A New Milrinone Analog: Role of Binding to A1 Adenosine Receptor in its Positive Inotropic Effect on Isolated Guinea Pig and Rat Atria

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Accepted for publication July 28, 1997

ABSTRACT

In electrically driven left atria isolated from guinea pig and rat, a new milrinone analog, 6-ethyl-5-propionyl-1,2-dihydro-2-oxo-3-pyridine carbonitrile, produced a positive inotropic effect that was not dependent on adrenergic mechanisms and was more marked than that exerted by the parent compound. Its inotropic action was almost completely abolished by pretreatment of atria with adenosine deaminase and correlated well with its binding ability to the cardiac adenosine A1 receptor. In this regard, the analog showed a 100-fold higher affinity for adenosine receptor than that of milrinone. It shifted to the right the concentration-response curves for the negative inotropic action of the stable adenosine receptor agonist R-phenylisopropyladenosine. The new analog behaved as a competitive inhibitor of Type III phosphodiesterase isolated from both guinea pig and rat, although its $K_i$ value was 10 times higher than that of milrinone. However, an increase in cAMP levels does not seem to be involved in the mechanism of action of the new compound, because the presence of carbachol did not decrease the extent of the positive inotropic effect of the analog and did not modify its $EC_{50}$ in either guinea pig or rat myocardial preparations. Taken together, these results suggest that the milrinone structure can be modified, giving rise to a more active compound whose inotropic effect in both guinea pig and rat appears to be more clearly related to antagonism toward endogenous adenosine than to Type III phosphodiesterase inhibition.

The treatment of congestive heart failure is largely based on the use of cardiac glycosides, diuretics and vasodilators. Although digoxin is one of the most commonly prescribed drugs, the role of digitalis in the management of the disease remains at the center of the oldest continuing controversy in the history of medicine (Packer, 1997). The pronounced toxic effects and low therapeutic index of digitalis glycosides (Marchisio, 1971; Lathers and Roberts, 1980) have stimulated the development of new agents. In particular, antagonism toward endogenous adenosine at the cardiac A1 inhibitory receptor has been suggested (Farah and Alousi, 1978; Alousi et al., 1979; Ward et al., 1983). These drugs have both inotropic and peripheral vasodilator activities and therefore enhance cardiac output by increasing cardiac contractility and simultaneously reducing impedance to ventricular ejection (Miller et al., 1981; Taylor et al., 1982).

Received for publication April 21, 1997.

ABBREVIATIONS: ADA, adenosine deaminase; $[^3H]$DPCPX, $[^3H]$8-cyclopentyl-1,3-dipropylxanthine; R-PIA, R-phenylisopropyladenosine; PDE, phosphodiesterase; DMSO, dimethylsulfoxide; Tris, Tris(hydroxymethyl)aminomethane; Ca$^{2+}$/ATPase, Ca$^{2+}$-activated adenosine triphosphatase; Na$^{+}$/K$^{+}$ ATPase, Na$^{+}$/K$^{+}$-activated adenosine triphosphatase.
amounts during heart failure (Newman et al., 1984), may further damage the heart by slowing conduction in the sinoatrial and AV nodes and reducing atrial contractility. For these reasons, the search continues for new adenosine antagonists more active than amrinone and milrinone.

In our previous studies (Dorigo et al., 1991, 1992, 1993, 1996), we used milrinone as the parent compound, variously modified its molecule and obtained several compounds that enhanced cardiac contractility to various extents. A linear relationship was observed between the ability of these new compounds to displace adenosine from its receptor and their inotropic action (Dorigo et al., 1992, 1997). Taking into account some observations of a structure-activity relationship (Dorigo et al., 1997), we designed and synthesized a new milrinone analog, 6-ethyl-5-propionyl-1,2-dihydro-2-oxo-3-pyridine carbinitrile, that is closely related to the parent compound. In the present study, we investigated and characterized the cardiac effects of this new molecule on isolated guinea pig and rat atria. In order to understand the biochemical mechanisms responsible for the cardiac effects of the new milrinone analog, we also determined the binding of the compound to cardiac A1 receptors and its inhibitory effect on Type III PDE isolated from guinea pig and rat ventricular tissue. All studies were carried out on tissues isolated from both guinea pig and rat, because Azari and Huxtable (1980) observed a species difference in the action of the parent drug amrinone on Langendorff perfused heart isolated from guinea pig and rat.

