Structure-Activity Relationships of Milrinone Analogues Determined *in Vitro* in a Rabbit Heart Membrane Ca^{2+}-ATPase Model

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The cardiac activity of a series of analogues of the positive inotropic bipyridines amrinone (5-amino-[3,4′-bipyridin]-6(1*H*)-one) and milrinone (2-methyl-5-cyano-[3,4′-bipyridin]-6(1*H*)-one) was evaluated *in vitro* in a rabbit myocardial membrane Mg^{2+}-dependent, Ca^{2+}-stimulable adenosine triphosphatase (Ca^{2+}-ATPase) model, and structure-activity relationships were compared for nine closely related derivatives. In the present studies, a 5-bromo analogue of milrinone stimulated myocardial membrane Ca^{2+}-ATPase significantly (10^{-7} M; $P < 0.001$ vs control, with 67% of the activity of milrinone), whereas a 2′-methyl-2*H*-milrinone derivative was inactive. Although amrinone was inactive in this assay, its 2-methyl analogue was stimulatory. However, analogues lacking a 2-substituent (with or without a 5-cyano group) or with the 3-N position blocked by a methyl group did not stimulate myocardial membrane Ca^{2+}-ATPase activity. Structural data for these bipyridines show that those with either a 2- or 2′-methyl substituent have a twist conformation, whereas those without are nearly planar. Activity data reveal that those bipyridines with a nonplanar conformation are more active in the Ca^{2+}-ATPase assay. Further study of milrinone analogues with a 2′-methyl substituent shows that even though the effect on the twist angle is equivalent to that of 2-methyl substitution, these analogues are less potent. Data for this series reveal that the prerequisites for Ca^{2+}-ATPase stimulation include not only a 2-methyl to maintain a twist conformation but also a free 3-N position and a 5-substituent. This model for optimal activity in the myocardial membrane Ca^{2+}-ATPase system differs from those proposed for phosphodiesterase enzyme receptor recognition only in the requirement for a nonplanar molecule. We have previously shown that milrinone, but not amrinone, shares structural homology with thyroxine and was able to stimulate myocardial membrane Ca^{2+}-ATPase activity in a manner similar to the thyroid hormone. Additionally, milrinone, but not amrinone, was an effective competitor for thyroxine binding to the serum transport protein transthyretin. Analysis of the milrinone-transthyretin crystal complex confirms the structural homology between milrinone and thyroid hormone which is not shared by amrinone. Modeling studies of the binding interactions of milrinone analogues indicate that the 2-desmethylmilrinone analogue, the most inhibitory analogue, lacks the hydrophobic contacts present in milrinone in its transthyretin-bound complex. These findings correlate with their structure-activity relationships in the Ca^{2+}-ATPase assay. On the basis of these data, it is predicted that increased inhibition of Ca^{2+}-ATPase activity could be achieved by the addition of a 2′-methyl to the 2-desmethylmilrinone analogue, the most inhibitory in this series. However, such an analogue would be a weaker competitor for the binding of thyroxine to transthyretin.

Introduction

Over the last several years new classes of compounds have been developed for the management of congestive heart failure by favorably altering ventricular loading conditions and myocardial contractility (refs 1 and 2 and references cited therein). The bipyridines milrinone (2-methyl-5-cyano-[3,4′-bipyridin]-6(1*H*)-one) (Figure 1) and amrinone (5-amino-[3,4′-bipyridin]-6(1*H*)-one) belong to a class of nonglycosidic positive inotropic agents whose inotropic and vasodilator effects have not been completely elucidated.[3] Among their modes of action are selective inhibition of specific cardiac cAMP phosphodiesterase activity,[4-6] altered intracellular Ca^{2+} compartmentalization, and activation of the Ca^{2+} release channel and Ca^{2+} pump.[6-9] We have reported that milrinone, but not amrinone, stimulates myocardial Ca^{2+}-stimulable adenosine triphosphatase (Ca^{2+}-ATPase) in a manner similar to the thyroid hormones L-thyroxine (T_4) and 3,5,3′-L-triiodothyronine (T_3).[9,10] In other studies we have shown that milrinone displaces labeled T_4 from its binding sites on human serum transthyretin (61% reduction in T_4 binding by 10 μM milrinone), whereas amrinone was ineffective.[11] Analysis of the molecular structure of milrinone revealed homology with thyroxine,[9] and a model of the interaction of milrinone in the transthyretin hormone binding site was proposed from computer modeling studies.[11] This model was validated by crystallographic determination of the milrinone-bound complex of human transthyretin.[12] In skeletal muscle sarcoplasmic reticulum

