.2 mM $ATPNa_2$, 2.5 mM EGTA, 10 mM sodium azide and 20 mM maleic acid buffered to pH 7.4 with Tris.

Inhibition curves were obtained in the presence of increasing concentrations of PCALC36 (1–100 μM for brain and kidney) or ouabain (0.01–10 μM for brain and 50–1000 μM for kidney).

2.4. Binding of [^{3}H]ouabain

The incubation was carried out at 37°, in a Mg–ATP–Na medium (3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 10 mM sodium azide, 3 mM $ATPNa_2$, 20 mM maleic acid–Tris pH 7.4) in the presence of 10 nM [^{3}H]ouabain (15 Ci/mmol, DuPont NEN).

Saturation experiments were performed by the addition of increasing concentrations of unlabeled ouabain (10–200 nM), since this protocol, classically referred as a competition experiment, allows a great economy of radioligand and preparation. The non-specific binding was determined in the presence of 1 mM ouabain. The reaction was started by the addition of 15 μg of brain hemispheres preparation, stopped after 2 hr by the addition of an ice-cold buffer (5 mM Tris–HCl, pH 7.4) and rapidly filtered under vacuum through glass fiber filter (Filtrak GMF 3).

Filters were washed twice with 10 mL of the same buffer, dried and added to 10 mL of scintillation cocktail consisting of toluene, 0.1 g/L POPOP and 4.0 g/L PPO. The radioactivity retained in the filters was counted in a Tri–Carb liquid scintillation analyser (Packard Instrument Co.).

In the dissociation assay, about 165 μg of brain protein were incubated at 37° in Mg–ATP–Na medium containing 10 nM [^{3}H]ouabain (final volume 5.5 mL) for 2 hr. The incubation was followed by isotopic dilution with 100 μM ouabain alone (control) or with 30 μM of PCALC36. Aliquots of 500 μL were filtered after established periods of time, as described above.

To investigate the stability of the PCALC36–Na^+,K^+-ATPase complex, the brain preparation was pre-incubated at 37° in 14 mL of Mg–ATP–Na incubation medium in the absence (control) or presence of PCALC36 (30 μM) and diluted to 30 mL with Mg–ATP–Na medium after 2 hr. The protein was recovered by centrifugation at 100,000 *g* for 1 hr, washed by resuspension in Mg–ATP–Na medium and incubated in 30 mL of the same medium for 30 min at 37° followed by a second centrifugation under the same conditions described above [17]. Final pellets were used to measure the binding of [^{3}H]ouabain as previously described.

2.5. Analysis of the results

Binding data were graphically represented using Scatchard plot. The parameters B_{max} and K_d were calculated using a computerized non-linear regression analysis of the untransformed data (Prism®, GraphPad Software Inc., version 1.03), assuming a single population of binding sites. The same program was used to fit inhibition curves, assuming a sigmoidal dose–response curve model.

3. Results

3.1. Selective inhibition of Na^+,K^+-ATPase isoforms

In order to evaluate the potency of PCALC36 to inhibit the α2/α3 and α1 Na^+,K^+-ATPase isoforms, we performed inhibition assays using preparations enriched in Na^+,K^+-ATPase from rat brain and kidney, respectively. As shown in Fig. 2A, ouabain exhibited a higher potency to inhibit the Na^+,K^+-ATPase present in the brain preparation than in the kidney (IC_{50} = 0.0886 ± 0.0075 μM and 69.94 ± 8.52 μM, respectively). The full inhibition of kidney Na^+,K^+-ATPase is compatible with the presence of only the low-affinity isoform α1 in this tissue, whereas the nearly full inhibition of brain Na^+,K^+-ATPase at low ouabain concentrations indicates that our brain preparation contains mainly the high-affinity isoforms α2/α3. In contrast with ouabain, PCALC36 inhibited all isoforms with similar potency, as indicated by IC_{50} values of 4.333 ± 0.898 μM and 11.04 ± 0.861 μM for brain and kidney, respectively (Fig. 2B).

