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Laboratory notes

Novel milrinone analogs of pyridine-3-carbonitrile derivatives as promising cardiotonic agents

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Abstract

In an attempt to design new inotropic drugs for congestive heart failure (CHF) with less proarrhythmic potential, three series of compounds analogous to milrinone were prepared, namely, 4-aryl-6-(4-pyridyl)-2-oxo-1,2-dihydropyridine-3-carbonitriles **2a–g**, 4-aryl-6-(4-pyridyl)-2-thioxo-1,2-dihydropyridine-3-carbonitriles **3a–g** and 2-amino-4-aryl-6-(4-pyridyl)-pyridine-3-carbonitriles **4a–g**. The first series was prepared by reacting 4-acetyl pyridine with the appropriate aldehyde, ethyl cyanoacetate and ammonium acetate in ethanol. Reaction of **2a–g** with phosphorus pentasulfide afforded the second series **3a–g**. The third target compounds **4a–g** were prepared applying the same procedure used to synthesize **2a–g** using malononitrile instead of ethyl cyanoacetate. All the newly synthesized compounds were evaluated for their cardiotonic activity and their in vivo cardiovascular effects. In addition, their oral and parentral acute toxicity were determined. Compounds **2a**, **2b**, **2c**, **4c** and **4f** proved to exert cardiotonic activity comparable to that of milrinone using spontaneously beating atria model from reserpine-treated guinea pigs. In addition these compounds proved to be non-toxic and well tolerated by mice up to 250 mg kg^{-1} orally and up to 125 mg kg^{-1} through parenteral route.

Keywords: Pyridine-3-carbonitrile; Milrinone analogs; Cardiotonics; Acute toxicity

1. Introduction

Congestive heart failure (CHF) is an increasingly prevalent problem in cardiology. The annual mortality rate of patients who have New York Heart Association (NYHA) functional class IV CHF remains high regardless of therapy with a variety of drugs [1]. Therapy with cardiotonic agents for patients who have CHF has been shown to increase cardiac output, reduce preload and afterload, and has life-saving potential [2]. Cardiotonic agents currently available for the treatment of hemodynamic crisis in CHF are cardiac glycosides or sympathomimetic catecholamines. Cardiac glycosides have an extremely narrow safety margin and high incidence of arrhythmogenicity [3,4]. Sympathomimetic agents (e.g. dobutamine and dopamine) are orally inactive and may lead to tachyphylaxis. Even worse is that sympathomimetic agents may increase the mortality of patients with acute CHF [5]. In the past two decades, novel non-glycosidic, non-sympathomimetic cardiotonic agents that have selective PDE III inhibitory action have been developed, e.g. amrinone [6] and milrinone [7].

In this work, we present the synthesis, characterization and biological evaluation of novel milrinone analogs, in which the 2-position of the pyridine has oxo, thioxo or amino group. The 4-position is substituted by aryl substituent, while the 5-position is unsubstituted so as to minimize the steric influence upon the 4-pyridyl group in the 6-position (Fig. 1). The study aims to rationalize the effect of locating pyridyl group in the 6-position instead of methyl group in milrinone and to check how other structural modifications would influence the cardiotonic activity and cardiovascular effects of the newly synthesized compounds.

2. Chemistry

Reactions outlined in Fig. 2 were adopted to synthesize the desired compounds **2a–g, 3a–g** and **4a–g**. Reaction of

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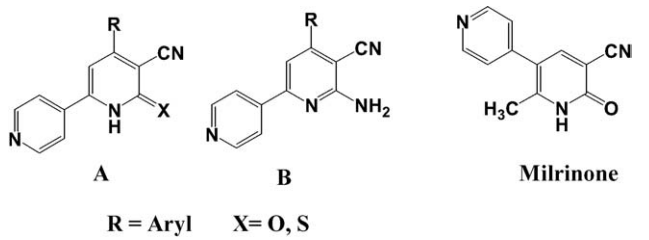

Fig. 1. Structures of milrinone and the novel series of milrinone analogs **A** and **B**.

4-acetylpyridine with the selected aldehyde, ethyl cyanoacetate and ammonium acetate in ethanol afforded 4-aryl-6-(4-pyridyl)-2-oxo-1,2-dihydropyridine-3-carbonitriles **2a**–**g**. The thioxo derivatives, 4-aryl-6-(4-pyridyl)-2-thioxo-1,2-dihydropyridine-3-carbonitriles **3a**–**g** were obtained by the reaction of **2a**–**g** with phosphorus pentasulfide in pyridine. The target compounds; 2-amino-4-aryl-6-(4-pyridyl)-pyridine-3-carbonitriles **4a**–**g** were synthesized applying the same procedure used to synthesize **2a**–**g** except that ethyl cyanoacetate was replaced by malononitrile (Table 1 and Fig. 2). The synthetic procedures applied in the present work are considered to be multicomponent reactions (MCRs), where at least three starting materials react together in one pot to give a product incorporating part of all starting materials. This is in contrast to classical multi-step reactions, where only two starting materials react. Whenever a MCR can be applied in chemistry, this is preferred since it is easier to perform, gives higher overall yields and is less time consuming [8].

3. Statistical analysis

Data were expressed as mean ± standard error (S.E.). *t*-Test was used to compare between two groups. One-way analysis of variance (ANOVA) techniques were used to examine the studied parameters among more than two groups. For pairwise comparisons among groups, the least significance difference (LSD) test was used [9].