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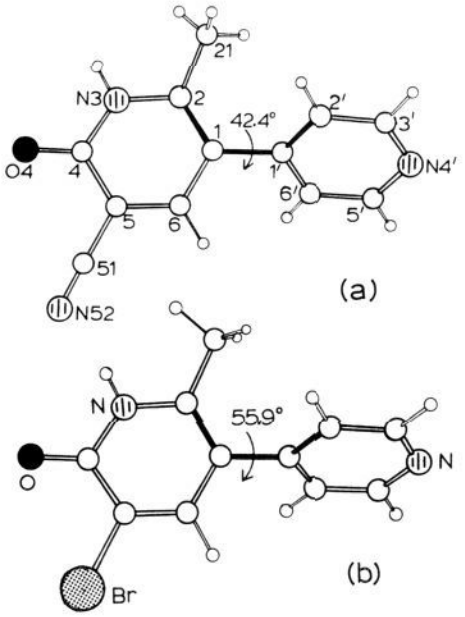


Figure 1. Molecular structure of (a) milrinone and (b) its 5-bromo analogue. The torsion angle C(2)–C(1)–C(1′)–C(2′) is shown.

(SR) membranes, milrinone also displaced radiolabeled T_4 from binding sites in concentrations equimolar to unlabeled T_4. In addition, milrinone and the 5-bromo and 5-amino analogues of milrinone all stimulated skeletal muscle SR membrane Ca^{2+}-ATPase activity.[13]

To understand the mechanism of action of positive inotropic bipyridine agents on the selective inhibition of cAMP phosphodiesterase, models of the active site interactions have been proposed[14–17] which reveal the prerequisite features for maximal enzyme effects. These early models were refined as second-generation phosphodiesterase inhibitors were designed.[14,17] These models suggested that potent inhibitors require a dipole or heteroatom cluster, a lone pair or phenyl ring, and an electron-rich region as depicted by imidazole.[17] Pharmacophore requirements were also derived on the basis of structural and electronic similarities[15] which showed that, for milrinone and other bipyridines, the primary features needed for positive inotropic action are an aromatic hydrophobic area and an electronegative region produced by a pyridyl N or amide system capable of tautomerization. These models show that two sites of recognition are by means of hydrogen bond donor and acceptor positions.[17] Other studies revealed that methylation in the 3-N position of the pyridone ring leads to a striking reduction in activity.[16] Many of these characteristics are also exhibited by the milrinone derivatives under study here.

To evaluate the molecular features required for biochemical activity of inotropic bipyridines, the conformational, geometric, and electrostatic properties of inotropic bipyridines have been investigated in several laboratories.[15,17–22] Geometry optimizations based on *ab initio* calculations show that amrinone and milrinone prefer a twist conformation as the minimum energy structure with the torsion angle C(2)–C(1)–C(1′)–C(2′) of 37° for amrinone and 67° for milrinone, respectively. In the case of amrinone, the torsion energy has a broad shallow profile with a barrier of about 2 kcal/mol, while

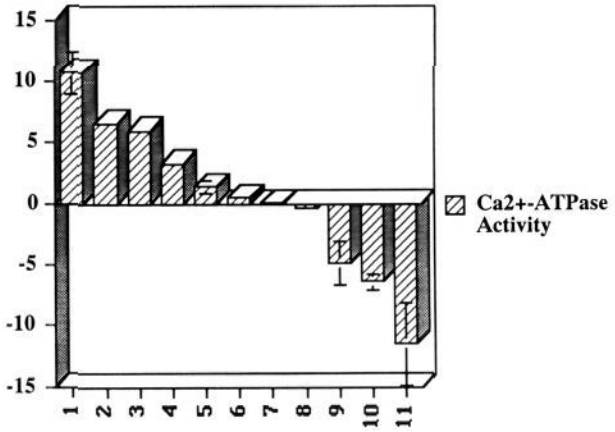


Figure 2. Comparison of the change in Ca^{2+}-ATPase stimulation (μM P_i/mg/60 min) with the addition of each analogue, 10^{-7} M. See Table 1 for analogue definitions.

that for milrinone has a steeper narrow profile with a barrier of about 12 kcal/mol.[19,20]

Calculations of the molecular electrostatic potential for amrinone and milrinone show that there are negative potential zones around the pyridine 4′-N and the 4-keto and 5-substituent of both molecules. These zones around the pyridone ring broaden on going from a planar to a twist conformation with those for amrinone having a deeper negative potential. These data suggest that the twisted conformations are more active.[19,20] A model for putative receptor interactions has been proposed based on the results of similar electrostatic potential calculations carried out on other positive inotropes which requires an aromatic hydrophobic area and an electronegative region produced by a pyridyl N or an amide tautomeric system.[15,17,18,22]

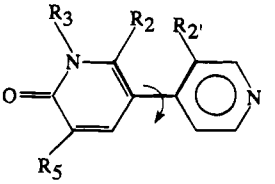
As part of a program to define the thyromimetic properties of inotropic bipyridines, we report the Ca^{2+}-ATPase activity for a series of nine bipyridine analogues of milrinone and amrinone in myocardial membranes and rabbit skeletal muscle sarcoplasmic reticulum membranes.[13] We also report the crystal structure of the 5-bromo analogue of milrinone and compare its structure with structures of other analogues in this series.[23–27] We also have investigated inotrope interactions in the milrinone–human transthyretin crystal complex and propose a qualitative model of the bipyridine inotrope binding site interactions necessary for myocardial membrane Ca^{2+}-ATPase activity, as well as for the competition with thyroxine binding to transthyretin.

