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Synthesis and Pharmacological Characterization of Functionalized 2-Pyridones Structurally Related to the Cardiotonic Agent Milrinone

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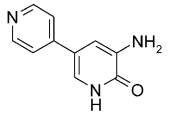
Abstract—A new class of cardiotonic agents characterized by a 2-pyridone structure was synthesized. Appropriate *sym*-2-dimethylaminomethylene-1,3-diones reacted with methylcyanoacetate to afford the desired compounds. These derivatives were evaluated for their ability in inducing cardiotonic response on guinea pig isolated myocardial preparations. Compound **8b** increased atrial contractility to an extent which is significantly higher than that of milrinone, the parent drug used as a reference compound. The pharmacological characterization and the docking studies performed on **8b** highlighted its selective mechanism of action via type 3 PDE (PDE3) inhibition.

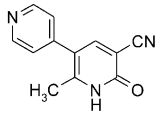
Introduction

Congestive heart failure (CHF) is a major cause of death in patients with heart disease. For many years, digitalis glycosides have been used for the treatment of CHF. This use, however, is limited because of their narrow therapeutic index and their propensity to cause life-threatening arrhythmias (arrhythmogenic lability). The search for orally active 'non-glycoside' cardiotonic drugs displaying a greater safety profile and improved efficacy on patient survival resulted in a new class of cardiotonics endowed with a different mechanism of action. Amrinone and milrinone[1] are the prototypes of a series of analogues, proposed as a replacement for digitalis, referred to as inhibitors of cGMP-inhibited cAMP phosphodiesterase (PDE), also called Type 3 PDE or PDE3.[2] In particular, milrinone, which has been approved by the United States Food and Drug Administration and is used in the clinical treatment of patients with severe heart failure,[3] improves the hemodynamic status of heart failure via inotropic/vasodilatory effects attributable to the increase in intracellular cAMP level.[4] This mechanism of action seems related to its ability in mimicking the structural and electronic features of cAMP at the active site of the enzyme, thus inhibiting conversion of cAMP to 5′-AMP.[5] However, some evidence suggests that these drugs can cause inotropic action also through antagonism towards endogenous adenosine at the cardiac A_1 receptor.[6] Until now, a positive inotropic agent with exclusive high affinity for PDE3 or for A_1 receptor has not yet been synthesized.

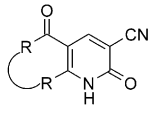
In previous studies, we used milrinone as the template for the synthesis of a number of 6-substituted 5-acyl-1,2-dihydro-2-oxo-3-pyridinecarbonitriles **1**[7] and related derivatives in which the cyano group was substituted with an acetyl function **2**.[8]



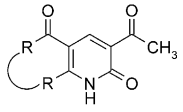
amrinone



milrinone



1a R = CH_3



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Among these derivatives, ketone **1a**[9] is the most interesting inducing a significant increase in contractile force of both guinea pig spontaneously beating atria and electrically driven left atria, comparable to that of milrinone, particularly at higher concentrations, while the 3-acetyl analogues **2** showed poor cardiac activity.[8]

The mechanism of action of **1a** refers to a competitive inhibition of the PDE3, even if an antagonism towards endogenous adenosine also seems to be involved.[10]

Moreover, as further investigation on the molecular mechanism that leads to the effective PDE3 inhibition, without antagonism at A_1 receptor, we have recently built a theoretical model of the catalytic site of PDE3A by comparative modelling[11] from the crystallographic structure of the catalytic site of PDE4B2B and the site-directed mutagenesis data available for PDE3A. On this model, docking studies performed with milrinone and different PDE3 inhibitors chosen from literature, let us derive some useful information regarding the structural requirements for selective enzymatic inhibition. The lactamic function was confirmed by our study as the primary binding site and as common anchor point for all inhibitors: it displayed two hydrogen bonds with the amino group of Lys947 side chain and with the backbone carbonyl group of Thr908. In particular, as concern 2-pyridone derivatives, the substituent on position 3 seemed to be conditioned by the size of the hydrophobic pocket where the 2-pyridone ring is inserted (residues Thr908, Leu910, His913, Lys947 and Ile951), while the substituent on position 5 resulted free to occupy a wide portion of the catalytic site.

Thus, to better characterize the binding site and to visualize the nature of the molecular contacts between 2-pyridone inhibitors and PDE3A, a more detailed knowledge of the interactions between this class of ligands and the enzyme is required, especially as concern substitution on positions 3 and 5. In fact, concerning positions 4 and 6, a number of SAR and QSAR studies have fully defined their structural characteristics.[12a,b]

Taking into account the foregoing statements and in order to obtain new milrinone analogues with a better cardiotonic activity due to a unique mechanism of action, we planned the synthesis of methyl esters of 6-substituted 5-acyl-1,2-dihydro-2-oxopyridine-3-carboxylic acids **4a–c**, **e**, 1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxilic acids **4f–i**, 2,5-dihydro-2,5-dioxo-1*H*-[1]pyrindine-3-carboxylic acid **4j**, 2,5-dihydro-2,5-dioxo-1*H*-indeno[1,2-*b*]pyridine-3-carboxilic acid **4k** and also the synthesis of 6 - ethyl - 5 - propionyl - 1*H* - pyridine - 2 - one **8b** and 1*H*-indeno[1,2-*b*]pyridin-2,5-dione **8k**.

In these new milrinone analogues, the 2-pyridone nucleus is characterized by the presence of the ester instead of the cyano or acetyl function in position 3, whereas substituents in positions 5 and 6 are varied by homologation, ramification and also aromatic substitution, or are fused generating a rigidified bicyclic or tricyclic structures. Position 4 has been maintained unsubstituted in accordance with literature.[12a,b]

The cardiac effect of all new derivatives has been investigated on guinea pig isolated myocardial preparations, using milrinone as the reference compound. In the case of **8b**, which revealed itself as the most active inotropic agent, the biochemical mechanism responsible for its cardiac action has been also examined.

In addition, in order to give further proof to the mechanism of action for **8b** and **4b**, molecular models of the complex enzyme-inhibitor were generated for **8b**, **4b**, cilostamide,[13] a highly potent PDE3 inhibitor and milrinone, using the theoretical model of PDE3A.[11] A preliminary study was also performed docking **8b** and milrinone in the virtual model of A_1 adenosine receptor[14] in order to assess the binding data obtained on A_1 receptor.

