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# DIFFERENT ACTION OF MILRINONE ANALOGS IN GUINEA-PIG ATRIA

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**Abstract**—1. Three new milrinone analogs, esters of 2-substituted 5-cyano-1,6-dihydro-6-oxo-3-pyridine carboxylic acids [compounds **I**, **II** and **III**] displaced 3H-CHA ($N^6$-cyclohexyl[$^3$H]-adenosine) from its binding sites to $R_i$ receptors in the rat brain.

2. When tested on the contractile activity of electrically driven left atrium from reserpine-treated guinea-pigs, **I** induced marked positive inotropic activity, whereas the most lipophilic compounds **II** and **III**, induced negative inotropic effects.

3. These results suggest that positive inotropic agent may act by displacing endogenous adenosine from its $R_i$ inhibitory receptors in the atria, whereas the negative inotropic agents may act as agonists at the same adenosine receptor.

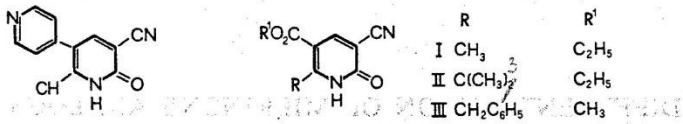
## INTRODUCTION

An antagonism towards endogenous adenosine (Dorigo and Maragno, 1986; Earl *et al.*, 1986; Parsons *et al.*, 1988), and a selective phosphodiesterase III inhibition (Kariya *et al.*, 1982; Endoh *et al.*, 1986), are part of the complex mechanism of action of new cardiotonic agents, such as amrinone, milrinone, enoximone, sulmazole and pimobendan. Adenosine released in the heart is believed to be an important regulator of cardiac functions (Berne, 1963). It decreases atrial force of contraction (Collis and Saville, 1984; Endoh *et al.*, 1985), shows sinus rate (Belardinelli *et al.*, 1983; Endoh *et al.*, 1985) and atrio-ventricular conduction (Belardinelli *et al.*, 1983). A direct inhibition of L-type $Ca^{2+}$ channels (Ribeiro and Sebastiao, 1986; Cerbai *et al.*, 1988) and a direct cAMP and cGMP-independent activation of a $K^+$ conductance were shown to be involved in the reduction of pacemaker activity and conductance caused by adenosine (Schmitz *et al.*, 1981; Belardinelli and Isenberg, 1983; Jochem and Nawrath, 1983; Brückner *et al.*, 1985). A direct cAMP and cGMP-independent decrease in atrial force of contraction have also been reported for adenosine in different animal species (Schmitz *et al.*, 1981; Belardinelli and Isenberg, 1983; Endoh *et al.*, 1983; Jochem and Nawrath, 1983; Brückner *et al.*, 1985; Scholz *et al.*, 1987). All these cardiac effects are mediated by an interaction of adenosine with $R_i$ inhibitory receptors present on the cell membrane (for review see Schutz and Freissmuth, 1985; Brückner *et al.*, 1985; Scholz *et al.*, 1987). Consequently, in some animal species, an inhibition of adenosine binding to $R_i$ receptors may lead to positive inotropic and chronotropic effects without variations in cyclic nucleotide level (Böhm *et al.*, 1984; Schmitz *et al.*, 1981). This mechanism of action is particularly interesting for the practical consequences, since increases in cyclic AMP concentration are known to be associated not only with contractile effects but also with the genesis of harmful arrhythmias (Brodde *et al.*, 1987). The search for a more specific and effective pharmacological treatment for heart failure has lead to the recent development of several novel milrinone analogs (Mosti *et al.*, 1989). Some of these agents, methyl and ethyl esters of 2-substituted 5-cyano-1,6-dihydro-6-oxo-3-pyridine carboxylic acids (Fig. 1), were selected for the present study. Their influence on heart contractile activity was studied in guinea-pig atria where increases in cyclic AMP concentration in response to the inhibition of phosphodiesterase III do not necessarily cause increases in contractility (Weishaar *et al.*, 1987) and where adenosine receptors are not functionally related to adenylate cyclase (Schutz and Tuisle, 1981; Schmitz *et al.*, 1981). In this preparation milrinone analogs induce positive inotropic effects by inhibiting the binding of endogenous adenosine to its $R_i$ receptors without variations in the cellular cyclic AMP content (Dorigo *et al.*, 1987). The contractile activity of the studied compounds was compared with their ability to inhibit the specific binding of [$^3$H]CHA to $R_i$ inhibitory receptors in rat brain and with their lipophilia. The present study offers a new insight into the nature and function of $R_i$ adenosine receptors in the guinea-pig atria.