Results

The schematic structures, molecular conformation, and bioactivities, as measured by the change in Ca^{2+}-ATPase activity which they induce, of 11 bipyridine inotropic agents with substitutions in the 2-, 3-, 5-, and 2′-positions are illustrated in Figure 2 and Table 1. The *in vitro* analogue concentration was 10^{-7} M, the concentration at which milrinone has the greatest enzyme stimulating effect.

As illustrated by these structure–activity data, milrinone (Figure 2 and Table 1, compound **1**) has the greatest stimulatory effect on Ca^{2+}-ATPase activity while that of its 2-desmethyl analogue (**11**) was the most inhibitory, indicating the crucial importance of the

Table 1. Change in Myocardial Membrane Ca^{2+}-ATPase Activity for a Series of Positive Inotropic Milrinone Derivatives



analogue	R_2	R_3	R_5	$R_{2'}$	activity[a]	torsion[b]	ref
1	CH_3	H	CN	H	10.7 ± 1.7	45.2; 62.2	23, 28
2	CH_3	H	Br	H	6.6 ± 0.4	55.9	
3	CH_3	H	NH_2	H	6.0 ± 0.4	−38.5; 36.8	27
4	CH_3	H	CN	CH_3	3.3 ± 0.1	74.3	27
5	H	H	NH_2	H	1.4 ± 0.5	13.4; 8.7; −11.8; 21.7; −0.8	23, 28
6	H	H	CN	CH_3	0.5 ± 0.3	143.9	27
7	H	H	NH_2	CH_3	0.03 ± 0.3	59.4	27
8	CH_3	H	NH_2	CH_3	−0.3 ± 0.2		
9	CH_3	H	H	H	−4.9 ± 1.8	58.8; 55.9	25
10	CH_3	CH_3	CN	H	−6.4 ± 0.7	43.7; 66.6	24
11	H	H	CN	H	−11.5 ± 3.4	3.3; −2.2	26

[a] Enzyme activity is in μmol of P_i/mg/60 min and expressed as mean ± SE of three or more experiments. [b] Torsion angle C(2)−C(1)−C(1′)−C(2′).

Table 2. Average Bipyridine Ring Geometries

	average (Å)				average (deg)		
bond	overall	free base	protonated	angles	overall	free base	protonated
C1−C2	1.377	1.370	1.387	C6−C1−C2	118.16	117.85	118.56
C1−C6	1.414	1.412	1.417	C1−C2−N3	119.23	119.80	118.50
C2−N3	1.362	1.363	1.362	C2−N3−C4	125.53	125.34	125.77
N3−C4	1.374	1.373	1.376	N3−C4−C5	114.94	114.81	115.09
C4−C5	1.438	1.432	1.445	C4−C5−C6	120.44	120.62	120.22
C5−C6	1.366	1.371	1.361	C5−C6−C1	121.49	121.51	121.46
C1′−C2′	1.396	1.392	1.400	C6′−C1′−C2′	117.55	117.25	117.94
C1′−C6′	1.392	1.392	1.391	C1′−C2′−C3′	119.01	118.74	119.34
C2′−C3′	1.386	1.386	1.385	C2′−C3′−N4′	122.74	124.44	120.56
C3′−N4′	1.333	1.336	1.342	C3′−N4′−C5′	118.55	116.36	121.38
N4′−C5′	1.335	1.331	1.328	N4′−C5′−C6′	122.31	123.78	120.42
C5′−C6′	1.384	1.386	1.382	C5′−C6′−C1′	119.61	119.40	119.88
C4−O4	1.244	1.248	1.234				
C1−C1′	1.483	1.486	1.480				

2-methyl constituent on enzyme activity. The addition of a 2′-methyl group ot the milrinone pyridine ring (**4**) decreases the milrinone effect significantly (10.7 vs 3.3 μmol of P_i/mg/60 min), whereas this substitution in compound **6** blocks most of the enzyme inhibitory effect observed with compound **11**. The significant stimulatory effect of the 5-bromo analogue **2** further indicates the importance of an electronegative substituent at this position which corresponds to the phenolic iodine of thyroxine.[9]

These data also show that the parent inotrope, amrinone (**5**), has little effect in this system whereas the addition of a 2-methyl to amrinone (**3**) significantly increases its stimulatory potency (6.0 vs 1.4 μmol of P_i, respectively). However, the addition of a 2′-methyl in the pyridine ring of amrinone tends to decrease potency as shown by the negligible stimulatory activity of compound **7**. Surprisingly there is little effect with the addition of a 2-methyl to this analogue (**8**), which only minimally inhibits enzyme activity.