Results and Discussion

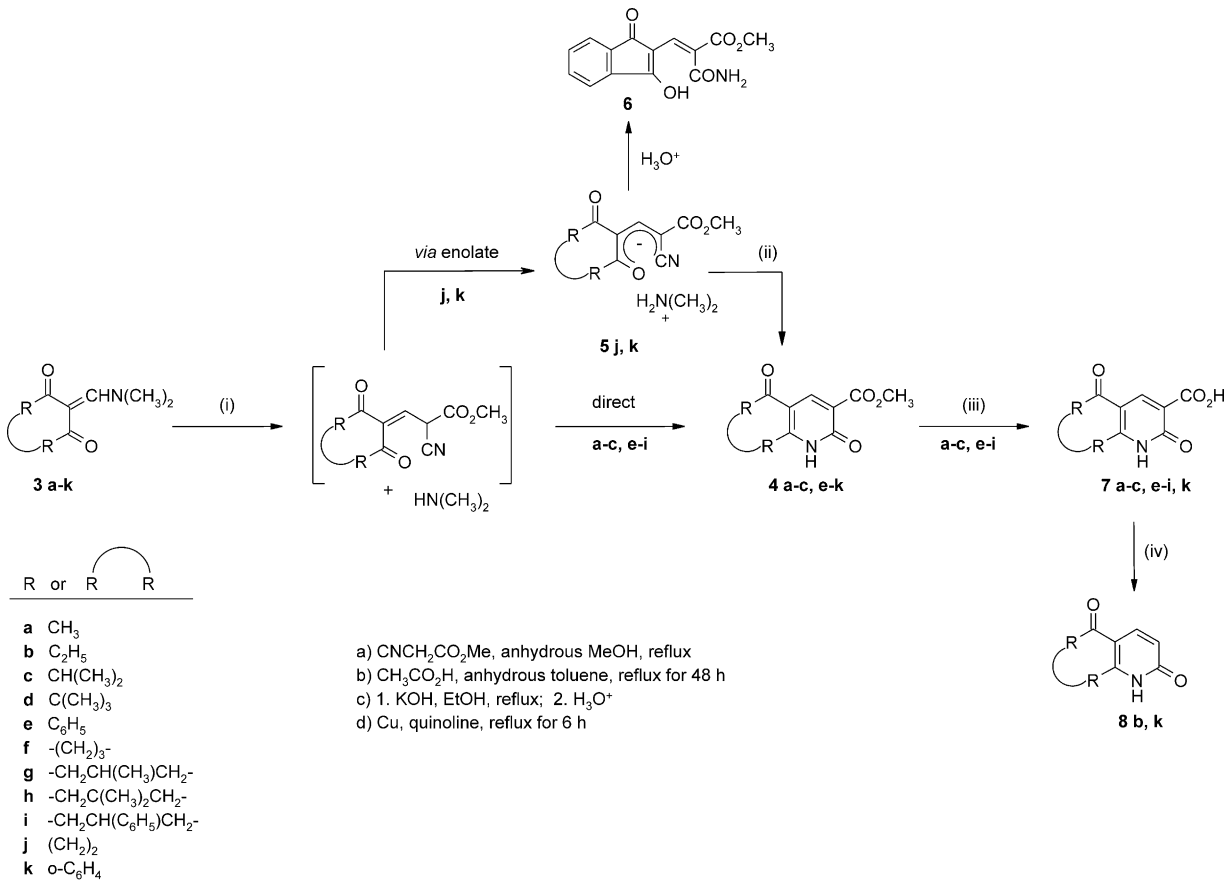
Chemistry

In a previous paper,[7] we reported the facile reaction of open-chain and cyclic *sym*-2-dimethylaminomethylene-1,3-diones with various dinucleophiles to give functionalised pyrazoles, isoxazoles, pyrimidines and pyridines. For the synthesis of the esters **4a–c**,**e–k** (Scheme 1), we adopted this fruitful reaction of both open-chain and cyclic synthons **3a–k**,[15] obtained by us in very high yields by refluxing a solution of the suitable 1,3-diones in *N*,*N*-dimethylformamide dimethylacetal, and a 1,3-dinucleophile with *C*–C–*N* structure, namely the methyl cyanoacetate.

This reaction usually occurs, first, between the nucleophilic methylenic group of the 1,3-dinucleophile and the electrophilic extra-chain carbon atom of the synthons **3a–k**. Subsequently, the dimethylamine, when released in situ, mostly cause the reaction to proceed towards the direct closure of the 2-pyridone ring without isolation of intermediates. There were no problems concerning the regiospecificity of the cyclization step, due to the symmetrical nature of the 1,3-dione. The cyclization step afforded the desired esters **4**, generally as a sole product, in good yield. Only in the case of **3d**, did the above reaction not occur because of the strong steric hindrance caused by the *tert*-butyl group.

In a few cases, we have been able to isolate intermediate that confirm the direct intervention of the dimethylamine which, in particular cases, may act as a base to form water soluble salts. In fact, the formation of enolates **5j**,**k** is probably due to a stronger acidity of the enol form involved, which protonates the released dimethylamine thus forming a stable salt. Enolates **5** always gave the desired 2-pyridones **4** by refluxing in anhydrous toluene with a small quantity of acetic acid.

To our knowledge, between the title compounds, the only one described in literature was **4a**, obtained in 68% yield by reaction of methyl 3-methoxymethylene-2,4-pentanedione with methyl cyanoacetate in the presence of sodium methoxide.[16]



Scheme 1.

Only **5k** was isolated, purified and characterized as a solid product, which resulted very soluble in water. The careful acidification of **5k** gave the corresponding methyl-2-aminocarbonyl-3-(3-hydroxy-1-oxo-1*H*-inden-2-yl)acrylate **6**. All the attempts to isolate and identify **5j** failed due to the difficulty in purifying the crude vitreous reaction residue. Esters **4** were converted into carboxylic acids **7** by saponification with potassium hydroxide in boiling ethanol followed by acidification. **7j** was not obtained due to the lability of **4j** in the experimental conditions.

Finally, the carefully decarboxylation of acids **7** by reflux in quinoline containing a catalytic amount of copper powder afforded to 6 substituted-5-acyl-1*H*-pyridin-2-ones **8**.

Acids **7a**,**c**,**e**–**i** were already prepared, with a different procedure, by refluxing a solution of the corresponding amides in concentrated hydrochloric acid for a certain time.[7] None of these acids showed inotropic or chronotropic activity in the isolated atria preparations.[9] Pyridones **8a**,**c**,**e**–**i** were also obtained by decarboxylation of the corresponding pyridine and quinoline carboxylic acids.[7] Among these pyridinones only **8h**, characterized by a quinolinedione structure, induced a positive inotropic effect, particularly at higher concentrations. The pharmacological characterization of **8h** suggested that an antagonism towards endogenous adenosine seemed to be involved in its contractile effect.[9]

Pharmacological characterization

The newly synthesized analogues **4a**–**c**,**e**–**k**, **7b**,**k** and **8b**,**k** were tested for their effects on the force of contraction and frequency rate of guinea pig spontaneously beating atria in comparison with the parent drug milrinone (Tables 1 and 2). Milrinone and the new analogues were always tested on myocardial preparations obtained from reserpine-treated animals in order to avoid a catecholamine release from adrenergic stores which could mask tissue responses to experimental drugs. Among the tested compounds, **4c**,**f**,**g**,**h**, **7k** and **8b** increased the force of contraction of atria in a concentration-dependent way (from 1 μM to 1 mM). Even at the highest concentrations tested (1 mM), the analogues did not cause arrhythmias or any increase in resting tension. Moreover, the effects of the new compounds were completely reversible, since washout of myocardial preparations restored pre-drug cardiac contractility. The results in Table 1 show that the inotropic response was particularly evident at the highest concentrations tested for **4c**,**f**,**g**,**h** and **7k**. **8b** was the most active inotropic agent, increasing atrial contractility to an extent significantly higher than that of milrinone. The new compounds did not significantly affect the cardiac frequency, only **8b** caused a chronotropic effect comparable to that exerted by milrinone (Table 2). For the sake of clarity, it must be noted that, in the same myocardial preparations, the full β-adrenoceptor agonist isoprenaline (0.2 μM) caused an increase of 262 ± 4.8% in the force of contraction and an

Table 1. Effect of compounds **4a–c,e–k**, **7b,k** and **8b,k** on the force of contraction of spontaneously beating atria isolated from reserpine-treated guinea pigs: comparison with milrinone