4. Results and discussion

4.1. In vitro studies

4.1.1. Cardiotonic effect on isolated atria

Biological evaluation of the test compounds as cardiotonic agents was determined using spontaneously beating atria model from reserpine-treated guinea pigs to eliminate the influence of noradrenaline upon contractility [10,11]. Inotropic and chronotropic activities were expressed as percentage change in the force of contraction and frequency rate over control (Table 2).

In general, at concentrations of 10^{-5} and 5×10^{-5} M most test compounds showed a minimal response, whereas, most test compounds in a final concentration of 5×10^{-4} M, caused an increase in the force of contraction of spontaneously beating guinea pig atria (Fig. 3). The highest concentration examined was 5×10^{-4}M because some of the test compounds produced insoluble precipitation at 10^{-3}M. Substitution of the oxo group at 2-position of pyridine by thioxo group abolished or reduced the activity. On the other hand, some 2-amino derivatives **4a**–**g** possessed good contractile activity. Compounds **2a**, **2b**, **2c**, **4c** and **4f** (% change of developed tension over control = 94.4 ± 5.2, 89.2 ± 4.7, 87.5 ± 5.1, 89.2 ± 5.1 and 80.6 ± 4.2, respectively) showed contractile activity higher or comparable to milrinone (% change of developed tension over control = 84 ± 1.6). Contrary to mil-

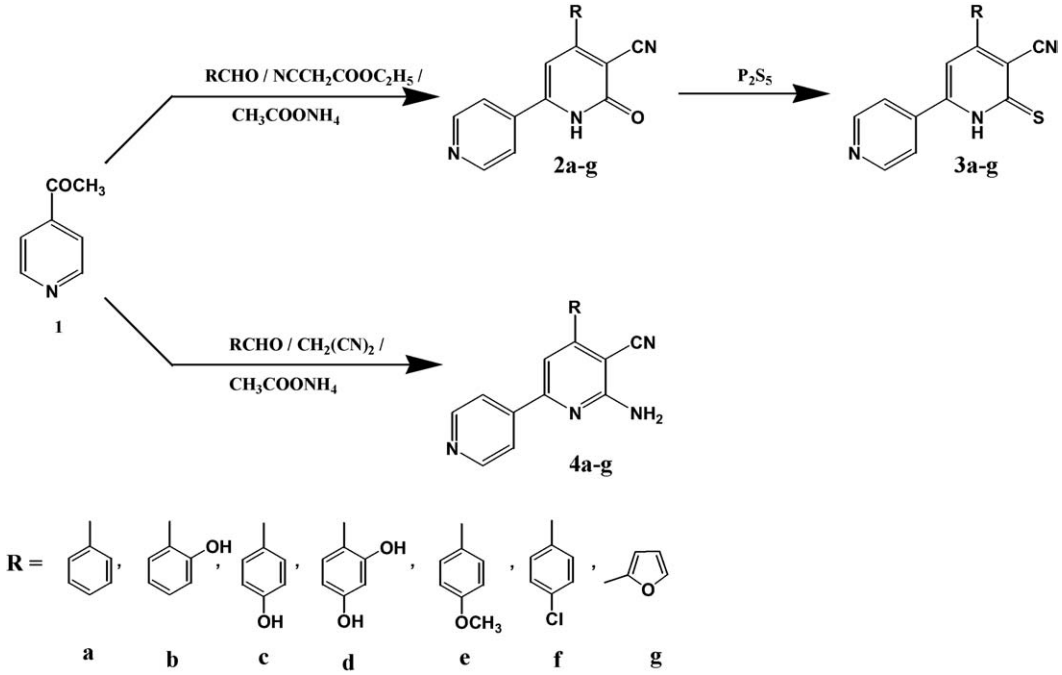

Fig. 2. Synthesis of target compounds.

Table 1
Physical and analytical data of the test compounds

Compound number	Yield (%)	m.p °C	Molecular formula (molecular weight)
2a	76	> 300	$C_{17}H_{11}N_3O$ (273.28)
2b	84	> 300	$C_{17}H_{11}N_3O_2$ (289.28)
2c	82	> 300	$C_{17}H_{11}N_3O_2$ (289.28)
2d	73	>300	$C_{17}H_{11}N_3O_3$ (305.28)
2e	78	284–286	$C_{18}H_{13}N_3O_2$ (303.31)
2f	81	> 300	$C_{17}H_{10}ClN_3O$ (307.73)
2g	78	> 300	$C_{15}H_9N_3O_2$ (263.25)
3a	69	> 300	$C_{17}H_{11}N_3S$ (289.35)
3b	58	> 300	$C_{17}H_{11}N_3OS$ (305.35)
3c	67	> 300	$C_{17}H_{11}N_3OS$ (305.35)
3d	66	> 300	$C_{17}H_{11}N_3O_2S$ (321.05)
3e	68	296–298	$C_{18}H_{13}N_3OS$ (319.38)
3f	59	> 300	$C_{17}H_{10}ClN_3S$ (323.80)
3g	62	> 300	$C_{15}H_9N_3OS$ (279.31)
4a	85	295–297	$C_{17}H_{12}N_4$ (272.30)
4b	76	260–262	$C_{17}H_{12}N_4O$ (288.10)
4c	75	> 300	$C_{17}H_{12}N_4O$ (288.10)
4d	79	> 300	$C_{17}H_{12}N_4O_2$ (304.30)
4e	86	294–296	$C_{18}H_{14}N_4O$ (302.33)
4f	88	258–260	$C_{17}H_{11}ClN_4$ (306.74)
4g[34]	78	> 300	$C_{15}H_{10}N_4O$ (262.26)

rinone, most of the tested compounds, at concentrations up to 5×10^{-4}M did not increase, but rather decreased the spontaneous rate of contractions of guinea pig atria. The lack of positive chronotropic effect by these compounds may be beneficial for its use in patients with CHF because any unnecessary increases in oxygen consumption could be avoided. Increases in oxygen consumption may constitute an important factor, which exacerbates the heart failure syndrome. In patients with severe CHF the positive force-frequency relationship disappears; or is inverted to the negative [12]. Millimolar concentrations of test compounds evoked signs of toxicity, such as reduction of contractile force, together with an increase in the frequency and sometime the appearance of