**Materials and Methods**

**Myocardial preparations.** Normal or reserpine-treated (2 mg/kg i.p. daily for 2 days) guinea pigs (300–500 g b.wt.) and rats (150–200 g b.wt.) were killed by a blow to the head followed by exsanguination. The atria were separated from the ventricles and suspended vertically in a 30-ml organ bath containing a physiological salt solution constantly gassed by 95% O2 and 5% CO2 at 29°C. The bath solution contained (mM): NaCl 120, KCl 2.7, CaCl2 1.36, MgCl2 0.99, NaH2PO4 0.4, NaHCO3 12 and glucose 5.5.

The atria were electrically driven at 1 Hz by square-wave pulses just above threshold voltage, 0.6 to 0.9 msec in duration (S44 stimulator, Grass Instrument Corporation, Quincy, MA). The force of contraction was recorded on an isometric force transducer (7003 Basile, Comerio, Varese, Italy) connected to a rectilinear recorder (KV 135 Battaglia Rangoni, Casalecchio di Reno, Bologna, Italy). The initial equilibration period was 40 to 60 min for each preparation. Resting tension was adjusted to about 5 mN.

In myocardial preparations isolated from reserpine-treated animals, depletion of catecholamines was confirmed by the lack of any positive inotropic effect of tyramine (15 μM). Where indicated, propranolol (1 μM), carbachol (50 nM) or ADA (2 U/ml) was added to the bath medium 20 min before addition of the drugs.

In each atrial preparation, the response to isoprenaline (0.2 and 0.02 μM for guinea pig and rat atria, respectively) was determined before addition of the test compounds and was considered the maximum positive inotropic effect (E<sub>max</sub>) obtainable in the atria. Milrinone analog was added cumulatively, and the inotropic response caused by each drug concentration was recorded up to the maximum response before a higher concentration was added. The peak of the inotropic response caused by each concentration of analog was half the maximum effect obtainable with the drug. Milrinone and its analog were dissolved in DMSO, the final concentration of which in the medium did not itself influence the basal activity of the atrial preparations.

**Receptor binding assay.** Guinea pig and rat ventricular tissue was dissected in a Brinkman PT-10 Polytron (setting 6) in 25 volumes (w/v) of 50 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 40000 × g and resuspended in 50 mM Tris-HCl (pH 7.4) containing 2 U/ml ADA. After 30 min of incubation at 37°C, in order to metabolize endogenous adenosine, the membranes were centrifuged at 40000 × g and the pellets were stored at −80°C until used.

Binding of milrinone analog to cardiac A<sub>1</sub> adenosine receptor was determined by its ability to displace [3H]DPCPX, a specific antagonist for A<sub>1</sub> receptors. Binding experiments were carried out for 150 min at 0°C in 1 ml of buffer containing 1 nM [3H]DPCPX, membranes from 10 mg (wet weight) of tissue and increasing concentrations of milrinone analog. To determine IC<sub>50</sub> values (where IC<sub>50</sub> is the concentration that displaces 50% of the labeled ligand), six different concentrations (from 32 nM to 32 μM) of milrinone analog were added to the binding assay medium. All experiments were carried out in triplicate. K<sub>i</sub> values (K<sub>i</sub> = inhibitory binding constant) were calculated from the Cheng and Prusoff (1973) equation with K<sub>i</sub> = IC<sub>50</sub>/(1 + C/K<sub>i</sub>*), where C is the radioligand concentration used and K<sub>i</sub>* (1 nM) is its dissociation constant. Binding data were analyzed using the nonlinear regression curve-fitting computer program Ligand (Munson and Rodbard, 1980). Nonspecific binding was determined in the presence of 10 μM N<sub>6</sub>-cyclohexyladenosine (or 1 mM theophylline) and was routinely 30% of total binding. Bound and free [3H]DPCPX were separated using a Brandel cell harvester by rapid filtration under vacuum through Whatman GF/B glass-fiber filters and then were washed three times with ice-cold buffer, dried and counted in 5 ml of Istagel (Packard, Groningen, The Netherlands) in a Beckmann Liquid Scintillation Spectrometer, at a counting efficiency of about 55%.