Compd	Developed tension (% variation from basal value)						
	10^{-6} M	$3{\cdot}10^{-6}$ M	10^{-5} M	$3{\cdot}10^{-5}$ M	10^{-4} M	$3{\cdot}10^{-4}$ M	10^{-3} M
Milrinone	16.69±0.95	30.24±0.75	38.68±1.25	46.16±0.80	52.70±0.83	52.25±1.13	41.63±1.33
4a	6.94±0.76	10.49±0.26	4.47±0.35	−12.38±0.97	−30.43±0.64	−26.07±0.76	−30.60±1.45
4b	−3.33±0.21	−5.43±0.61	−4.68±0.71	−5.40±1.01	−5.40±1.81	−4.56±0.98	nd
4c	5.51±0.30	11.96±0.41	19.13±0.33	28.47±0.45	51.08±1.09	72.39±0.63	54.12±0.57
4e	−0.13±0.05	2.51±0.25	−1.27±0.18	−11.33±0.56	−33.12±0.47	−41.17±0.63	−45.80±0.70
4f	2.12±0.13	5.98±0.10	9.53±0.38	18.79±0.53	23.57±0.61	28.69±0.96	50.33±0.95
4g	2.04±0.08	4.48±0.36	7.25±0.32	12.45±0.27	17.67±1.07	25.11±0.85	45.05±0.60
4h	2.00±0.18	5.10±0.30	10.12±0.56	16.42±0.32	21.52±0.77	37.65 ±0.88	40.89±1.01
4i	−0.20±0.01	1.65±0.03	−4.02±0.05	−21.30±0.83	−59.82±0.45	−92.96±0.92	nd
4j	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.70±0.04	3.90±0.03	nd
4k	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	nd
7b	−0.94±0.02	−1.88±0.07	−1.88±0.07	−0.94±0.05	−0.94±0.05	−0.94±0.05	−0.17±0.01
7k	5.94±0.10	5.94±0.03	5.94±0.03	14.80±0.28	20.96±0.58	27.69±0.81	58.21±1.50
8b	18.96±0.40	27.09±3.06	40.13±3.03	57.19±5.80	80.40±5.94	106.79±6.15	145.64±8.41
8k	10.35±0.29	13.78±0.34	13.78±0.34	21.22±0.49	14.30±0.40	6.86±0.28	2.91±0.18

Milrinone and the new analogues were added cumulatively to the bathing fluid, and inotropic effect was recorded for 5 min after they reached maxima, before adding a higher concentration. The effect of each concentration of a compound was defined by the difference between the force of contraction, before and after its addition to the bathing fluid, and was expressed as a percent variation in developed tension in respect to the controls. The value of basal force of contraction was 8.01±0.56 mN. Results are means ±SE from six experiments for each compound carried out on six different myocardial preparations. Negative value indicates a negative inotropic effect.

Table 2. Effect of compounds **4a–c,e–k**, **7b,k** and **8b,k** on the frequency rate of spontaneously beating atria isolated from reserpine-treated guinea pigs: comparison with milrinone

Compd	Frequency (% variation from the basal value)						
	10^{-6} M	$3{\cdot}10^{-6}$ M	10^{-5} M	$3{\cdot}10^{-5}$ M	10^{-4} M	$3{\cdot}10^{-4}$ M	10^{-3} M
Milrinone	3.01±0.24	9.12±0.05	11.52±0.33	18.22±0.84	24.46±0.75	32.81±1.04	39.20±0.90
4a	−1.35±0.13	2.89±0.19	−6.71±1.26	−8.12±0.62	−5.63±0.53	−7.52±0.55	0.00±000
4b	−16.66±2.21	−19.44±1.45	−24.44±3.92	−24.44±3.21	20.11±2.31	19.56±2.44	nd
4c	−1.84±0.09	−35.3±0.29	−0.33±0.05	0.60±0.33	7.68±0.64	7.49±0.44	1.36±0.09
4e	−1.18±0.19	−1.36±0.15	−2.79±0.47	−11.49±0.60	−22.96±0.84	−38.83±0.87	−41.00±0.87
4f	−0.69±0.14	−0.44±0.06	−0.17±0.02	0.28±0.08	1.32±0.17	6.70±0.36	8.34±0.60
4g	0.05±0.02	1.07±0.35	3.24±0.17	−0.93±0.06	−0.59±0.07	0.77±0.13	0.99±0.05
4h	−0.06±0.06	−1.25±0.10	−1.98±0.12	0.06±0.03	2.60±0.23	3.83±0.23	10.60±0.75
4i	−0.17±0.05	−0.93±0.11	−10.46±0.94	−20.66±0.77	−36.10±0.71	−80.41±0.91	nd
4j	0.00±0.00	0.00±0.00	−3.03±0.00	−9.09±0.01	−9.09±0.32	−10.01±0.41	nd
4k	0.00±0.00	0.00±0.00	−6.46±0.51	−6.48±0.38	−0.00±0.00	0.00±0.00	nd
7b	−0.28±0.03	−0.28±0.04	−0.28±0.06	−0.28±0.04	−0.28±0.03	−0.28±0.04	−0.28±0.05
7k	−7.82±0.24	−7.82±0.64	−10.43±0.30	−9.21±0.25	−0.56±0.06	4.62±0.09	34.98±0.20
8b	1.14±0.03	3.85±0.10	8.34±0.12	17.26±1.18	21.94±2.23	32.88±2.29	41.28±3.42
8k	5.64±0.10	4.45±0.09	5.54±0.07	6.52±0.06	6.52±0.06	11.07±0.13	16.41±0.17

Milrinone and the new analogues were added cumulatively to the bathing fluid, and chronotropic effect was recorded for 5 min after they reached maxima, before adding a higher concentration. The effect of each concentration of a compound was defined by the difference between the frequency, before and after its addition to the bathing fluid, and was expressed as a percent variation in frequency in respect to the controls. The value of basal heart rates was 136±7 bpm. Results are means ±SE from six experiments for each compound carried out on six different myocardial preparations. Negative value indicates a negative chronotropic effect.

increase of 57±3.0% in the heart rate over the basal values, respectively. Since, among the tested compounds, **8b** seemed to be the most interesting one, further experiments were carried out in order to characterize its pharmacological action.

Firstly, as even the small effect of a compound on heart frequency could influence the action of the substance on the force of contraction of myocardial preparations, **8b** was studied in left atria electrically driven at 1 Hz. Results (Table 3) confirmed that, also in these experimental conditions, the milrinone analogue had a positive inotropic effect higher than that of milrinone. However, for **8b**, a higher inotropic activity did not correspond to a higher potency compared to that of milrinone. EC_{50} values for **8b** and milrinone, calculated from these concentration–effect curves, were 126±4.10 and 27.35±0.10 μM, respectively.