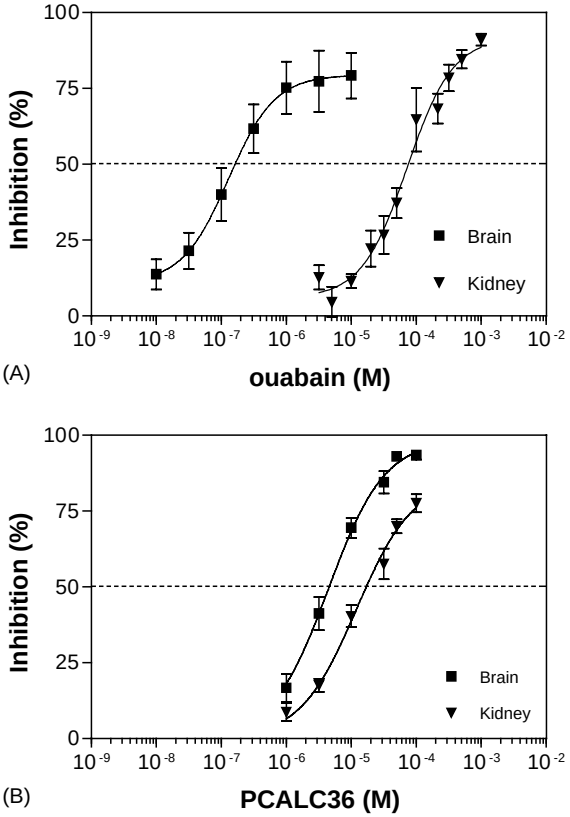


Fig. 2. Inhibition curves of Na^+/K^+-ATPase from brain and kidney preparations by ouabain (A) and PCALC36 (B). Results were expressed as percent of the inhibition measured in the presence of 1 mM ouabain (mean ± SEM) and were obtained from four experiments performed in triplicate. Curves were drawn using the parameters fitted by non-linear regression analysis with the model of sigmoidal dose–response curve.

3.2. Potassium effect

Antagonism by K^+ is a classical mechanism to decrease the biochemical and clinical effects of cardiac glycosides [18–20]. As shown in Fig. 3A, increasing K^+ concentration from 1 to 10 mM progressively antagonized ouabain effect, as evidenced by a shift to the right of the ouabain inhibition curves (increase of IC_{50} from about 30 to 100 μM). On the contrary, the increasing of K^+ concentration did not affect the inhibition promoted by PCALC36, as evidenced by the overlap of the inhibition curves (Fig. 3B).

3.3. Inhibition by vanadate

Vanadate, a classical inhibitor of P-type ATPases, is more potent to inhibit Na^+,K^+-ATPase when in the E2 conformation [16,18], that predominates in the presence of low Na^+, high Mg^{2+} and high K^+ concentrations. Figure 4A shows that a difference of potency of about 25-fold is observed for vanadate when comparing the ionic conditions favouring E1 or E2 conformations. On the other hand, inhibition curves of PCALC36 were identical in both ionic conditions (Fig. 4B).

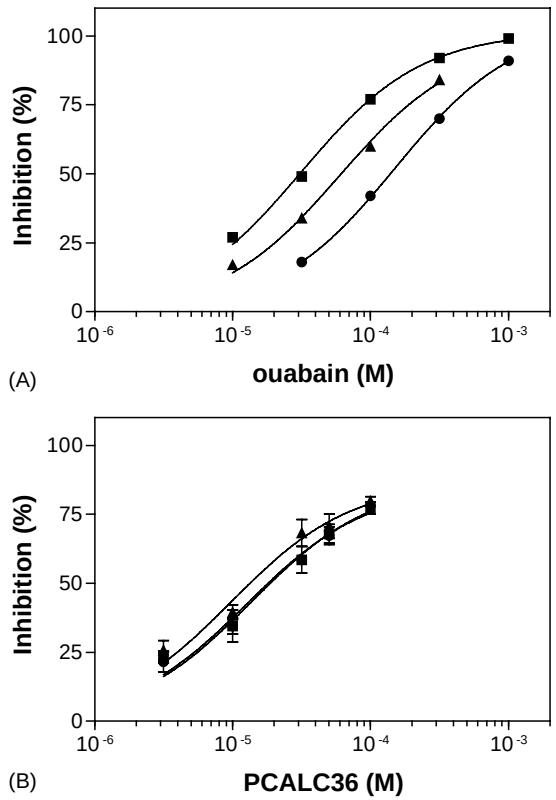


Fig. 3. Effect of potassium on inhibition curves of rat kidney Na^+/K^+-ATPase by ouabain and PCALC36. Effects of ouabain (A) and PCALC36 (B) were measured at three different concentrations of KCl, namely 1 mM (■), 3 mM (▲) and 10 mM (●). Results were expressed as percent of the inhibition measured in the presence of 1 mM ouabain (mean ± SEM) and were obtained from four experiments performed in triplicate. The fitted curves were obtained by non-linear regression using the model of sigmoidal dose–response curve.