Table 2
Effect of the test compounds upon contractile activity of spontaneously beating atria from reserpine-treated guinea pigs at 5×10^{-4} M concentration

Compound numbers	Developed tension (% change over control)[a]	Frequency rate (% change from control)[a]
Milrinone	84.0 ± 4.6	38 ± 3.2
2a	94.4 ± 5.2	–4.66 ± 2.1
2b	89.2 ± 4.7	–3.33 ± 3.6
2c	87.5 ± 5.1	–8.33 ± 3.8
2d	63.5 ± 3.7	[b]
2e	24.1 ± 3.9	[b]
2f	11.2 ± 2.1	[b]
2g	35.4 ± 3.8	[b]
3a	11.7 ± 2.6	[b]
3b	[b]	[b]
3c	[b]	[b]
3d	[b]	[b]
3e	[b]	[b]
3f	18.3 ± 1.4	–3.21 ± 2.4
3g	[b]	[b]
4a	19.7 ± 2.4	[b]
4b	77.3 ± 4.5	[b]
4c	89.2 ± 5.1	[b]
4d	[b]	[b]
4e	[b]	[b]
4f	80.6 ± 4.2	–16.66 ± 1.4
4g	33.4 ± 2.1	[b]

[b] No inotropic or chronotropic effect.
[a] Mean ± S.E.M. from four atria.

moderate arrhythmias. These toxic effects were completely reversed by washing the heart preparation.

The contractile activity of the most active test compounds, namely **2a**, **2b**, **2c**, **4c** and **4f** as well as of milrinone does not involve receptor activation. The direct interaction of the test compounds with β_1-adrenergic receptors was excluded, as propranolol at concentration of 0.1 μM did not affect their contractile activity. It is worth-mentioning that propranolol at this concentration abolished maximal contractile response

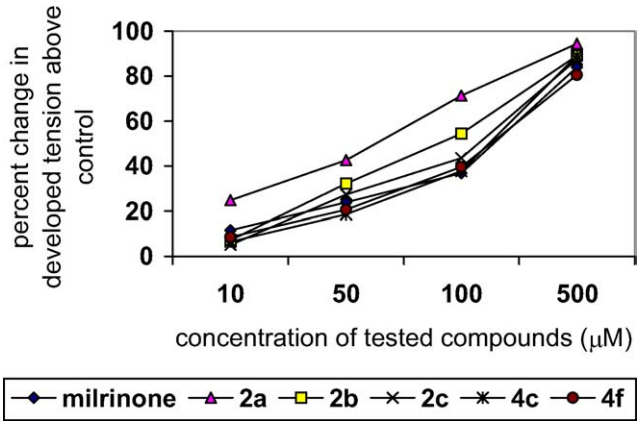

Fig. 3. Concentration-response curves for the developed tension of the tested compounds on isolated guinea pig atria. Effects, measured at time of maximum effect, were calculated as differences in developed tension before and after drug addition and were expressed as percent change over control. Values are mean from four experiments carried out on different myocardial preparations ($N = 8$).

Table 3
Developed tension (% change over control)[a] of the test compounds in the presence of propranolol, pyrilamine, ranitidine and carbachol

Compound number	-----	Propranolol (0.1 μM)	Pyrilamine (0.1 μM)	Ranitidine (10 μM)	Carbachol (50 μM)
Milrinone	84.0 ± 4.6	82.1 ± 2.6	79.5 ± 5.1	82.3 ± 3.2	4.6 ± 0.2[b]
2a	94.4 ± 5.2	92.8 ± 4.6	91.2 ± 3.9	93.8 ± 5.1	46.9 ± 7.8[b]
2b	89.2 ± 4.7	89.2 ± 3.6	87.5 ± 2.8	86.3 ± 4.6	55.2 ± 10.9[b]
2c	87.5 ± 5.1	86.4 ± 5.3	87.5 ± 6.2	89.2 ± 3.2	39.4 ± 6.2[b]
4c	89.2 ± 5.1	85.5 ± 6.2	86.3 ± 5.2	90.3 ± 4.3	45.3 ± 8.9[b]
4f	80.6 ± 4.2	80.7 ± 4.2	81.1 ± 3.4	78.6 ± 2.9	32.7 ± 5.3[b]

-----: in absence of receptor blocker or carbachol.
[a] Mean ± S.E.M. from four atria.
[b] $P < 0.01$ compared to developed tension recorded in absence of receptor blocker or carbachol.

to isoprenaline in the same heart preparation [11]. Moreover, the activity was not affected by 0.1 μM pyrilamine or 10 μM ranitidine, excluding an interaction with H_1- and H_2-histamine receptors, respectively (Table 3).

4.1.2. Influence of carbachol on the positive inotropic effect of the studied compounds

The positive inotropic activity of compounds **2a**, **2b**, **2c**, **4c** and **4f** as well as of milrinone was inhibited by carbachol at a concentration of 50 μM, which has been reported to inhibit the inotropic responses induced by increasing intracellular cAMP in the same preparation most probably due to stimulation of muscarinic M_2 receptors in the heart, which are linked to inhibition of cAMP [11]. Thus indicating that the positive inotropic effect of the test compounds is possibly partially mediated by an increase in cAMP (Table 3).