**Assay of soluble Type III PDE activity from guinea pig and rat heart.** Type III PDE was isolated from guinea pig and rat heart using the procedure described by Weishaar et al. (1986). Type III PDE from guinea pig and rat heart had apparent K<sub>m</sub> values for cAMP of 1.33 ± 0.15 and 1.37 ± 0.35 μM and V<sub>max</sub> values of 4.54 ± 0.29 and 4.20 ± 0.12 nmol/mg protein/min, respectively. When assayed at 0.4 μM cAMP, the activities of guinea pig and rat cardiac Type III PDE were inhibited by about 80% by 4 μM cGMP. Both fractions were insensitive to calmodulin and were only slightly inhibited by 100 μM ralipamil, the specific inhibitor of Type IV PDE.

PDE activity was measured by the two-step procedure of Thompson et al. (1974), as previously described (Dorigo et al., 1992).

**Assay of ATP-dependent 45Ca<sup>2+</sup> uptake by cardiac sarcoplasmic reticulum vesicles.** A crude cardiac membrane vesicle preparation enriched in sarcoplasmic reticulum was obtained by the method of Jones et al. (1977) from guinea pig and rat ventricular tissue. Ca<sup>2+</sup> uptake was determined as previously described (Floreni et al., 1996). Milrinone and milrinone analog were dissolved in DMSO; the same amount of DMSO was always added to the controls.

**Assay of Na<sup>+</sup>/K<sup>+</sup> ATPase, Ca<sup>2+</sup> ATPase and Na<sup>+</sup>/Ca<sup>2+</sup> exchange carrier activities in cardiac sarcosomel membrane vesicles.** Cardiac sarcosomel vesicles were prepared from guinea pig and rat ventricular tissue by the method of Slaughter et al. (1983). Na<sup>+</sup>/K<sup>+</sup> ATPase, Ca<sup>2+</sup> ATPase and Na<sup>+</sup>/Ca<sup>2+</sup> exchange carrier activities were measured as previously described (Floreni et al., 1996).

**Protein assay.** Protein content was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

**Statistical analysis.** Data are expressed as arithmetic means ± S.E.; Student’s two-tailed t test was used for statistical analysis.

**Chemicals.** The following drugs and chemicals were used in this study: milrinone (Sanofi-Winthrop, Collegeville, PA), milrinone analog (L. Mosti, Institute of Pharmaceutical Sciences, University of Genova, Italy), reserpine, tyramine, propranolol, isoprenaline, R-
PIA, carbamylcholine chloride (carbachol), ADA (type VI, from calf intestinal mucosa), Tris, ATP, GMP, EGTA, DMSO, N6-cycloexyladenosine, Dowex 1 × 2, DEAE cellulose and 5'-nucleotidase (grade II, from Crotalus atrox) (Sigma Chemical Co., St. Louis, MO), [3H]DPCPX (NEN New England Nuclear, Florence, Italy) and 8-[3H]cAMP (Amersham Italia, Milan, Italy). All other reagents were of analytical grade.

Results

The newly synthesized analog of milrinone was closely related to the parent drug. As shown in figure 1, the basic structure of dihydro-oxo-pyridine carbonitrile was maintained, whereas the 4-pyridinyl moiety in C5 and the methyl group in C6 were substituted with a propionyl group and an ethyl group, respectively.