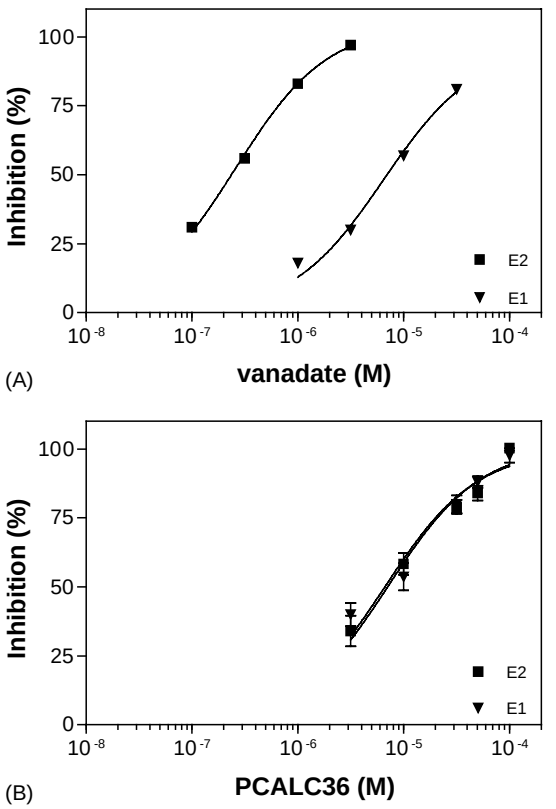


Fig. 4. Inhibition curves of rat kidney Na^+/K^+-ATPase by vanadate and PCALC36. Two different ionic conditions were used in order to favour E1 (▼) or E2 (■) conformation of the enzyme: 100 mM NaCl, 3 mM $MgCl_2$ and 3 mM KCl (E1); 30 mM NaCl, 15 mM $MgCl_2$ and 20 mM KCl (E2). In both ionic conditions 3 mM ATP was used. The effect was reported as percent of Na^+,K^+-ATPase inhibition obtained in the presence of 1 mM ouabain. Curves were fitted by non-linear regression analysis using the model of sigmoidal dose–response. Each point represents the mean ± SEM of three experiments performed in triplicate.

3.4. Effect of PCALC36 on [^{3}H]ouabain binding at equilibrium

As biochemical results suggested that PCALC36 has a different pattern of inhibition from classical inhibitors, we performed binding studies in order to investigate the mechanism of PCALC36 interaction with the Na^+,K^+-ATPase. Saturation curves of [^{3}H]ouabain were carried out in the absence and presence of increasing concentrations of PCALC36, and these results were expressed as Scatchard plots (Fig. 5). As the concentration of PCALC36 increased, a parallel shift to the left was observed, indicating a decrease of apparent B_{max}, without significant alteration of the K_d value for ouabain (Table 1). This experiment was done three times with qualitatively similar results.

3.5. Influence of PCALC36 on [^{3}H]ouabain dissociation kinetics

The dissociation promoted by unlabeled ouabain was not affected by the addition of PCALC36 30 μM, as shown in Fig. 6. The values of the dissociation constants were

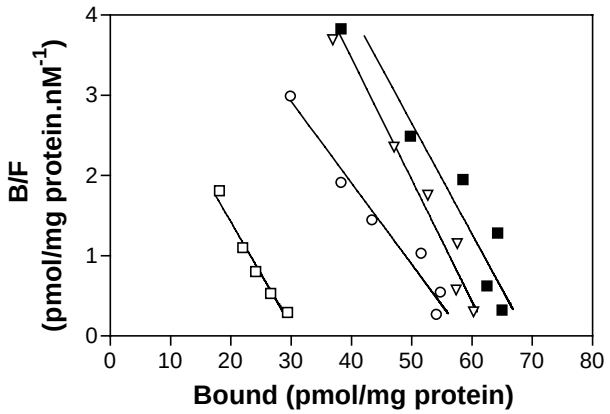


Fig. 5. Typical Scatchard plot for specific binding of [^{3}H]ouabain in rat brain preparation, in the absence (control, ■) or presence of increasing PCALC36 concentrations: 2 μM (▽), 5 μM (○) and 10 μM (□). The fitted curves were obtained by non-linear regression of the untransformed data using the model of a single class of binding sites. Each point represents the mean of triplicate determinations in a typical experiment.