In order to better elucidate the mechanisms responsible for the positive inotropic effect of this new milrinone analogue, its effect was also determined in the presence of the β-adrenoceptor antagonist propranolol (10^{-7} M), α_1-adrenoceptor antagonist prazosin (5×10^{-9} M), H_2-histaminoceptor antagonist ranitidine (10^{-5} M), H_1-histaminoceptor antagonist pyrilamine (10^{-7} M), Ca^{2+}-channel blocker verapamil (10^{-7} M), and carbachol (5×10^{-8} M), an agent known to selectively abolish the increase in contractility of the atria induced by a rise of cAMP levels.[17,18] Moreover, the effect of **8b** was also

Table 3. Effect of compund **8b** on the force of contraction of electrically driven left atria isolated from reserpine-treated guinea-pigs

Compd	Developed tension (% variation from the basal value)						
	10^{-6} M	$3{\cdot}10^{-6}$ M	10^{-5} M	$3{\cdot}10^{-5}$	10^{-4}	$3{\cdot}10^{-4}$ M	10^{-3} M
Milrinone	1.23±0.01	4.92±0.03	13.25±0.31	22.83±0.31	34.72±0.38	44.72±0.38	41.96±0.32
8b	9.84±0.27	11.90±0.37	16.77±1.38	25.97±2.67	36.83±2.94	52.64±3.67	70.93±5.64

Milrinone and the new analogues were added cumulatively to the bathing fluid, and inotropic effect was recorded for 5 min after they reached maxima, before adding a higher concentration. The effect of each concentration of a compound was defined by the difference between the force of contraction, before and after its addition to the bathing fluid, and was expressed as a percent increase in developed tension over controls. The value of basal force of contraction was 4.91±0.42 mN. Results are means ±SE from six experiments for each compound carried out on six different myocardial preparations.

tested in the presence of adenosine deaminase (ADA, 2 U/mL), the enzyme that inactivates adenosine by metabolising it to inosine. As shown in Table 4, the cardiotonic action of **8b** does not involve activation of adrenoceptors (both α- and β-adrenoceptors) or histaminoceptors (both H_1- and H_2-histaminoceptors), since its effect was not influenced by the presence of prazosin, propranolol, pyrilamine or ranitidine, respectively. An increased uptake of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels, with consequent increase in intracellular Ca^{2+} availability for the contractile machinery, could be involved in the inotropic response to **8b**. In fact, verapamil, at a concentration sufficiently high to block slow-Ca^{2+} channels, inhibited the effect of the new analogue. In Table 4, it is also shown that the increase in cardiac developed tension, evoked by the new milrinone analogue, was not modified by the presence of adenosine deaminase. As a means of comparison, it can be observed that, under the same experimental conditions, the presence of adenosine-deaminase decreases the positive inotropic effect of milrinone by about 90%.[19] This interesting finding clearly suggests that the effect of **8b** does not originate from its ability to antagonize the influence exerted by endogenous adenosine on the heart. Such a hypothesis is supported by the results of some experiments carried out to evaluate the affinity of **8b** towards A_1 and A_{2A} receptors. Studies of displacement of [^{3}H]CHA from rat cortical membranes and of [^{3}H]CGS21680 from rat striatal membranes indicate very low or no affinity of **8b** for A_1 (percent inhibition of specific binding at a concentration of 10 μM, %I = 10.2) and A_{2A} adenosine receptors (percent inhibition of specific binding at a concentration of 10 μM, %I = 0.0).

However, the main finding of this set of experiments is represented by the data obtained in the presence of carbachol (see Table 4), which clearly suggest an important involvement of cAMP in the cardiac action of **8b**, since, at all tested concentrations, the effect of the new analogue was significantly reduced when left atria were pre-incubated with carbachol. This finding is confirmed by the results reported in Figure 1, in which the inhibitory effects of both milrinone and **8b** on the activity of a PDE3 isolated and partially purified from guinea pig cardiac tissue are shown. **8b** inhibited in a concentration-dependent way the enzyme activity. When compared to the parent drug, the new analogue appeared rather similar to milrinone in its activity, its maximal inhibition against PDE3 activity being 81.93±0.29% versus 100% of milrinone. The potency of the new compound was also quite similar to that of the parent drug, as indicated by the comparison of K_i values (4.67±0.04 and 8.64±0.22 μM for milrinone and **8b**, respectively), calculated by means of Cheng and Prusoff's equation[20] from the reported concentration–effect curves. These results, together with those obtained in the presence of carbachol, indicate that the positive inotropic effect exerted by this new milrinone analogue is related to an increase in cAMP levels obtained through inhibition of PDE3 activity, even if the ability of the compound to increase Ca^{2+} availability through voltage-dependent Ca^{2+} channels cannot be excluded. In this regard, an increase in Ca^{2+} entry through Ca^{2+} channels has been already proposed for milrinone.[21] However, this effect may be a consequence of cAMP increase due to PDE inhibition, as recently clearly reviewed.[22]

Table 4. Effect of various antagonists on the positive inotropic effect exerted by increasing concentrations of compound **8b** on the force of contraction of electrically driven left atria isolated reserpine-treated from guinea pigs

Inhibitor (M)		Developed tension (Δ% over the basal value)		
		$3{\cdot}10^{-6}$ M **8b**	10^{-5} M **8b**	$3{\cdot}10^{-3}$ M **8b**
—		11.90±0.30	36.83±2.94	75.45±6.12
Propranolol	(10^{-7} M)	11.61±0.25*	35.41±2.32*	73.45±6.12*
Prazosine	($5{\cdot}10^{-9}$ M)	10.31±0.99*	37.53±3.21*	76.37±3.22*
Rantidine	(10^{-5} M)	12.61±1.34*	34.98±3.01*	74.89±3.45*
Pyrilamine	(10^{-7} M)	12.45±1.55*	34.79±3.65*	75.21±4.01*
Verpamile	(10^{-7} M)	4.76±0.23 (−60%)**	16.58±1.57 (−55%)**	41.49±2.31 (−45%)**
Carbachol	($5{\cdot}10^{-8}$ M)	7.14±1.03 (−40%)**	23.93±2.05 (−35%)**	52.81±3.54 (−30%)**
Adenosine-deaminase	(2 U/mL)	11.63±0.98*	36.83±2.45*	73.78±5.74*

Results are means ±SE from six experiments for each compound carried out on six different myocardial preparations. *Not statistically significant ($p > 0.05$); **statistically significant ($p < 0.001$) versus values obtained with the same concentration of **8b** in the absence of antagonists.

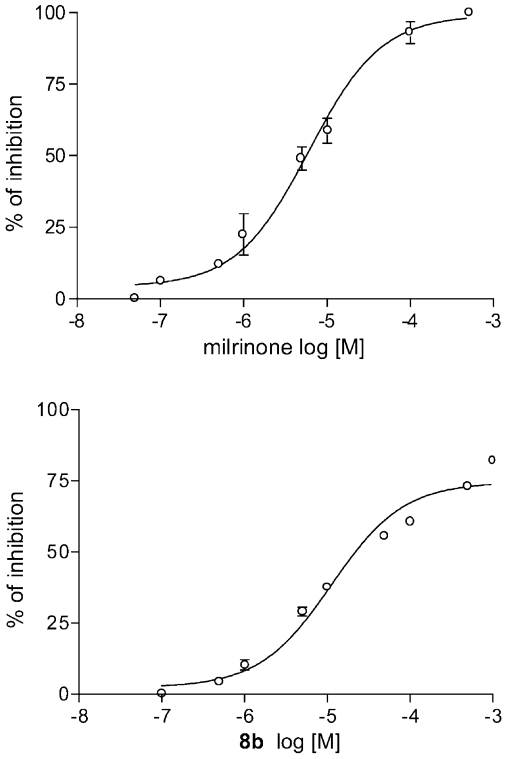


Figure 1. Concentration–effect curves for the inhibition of the activity of PDE3 partially purified from guinea pig cardiac tissue by milrinone and its analogue **8b**. PDE3 activity was measured by the two-step method,[27] as previously described.[10] Results are means ± SE from four experiments in duplicate. Data are expressed as percent inhibition of the basal enzyme activity (1.14 ± 0.02 nmol cAMP hydrolyzed/mg of protein/min).