Effect of milrinone analog on atria contractility. We determined the effect of the new analog on the contractility of isolated guinea pig and rat atria (n = 8) electrically driven at 1 Hz. It caused a concentration-dependent increase in the force of contraction of atria from both species. The increase was quite rapid in onset and reached its peak within 10 min. Figure 2 shows the cumulative concentration-response curves for the positive inotropic effect of milrinone analog in guinea pig (panel A) and rat (panel B) electrically driven left atria, compared with the effect of the parent drug. When the extent of contraction induced by the drugs was referred to the maximum contraction induced by isoprenaline in the same experimental conditions in both guinea pig and rat atria, the highest concentrations of the analog appeared to be more active than those of the parent compound. Moreover, the analog caused a more marked increase in the force of contraction of rat atria than of guinea pig atria. The EC50 values for milrinone and its analog, calculated from the cumulative concentration-response curves, indicate that the potency of the analog was quite similar to that of the parent compound in both species. The effect of the new compound was not modified in catecholamine-depleted atria or in atria pre-treated with 1 µM propranolol (data not shown).

Even at the highest concentration tested (1 mM), the analog did not have toxic effects on the myocardial preparations; it did not cause arrhythmias or any increase in resting tension. Moreover, the effect of the analog was completely reversible; washout of myocardial preparations restored the pre-drug force of contraction of atria.

When tested on spontaneously beating guinea pig and rat atria, the new compound exerted a positive inotropic action quantitatively similar to that observed in electrically driven left atria (data not shown).

Because antagonism toward endogenous adenosine has been suggested as one of the mechanisms responsible for the positive inotropic effect of some milrinone analogs (Dorigo et al., 1992, 1997), 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) to left atria isolated from both guinea pig and rat evoked by itself a sustained increase in force of contraction that lasted for 15 to 20 min and left the heart preparation stabilized at a higher contractile level than in controls (+15% and +30% in guinea pig and rat, respective-
ly). Pretreatment of myocardial preparations with ADA markedly decreased the effect of the milrinone analog (table 1). As previously reported (Dorigo et al., 1990), 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 (Dorigo et al., 1990).

To elucidate the involvement of adenosine antagonism further, we evaluated the influence of the new compound on the negative inotropic effect induced in atria by R-PIA, a stable adenosine receptor agonist. As shown in figure 3, the analog caused a rightward shift of the concentration-response curve for R-PIA. This effect was more pronounced in rat (panel B) than in guinea pig (panel A).

To evaluate the involvement of cAMP increase in the mechanism of the cardiotonic action of the new analog, we tested the influence of carbachol (50 nM) on the positive inotropic effect caused by the compound. The results indicated that the presence of carbachol did not modify the EC50 values for the positive inotropic effect of the analog in either guinea pig (EC50 = 40 ± 3 µM) or rat (EC50 = 61 ± 5 µM) heart. Moreover, the Emax of the analog was slightly enhanced, rather than decreased, by pretreatment of myocardial preparations with carbachol. This enhancement of Emax was probably related to the decrease in basal contractility observed in the presence of carbachol.

**Binding of milrinone analog to cardiac A1 receptors.**

To confirm the involvement of antagonism toward endogenous adenosine in the positive inotropic action of the new milrinone analog, we determined the effect of the compound on the specific binding of an adenosine A1 receptor antagonist, [3H]DPCPX, in membranes from guinea pig and rat cardiac tissue. Figure 4 clearly shows that the analog displaced [3H]DPCPX from its binding sites to isolated cardiac membranes. Using the Cheng and Prusoff (1973) equation, we obtained Ki values of 1.30 ± 0.28 and 0.66 ± 0.03 µM in guinea pig and rat cardiac membranes, respectively.

**TABLE 1**

Effect of ADA on the positive inotropic effect caused by milrinone analog in guinea pig and rat electrically driven left atria

ADA (2 U/ml) was added to the bathing medium of electrically driven left atria 20 min before the addition of the milrinone analog. Because ADA itself increased the force of contraction of the atria, the effect of the analog was evaluated by considering that the basal level of force of contraction was that reached when the effect of ADA had become stable. The effects, measured at the time of maximum effect, were expressed as percentages of maximum increase in force of contraction [Emax] induced by isoprenaline in the same preparation. The values are means ± S.E. from four experiments carried out on different myocardial preparations (n = 4).