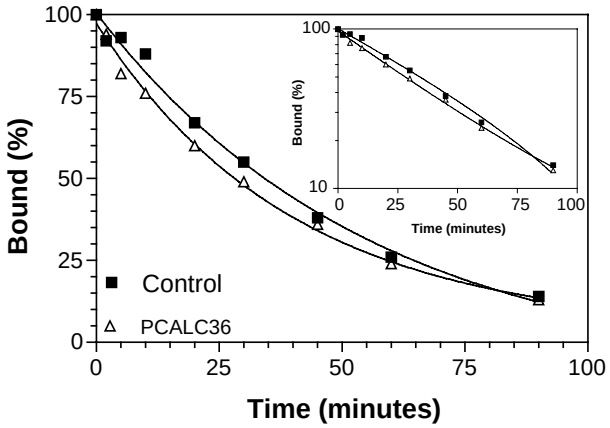


Fig. 6. Influence of PCALC36 upon [^{3}H]ouabain dissociation kinetics in rat brain preparation. After 2 hr incubation, the dissociation of [^{3}H]ouabain was initiated by isotopic dilution with 100 μM ouabain alone (■, control) or with 30 μM PCALC36 (△). Inset: semi-logarithmic representation of the data. The fitted curves were obtained by non-linear regression using the model of one phase exponential decay. Each point represents the mean of triplicate determinations in a typical experiment.

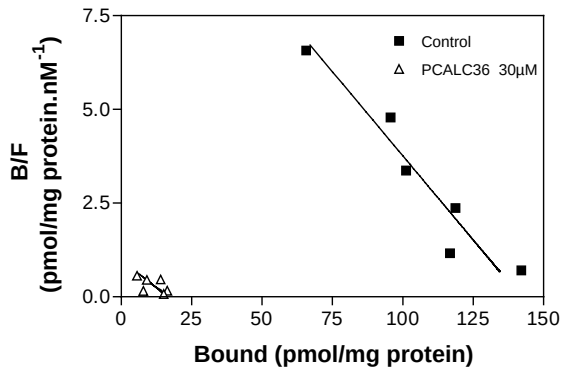


Fig. 7. Reversibility of PCALC36 inhibition of ouabain binding. Scatchard plot for specific binding of [^{3}H]ouabain in rat brain preparation pre-treated with PCALC36 30 μM (△) or water (■, control) and extensively washed (see Section 2). The fitted curves were obtained by non-linear regression using the model of a single class of binding sites. Each point represents the mean of triplicate determinations in a typical experiment.

obtained from a typical experiment, reproduced once with similar results. Comparing with the control, the dissociation constant of [^{3}H]ouabain ($k_{-1} = 0.018 \pm 0.0033$ min^{-1}) was not significantly changed by addition of PCALC36 ($k_{-1} = 0.025 \pm 0.0028$ min^{-1}). This result supports the previous observation that the addition of PCALC36 did not interfere with ouabain affinity.

3.6. Stability of the PCALC36–Na^+,K^+-ATPase complex

As an irreversible binding of PCALC36 to ouabain binding sites could explain the decrease of B_{max} for ouabain (Fig. 5), we carried out experiments to investigate the stability of the complex PCALC36–Na^+,K^+-ATPase. In this protocol, we compared the binding of [^{3}H]ouabain to a brain preparation after pretreatment with 30 μM PCALC36 or with water (control) and extensive washing to allow the dissociation of reversible ligands. Analysis of the Scatchard plot (Fig. 7) indicates that the number of [^{3}H]ouabain binding sites ($B_{max} = 16.7 \pm 3.17$ pmol/mg protein) decreased comparing with control ($B_{max} = 142 \pm 6.62$ pmol/mg protein), without alteration of the K_d value (11.1 ± 2.26 μM and 16.7 ± 11.6 μM for control and PCALC36, respectively) suggesting that the binding of PCALC36 to Na^+,K^+-ATPase is very stable (very slow dissociation) and can be considered as ''irreversible''. Note that the 90% decrease of ouabain B_{max} is compatible with the 70% inhibition exerted by the same concentration of PCALC36 in the activity assays (Figs. 2–4).