Other requisite features of the inotrope analogues for Ca^{2+}-ATPase effect were probed by compounds **9** and **10**. Data for **9** reveal the role of the 5-substituent in altering enzyme activity, whether electron withdrawing (CN, Br) or electron releasing (NH_2), as the absence of a 5-substituent causes inhibition of enzyme activity by that analogue (comparing **9** with **1**−**3**). These data also show that when the 3-NH pyridone position is blocked with a methyl group (**10**) there is inhibition of enzyme activity by the compound; this suggests the importance of a free 3-NH to participate in hydrogen bonding and other electrostatic interactions with the enzyme. Both of these changes results in enzyme inhibition and point to the importance of these positions in reversing Ca^{2+}-ATPase stimulation.

The molecular conformations of the 11 inotropic bipyridines under study are characterized by the torsion angle C(2)−C(1)−C(1′)-C(2′) (Figure 1) which are also listed in Table 1. These data show a wide variation in torsion angle, ranging from 0° to 144°, which is reflective of bipyridine ring substitution patterns. For example, methylation at the 2- or 2′-position of the bipyridine causes the molecule to deviate from planarity. The increased activity of the methylated molecules, compared to more planar nonmethylated parent compounds, suggests an influence on biochemical activity.

Geometry Correlations. To understand the influence of geometry on conformation and activity, analyses of the geometries of these inotropes have also been carried out (Table 2, Figure 3). The bipyridine geometries are reflective of their protonation state in the crystal lattice, and although there are only small differences in the bonds and angles of the free base versus protonated structures, these changes are focused at specific atoms in the bipyridine ring structure. For example (Table 2, Figure 3), when the average bond

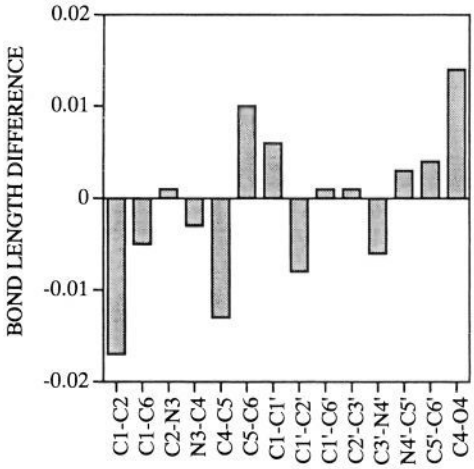


Figure 3. Bar graph showing differences between the average bipyridine ring bond lengths (Å) between free base and protonated structures, respectively, for the structures listed in Table 1.

lengths in the pyridone ring are compared with those for the free base or protonated structures, it can be seen that the largest differences are observed for the bond lengths of C(1)−C(2), C(4)−C(5), and C(4)−O(4) of the pyridone ring and the geometry around 4′-N of the pyridine ring (Figure 3). The most pronounced differences involve the keto function (1.234 vs 1.248 Å for protonated and free base, respectively). While the changes in bond distances between electronic species are not large, the difference in the bond angle at 4′-N is significant (121.4° vs 116.4° for protonated and free base, respectively). These changes are in response to protonation at 4′-N of the pyridine ring.

The pyridone ring in all structures of bipyridine inotropic agents is nearly planar with the greatest individual variation observed for the C(4)−C(5) bond. There is significant variation in the pyridone ring torsion angles of any one compound although the overall average is nearly planar. These changes are reflective of the ring substitutents and the protonation state of the molecule. The pyridone ring substitution in positions 2, 3, and 5 causes the variation of bond distances and angles and influences the electron delocalization in the ring (Table 2).

Hydrogen Bonding. This series of positive inotropic bipyridines is further characterized by a network of intermolecular hydrogen bonds with 4-O and 4′-N acting as acceptors and 3-NH and 5-NH_2 acting as donors (Table 3). These structures can be divided into two groups: those which are free bases and those which are bipyridinium salts. The salt series include structures of **1**, **3**, **5**, **10**, and **11**. In all structures the single anion (Cl^-, Br^-) is coupled to the bipyridinium monocation. The geometry of the unprotonated 2,3-dimethyl 5-cyano analogue **10**, compared with that of its protonated form, reveals significant differences in the C(3′)−N(4′)−C(5′) geometry of the pyridine ring. All bipyridine inotropic agents reported here also participate in stacking interactions involving both bipyridine rings, with ring−ring distances varying from 3.3 to 3.5 Å. In some structures this ring stacking results is a hyperparallelism of the ring layers and the addition of noncrystallographic symmetry elements in others.[27]

Further analysis reveals that the packing of the free base analogues form cyclic symmetrical hydrogen bonds between 3-N-H···4-O, while those which are salts have linear 4′-N···4-O interactions (Table 3). In some cases, the hydrogen bonding patterns are more complex, depending on the number of independent conformers in the asymmetric unit or the number of water and anions in the lattice. Compounds **5A** and **6** (Table 3) are the only two structures to utilize N·N hydrogen bonds. The packing pattern of **5A** is complex as the four independent molecules are involved in all observed hydrogen bond patterns in this series, as well as ring−ring stacking.