4.1.3. Influence of adenosine deaminase (ADA) on the positive inotropic effect of the studied compounds

The contractile activity of the test compounds **2a**, **2b**, **2c**, **4c** and **4f** as well as of milrinone involves antagonism toward endogenous adenosine because it was modified by preincubation of atria with ADA (2 U ml^{-1}), the enzyme that inactivates adenosine by metabolizing it to inosine [13] (Table 4).

4.1.4. Effects of the studied compounds on vascular smooth muscles

Compounds **2a**, **2b**, **2c** and **4c** but not **4f** inhibited the sustained vascular contraction induced by 10 μM noradrenaline in helical strips of guinea pig thoracic aorta. The relaxant effect of the test compounds was less potent than milrinone. Elevation of cAMP levels due to PDE III inhibition by the studied compounds in vascular smooth muscle cells may be responsible for the vascular relaxation. It is noteworthy that the studied compounds show a higher cardiac vs. vascular selectivity compared to milrinone. The small amount of vasodilatation induced by these compounds may be beneficial for the treatment of heart failure because it reduces cardiac afterload without triggering excessive neurohumoral activation (Fig. 4).

Table 4
Developed tension (% change over control)[a], of the test compounds in the absence and presence, of ADA

Compound number	-----	ADA (2 U ml^{-1})
Milrinone	84.0 ± 4.6	60.4 ± 6.4[b]
2a	94.4 ± 5.2	49.2 ± 5.6[b]
2b	89.2 ± 4.7	40.7 ± 4.9[b]
2c	87.5 ± 5.1	39.3 ± 5.1[b]
4c	89.2 ± 5.1	51.4 ± 6.4[b]
4f	80.6 ± 4.2	44.9 ± 4.1[b]

-----: in absence of ADA.
[a] Mean ± S.E. from four atria.
[b] $P < 0.01$ compared to developed tension recorded in absence of ADA.

4.2. In vivo studies

4.2.1. Cardiovascular effects of the studied compounds in anaesthetized dogs

In anaesthetized dogs, the test compounds elicited almost no effect on the heart rate (HR) at any dose. At doses less than 10^{-5} M kg^{-1} all test compounds did not cause a significant change in mean blood pressure (MBP). At a dose of 10^{-5} M kg^{-1}, or higher, compounds **2a** and **4c** induced a mild decrease in MBP (Table 5).

4.2.2. Effects of the studied compounds in experimental model of arrhythmia

The test compounds did not cause arrhythmias under control conditions in anaesthetized dogs nor did they exacerbate adrenaline-induced arrhythmia [14] at a dose of 10^{-5} M kg^{-1}. At a dose of 2×10^{-5} M kg^{-1} only compounds **2b** and **4f** exacerbated adrenaline-induced arrhythmias. In this series of experiments, compounds **2b** and **4f** induced ventricular fibrillation (VF) in one out of six dogs during infusion of epineph-

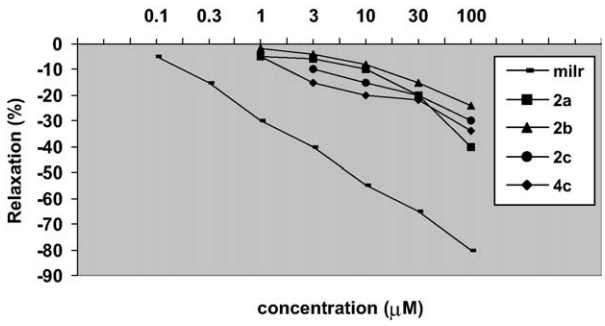

Fig. 4. Relaxant effect of compounds **2a**, **2b**, **2c**, **4c** and milrinone on the sustained contraction induced by 10 μM norepinephrine in helical strips of guinea pig thoracic aorta. Relaxation is expressed as a percentage of the response to 100 μM papaverine. Each point represents the mean ($N = 6$). IC_{50} of compounds **2a**, **2b**, **2c** and **4c** > 100 μM. IC_{50} of milrinone 6.5 μM.

Table 5
Effect of the test compounds on MBP and HR in anaesthetized dogs

Compound number	Dose (M kg^{-1} i.v.)	MBP (mmHg)	HR (Beats min^{-1})
Control		115 ± 7	98 ± 7
Milrinone	1×10^{-6}	110 ± 9	102 ± 9
	1×10^{-5}	80 ± 5[b]	142 ± 12[b]
2a	1×10^{-5}	112 ± 6	100 ± 5
	2×10^{-5}	100 ± 4[a]	96 ± 6
2b	1×10^{-5}	114 ± 7	99 ± 6
	2×10^{-5}	109 ± 9	95 ± 8
2c	1×10^{-5}	111 ± 10	94 ± 6
	2×10^{-5}	107 ± 8	96 ± 4
4c	1×10^{-5}	108 ± 10	102 ± 7
	2×10^{-5}	97 ± 8[a]	104 ± 8
4f	1×10^{-5}	113 ± 6	94 ± 8
	2×10^{-5}	105 ± 7	93 ± 7

[a] $P < 0.05$ vs. control.
[b] $P < 0.01$ vs. control.

rine. In the same series of experiments, the arrhythmiogenic potency of these compounds was lower than that of milrinone. At 10^{-5} M kg^{-1} i.v. milrinone induced VF in five out of six dogs examined (Table 6).