For complete in vitro study of this new analogue, **8b** was also tested on other enzymes involved in the control of cardiac contractility, that is sarcolemmal Na^+/K^+ATPase and Ca^{2+}ATPase, and sarcoplasmic reticulum Ca^{2+}ATPase. The analogue was devoid of any effect on both Ca^{2+}ATPases, whereas, at the highest concentration tested (10^{-3} M), it inhibited the activity of Na^+/K^+ATPase of 18 ± 2% in respect of control values.

Docking studies

Results of docking studies on the theoretical model of PDE3A[11] for structure **8b**, the most active compound among those studied (Fig. 2), highlighted that this derivative shares a common binding mode with milrinone, inserting its lactamic ring in the hydrophobic pocket defined by Thr908, Leu910, His913, Lys947 and Ile951. The lactamic function, as in the case of milrinone, displays two hydrogen bonds with the biological counterpart: the first with the amino group of Lys947 sidechain and the second with the backbone carbonyl group of Thr908. On position 3, the absence of the cyano moiety does not seem to positively or negatively influence the binding mode of **8b**. This result could be explained by taking into account that 2-pyridone derivatives direct the substituent on position 3 towards a pocket defined mainly by non-polar residues such as Leu910, Ile951, Phe1004 and Leu1012. As a consequence, the cyano group, which does not interact with the biological counterpart due to its polar nature, could be replaced by a hydrogen.

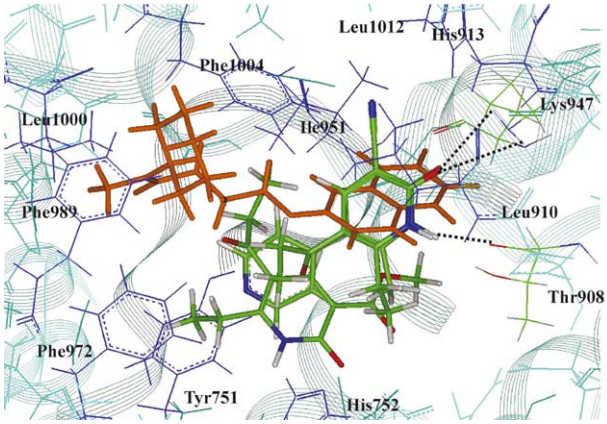


Figure 2. Binding of milrinone, **4b**, **8b** and cilostamide into the catalytic site of PDE3A. The protein is shown as ribbon. Milrinone, **4b**, **8b** (sticks) and PDE3A residues involved in hydrogen bonding are coloured as follows: green, carbon atom; red, oxygen atom; blue, nitrogen atom; grey, hydrogen atom. For clarity, cilostamide (sticks) is coloured in orange. Other PDE3A residues mentioned in the text and not involved in hydrogen bonding, are coloured in blue.

Regarding the substituent in position 5, the acyl moiety occupies a portion common to the pyridine ring of milrinone. The carbonyl group, according to our calculations, is not able to display any hydrogen bond or polar interactions with the enzymatic counterpart, such as in the case of the pyridine ring of the reference compound. However, it should be noted that the pyridine ring on milrinone is able to partially shield residues Tyr751 and His752, described as extremely important for catalysis by site directed mutagenesis experiments.[23] This finding contributes to justify on molecular basis, the slightly higher potency of milrinone as inhibitor in comparison with **8b**, as pointed out by the pharmacological results. In addition, docking studies highlight that the substituent on position 5 in 2-pyridone derivatives has the possibility to occupy a large pocket making extra hydrogen bonds or additional hydrophobic interactions into the PDE3A catalytic site. This finding could help in explaining the reduced potency displayed by milrinone and **8b** in comparison with inhibitors with a longer molecular scaffold. For example cilostamide, a well-known potent PDE3 inhibitor (K_i value vs PDE3 = 5 nM),[13] properly occupies the catalytic site showing a similar hydrogen bonding network than milrinone and **8b** plus extensive van der Waals interactions with Leu910 and Ile951 and a number of hydrophobic interactions with the side chains of residues Phe989, Leu1000 and Phe1004.

Furthermore, in order to give a preliminary investigation whether a negative interaction or a non-interaction with the enzyme could help in explaining the lacking of pharmacological activity in derivative **4b**, we also applied our docking procedure in this case. The results obtained with **4b** (Fig. 2), suggest that substitution of

the cyano with an ester function is detrimental, since it produces a different binding mode, probably due to the steric hindrance of the ester function, longer than a cyano moiety. The ester function, according to the results of our calculations, makes the pyridone unable to fully accommodate into the hydrophobic pocket formed by Thr908, Leu910, His913, Lys947 and Ile951. The molecule does not display hydrogen bonds with the counterpart and occupies a part of the enzymatic pocket closer to residues Tyr751 and Phe972. The consequent lost of the interaction with residues Lys947 and Thr908 combined with the pharmacological profile displayed by compound **4b** could be seen as a further proof of the key role played by these two amino acid residues, specific for binding to the inhibitor.

In addition, since in vitro binding results on PDE3 highlight the enzyme inhibition as the unique mechanism of action of **8b** in comparison with milrinone, which is reported to act also via antagonism at cardiac A_1 adenosine receptor, we find it interesting to verify whether these biological data could be supported by the results of an 'in silico' approach. Regarding the catalytic site of PDE3A, the similar binding mode displayed by milrinone and **8b**, does not highlight any peculiar interaction able to explain the pure enzymatic mechanism of action of this analogue. We thus performed docking experiments on milrinone and **8b**, using a theoretical model of the human A_1 adenosine receptor recently developed by us.[14] The results obtained (Fig. 3) show that **8b** does not have any particular affinity towards the biomolecule, since it accommodates into the binding site of the receptor without displaying any interaction with the counterpart. Interestingly, milrinone is able to establish a hydrogen bond between the nitrogen of lactamic function and the backbone carbonyl group of residue Thr91 and an additional polar interaction between the cyano group in position 3 and the amino groups (backbone or, alternatively, side chain) of His278. These data are in agreement with results of site-directed mutagenesis experiments,[24] which have fully demonstrated the involvement of these two amino acid residues in the antagonist binding.

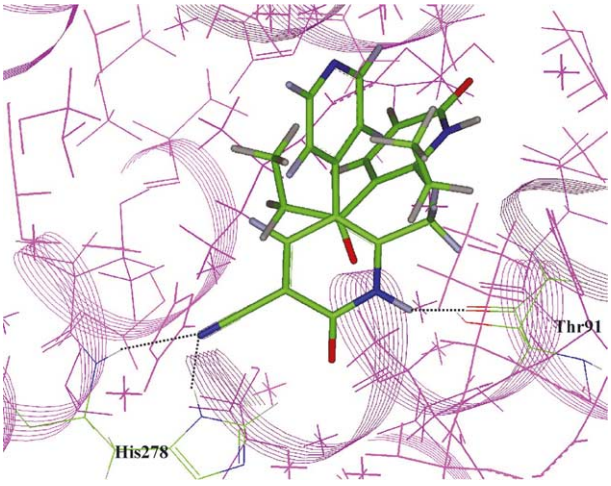


Figure 3. Binding of milrinone and **8b** into the active site of human A_1 adenosine receptor. The protein is shown as ribbon. Ligand (sticks) and A_1 residues involved in hydrogen bonding are coloured as follows: green, carbon atom; red, oxygen atom; blue, nitrogen atom; grey, hydrogen atom.