Structural data for these compounds reveal that the presence of the 2-methyl substituent in these bipyridine structures causes the molecule to have a twist conformation as compared to the nearly planar conformation of amrinone.[23−28] The molecular conformation of the 5-bromo analogue of milrinone (Figure 1) shows that the bromopyridone ring has a greater twist, as defined by the torsion angle C(2)−C(1)−C(1′)−C(2′), than observed in the structure of milrinone.[23] The crystal packing of the 5-bromo analogue reveals a network of hydrogen bonds involving N(3)···O(4) (2.76 Å) forming a symmetric dimer pairs and Br···N(3) contacts (3.47 Å), shorter than the sum of the van der Waals radii (3.60 Å), connecting layers of molecules. Such close intermolecular contacts have been observed in other halogenated structures, in particular the thyroid hormones.[29] There are also ring−ring stacking interactions between the molecular layers, a feature common to most of the structures in this series.[27]

Discussion

The data shown in Table 1 for this series of 11 inotropic bipyridines reveal correlations between molecular structure and substituent pattern at positions 2, 5, and 2′ and their activity in the rabbit myocardial membrane Ca^{2+}ATPase assay system. As illustrated, analogues **1**−**4** stimulate the enzyme, with milrinone (**1**) having the greatest activity; analogues **5**−**8** have little or no effect, as reflected by amrinone (5); and the remaining analogues, **9**−**11**, are inhibitory in this system. By comparison of pairs of compounds, the relative contribution of each substituent or conformational feature can be assessed.

The nature of the 5-substituent is coupled to the presence of the 2-methyl for optimal effect in the myocardial membrane Ca^{2+}-ATPase system as all combinations of 5-CN and NH_2 have activity. Comparison of milrinone (Table 1, **1**) and its 5-bromo analogue (**2**) suggests that the effect of changing the electrostatic character of the 5-CN to 5-Br is to decrease stimulatory activity. The relative effect of the 5-CN alone is to increase the stimulatory activity as seen by comparison of **1** and **9** which have similar conformations. Moreover, comparison of **9** and **3** shows the relative importance of the 5-amino substituent alone for enzyme activity.

The calculation of the effect of the 2-methyl substituent on activity is more indirect since in most comparisons thre is a change in conformation as well as substituent. Comparison of **1**, the most stimulatory, with **11**, the most inhibitory, shows that the combined effect of the 2-methyl and the change in molecular conformation is a complete reversal of action on Ca^{2+}-ATPase activity. However, comparison of **6** and **11** implies that the 2-CH_3 alone decreases potency while

Table 3. Hydrogen-Bonding Patterns of Inotropic Bipyridines[a]

analogue	A···D	analogue	A···D	analogue	A···D
1A:W		**4**		**7**	
N(3)···O(4)	2.76	N(3)···O(4)	2.74	N(3)···O(4)	2.76
O(4)···W	3.12	**5A**, $z = 4$		N(51)···O(4)	2.93
W···W	2.16	N(3)···N(4′)	2.85	**9A**	
1B:HCl		N(51)···O(43)	3.02	N(3)···O(4)	2.77
N(3)···Cl	3.06	O(4)···N(51*)	3.06	**9B**	
N(4′)···O(4)	2.64	O(4)···N(513)	3.00	N(3)···O(4)	2.76
2		N(4′*)···N(33)	2.80	**10A**:HBr2W	
N(3)···O(4)	2.76	N(3*)···N(4′3)	2.82	N(4′)···W1	2.69
3:2HCl,4W		O(4*)···N(34)	2.73	N(4′)···W2	2.62
N(51)···W1	3.12	N(513)···O(43)	3.16	O(4)···W1	3.01
N(4′)···O(4)	2.60	O(43)···N(514)	3.04	Br···W1	3.38
N(4′*)···O(4*)	2.61	**5B**:2HCl,W		Br···W2	3.48
N(3*)···W2	2.77	N(3)···Cl1	3.09	**10B**	
N(51*)···W1	3.05	N(51)···Cl1	3.11	**11**:2HBr	
Cl1···W2	3.16	N(51)···Cl2	3.04	N(4′)···O(4)	2.83
Cl1···W4	3.14	N(51)···W	3.13	N(4′*)···O(4*)	2.70
Cl2···W1	3.17	N(4′)···O(4)	2.68	Br1···N(4′)	3.37
Cl2···W3	3.16	Cl2···W	3.04	Br2···N(3*)	3.26
Cl2···W4	3.12	Cl2···W	2.87		
W1···W3	2.77	**6**			
W2···W4	2.69	N(3)···N(4′)	2.75		

[a] See Table 1 for inotrop description.

the effect of conformational change increases potency. The only inconsistencies observed for the 2-methyl assignment come from comparison of compounds **3**, **5**, and **8**, all of which have a 5-amino substituent. These inconsistencies suggest that the differences in the electrostatic properties of CN and NH_2 also have an influence and that the contribution of the 2-methyl must be taken together with that at the 5-position.