4.2.3. Effects of the studied compounds in experimental model of heart failure

Fifty rats were subjected to ligation of the left coronary artery as described by Gomej et al. [15]. Eight days after surgical intervention, animals that survived were grouped into seven groups, the first group received no drugs, whereas groups II–VII received milrinone and the test compounds **2a**, **2b**, **2c**, **4c** and **4f**, respectively, orally daily in a dose of 10^{-4} M kg^{-1} b.wt for 6 weeks starting from postligation day 8. At the end of the experimental period, each rat was sacrificed, the heart was rapidly excised and left ventricular papillary muscles were isolated for determination of isometric force [16]. Milrinone, compounds **2a**, **2b**, **2c**, **4c** and **4f** significantly increased isometric force (reflected as developed tension) of isolated left ventricular papillary muscles compared to non-treated coronary artery ligated rats, with a significant difference between rats that received milrinone and those that received the other test compounds (Table 7).

4.3. Acute toxicity

The active compounds **2a**, **2b**, **2c**, **4c** and **4f** were further evaluated for their oral acute toxicity in male mice using a literature method [17,18]. The results indicated that test compounds proved to be non-toxic and well tolerated by the experimental animals up to 250 mg kg^{-1}, although no mortality was recorded at 500 mg kg^{-1}. Moreover, these compounds were tested for their toxicity through parenteral route [19]. The results revealed that all the test compounds were non-toxic up to 125 mg kg^{-1}.

Table 6
Effect of the test compounds in adrenaline-induced arrhythmia in dogs

Compound number	Dose (M kg^{-1} i.v.)	Incidence of VF
Milrinone	1×10^{-6}	0/6
	1×10^{-5}	5/6
2b	1×10^{-5}	0/6[a]
	2×10^{-5}	1/6[a]
4f	1×10^{-5}	0/6[a]
	2×10^{-5}	1/6[a]

VF: ventricular fibrillation.
[a] $P < 0.01$ vs. milrinone at 1×10^{-5} M kg^{-1} i.v.(Fisher's exact test).

Table 7
Developed tension [DT] (Mean ± S.E.) of isolated left ventricular papillary muscles, 6 weeks after coronary artery ligation in rats

Group	DT(g mm^{-2})
Non-treated coronary artery ligated $N = 6$	2.76 ± 0.13
Coronary artery ligated and treated with milrinone $N = 6$	3.29 ± 0.24[a]
Coronary artery ligated and treated with compound **2a** $N = 6$	4.10 ± 0.32[b,c]
Coronary artery ligated and treated with compound **2b** $N = 6$	3.91 ± 0.21[b,c]
Coronary artery ligated and treated with compound **2c** $N = 6$	3.85 ± 0.49[b,c]
Coronary artery ligated and treated with compound **4c** $N = 6$	3.94 ± 0.29[b,c]
Coronary artery ligated and treated with compound **4f** $N = 6$	3.77 ± 0.35[b,c]

[a] $P < 0.05$ compared to non-treated coronary artery ligated rats.
[c] $P < 0.01$ compared to non-treated coronary artery ligated rats.
[b] $P < 0.01$ compared to milrinone-treated coronary artery ligated rats.

5. Conclusion

The cardiotonic effect on isolated guinea pigs atria clearly showed that compounds derived from series **A** (X = O); 4-aryl-6-(4-pyridyl)-2-oxo-1,2-dihydropyridine-3-carbonitriles **2a–g** exhibited better cardiotonic activity than their structurally related analogs **A** (X = S); 4-aryl-6-(4-pyridyl)-2-thioxo-1,2-dihydropyridine-3-carbonitriles **3a–g** (Fig. 1). Within series **A** (X = O), compounds having phenyl, 2-hydroxyphenyl or 4-hydroxyphenyl substituent at 4-postion of pyridone ring system (**2a**, **2b** or **2c**) showed remarkable cardiotonic activity comparable to that of milrinone. Introduction of more polar 2,4-dihydroxyphenyl, bulky 4-methoxyphenyl or small sized furyl moiety at 4-postion of pyridone ring system (**2e**, **2f** or **2g**) resulted in sharp reduction of the activity. On the other hand, the activity of compounds derived from series **B** is lower than that of compounds derived from series **A** (X = O) (Fig. 1). Compounds having 4-hydroxyphenyl or 4-chlorophenyl substituent at 4-position of 2-aminopyridine ring system showed the highest activity within this series.

As a result, the studied milrinone analogs **2a**, **2b**, **2c**, **4c** and **4f** proved to exert cardiotonic activity. The presence of oxo group at 2-position and phenyl or hydroxyphenyl moiety at 4-position of pyridine ring system are essential for the cardiotonic activity. The cardiac activity of these active compounds does not involve catecholamine release because the compounds were tested in atria isolated from reserpine-treated guinea pigs not responding to the tyramine test. Nei-

ther H_1- nor H_2-histamine receptors were involved in the contractile activity of the test compounds, which remained insensitive to pyrilamine and ranitidine. Increases in intracellular cAMP content seem to be involved, since the contractile effect was inhibited by carbachol, an agent that selectively abolishes the elevation of heart contractility sustained by increases in cAMP levels induced either by adenylcyclase stimulation or PDE III inhibition in different preparation [20]. Our data demonstrate that the cardiotonic activity of our new synthesized milrinone analogs also involves antagonism toward endogenous adenosine. The negative inotropic effect of adenosine is ascribed to reduce calcium entry into cells as a consequence of direct K^+ channel activation [21], so an antagonist with high affinity for the receptor of endogenous adenosine may increase cardiac contractility without risk of the arrhythmias that always result from the use of PDE III inhibitor. The lack of positive chronotropic effect by these compounds may be beneficial for their use in patients with CHF. The proarrhythmic potential of the studied compounds was low representing a therapeutic advantage for their use in heart failure. These compounds represent good leads and an intensive investigation is currently undertaken to identify their exact mechanism of action and optimum dosing.