Thus, the findings of our computational approach support the inhibition of PDE3 as the unique mechanism of action for **8b** and support the hypothesis of a double mechanism of action for milrinone. Moreover, they suggest an essential role in 2-pyridone derivatives for the cyano moiety, which probably is one of the switch able to turn their biological activity mainly towards the adenosine receptor antagonism.[19]

Conclusion

The present paper presents a combination of synthetic, pharmacological and computational methods to gain further insights into the molecular requirements for selectively targeting 2-pyridone derivatives towards PDE3 inhibition or A_1 adenosine receptor antagonism.

Interestingly, through this approach, for the first time, a new milrinone analogue has been synthesized, that is **8b**, endowed with a positive inotropic effect higher than that of the parent drug but, differently from milrinone, provided with high selectivity towards cardiac PDE3. Pharmacological characterization and computational results in fact, suggest PDE3 inhibition as a unique mechanism of **8b** cardiac actions. In addition, these docking studies give further evidence to the key role played in PDE3 by residues Thr908 and Lys947 in the binding with the inhibitor. Differences in the interaction of 2-pyridone derivatives with the enzyme and the A_1 receptor, depending on the nature of substituent on position 3, have been pointed out. Furthermore, some considerations on the characteristics of substituent on position 5 of the 2-pyridone structure, in order to increase its inhibitory potency towards PDE3, have been drawn. QSAR and 3-D-QSAR studies on this class of milrinone analogues are in progress.

Experimental

Chemistry

Melting points were determined with a Fisher–Johns apparatus and are uncorrected. IR spectra were registered on a Perkin-Elmer 398 spectrophotometer and are expressed in cm^{-1}. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 200 (200 and 50.30 MHz, respectively) spectrometer. Chemical shifts are reported as δ values (ppm) downfield from internal Me_4Si in the solvent shown. Coupling constants (*J*) are expressed in Hertz (Hz). The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), ar (aromatic proton), ex (exchangeable with D_2O). Elemental analyses for C, H, N, were performed on a Model EA 1110 Elemental Analyzer (Carlo Erba Strumentazione) and results agree within ±0.3% with calculated values. All reagents were of analytical grade.

Methyl esters of 6-substituted 5-acyl-1,2-dihydro-2-oxopyridine-3-carboxylic acids 4a–c,e and 1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxylic acids 4f-i: general direct procedure

Methyl cyanoacetate (9,9 g, 100 mmol) was added to a solution of the suitable *sym*-2-dimethylaminomethylene-1,3-diones **3** (10 mmol) in anhydrous MeOH (20 mL). The mixture was stirred at reflux at different times (**4a**,**f**,**g**,**h**,**i**, 7 h; **4b**,**c**,**e**, 24 h), then the formed crude solid was filtered, washed thoroughly with anhydrous Et_2O and recrystallized (**4a**,**f**,**g**,**i** from EtOH; **4b**,**c**,**e**,**h**, from EtOAc).

Methyl 5-acetyl-6-methyl-1,2-dihydro-2-oxopyridine-3-carboxylate (4a). (72%); mp 202–203 °C; IR ($CHCl_3$): 3350–2500, 1739, 1705, 1655 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.54 (s, 3H, CH_3), 2.80 (s, 3H, CH_3CO), 3.93 (s, 3H, CH_3O), 8.72 (s, 1H, CH-4), 12.94 (br s, 1H, NH ex).[16]

Methyl 5-propionyl-6-ethyl-1,2-dihydro-2-oxopyridine-3-carboxylate (4b). (88%); mp 178–180 °C; IR ($CHCl_3$): 3360–2500, 1735, 1702, 1653 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.18 (t, $J=7$, 3H, CH_2CH_3), 1.34 (t, $J=7$, 3H, CH_2CH_3), 2.50–3.35 (m, 4H, 2 CH_2CH_3), 3.93 (s, 3H, CH_3O), 8.75 (s, 1H, CH-4), 12.60 (br s, 1H, NH ex).

Methyl 5-isobutyryl-6-isopropyl-1,2-dihydro-2-oxopyridine-3-carboxylate (4c). (83%); mp 204–205 °C; IR ($CHCl_3$): 3370, 3300–2500, 1738, 1705, 1658 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.17 (d, $J=7$, 6H, $CH(CH_3)_2$), 1.40 (d, $J=7$, 6H, $CH(CH_3)_2$), 3.00–3.80 (m, 2H, 2 $CH(CH_3)_2$), 3.95 (s, 3H, CH_3O), 8.61 (s, 1H, CH-4), 12.05 (br s, 1H, NH ex).

Methyl 5-benzoyl-6-phenyl-1,2-dihydro-2-oxopyridine-3-carboxylate (4e). (54%); mp 234–236 °C; IR ($CHCl_3$): 3350, 3200–2500, 1738, 1705, 1650 cm^{-1}. ^1H NMR ($CDCl_3$) δ 3.92 (s, 3H, CH_3CO), 7.20–7.70 (m, 10H, ar), 8.47 (s, 1H, CH-4), 12.30 (br s, 1H, NH ex).

Methyl 1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxylate (4f). (80%); mp 272–274; IR ($CHCl_3$): 3200–2500, 1738, 1680, 1640 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.90–2.40 (m, 2H, CH_2-7), 2.45–2.75 (m, 2H, CH_2-6), 2.95 (near t, $J=6$, 2H, CH_2-8), 3.89 (s, 3H, CH_3O), 8.72 (s, 1H, CH-4), 12.30 (br s, 1H, NH ex).

Methyl 7-methyl-1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxylate (4g). (98%); mp 263–265; IR ($CHCl_3$): 3470, 3300–2400, 1732, 1680, 1642 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.18 (d, $J=6$, 3H, CH_3-7), 2.12–2.55 (m, 2H, CH_2-8), 2.55–2.95 (m, 2H, CH_2-6), 2.95–3.25 (m, 1H, CH-7), 3.87 (s, 3H, CH_3O), 8.75 (s, 1H, CH-4), 13.03 (br s, 1H, NH ex).

Methyl 7,7-dimethyl-1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxylate (4h). (70%); mp 243–245; IR ($CHCl_3$): 3100–2400, 1738, 1680, 1642 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.16 (s, 6H, $2CH_3$), 2.48 (s, 2H, CH_2-8), 2.95 (s, 2H, CH_2-6), 3.92 (s, 3H, CH_3O), 8.83 (s, 1H, CH-4), 12.80 (br s, 1H, NH ex).

Methyl 7-phenyl-1,2,5,6,7,8-hexahydro-2,5-dioxoquinoline-3-carboxylate (4i). (93%); mp 303–305; IR (KBr): 3300–2500, 1700, 1648, 1587 cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.40–3.60 (m, 5H, CH-7 + CH_2-6 + CH_2-8), 3.79 (s, 3H, CH_3O), 7.4 (s, 5H, ar), 8.47 (s, 1H, CH-4), 12.70 (br s, 1H, NH ex).

Dimethyl ammonium salts of enolate anions 5j,k: general procedure. As mentioned above, during the synthesis of pyridones **4j** and **4k**, the corresponding dimethylammonium salts **5j** and **5k** were obtained as main products. Their isolation from thick crude oily residues of the reaction was carried out with a thorough washing and handling with Et_2O/EtOAc (3:1) at rt. The organic layer was dried, filtered and evaporated under reduced pressure: the solid residue was finally recrystallized from EtOH.