The effect of the 2′-methyl substituent is to decrease the stimulatory activity as reflected in the comparison of **3** and **8**. However, since the crystal structure of **8** have not been determined, its conformation can only be assumed to be similar to that of **3**, in which case the change in activity can be assigned entirely to the 2′-methyl on the pyridine ring. The difference in activity between compounds **1** and **4** is consistent with this assignment. Comparison of **5** and **7** may reveal an added effect for the change in torsion angle, and their activity differences are the combination of a positive effect for torsion change and a negative effect for the 2′-methyl.

On the basis of these studies, it was verified that the 3-NH pyridone ring position should be free as methylation results in a significant reduction in Ca^{2+}-ATPase stimulation, similar to that observed for phosphodiesterase action.[16,17] This suggests either steric constraints in the binding site of Ca^{2+}-ATPase or the requirement for hydrogen bond formation for activity. In addition, maximum stimulation of Ca^{2+}-ATPase activity is obtained with 2-methyl compounds such as milrinone which have a twist conformation. Amrinone, which lacks this 2-methyl group and thus have a nearly planar conformation, in inactive in this system. The structure–activity relationships observed for these milrinone analogues are schematically summarized in Figure 4. Those analogues which are stimulatory (Table 1, **1**–**4**) in the Ca^{2+}-ATPase model have a 2-methyl substituent which causes the bipyridine ring system to have a twist angle > 40°, a hydrogen at position 3 which permits hydrogen-bonding interactions with the enzyme system, and at position 5 either a cyano, bromo, or amino group. When these conditions are not present, the analogues are not stimulatory. The absence of a

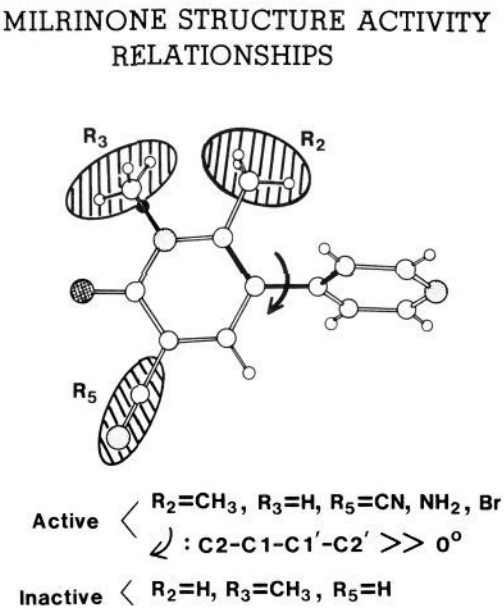


Figure 4. Model of the requirements for maximal stimulation of Ca^{2+}-ATPase in a rabbit myocardial membrane assay. This model shows that maximal stimulation requires a 2-CH_3, a 3-NH, an electronegative 5-substituent, and a twist angle greater than 40° between rings.

methyl group at position 2 results in a planar bipyridine ring system; a 3-methyl group blocks hydrogen-bonding interactions; and a hydrogen at the 5-position is not large enough for optimal interactions in the enzyme model. A 2′-methyl milrinone analogue lacking a 2-methyl group, although it has a significant twist angle, does not stimulate enzyme activity. These data also suggest that by appropriate combinations of substituents at the pyridone 2, 3, and 5 positions by bipyridine can have stimulatory (analogues **1**–**4**), inhibitory (**9**–**11**), or no (**5**–**8**) effects in the myocardial membrane Ca^{2+}-ATPase assay system.

This model showing stereochemical requirements for maximal stimulatory effects of inotropic bipyridines on Ca^{2+}-ATPase activity is similar to that proposed for maximal phosphodiesterase III inhibition.[17,18] As indi-