<table>
<thead>
<tr>
<th>ADA Concentration</th>
<th>Rat Left Atria</th>
<th>Guinea Pig Left Atria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Emax 2 U/ml ADA</td>
<td>%Emax 2 U/ml ADA</td>
</tr>
<tr>
<td>5 × 10⁻⁶ M</td>
<td>5.52 ± 0.31 0</td>
<td>0</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>10.15 ± 0.30 2.3 ± 0.4*</td>
<td>19.05 ± 0.41 3.8 ± 0.4*</td>
</tr>
<tr>
<td>2 × 10⁻⁵ M</td>
<td>0.14 ± 4.39 0</td>
<td>19.05 ± 0.41 3.8 ± 0.4*</td>
</tr>
<tr>
<td>5 × 10⁻⁵ M</td>
<td>36.60 ± 6.18 14.4 ± 1.4*</td>
<td>30.24 ± 0.95 4.2 ± 0.3*</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>77.90 ± 6.07 16.4 ± 1.9*</td>
<td>40.71 ± 1.02 2.7 ± 0.5*</td>
</tr>
<tr>
<td>2 × 10⁻⁴ M</td>
<td>91.51 ± 8.53 11.6 ± 3.5*</td>
<td>40.71 ± 1.02 2.7 ± 0.5*</td>
</tr>
<tr>
<td>5 × 10⁻⁴ M</td>
<td>109.63 ± 15.8 7.8 ± 4.3*</td>
<td>40.71 ± 1.02 2.7 ± 0.5*</td>
</tr>
<tr>
<td>10⁻³ M</td>
<td>100.00 ± 3.8 5.8 ± 2.1*</td>
<td>44.25 ± 0.87 1.8 ± 0.7*</td>
</tr>
</tbody>
</table>

* P < .001, calculated vs. the value obtained with the same concentration of milrinone analog in the absence of ADA.
from guinea pig and rat cardiac tissue. It inhibited, in a concentration-dependent way, the activity of the enzyme isolated from the cardiac tissue of both animal species. $K_i$ values of 11 $\mu$M and 19 $\mu$M were calculated for guinea pig and rat, respectively, as shown by the Dixon plots (Dixon, 1953) reported in figure 5. Analysis of the data according to Lineweaver and Burk (1934) (fig. 6) clearly indicates that the analog behaved as a competitive inhibitor for Type III PDE activity.

### Effect of milrinone analog on other enzymes and transport systems involved in cardiac contractility.

The milrinone analog was also tested on other enzymes and transport systems involved in the control of cardiac contractility, such as the sarcolemmal Na\(^+/K^+\) ATPase, Ca\(^{2+}\) ATPase and Na\(^+\)/Ca\(^{2+}\) exchange carrier activities and the sarcoplasmic reticulum Ca\(^{2+}\) pump. It had no effect on them (data not shown).

### Discussion

The present results show that a new milrinone analog, 6-ethyl-5-propionyl-1,2-dihydridro-2-oxo-3-pyridine carbonitrile, closely related to the parent compound, increases in a concentration-dependent way the force of contraction of isolated guinea pig and rat atria. The new compound is more active than the parent drug in both types of atria, although its potency is quite similar to that of milrinone. Its cardiac action is completely independent of direct or indirect adrenergic mechanisms. In fact, it is not modified by pretreatment of myocardial preparations with the beta blocker propranolol or by depletion of cardiac adrenergic stores by reserpine. Our data suggest that the main mechanism responsible for the positive inotropic effect of this milrinone analog is antagonism toward endogenous adenosine.