Dimethyl ammonium salt of enolate anion of methyl 2-cyano-3-(2-hydroxy-5-oxocyclopenten-1-yl)acrylate (5j). 63%); mp 154–156 °C; IR (KBr): 3300–2700, 2478, 2200, 1708, 1675 cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.22 (s, 4H, $2CH_2$), 2.58 (s, 6H, $N(CH_3)_2$), 3.67 (s, 3H, CH_3O), 7.65 (s, 1H, CH), 8.22 (near s, 2H, OH + NH ex).

Dimethyl ammonium salt of enolate anion of methyl 2-cyano-3-(3-hydroxy-1-oxo-1*H*-inden-2-yl)acrylate (5k). (70%); mp 166–167 °C; IR (KBr): 3200–2700, 2480, 2198, 1706, 1662 cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.57 (s, 6H, $N(CH_3)_2$), 3.69 (s, 3H, CH_3O), 7.49–7.63 (m, 4H, ar), 7.81 (s, 1H, CH), 8.18 (near s, 2H, OH + NH ex). ^{13}C NMR (DMSO-d_6) δ 34.50, 52.44, 81.52, 107.91, 109.79, 120.53, 132.99, 138.65, 143.08, 169.50, 193.08.

Methyl 2-aminocarbonyl-3-(3-hydroxy-1-oxo-1*H*-inden-2-yl)acrylate 6. An aqueous (20 mL) solution of the salt **5k** (3 g, 10 mmol) was carefully acidified with 6 M HCl at 0 °C. The amorphous precipitate was filtered, washed thoroughly with water, dried and purified by recrystallization from MeOH. Yield 65%; mp 165–167 °C; IR (KBr): 3330, 3190, 1690, 1640, 1568 cm^{-1}. ^1H NMR (DMSO-d_6) δ 3.72 (s, 3H, CH_3), 7.50–7.70 (m, 4H, ar), 7.84 (s, 1H, CH), 7.30–9.30 (very br s, 3H, OH + NH_2, ex). ^{13}C NMR (DMSO-d_6) δ 51.85, 106.00, 106.93, 120.70, 132.93, 137.19, 138.86, 168.11, 171.50, 189.06.

Methyl 2,5-dihydro-2,5-dioxo-1*H*-[1]pyrindine-3-carboxylate (4j) and methyl 2,5-dihydro-2,5-dioxo-1*H*-indeno[1,2-b]pyridine-3-carboxylate (4k): procedure via enolate intermediates. A solution of suitable salts **5** (10 mmol) and glacial AcOH (0.6 g, 10 mmol) in anhydrous toluene (200 mL) was refluxed in a Dean-Stark apparatus for 48 h. The mixture was washed with 1 M aqueous NaOH solution and water, dried and evaporated under reduced pressure. The residue was recrystallized from EtOH.

Methyl 2,5-dihydro-2,5-dioxo-1*H*-[1]pyrindine-3-carboxylate (4j). (57%); mp 299–301 °C; IR (KBr): 3200–2400, 1735, 1662, 1648 cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.50–2.80 (m, 2H, CH_2-7), 2.90–3.20 (m, 2H, CH_2-6), 3.88 (s, 3H, CH_3O), 8.21 (s, 1H, CH-4), 12.90 (br s, 1H, NH ex).

Methyl 2,5-dihydro-2,5-dioxo-1*H*-indeno[1,2-*b*]pyridine-3-carboxylate (4k). (98%); mp 310–312 °C; IR (KBr): 3100–2500, 1715, 1690, 1638 cm^{-1}. ^1H NMR (DMSO-d_6) δ 3.78 (s, 3H, CH_3O), 7.55–7.75 (m, 3H, ar), 7.95–8.05 (m, 1H, CH-6), 8.18 (s, 1H, CH-4), 13.75 (br s, 1H, NH ex).

6-Substituted 5-acyl-1,2-dihydro-2-oxopyridine-3-carboxylic acids (7b) and 2,5-dihydro-2,5-dioxo-1*H*-indeno[1,2-b]pyridine-3-carboxylic acid (7k): general procedure. Potassium hydroxide (1.68 g, 30 mmol) dissolved in 95% EtOH (20 mL) was added to a solution of the corresponding ester **4** (10 mmol) in the same solvent (30 mL). The resulting solution was boiled for 24 h (48 h in the case of **4k**), the solvent was evaporated under reduced pressure and the residue was dissolved with water (20 mL). The solution was washed with Et_2O and acidified with 6 N HCl at 0 °C (pH ~1) and the solid residue was filtered, washed with water, dried in a vacuum oven at 100 °C and purified by recrystallization from EtOH (**7k**) or from EtOAC (**7b**).

7a,**c**,**e**–**i**, already prepared by us following a different synthetic way,[7] were also synthesized according the above procedure thus confirming not only the satisfactory yield but also further proving their structure.

5-Propionyl-6-ethyl-1,2-dihydro-2-oxopyridine-3-carboxylic acid (7b). (81%); mp 185–187 °C; IR ($CHCl_3$): 3500–2500, 1735, 1690, 1630 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.21 (t, $J=7$, 3H, CH_2CH_3), 1.40 (t, $J=7$, 3H, CH_2CH_3), 2.75–3.50 (m, 4H, $2CH_2CH_3$), 9.04 (s, 1H, CH-4), 12.60 (br s, 1H, NH ex), 13.22 (near s, 1H, CO_2H ex).

2,5-Dihydro-2,5-dioxo-1*H*-indeno[1,2-*b*]pyridine-3-carboxylic acid (7k). (99%); mp >465 °C; IR (KBr): 3300–2500, 1725, 1697, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 7.60–7.80 (m, 3H, ar), 8.05–8.15 (m, 1H, CH-6), 8.32 (s, 1H, CH-4).

6-Substituted-5-acyl-1*H*-pyridin-2-ones (8b,k). The title pyridinones were prepared according to the procedure already described by us.[7] A solution of **7b**,**k** (10 mmol) in quinoline (15 mL) containing copper powder (0.20 g) was refluxed for 6 h. The mixture was filtered hot, the liquid was cooled and $CHCl_3$ (25 mL) was added. The added $CHCl_3$ caused the immediate precipitation of the product, which was filtered and recrystallized from Et_2O (**8b**) or EtOH (**8k**).

6-Ethyl-5-propionylpyridine-2(1*H*)-one (8b). (40%); mp 140–142 °C; IR ($CHCl_3$): 3100–2600, 1680, 1650 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.17 (t, $J=7$, 3H, CH_2CH_3), 1.32 (t, $J=7$, 3H, CH_2CH_3), 2.82 (q, $J=7$, 2H, CH_2CH_3), 3.05 (q, $J=7$, 2H, CH_2CH_3), 6.45 (d, $J=10$, 1H, CH-3), 7.89 (d, $J=10$, 1H, CH-4), 12.75 (br s, 1H, NH ex).

1*H*-Indeno[1,2-*b*]pyridine-2,5-dione (8k). (20%); mp 352–355 °C; IR (KBr): 3000–2400, 1710, 1658, 1640 cm^{-1}. ^1H NMR (DMSO-d_6) δ 7.45–7.72 (m, 4H, ar), 8.07 (s, 1H, CH-4).