4. Discussion

In the present study, we investigated the effect of PCALC36, an original molecule with a non-steroidal skeleton, on Na^+,K^+-ATPase, the receptor of cardiac glycosides.

Analyzing the results of the biochemical assays, we conclude that PCALC36 exhibits a different pattern of Na^+,K^+-ATPase inhibition than ouabain, a cardiac glycoside routinely used *in vitro*. The first evidence was the absence of selectivity for inhibiting the different rat isoforms, whereas ouabain is about one thousand times more potent to inhibit brain isoforms (α2/α3) than kidney isoform (α1) ([21] and Fig. 2A of present work). Besides that, the inhibition exerted by PCALC36 was not antagonized by potassium, another classical feature of cardiac glycosides ([18–20] and Fig. 3A of present work). This K^+ antagonism is explained by the fact that K^+ favours the dephosphorylation of the enzyme and the stabilization of

Table 1
Effect of PCALC36 on ouabain binding at equilibrium

	Control	PCALC36 2 μM	PCALC36 5 μM	PCALC36 10 μM
B_{max} (pmol/mg protein)	76.6 ± 6.2	66.3 ± 3.7	61.1 ± 4.0	44.0 ± 7.8*
K_d (nM)	13.6 ± 7.9	10.8 ± 4.6	14.4 ± 4.3	9.92 ± 2.33

B_{max} and K_d values for ouabain, measured in the absence (control) and presence of PCALC36, were calculated by non-linear regression of the untransformed data using the model of one single binding site. Parameters are expressed as means of three experiments each performed in triplicate (mean ± SEM).

* Significantly different from control ($P < 0.05$, one-way ANOVA, followed by Dunnett's test).

the E2K conformation, which has less affinity for ouabain than the E2P conformation [18]. As a conclusion, PCALC36 appears to have no preference for a specific conformation of the enzyme, at least with respect to the two more stable ones described so far (E1 and E2). When we used two different ionic compositions of the medium in order to favour either E1 or E2 conformation of the enzyme, the inhibition promoted by PCALC36 was not affected. This suggests that PCALC36 acts differently than vanadate, a classical inhibitor of P-type ATPases which is more potent to block the enzyme when in the E2 conformation. In addition, this result further indicates that PCALC36 has a mechanism of inhibition different from cardiac glycosides since ouabain is 20 times more potent to inhibit (lamb) kidney Na^+,K^+-ATPase in the E2 than E1 conformation [16].

Binding studies revealed that increasing concentrations of PCALC36 did not change ouabain affinity but decreased its maximal capacity of binding (as measured by the B_{max}). This result is compatible with a stable covalent binding to the same site of ouabain or to an adjacent overlapping site (''irreversible competition'', [22]). On the other hand, (irreversible) binding of PCALC36 to a conformation different from E2P, with blocking of the enzyme turn-over and consequently decrease of the number of enzymes in the proper conformation for ouabain binding, cannot be ruled out. In order to further investigate the drug–receptor interaction, we performed a dissociation kinetics assay, an experimental tool that has been sucessfully used to reveal negative cooperativity between different sites present on the same macromolecule [23]. The dissociation rate constant of ouabain was not modified by the addition of PCALC36, reinforcing the previous observation that PCALC36 did not change ouabain affinity. This result further excludes the hypothesis of an allosteric interaction between ouabain and PCALC36 binding sites.

In order to test the hypothesis of formation of a stable (''irreversible'') complex between PCALC36 and Na^+,K^+-ATPase, we used a protocol that has been previously applied with success to confirm the pseudo-irreversible nature of the binding of a PAF antagonist [17]. As an extensive period of washing was not able to remove the inhibitory effect that PCALC36 exerts on ouabain binding (Fig. 7), we can conclude that this drug forms a very stable complex with its binding site. In fact the very slow dissociation of the complex PCALC36–Na^+,K^+-ATPase could explain the lack of K^+ effect on PCALC36 inhibition so that the lack of K^+ effect cannot rule out the hypothesis of an (irreversible) binding to the cardiac glycosides site. However, the absence of selectivity of PCALC36 with respect to the rat isoforms of Na^+,K^+-ATPase, characteristic of ouabain inhibition, suggests a molecular mechanism of inhibition different from the cardiac glycosides. As a consequence, our proposal that PCALC36 binds to, and freezes, a conformation different from E2P, blocking the enzyme turnover and inhibiting the ATPase activity is compatible with all the experimental data available at the moment.