Several observations support this hypothesis. First, the analog binds with high affinity to the A\(_1\) adenosine receptor present in membranes isolated from guinea pig and rat cardiac tissue, as demonstrated by its ability to displace \[^3H\]DPCPX, a specific adenosine antagonist, from its binding sites. Second, the cardiac action of the analog is markedly decreased when endogenous adenosine is removed by pretreatment of atria with ADA, the enzyme that converts adenosine to inactive inosine. The positive inotropic effect of the new analog is not affected by the presence of carbachol. According to Endoh (1979), this result excludes any involvement of cAMP in the mechanism of cardiotonic action. Moreover, the compound shifts rightward the cumulative concentration-response curves for the negative inotropic effect of R-PIA, a stable adenosine receptor agonist. These cardiac effects, i.e., positive inotropic effect and shift of R-PIA curves, are more pronounced in rat than in guinea pig atria. The increase in force of contraction caused by the analog is in fact 45% of $E_{\text{max}}$ in guinea pig atria and 100% of $E_{\text{max}}$ in rat atria. Furthermore, in guinea pig atria, 20 $\mu$M analog increases the EC\(_{50}\) value of R-PIA about 2.5 times, whereas in rat atria the same concentration increases the value 5-fold. It seems reasonable to suggest that the higher activity of the compound on rat than on guinea pig pig cardiac tissue is related to its higher affinity for rat cardiac adenosine receptor. We calculated $K_i$ values for displacement of \[^3H\]DPCPX of 0.66 $\pm$ 0.03 $\mu$M and 1.30 $\pm$ 0.28 $\mu$M in rat and guinea pig cardiac tissue, respectively. In line with these considerations, it is reasonable to assume that the analog is more active than milrinone in causing a positive inotropic effect in both guinea pig and rat atria. In our previous work on guinea pig cardiac tissue (Dorigo et al., 1992), we calculated a $K_i$ value of 100 $\mu$M for displacement of an adenosine agonist (\[^3H\]cycloexyladenosine) by milrinone. In rat cardiac tissue, too, the $K_i$ value for milrinone is similar (unpublished results). Therefore, the higher efficacy of the analog with respect to that of the parent compound is ascribed to its higher ability to displace endogenous adenosine.

The inhibition of soluble Type III PDE is generally considered part of the mechanism of the cardiac action of milrinone (Endoh et al., 1986; Weisshaar et al., 1986; Silver et al., 1988; Brunskhorst et al., 1989). In our conditions, milrinone inhibited guinea pig cardiac Type III PDE in a competitive way, with a $K_i$ value of 1.4 $\mu$M (Dorigo et al., 1992). Similar behavior and similar kinetic parameters are also evident against rat cardiac Type III PDE (data not shown). The present data show that the new analog inhibits both guinea pig and rat cardiac Type III PDE competitively. Dixon plot analysis of the data yielded $K_i$ values of 11 and 19 $\mu$M for Type III PDE isolated from guinea pig and rat heart, respectively. Thus milrinone, although it is a more potent inhibitor...
of cardiac Type III PDE than its analog, is less active as a positive inotropic agent in both species tested. Moreover, the experiments performed in the presence of carbachol (Dorigo and Maragno, 1986; present results) indicate that an increase in cAMP does not play any fundamental role in the mechanism of action of milrinone and its analog. This suggests that there is no relationship between the inhibition of cardiac Type III PDE and the positive inotropic effect of the two drugs. Because some degree of inhibition of cardiac Type III PDE is always caused by both milrinone and its analogs, although it is apparently not involved in their inotropic action, it must be assumed that in rat also (as documented in guinea pig atria; Weishaar et al., 1987), Type III PDE may be either biochemically uncoupled from myocardial contractile proteins or compartmentalized in the cytosol, so that increases in cAMP concentrations do not influence cardiac contractility. The present results further support our previous hypothesis (Dorigo et al., 1992) that milrinone and its analogs may exert cardiac effects mainly through antagonism toward adenosine rather than through inhibition of Type III PDE activity.

In conclusion, our data demonstrate that our new synthesized milrinone analog has higher affinity for cardiac A1 adenosine receptor, and consequently a higher positive inotropic effect, than milrinone. We are thus encouraged to search for new analogs with even more pronounced characteristics as antagonists toward endogenous adenosine, in an attempt to create a safe cardiotonic drug devoid of any dangerous effect on intracellular cAMP levels. The negative inotropic effect of adenosine is ascribed to reduced calcium entry into cells as a consequence of direct K+-channel activation (Belardinelli and Isenberg, 1983), 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 a Type III PDE inhibitor.

References


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