6. Experimental protocols

6.1. Chemistry

Melting points were determined in open glass capillaries using a Thomas capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on 470-Shimadzu IR spectrophotometer using the KBr disc technique. ^{1}H-NMR spectra were recorded on Jeol-400 MHz spectrometer (DMSO-d_6), and the chemical shifts are given in δ (ppm) downfield from tetramethylsilane (TMS) as an internal standard. Splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet. Elemental analyses were performed on Perkin–Elmer 2400 elemental analyzer, and the found values were within ± 0.4% of the theoretical values. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-protected aluminium sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for few seconds.

*6.1.1. 4-Aryl-6-(4-pyridyl)-2-oxo-1,2-dihydropyridine-3-carbonitriles (**2a–g**)*

A mixture of 4-acetylpyridine (1.21 g, 10 mmol), ethyl cyanoacetate (1.13 g, 10 mmol), the appropriate aldehyde (10 mmol) and ammonium acetate (6.16 g, 80 mmol) in ethanol (50 ml) was heated under reflux for 6–8 h. the reaction mixture was cooled and the formed precipitate was filtered, washed with ethanol, then washed successively with water, dried and crystallized from methanol (Table 1). IR (cm^{-1}): 3285–3260 (NH), 2220–2210 (CN), 1654–1647 (C = O). ^{1}H-NMR of compound **2a**: δ 7.11 (s, 1H, cyanopyridine C_5-H), 7.58–7.59 (m, 3H, phenyl-H), 7.74–7.76 (m, 2H, phenyl-H), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.74 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.92 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2b**: δ 7.13 (s, 1H, cyanopyridine C_5-H), 7.62–7.72 (m, 4H, phenyl-H), 7.91 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.76 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.33 (s, 1H, OH, D_2O exchangeable) 12.92 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2c**: δ 7.11 (s, 1H, cyanopyridine C_5-H), 7.43 (d, J = 5.92 Hz, 2H, phenyl $C_{3,5}$-H), 7.58 (d, J = 5.92 Hz, 2H, phenyl $C_{2,6}$-H), 7.93 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.79 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.42 (s, 1H, OH, D_2O exchangeable), 12.91 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2d**: δ 6.34 (s, 1H, phenyl C_3-H), 6.45 (d, J = 7.2 Hz, 1H, phenyl C_5-H), 6.98 (d, J = 7.2 Hz, 1H, phenyl C_6-H), 7.09 (s, 1H, cyanopyridine C_5-H), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.37 (s, 1H, OH, D_2O exchangeable), 10.42 (s, 1H, OH, D_2O exchangeable), 12.93 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2e**: δ 3.85 (s, 3H, OCH_3), 7.05 (s, 1H, cyanopyridine C_5-H), 7.13 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.74 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.91 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.74 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.91 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2f**: δ 7.15 (s, 1H, cyanopyridine C_5-H), 7.32 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.46 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.91 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **2g**: δ 6.63 (dd, J_1 = 1.7, J_2 = 3.4 Hz, 1H, furyl C_4-H), 7.12 (s, 1H, cyanopyridine C_5-H), 7.28 (d, J = 1.7 Hz, 1H, furyl C_3-H), 7.82 (d, J = 3.4 Hz, 1H, furyl C_5-H), 7.93 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.79 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.91 (br s, 1H, NH, D_2O exchangeable).

*6.1.2. 4-Aryl-6-(4-pyridyl)-2-thioxo-1,2-dihydropyridine-3-carbonitriles (**3a–g**)*

To a solution of the appropriate **2a–g** (10 mmol) in pyridine, was added P_2S_5 (2 g). The reaction mixture was heated under reflux for 8 h, then poured into hot water. The separated solid product after cooling, was filtered, washed successively with water, dried and crystallized from ethanol (Table 1). IR (cm^{-1}): 3255–3190 (NH), 2224–2217 (CN), 1528-1520,1236–1222, 1146-1138 and 912–898 (NCS amide I, II, III and IV bands, respectively). ^{1}H-NMR of compound **3a**: δ 7.13 (s, 1H, cyanopyridine C_5-H), 7.58–7.61 (m, 3H, phenyl-H), 7.72–7.77 (m, 2H, phenyl-H), 7.91 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.72 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.90 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3b**: δ 7.12 (s, 1H, cyanopyridine C_5-H), 7.61–7.73 (m, 4H, phenyl-H), 7.92 (d, J = 5.88 Hz,

2H, pyridine $C_{3,5}$-H), 8.75 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.32 (s, 1H, OH, D_2O exchangeable) 12.91 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3c**: δ 7.13 (s, 1H, cyanopyridine C_5-H), 7.44 (d, J = 5.92 Hz, 2H, phenyl $C_{3,5}$-H), 7.57 (d, J = 5.92 Hz, 2H, phenyl $C_{2,6}$-H), 7.94 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.43 (s, 1H, OH, D_2O exchangeable), 12.92 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3d**: δ 6.36 (s, 1H, phenyl C_3-H), 6.44 (d, J = 7.2 Hz, 1H, phenyl C_5-H), 6.96 (d, J = 7.2 Hz, 1H, phenyl C_6-H), 7.11 (s, 1H, cyanopyridine C_5-H), 7.91 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.79 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.35 (s, 1H, OH, D_2O exchangeable), 10.44 (s, 1H, OH, D_2O exchangeable), 12.92 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3e**: δ 3.84 (s, 3H, OCH_3), 7.07 (s, 1H, cyanopyridine C_5-H), 7.11 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.76 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.93 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.76 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.90 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3f**: δ 7.16 (s, 1H, cyanopyridine C_5-H), 7.34 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.48 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.94 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.79 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.93 (br s, 1H, NH, D_2O exchangeable).