## METHODS

### *Isolated atria preparations*

Male guinea-pigs (300–500 g) were killed by a blow to the head followed by exanguination and the atria were separated from the ventricles and suspended vertically in a bath containing 30 ml of physiological salt solution of the following composition (mmol.$l^{-1}$): NaCl 120, KCl 2.7, $MgCl_2$ 0.9, $NaH_2PO_4$ 0.4, $CaCl_2$ 1.37, $NaHCO_3$ 11.9, glucose 5.5. The solution was maintained at 29°C and was bubbled vigorously with a mixture of 95% $O_2$ and 5% $CO_2$




MILRINONE ESTERS OF 2-SUBSTITUTED 5-CYANO-1,6-DIHYDRO -6-OXO-3PYRIDINECARBOXYLIC ACIDS

Fig. 1. Structure of different milrinone analogs.

which produced a pH of 7.5. The left atrium was mounted on punctate electrode with a load of 0.5 g and stimulated at a frequency of 1.5 Hz by square wave electrical pulses of 3 msec duration and a voltage 10–20% greater than threshold delivered by a Grass stimulator (Mod. 24KR). The control developed tension ranged from 0.09 to 0.20 mN. Changes in developed tension were registered by a writing oscillograph (Basile Unirecord System, Mod. 7050). The electrical stimulation was performed in order to eliminate any influence on contractile activity due to variations in frequency rate.

*Inotropic activity*

The experiments were performed on atria obtained from reserpine-treated animals. Reserpine (2 mg/Kg$^{-1}$, i.p.) was given at 48 and 12 hr before the animals were killed, in order to eliminate the influence of noradrenaline which might be released from sympathetic nerve terminals (Temma *et al.*, 1977). Noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (2 $\mu$g/ml$^{-1}$) before starting the experiments. The drugs were added to the perfusion fluid after 90 min of equilibration. Inotropic agents were added cumulatively and the inotropic effect was recorded for 5 min after it reached its maximum before washing the preparations or before adding a higher concentration.

*Receptor binding assays*

[$^3$H]CHA ($N^6$-cyclohexyl[$^3$H]adenosine) binding assay. For receptor binding assays male Wistar rats (150–200 g) were decapitated and the whole brain (minus brainstem and cerebellum) was dissected on ice. The tissue was disrupted in a Polytron (setting 5) in 20 vol of 50 mM Tris–HCl pH 7.4. The homogenate was centrifuged at 48,000 *g* for 10 min and the pellet was suspended in buffer, centrifuged and resuspended in Tris–HCl containing 2 IU of adenosine deaminase per ml. After 30 min incubation at 37°C membranes were centrifuged and pellet was stored at −70°C. Binding experiments with [$^3$H]CHA (13.5 Ci/mMol) (Bruns *et al.*, 1980) were performed in 1 ml of buffer which contained 1 nM [$^3$H]CHA, membranes from 15 mg (wet wt) of tissue and the compounds to be tested. After 120 min incubation at 23°C separation of bound from free ligand was performed by rapid filtration through Whatman GF/B filters which were washed 3 times with ice-cold buffer, dried and counted in 5 ml of acidified Instagel. Unspecific binding was defined as binding in the presence of 10 $\mu$l L-PIA (*N*-(L-phenylisopropyl)adenosine) and was <10% of total binding. To determine $IC_{50}$-values, the test compounds were added in triplicate to the binding assays at least six different concentrations and the $IC_{50}$s were calculated by probit analysis.

*Drugs and chemicals*

The agents that we investigated and their sources of supply were as follows: milrinone (Sterling Winthrop), adenosine-deaminase (Sigma), [$^3$H]CHA (New Research Products), instagel (Packard), L-PIA (RBI).

*Calculations*

Data are shown as mean ± SEM. Statistical evaluation of the data was calculated by Student's *t*-test for paired observations. Means were considered statistically different when *P* was lower than 0.05.