Another point raised by the irreversible nature of the enzyme block is the possibility that PCALC36 could act unspecifically by partition into the lipid phase disturbing the microenvironment of the enzyme, with consequence on the enzyme activity which depends critically on association with phospholipids [24]. In fact, some reports describe the inhibition of Na^+,K^+-ATPase activity by lipophilic compounds like cholesterol and fatty acids by altering the membrane fluidity and consequently the catalytic activity of the Na^+,K^+-ATPase [25,26]. As PCALC36 is a lipophilic molecule, the possibility of such an unspecific inhibition cannot be ruled out *a priori*, even if PCALC36 was active at micromolar concentrations. As a consequence, further experiments are needed in order to investigate the selectivity of this drug.

As an attempt to compare PCALC36 with other compounds that have been developed for Na^+,K^+-ATPase, we first have to mention that no other coumestan has been so far reported as inhibitor of Na^+,K^+-ATPase, besides our first description of an isomer of PCALC36 [12]. On the other hand, some works already reported an inhibition of Na^+,K^+-ATPase by flavonoids, either natural occurring flavonoids like quercetin [27] or synthetic ones [28,29]. The inhibition promoted by quercetin is probably not related to an effect at the cardiac glycosides binding site, since the addition of quercetin increases the formation of the E1P intermediate and not E2P, as observed with ouabain [27]. With respect to other synthetic compounds structurally unrelated to PCALC36, different altered steroid-like compounds have been developed as new inhibitors of Na^+,K^+-ATPase. Gobbini *et al*. [9] reported that seco-D steroids (i.e. compound in which the D ring of the steroid skeleton is broken) bearing an (aminoethoxy)imino chain at position 17 exhibit affinity for dog kidney Na^+,K^+-ATPase in the micromolar range, although the stereochemistry of the classical digitalis skeleton was lost. Another approach [10,11] was the synthesis of derivatives with a hydroindene skeleton (i.e. compounds preserving the most distinctive part of the digitalis skeleton, i.e. the C and D rings with a *cis* junction). These compounds inhibited [^{3}H]ouabain binding to dog kidney [10] or rat and human Na^+,K^+-ATPase [11] and exhibited some inotropic effect [10,11]. As these molecules are structurally related to cardiac glycosides they are supposed to bind at the same site, hypothesis supported by the difference of potency observed towards the high- and low-affinity isoforms present in rat [11], as occurred with ouabain (but not PCALC36). For the same reason, LND-623, an aminosteroid which appears to exert its inotropic activity via a direct and relatively selective inhibition of the high-affinity isoform present in the heart [30,31], is also supposed to bind at the glycoside site of the Na^+,K^+-ATPase.

The best strategy for searching new Na^+,K^+-ATPase inhibitors as putative ideal inotropic drugs is a matter of

discussion. An interesting idea should be to develop selective inhibitors, able to differentiate the isoforms and helpful to elucidate which isoforms are involved in the positive inotropic effect and those linked to toxicity [8,32]. Based on experiments with hearts from mice genetically modified to express reduced levels of either α1 or α2 isoform, it was concluded that α2 was responsible for the control of calcium concentration in the heart and associated to the positive inotropic effect of cardiac glycosides, whereas inhibition of α1 isoform should lead to the cardiotoxic effects of cardiac glycosides [33]. In fact, such hypothesis had already been proposed 20 years ago, when we showed that concentrations of ouabain lower than 10 μM, supposed to bind to the high affinity sites present in rat heart ventricles (mainly α2, as actually known) produced inotropic effects without tonotropic (toxic) effect [34].

In contrast with the above putative requirements for new Na^+,K^+-ATPase inhibitors, based on rodent ATPases, PCALC36 is far less selective for α2 than ouabain. Nevertheless, it is important to emphasize that besides its great selectivity in rodents, cardiac glycosides are very poorly selective for human isoforms, with even a slight inversion of selectivity since ouabain is 2–4 times more potent to inhibit α1 than α2 isoform [35]. With this in mind, we cannot rule out that PCALC36 should present a more favourable selectivity for α2 isoform in human.

In conclusion, the main contribution of this work is the characterization of a new non-steroidal molecular structure that has a different pattern of Na^+,K^+-ATPase inhibition than cardiac glycosides and could thus serve as a structural paradigm to develop new inotropic drugs necessary for a safer therapeutical approach of heart failure treatment.

Acknowledgments

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