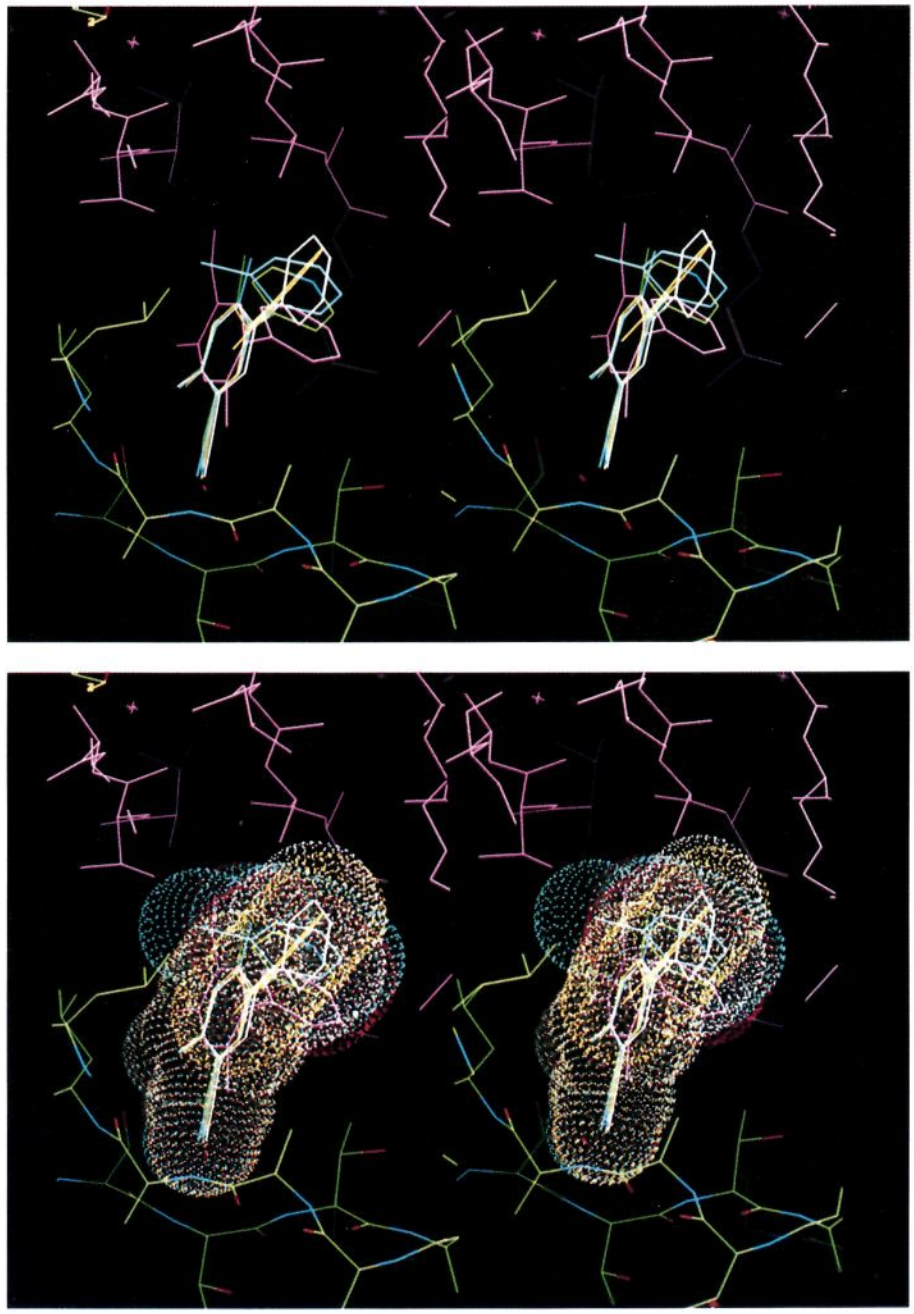


Figure 5. (Top) Stereodiagram of the binding site in the human transthyretin–milrinone complex.[12] Milrinone occupies the binding channel with a 50% positional disorder (magneta) along the crystallographic 2-fold axis in the binding channel which relates the independent monomers (colored by atom type) with their symmetry related monomers (magenta). Superimposed are the structures of analogues **4** (cyan), **6** (yellow), and **11** (white). These analogues were modeled by matching their respective pyridone ring with that of milrinone. (Bottom) Same stereoview with van der Waals surface added to illustrate close contacts.

cated for a 5-point pharmacophore model of the phosphodiesterase III, the positive inotrope should have a dipolar moiety with an adjacent acidic proton and a small lipophilic space across the ring from the pyridine N, hydrogen bonding distal from the dipolar moiety and generally flat topology.[18] These features are present in this series of bipyridines under study as revealed by the need for an acidic proton at the 3-NH position with an adjacent dipolar group at the 4-keto, a lipophilic group at the 5-position, and a hydrogen-bonding group distal to the keto group at the 4′-N position. These models differ in the details needed for enzyme selectivity. For maximum Ca^{2+}-ATPase stimulation, the bipyridine should have twist conformation and a 2-methyl substituent as found in milrinone, the most stimulatory analogue in this series.

Thyromimetic Properties. Structure–activity relationships reveal that the inotropic bipyridines share structural homology with thyroxine as shown by their stimulation of Ca^{2+}-ATPase activity in the same manner as the thyroid hormones.[9–11,13] Further study showed that milrinone, but not amrinone, could displace radiolabeled thyroxine from its serum transport protein, transthyretin (TTR).[11] Modeling studies of the binding of milrinone in the thyroxine binding site of transthyretin were confirmed by crystal structure analysis of a human TTR–milrinone complex.[12] These data show that milrinone occupies the thyroxine binding channel

with its cyanopyridone ring bound in the hormone halogen phenolic ring pocket. Modeling studies of amrinone bound in the transthyretin channel with the pyridone ring matching that of milrinone[12] showed that the 5-amino group has only three contacts shorter than 3.8 Å, with the shortest N···C distance of 3.3 Å; in contrast, the milrinone cyano group is involved in as many as 15 contacts with the surrounding residues. The lack of 2-methyl interactions formed by amrinone further weakens the binding of amrinone to TTR.