## RESULTS AND DISCUSSION

The influence of different milrinone analogs, namely compounds **I**, **II** and **III**, was studied in electrically driven left atrium from reserpine-treated guinea-pigs. In this preparation **I** exerted a more significant increase of developed tension in comparison to milrinone. The maximum positive effect was induced by a drug concentration of $10^{-4}$ M. Higher concentrations ($2 \cdot 10^{-3}$ M) of this compound caused a reduction in the contractile force that was more pronounced than that induced by milrinone (Table 1). The negative inotropic effect was the only response to **II** and **III** (Table 1). Taking into consideration the fact that amrinone and milrinone inhibit the binding of adenosine analogs to $R_i$ inhibitory receptors in different tissues (Earl *et al.*, 1986; Parsons *et al.*, 1988) and that in guinea-pig atria the cardiotonic activity of bipyridine compounds is related to an inhibition of the negative influence exerted by endogenous adenosine on the heart (Dorigo and Maragno 1986; Dorigo *et al.*, 1987), the influence of **I**, **II** and **III** on [$^3$H]CHA binding to $R_i$ receptors of the rat brain was studied. [$^3$H]CHA is a specific agonist for these receptors (Lewis *et al.*, 1981) and its binding parameters in rat brain can be related to physiological responses in the heart (Oei *et al.*, 1988). From the binding experiments the affinity of

Table 1. Effects of esters **I**, **II**, **III** on contractile force of electrically driven left atrium from reserpine-treated guinea-pigs: comparison with milrinone

| Inotropic agent | Developed tension (% increase over control) | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-5}$ M | $5 \cdot 10^{-5}$ M | $10^{-4}$ M | $5 \cdot 10^{-4}$ M | $10^{-3}$ M | $2 \cdot 10^{-3}$ M |
| Milrinone | 6.46 ± 0.54 | 26.21 ± 2.03 | 40.39 ± 0.71 | 42.04 ± 3.64 | 39.26 ± 2.80 | 26.29 ± 2.59 |
| **I** | 23.18 ± 0.12 | 51.33 ± 3.01 | 55.86 ± 4.06 | 47.86 ± 4.50 | 24.12 ± 1.77 | −8.99 ± 1.69 |
| **II** | 4.45 ± 0.60 | −28.38 ± 2.46 | −39.78 ± 3.92 | −58.56 ± 6.36 | −63.70 ± 7.38 | −65.28 ± 5.67 |
| **III** | 7.49 ± 1.98 | −32.23 ± 0.89 | −37.97 ± 0.43 | −50.84 ± 4.10 | −59.36 ± 2.77 | −71.58 ± 3.04 |

The compounds were added cumulatively to the perfusion medium of the atrium. Each data is mean ± SE of 8 determinations from 8 different experiments.

milrinone analogs for $R_i$ receptors was evaluated on the bases of the $IC_{50}$s that were $1.77 \pm 45 \cdot 10^{-4}$ M for **I**, $1.09 \pm 37 \cdot 10^{-3}$ M for **II** and $1.15 \pm 15 \cdot 10^{-4}$ M for **III**. These concentrations, correspond well to the ones that were highly active on the guinea-pig left atrium. Thus as in the case of the parent bypiridine agents compound **I** may induce its positive inotropic effect by inhibiting the binding of endogenous adenosine to $R_i$ receptors in the cardiac cell whereas compounds **I** and **II** may bind to and activate these receptors, thus evoking an adenosine-like negative inotropic response.

To further elucidate this point the effect of **I** and of milrinone on contractile force of the guinea-pig left atrium was studied in the presence of **II** and **III** in order to stress any possible interaction between the two types of compounds.

When **I** was added to the atrium preincubated with increasing concentrations of **II** (Fig. 2) or of **III** (Fig. 3), a progressive increase of the negative inotropic effect induced by **I** was evident while the positive response was depressed and finally abolished. **II** and **III** caused similar changes in the effect of milrinone thus suggesting the possibility of a different binding site for the various milrinone analogs at receptor level. This hypothesis is supported by the differences in lipophilia among the studied agents (Table 2).