Pharmacological studies

Animals. Dunkin–Hurtley male guinea pigs (350–400 g), obtained from Harlan Italy (S. Pietro al Natisone, Italy), were kept in controlled environmental conditions (temperature: 23 ± 2 °C; light–dark cycle: 7 am to 7 pm). Animals had free access to a standard laboratory diet and to water. All animal-use procedures described in this paper were in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and comply with the ethical principles and guidelines adopted by the European Community, law 86/609/CEE. The experimental protocol was approved by the local veterinary committee.

In order to obtain myocardial tissues depleted in endogenous catecholamines, the animals were treated intraperitoneally daily for 2 days with reserpine (2 mg/kg) before sacrifice. At the time of sacrifice, the animals were anaesthetised with inhalation of methoxyflurane and then killed by cervical dislocation; the heart being rapidly removed.

Assessment of inotropic and chronotropic activities on isolated atria preparations. The atria were separated from ventricles and suspended vertically in a 30 mL organ bath containing a physiological salt solution constantly gassed by 95% O_2 and 5% CO_2, at 30 °C. The bathing solution contained (in mM): NaCl 120, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.09, NaH_2PO_4 0.4, $NaHCO_3$ 12 and glucose 5.5. When left atria were used, they were suspended vertically in the organ bath and electrically driven at 1 Hz by square-wave pulses just above threshold voltage, of 0.6–0.9-ms duration (S44 stimulator, Grass Instrument Corporation, Quincy, MA, USA).

Resting tension was adjusted to about 10 and 5 mN in spontaneously beating atria and electrically driven left atrium, respectively. The developed tension was recorded isometrically by means of a high-sensitivity force transducer (type DYO for isolated auricles; Basile, Comerio, Varese, Italy) connected to a writing oscillograph (Unirecord System, model 7050, Basile, Comerio, Varese, Italy). The initial equilibration period was 40–60 min for each preparation. Since the atria were isolated from reserpine-treated animals, before the beginning of the experiments, depletion of catecholamines was verified by lack of any positive inotropic effect induced by the addition of tyramine (1.5 μM). Experiments were performed only in preparations that did not respond to tyramine. Milrinone and its analogues were added cumulatively and the responses caused by each drug concentration were recorded up to the maximum response before a higher concentration was added. The effects of each compound on the force of contraction and the frequency were expressed as the percent increase over controls (Δ%). Milrinone and their analogues were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the medium did not influence the basal activity of the atrial preparations. Where indicated, propranolol (0.1 μM) or prazosin (5 nM) or ranitidine (10 μM) or pyrilamine (1

μM) or verapamil (1 μM) or carbachol (0.05 μM) or adenosine-deaminase (2U/mL) were added to the bathing medium 20 min before the inotropic agents.

Assay of soluble PDE3 activity from guinea pig heart. PDE3 was isolated and partially purified from guinea pig heart using the procedure by Weishaar et al.[25] Protein content was determined by the method of Lowry et al.[26] using serum albumin as a standard. PDE activity was measured by the two-step procedure of Thompson et al.[27] in a 0.4 mL medium containing 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM EGTA, 0.4 μM [^{3}H]cAMP and about 30–50 μg of proteins, as previously described.[10] PDE3 from guinea pig heart had apparent K_m for cAMP of 1.33±0.15 μM and a V_{max} of 4.54±0.29 nmol cAMP hydrolyzed/mg protein/min. K_i values were calculated according to Cheng and Prusoff's equation[20] [$K_i = IC_{50}/(1 + C/K_D)$] where IC_{50} is the concentration of the compound that gives 50% of the obtainable inhibition, C is the substrate concentration used (0.4 μM cAMP) and K_D (1.33 μM) its dissociation constant. When assayed at 0.4 μM cAMP, the activity of our preparations of PDE3 was 1.14±0.02 nmol cAMP hydrolyzed/mg of protein/min. Milrinone and **8b** analogue were dissolved in DMSO, the final concentration of which in the reaction mixture did not exceed 1%. A similar amount of DMSO was added to the control samples. This amount did not affect PDE 3 activity.

Assay of sarcolemmal Na^+/K^+ATPase and Ca^{2+}ATPase activity. Cardiac sarcolemmal vesicles were prepared from guinea pig ventricular tissue by the method of Slaughter et al.[28] Na^+/K^+ATPase and Ca^{2+}ATPase activities were measured as previously described.[29] Control values for Na^+/K^+ATPase and Ca^{2+}ATPase activities were about 220 and 58 nmol ATP hydrolyzed/mg protein/min, respectively.

Assay of sarcoplasmic reticulum Ca^{2+}ATPase activity. A crude cardiac membrane vesicles preparation enriched in sarcoplasmic reticulum was obtained by the method of Jones et al.[30]

Ca^{2+}-stimulated ATPase activity was assayed as previously described.[31] Control values for sarcoplasmic reticulum Ca^{2+}ATPase was about 290 nmol P_i formed/mg of protein/min.

Statistical analysis. Data are expressed as arithmetic means±SEM. Comparisons between means were calculated by one-way analysis of variance (ANOVA). A p value of less than 0.05 was considered statistically significant. The EC_{50} values (where EC_{50} values were the concentrations that gave half of the maximum effect obtainable with the compound) and IC_{50} values (see above) were calculated by means of GraphPad Prism 3.03 Software from the concentration–response data, evaluated by sigmoidal curve fitting.

Docking studies

Docking studies on PDE3A catalytic site and A_1 adenosine receptor were performed according to the computational procedure already described.[11,14] Molecular structures of ligands (**4b**, **8b**, milrinone, cilostamide) were built and energy minimized within MacroModel.[32] Atomic charges were determined with semi-empirical AM1 calculations, using Spartan 'O2.[33] Calculations were carried out on a SGI O2 5000 workstation and on a standard personal computer running under Linux.

Supporting Material

Compd	Formula	Analysis % calcd/found		
		C	H	N
4b	$C_{12}H_{15}NO_4$	60.75	6.37	5.90
		60.82	6.37	5.91
4c	$C_{14}H_{19}NO_4$	63.38	7.22	5.28
		63.37	7.21	5.22
4e	$C_{20}H_{15}NO_4$	72.06	4.54	4.20
		71.80	4.50	4.14
4f	$C_{11}H_{11}NO_4$	59.72	5.01	6.33
		59.53	5.11	6.14
4g	$C_{12}H_{13}NO_4$	61.27	5.57	5.95
		61.11	5.55	5.80
4h	$C_{13}H_{15}NO_4$	62.64	6.06	5.62
		62.20	6.07	5.45
4i	$C_{17}H_{15}NO_4$	68.68	5.08	4.71
		68.59	5.04	4.69
4j	$C_{10}H_9NO_4$	57.97	4.38	6.76
		58.15	4.46	6.73
4k	$C_{14}H_9NO_4$	65.88	3.55	5.49
		65.99	3.41	5.42
5j	$C_{12}H_{16}N_2O_4$	57.13	6.39	11.10
		57.01	6.45	11.02
5k	$C_{16}H_{16}N_2O_4$	63.99	5.37	9.33
		63.80	5.50	9.28
6	$C_{14}H_{11}NO_5$	61.54	4.06	5.13
		61.49	4.07	5.11
7b	$C_{11}H_{13}NO_4$	59.18	5.87	6.27
		59.32	5.92	6.26
7k	$C_{13}H_7NO_4$	64.74	2.93	5.81
		64.74	3.01	5.78
8b	$C_{10}H_{13}NO_2$	67.02	7.31	7.82
		67.27	7.24	7.95
8k	$C_{12}H_7NO_2 \cdot 1/H_2O$	69.90	3.91	6.79
		71.04	3.88	6.92

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