In the case of the 2-methyl analogue of amrinone (**3**, Table 1) bound in TTR, modeling studies showed that because its twist angle is similar to milrinone, it could form the same contacts via its methyl group as observed for milrinone; however, its amino group interactions would be weaker than those of the milrinone cyano. Similar studies modeling the interactions of the 5-bromo analogue **2** (Table 1) show that the 5-bromo substituent fits the same binding pocket as the 5-cyano of milrinone and that it more closely resembles the electrostatic nature of iodine in the thyroid hormones.

As shown (Figure 5), comparison of analogues **4**, **6**, and **11**, modeled so that their respective pyridone ring was matched to that of milrinone, reveals that in the case of the structure of **4** (cyan) there are unfavorable contacts between the 2′-methyl and the side chain of residue threonine-119 (2.2 Å). These clone contacts are highlighted in Figure 5, bottom, which shows the same view of the active site model with van der Waals surfaces added to each of the inotropes. When the torsion angle between the bipyridine rings is rotated 180°, the 2′-methyl makes even closer contacts to the protein at residue leucine-17 (1.9 Å). Similar, but less severe, close contacts are observed for the structure of **6** (yellow), where the closest contact of the 2′-methyl is to leucine-17 (2.8 Å) and the side chain of alanine-108 (2.7 Å) where the ring is rotated 180°. On the other hand, because of its planar conformation and the lack of a 2-methyl substituent, there is a loss in the number of binding interactions for **11** (white), which has the largest inhibitory activity in the Ca^{2+}-ATPase enzyme system. Although there may be some changes in the binding orientation of inotrope TTR crystallized complexes, these models probe the most relevant range of interactions.

On the basis of these assignments, one can predict the activity, either stimulatory or inhibitory, for several new analogues. Thus, the addition of a 2′-methyl to **11** should have the effect of substantially increasing the inhibitory potency of this series. However, such an analogue would be predicted to be a weaker competitor for the binding of thyroxine to transthyretin because of the unfavorable interactions of the 2′-methyl group.

Materials and Methods

Reagents and Analogues. Na_2ATOP and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO.) Milrinone, amrinone, and other analogues were kindly provided by Sterling-Winthrop Research Institute (Rensselaer, NY, and Collegeville, PA).

Myocardial Membranes. Rabbit myocardial membranes were prepared as previously described[10] using the differential centrifugation method of Jones, Besch, and co-workers[30] and yielded a homogeneous vesicular preparation without contractile elements.

Ca^{2+}-ATPase Activity. Membrane Ca^{2+}-ATPase activity was measured as the difference in ATP hydrolysis in the presence and absence of 20 μM free Ca^{2+} and is expressed as μmol of inorganic phosphate (P_i) liberated/mg of protein/60 min assay period.[31] Assays were conducted in duplicate, and results presented are means ± SEM of three or more experiments. Intra-assay and interassay coefficients of variation were 2% and 13%, respectively.

Effect of Milrinone Analogues on Membrane Ca^{2+}-ATPase Activity. Milrinone and its analogues were solubilized in DMSO and incubated with cardiac membranes for 60 min at 37 °C during the ATPase assay. The final concentration of DMSO in incubation samples with bipyridine or diluent alone (control) was 1%, and analogue concentration was 10^{-7} M. Results were analyzed by paired *t*-test, comparing enzyme activity with and without bipyridine analogue.

Crystallographic Data. Crystals of the 5-bromo derivative of milrinone were grown from ethanolic solutions, and X-ray diffraction data were collected on an Siemens P3 automated diffractometer using Nb-filtered MoKα radiation (λ = 0.71069 Å). Cell parameters were determined by least-squares refinement of 24 reflections. Crystal data showed the crystals were monoclinic, $C2/c$, $Z = 8$, with lattice parameters a = 24.813(3) Å, b = 6.9012(8) Å, c = 12.398(2) Å, and β = 98.51(1)°. No significant intensity variation for standards was detected, and absorption and extinction corrections were not applied. There were 3160 unique reflections collected and the data reduced using the programs of Blessing.[32] The structure was solved by direct methods using the programs MULTAN78[33] and NQEST.[34] The function minimized during refinement was $\sum w(|F_o| - |F_c|)^2$ where $w = 1/\sigma^2(F)$. The final residual was 0.047 for 3160 reflections with $I > 2\sigma$. The nonhydrogen atoms were refined anisotropically, while the hydrogen atom positions were calculated and their isotropic thermal parameters held fixed at 1 unit greater than the nonhydrogen positions to which they were attached.

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Supplementary Material Available: Crystal data for the 5-bromomilrinone analogue and atomic coordinates, as well as the geometry and anisotropic thermal parameters (4 pages). Ordering information is given on any current masthead page.

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