Recently a map of the $N^6$ region of adenosine R receptors has been planned by means of structure–activity relationship of a large number of adenosine analogs (Kusachi *et al.*, 1985; Daly *et al.*, 1986) and with the aid of computer graphics (Van Galen *et al.*, 1989). The molecular modelling studies indicate that $R_i$ receptor contain different areas which must be filled for inducing adenosine like-effects and that hydrophobicity besides stereoselectivity is a prominent characteristic of agonists at the receptor. In fact substituents with more hydrophilic character usually cause a considerable decrease in affinity (Daly *et al.*, 1986; Van Galen *et al.*, 1987). Furthermore, at least three regions exist in the $R_i$ receptor model whose occupation leads to diminished receptor affinity, the so-called forbidden areas. If this molecular model is

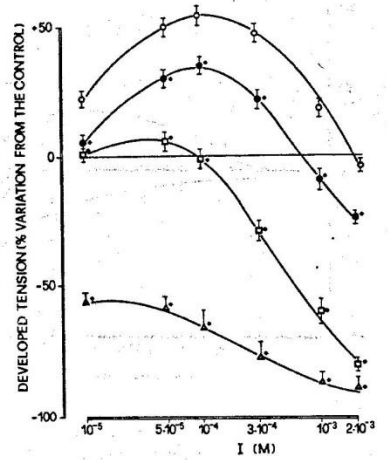



Fig. 3. Effect of compound **I** on electrically driven left atrium preincubated in the presence of compound **III**. Left atrium was obtained from reserpine-treated guinea-pig. Compound **III** was added to the perfusion medium 10 min before the addition of compound **I**. Each data is mean ± SE of 10 determinations from 10 different experiments. $*P < 0.001$; ○—○, **I**; ●—●, **I** + **III** $5 \cdot 10^{-5}$ M; □—□, **I** + **III** $10^{-4}$ M; △—△, **I** + **III** $5 \cdot 10^{-4}$ M.

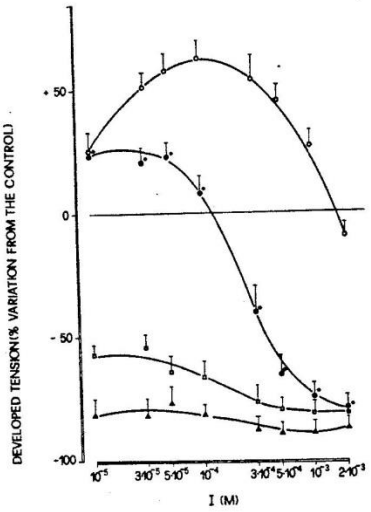



Fig. 2. Effect of compound **I** on electrically driven left atrium preincubated in the presence of compound **II**. Left atrium was obtained from reserpine-treated guinea-pig. Compound **II** was added to the perfusion medium 10 min before the addition of compound **I**. Each data is mean ± SE of 10 determinations from 10 different experiments. $*P < 0.001$; ○—○, **I**; ●—●, **I** + **II** $5 \cdot 10^{-5}$ M; □—□, **I** + **II** $10^{-4}$ M; △—△, **I** + **II** $5 \cdot 10^{-4}$ M.

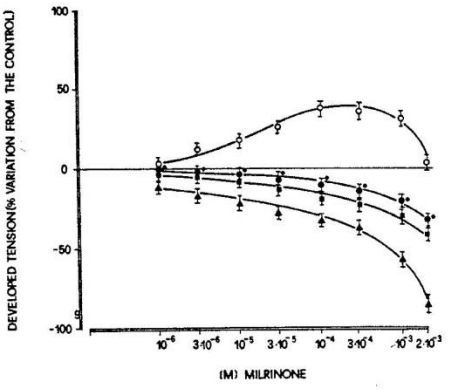



Fig. 4. Effect of milrinone on electrically driven left atrium preincubated in the presence of compound **II**. Left atrium was obtained from reserpine-treated guinea-pig. Compound **II** was added to the perfusion medium 10 min before the addition of milrinone. Each data is mean ± SE of 6 determinations from 6 different experiments. $*P < 0.001$; ○—○, milrinone; ●—●, milrinone + **II** $5 \cdot 10^{-5}$ M; ■—■, milrinone + **II** $10^{-4}$ M; △—△, milrinone + **II** $5 \cdot 10^{-4}$ M.