^{1}H-NMR of compound **3g**: δ 6.64 (dd, J_1 = 1.7, J_2 = 3.4 Hz , 1H, furyl C_4-H), 7.13 (s, 1H, cyanopyridine C_5-H), 7.31 (d, J = 1.7 Hz, 1H, furyl C_3-H), 7.81 (d, J = 3.4 Hz, 1H, furyl C_5-H), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.78 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 12.93 (br s, 1H, NH, D_2O exchangeable).

*6.1.3. 2-Amino-4-aryl-6-(4-pyridyl)-pyridine-3-carbonitriles (**4a–g**)*

The same procedure used to synthesize compounds **2a–g** was applied except that ethyl cyanoacetate was replaced by malononitrile. Compounds were crystallized from methanol. It is worth-mentioning that compound **4g** was previously synthesized [22] (Table 1). IR (cm^{-1}): 3510–3480, 3276–3268 (NH), 2220–2216 (CN).

^{1}H-NMR of compound **4a**: δ 6.86 (s, 1H, cyanopyridine C_5-H), 7.00 (s, 2H, NH_2, D_2O exchangeable), 7.53–7.54 (m, 3H, phenyl-H), 7.63–7.64 (m, 2H, phenyl-H), 7.66 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.74 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H).

^{1}H-NMR of compound **4b**: δ 6.88 (s, 1H, cyanopyridine C_5-H), 7.06 (s, 2H, NH_2, D_2O exchangeable), 7.64–7.71 (m, 4H, phenyl-H), 7.67 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.32 (s, 1H, OH, D_2O exchangeable).

^{1}H-NMR of compound **4c**: δ 6.89 (s, 1H, cyanopyridine C_5-H), 7.13 (s, 2H, NH_2, D_2O exchangeable), 7.44 (d, J = 5.92 Hz, 2H, phenyl $C_{3,5}$-H), 7.61 (d, J = 5.92 Hz, 2H, phenyl $C_{2,6}$-H), 7.69 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.78 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.41 (s, 1H, OH, D_2O exchangeable).

^{1}H-NMR of compound **4d**: δ 6.34 (s, 1H, phenyl C_3-H), 6.45 (d, J = 7.2 Hz, 1H, phenyl C_5-H), 6.98 (d, J = 7.2 Hz, 1H, phenyl C_6-H), 7.09 (s, 1H, cyanopyridine C_5-H), 7.11 (s, 2H, NH_2, D_2O exchangeable), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H), 10.37 (s, 1H, OH, D_2O exchangeable), 10.42 (s, 1H, OH, D_2O exchangeable).

^{1}H-NMR of compound **4e**: δ 3.85 (s, 3H, OCH_3), 7.05 (s, 1H, cyanopyridine C_5-H), 7.09 (s, 2H, NH_2, D_2O exchangeable), 7.13 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.74 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.91 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.74 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H).

^{1}H-NMR of compound **4f**: δ 7.11 (s, 2H, NH_2, D_2O exchangeable), 7.15 (s, 1H, cyanopyridine C_5-H), 7.32 (d, J = 8.80 Hz, 2H, phenyl $C_{3,5}$-H), 7.46 (d, J = 8.80 Hz, 2H, phenyl $C_{2,6}$-H), 7.92 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.77 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H).

^{1}H-NMR of compound **4g**: δ 6.63 (dd, J_1 = 1.7, J_2 = 3.4 Hz , 1H, furyl C_4-H), 7.11 (s, 2H, NH_2, D_2O exchangeable),7.12 (s, 1H, cyanopyridine C_5-H), 7.28 (d, J = 1.7 Hz, 1H, furyl C_3-H), 7.82 (d, J = 3.4 Hz, 1H, furyl C_5-H), 7.93 (d, J = 5.88 Hz, 2H, pyridine $C_{3,5}$-H), 8.79 (d, J = 5.88 Hz, 2H, pyridine $C_{2,6}$-H).

6.2. In vitro studies

The following drugs and chemicals were used in this study: milrinone (Boehringer Ingelheim Co, Ingelheim am Rhein), reserpine, tyramine, propranolol, isoprenaline, carbamylcholine chloride (carbachol), ADA (type VI, from calf intestinal mucosa), DMSO (Sigma Chemical Co., St. Louis, MO), All other reagents were of analytical grade.

6.2.1. Cardiotonic effect on isolated atria

Inotropic and chronotropic effects of the studied compounds in isolated guinea pig atria.

6.2.1.1. Preparation of isolated guinea pig atria. Reserpine-treated male guinea pigs (300–500 g) were killed by a blow to the head followed by exsanguination. The chests were opened via a midsternal incision, the pericardium and facia around the heart were removed and the heart was exposed. The hearts were rapidly placed in a Petri dish filled with physiological salt solution (PSS) and aerated with 95% O_2 and 5% CO_2 in which the atria were separated from ventricles. The atria were then placed in 10 ml organ baths containing PSS of the following composition: 120 mM NaCl, 2.7 mM KCl, 0.09 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.37 mM $CaCl_2$, 11.9 mM $NaHCO_3$, and 5.5 mM d-glucose. The solution was maintained at 34 °C and bubbled vigorously with a mixture of 95% O_2 and 5% CO_2 to produce a pH of 7.5. The resting tension was adjusted at 1 g and developed tension was recorded isometrically by means of a myograph F-60, Narco Bio-system isometric transducer. Signals from the force transducer were continuously recorded on a Narco Biosystem, MK-111 physiograph. After a period of stabilization of

60 min, control measurements of contractile force and frequency were made.