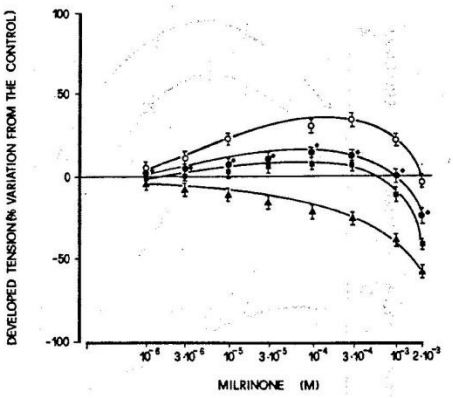



Fig. 5. Effect of milrinone on electrically driven left atrium preincubated in the presence of compound **III**. Left atrium was obtained from reserpine-treated guinea-pig. Milrinone was added to the perfusion medium 10 min before the addition of compound **III**. Each data is mean ± SE of 10 determination from 10 different experiments. $^*P < 0.001$; ○—○, milrinone; ●—●, milrinone + **III** $5 \cdot 10^{-1}$ M; ■—■, milrinone + **III** $10^{-4}$ M; △—△, milrinone + **III** $5 \cdot 10^{-4}$ M.

operative in the guinea-pig atria compound **I** may accommodate in one or more of the forbidden areas and diminish the receptor affinity, thus hindering the binding of endogenous adenosine to its cardiac receptor, whereas compounds **II** and **III** might reach and activate the subregions related to the transfer of an adenosine-like message into the cell.

The present results, together with the theoretical informations on receptor structure and function (Kusaki *et al.*, 1985; Daly *et al.*, 1986; Van Galen *et al.*, 1989), underline the complex nature of adenosine $R_i$ receptor and the possibility of multiple pharmacological interactions with different functional consequences.

## SUMMARY

1. The synthesis of ethyl and methyl esters of 2-substituted 5-cyano-1,6-dihydro-6-oxo-3-pyridine carboxylic acids gave rise to new milrinone analogs whose cardiac activity was studied on electrically driven left atrium from reserpine-treated guinea-pigs (Mosti *et al.*, 1989).

2. In guinea-pig left atrium compound **I** induced a marked and concentration dependent increase in developed tension that was followed by a phase of negative inotropism at the highest concentrations. This negative phase was more pronounced then that observed with milrinone. Compounds **II** and **III**, induced only a concentration-dependent negative inotropic action.

3. When guinea-pig left atrium was preincubated in the presence of **II** or **III**, a concentration-dependent inhibition of the stimulatory effects induced by **I** and by milrinone was observed, with consequent increase of the negative component.

4. In rat brain **I**, **II** and **III** inhibited the specific binding of $N^6$-cyclohexyl[$^3$H]adenosine ([$^3$H]CHA) to the adenosine $R_i$ inhibitory receptors with an $IC_{50}$ of $177 \pm 45\ \mu$M, $1.09 \pm 37$ mM and $115 \pm 15\ \mu$M, respectively, that are in the range of concentrations effective in the guinea-pig left atrium.

5. The determination of the lipophilia indicated that it increases in the following order **II** < **III** < **I**.

6. The present results suggest that a displacement of endogenous adenosine from its $R_i$ inhibitory receptors in the atria may be involved in the positive inotropic action of the least lipophylic compound, whereas the negative influence on cardiac contractility exerted by the most lipophilic compounds, **II** and **III** may be related to an activation of the same receptors.

Table 2. Lipophilia of substituents in the molecule of different milrinone analogs

| Compound | R | π | R′ | π′ | π + π′ |
|---|---|---|---|---|---|
| **I** | $-CH_3$ | 0.56 | $-C_2H_5$ | 1.02 | 1.58 |
| **II** | $-C(CH_3)_3$ | 1.98 | $-C_2H_5$ | 1.02 | 3.00 |
| **III** | $-CH_2C_6H_5$ | 2.01 | $-CH_3$ | 0.56 | 2.57 |

π (Hansch hydrophobic constant) of a substituent R is defined as the difference between the logP of $C_6H_5$-R and the logP of $C_6H_6$, where P is the octanol/water partition coefficient; a positive value for π means that, relative to H, the substituent R favors the octanol phase. π values were taken from substituent constants for correlation analysis in *'Chemistry and Biology* (Edited by Hansch C. and Leo A. J.), Wiley, New York (1979).

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