6.2.1.2. Effect on isolated atria. Experiments were performed on spontaneously beating atria obtained from reserpine-treated guinea pigs. Reserpine (2 mg kg^{-1} i.p.) was given 48 and 24 h before the animals were killed, to eliminate the influence of noradrenaline, which may be released from sympathetic nerve terminals. Noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (2 μg ml^{-1}) before starting the experiments. Experiments were performed only in preparations that did not respond to tyramine. All compounds were added to the perfusion fluid in an increasing concentrations starting by a concentration of 5×10^{-5}M and ending by a final concentration of 5×10^{-4} M. After each addition, the inotropic effect was recorded for 3 min before washing and replacement of the perfusion fluid.

All compounds were dissolved in dimethylsulfoxide (DMSO) and added to the bath in a volume of 100 μl, the same volume of DMSO did not produce any effect. Isoproterenol sulfate at a concentration of 5×10^{-4} M was used as a reference standard to be able to follow the changes in both the atria contractility and frequency rate.

Biological evaluation of the test compounds, as cardiotonic agents, was adopted, where inotropic and chronotropic activities were expressed as % change in the force of contraction and frequency rate over control.

To test the involvement of cardiac receptors on the contractile activity of the most active test compounds, the cardiotonic activity of compounds **2a**, **2b**, **2c**, **4c** and **4f** was examined in the presence of a number of receptor blockers, namely propranalol, pyrilamine and ranitidine that block beta adrenergic, H_1, H_2-histamine receptors, respectively. In addition, to test the role of cAMP in mediating the action of the studied drugs, their cardiotonic activity was examined in the presence of the muscarinic receptor agonist; carbachol at 50 μM concentration, which has been reported to inhibit the cardiotonic responses induced by increase in intracellular cAMP in the same preparation.

Because antagonism toward endogenous adenosine has been suggested as one of the mechanisms responsible for the positive inotropic effect of some milrinone analogs [23], some experiments were performed in the presence of ADA, the enzyme that inactivates endogenous adenosine by converting it to inosine. The addition of the enzyme (2 U ml^{-1}) to isolated guinea pig atria evoked by itself a sustained increase in force of contraction that lasted for 15–20 min and left the heart preparation stabilized at a higher contractile level than in controls (+15%). Pretreatment of myocardial preparations with ADA significantly decreased the effect of the test compounds. As previously reported, in the same experimental conditions, the effect of milrinone was also significantly diminished by depletion of endogenous adenosine. Control experiments confirmed that the treatment of atria with ADA did not alter the response of myocardial preparations to another agonist, isoprenaline [24].

6.3. In vivo studies

6.3.1. Cardiovascular effects of the studied compounds in anaesthetized dogs

Preparation of dogs: anesthesia was induced by inhalational ether and maintained with phenobarbitone sodium 80 mg kg^{-1} i.v. The trachea was intubated and the lungs were ventilated. Lactated Ringer's solution was infused at a rate of 5 ml kg^{-1} h^{-1} throughout the study via the femoral vein. A cannula, connected to a manometer, was introduced into the carotid artery and the MBP and HR were recorded.

6.3.2. Effects of the studied compounds in experimental model of heart failure

Fifty rats were subjected to ligation of the left coronary artery as described by Gomej et al. [15]. In brief, rats were lightly anaesthetized with ether. The trachea was intubated and the lungs were ventilated. The sternum was opened and the heart was briefly everted from the thoracic cavity. The left coronary artery was ligated by a tight slipknot with a 4-0 prolene suture from its origin. Coronary artery ligation was considered successful when the anterior wall of the left ventricle turned pale. The heart was then repositioned in the chest and the chest was closed in three layers(ribs, muscle and skin) with polyester 6-0 sutures. A plastic catheter connected to a 5 ml syringe was placed in the chest and was used to remove air from the chest after closure. 10 sham-operated were subjected to the same surgical procedure except that the suture, passed around the left coronary artery was not tied. Eight days after surgical intervention, animals that survived were grouped into seven groups, the first group received no drugs, whereas groups II–VII received milrinone and the test compounds **2a**, **2b**, **2c**, **4c** and **4f**, respectively, orally daily in a dose of 10^{-4} M kg^{-1} b.wt for 6 weeks starting from postligation day 8. At the end of the experimental period, each rat was sacrificed, the heart was rapidly excised and left ventricular papillary muscles were isolated for determination of isometric force [16].

6.3.3. Acute toxicity

The same active compounds **2a**, **2b**, **2c**, **4c** and **4f** were further investigated for their oral acute toxicity in male mice. Six groups of mice each consisting of six animals were used for each compounds. The compounds, or their vehicle 2% gum acacia (control), were given orally in doses of 1, 10, 100, 200, 250, 500 mg kg^{-1}, $N = 6$ each. Twenty-four hours later, the % mortality in each group and for each compound was recorded. Moreover, these compounds were tested for their toxicity through parenteral route. Six groups of mice each consisting of six animals were used for each compounds. The compounds, or their vehicle propylene glycol (control), were given by intraperitoneal injection in doses of 10, 50, 100, 125, 150 mg kg^{-1}, $N = 6$ each. Survival was followed up to 7 days.

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