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COMPETITION OF MILRINONE, A NON-IODINATED CARDIAC INOTROPIC AGENT, WITH THYROID HORMONE FOR BINDING SITES ON HUMAN SERUM PREALBUMIN (TBPA)*

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Abstract—Milrinone [2-methyl-5-cyano-(3,4′-bipyridin)-6(1*H*)-one] is a positive cardiac inotropic agent recently shown to have thyromimetic activity *in vitro* in a rabbit myocardial membrane Ca^{2+}-ATPase system [K. M. Mylotte *et al., Proc. natn. Acad. Sci. U.S.A.* **82**, 7974 (1985)]. In the present studies, milrinone was examined for activity as an inhibitor of iodothyronine binding by human serum thyroid hormone transport proteins, thyroxine-binding globulin (TBG), prealbumin (TBPA) and albumin. Polyacrylamide gel electrophoresis at pH 9.0 of sera equilibrated with [^{125}I]thyroxine showed that milrinone competed with L-thyroxine (T_4) for binding sites on TBPA (10 and 100 μM milrinone caused 61 and 73% reductions, respectively, in T_4 binding to TBPA, $P < 0.01$); T_4 displaced from TBPA was bound by TBG and albumin. Comparable reductions in T_4 binding to TBPA were observed in electrophoretic studies conducted at pH 7.4. Binding of triiodo-L-thyronine (T_3) to TBPA was electrophoretically confirmed and shown to be decreased in the presence of milrinone. Electrophoresis of purified TBPA also demonstrated that [^{14}C]milrinone co-migrated with this transport protein and that milrinone displaced tracer T_4 from TBPA. Amrinone, the 2-H-5-NH_2 analog of milrinone, had less than 5% of the activity of milrinone as an inhibitor of T_4 binding in electrophoretic studies. Scatchard analysis of T_4 and milrinone binding to purified TBPA, measured by equilibrium dialysis, showed two classes of binding sites, with association constants, respectively, of $6.1 \times 10^7\ M^{-1}$ and $1.6 \times 10^6\ M^{-1}$ for T_4, and $1.7 \times 10^6\ M^{-1}$ and $8.9 \times 10^2\ M^{-1}$ for milrinone. Computer graphic modeling of the binding of milrinone to the T_4 site in the crystal structure of TBPA showed that milrinone best occupied this site when the substituted bipyridine ring overlapped the phenolic ring of T_4. In this orientation the 5-cyano group, which has an electronegativity similar to that of iodine, occupied the same volume as the 5′-iodine of T_4. The 5-amino group of amrinone lacks these characteristics. In this orientation, the keto function of milrinone overlapped the T_4 4′-hydroxyl and could participate in similar intermolecular interactions. Thus, milrinone, a non-iodinated bipyridine, and thyroid hormone share structural and biochemical homologies and compete for the same binding site on TBPA.

Milrinone [2-methyl-5-cyano(3,4′-bipyridin)-6(1*H*)-one] is a nonglycosidic positive cardiac inotropic agent with a non-iodinated bipyridine structure (Fig. 1) [1]. X-ray crystallographic studies and computer graphic modeling of milrinone have shown structural homologies with thyroid hormone [2], and milrinone has been found to stimulate rabbit myocardial membrane Ca^{2+}-ATPase activity *in vitro* in a manner analogous to that of iodothyronines [2]. To further test milrinone for structure–activity relationships shared with thyroid hormone, we have examined the ability of milrinone to compete with L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) for their binding sites on human serum transport proteins, thyroxine-binding globulin (TBG), prealbumin (TBPA) and albumin. Milrinone has been compared in these experiments with amrinone (Fig. 1), its parent 2-H-5-NH_2 bipyridine analog [3].

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MATERIALS AND METHODS

Chemicals. Milrinone, amrinone and [^{14}C]milrinone were provided by Mr. A. E. Soria, Sterling-Winthrop Research Institute (Rensselaer, NY). The specific activity of the labeled milrinone was 26 μCi/mg. The purity of the milrinone was verified by gradient high pressure liquid chromatography in an ammonium acetate:acetonitrile system. Unlabeled T_4 and T_3 were purchased from Sigma (St. Louis, MO). [^{125}I]T_4 and [^{125}I]T_3 were obtained from New England Nuclear (Boston, MA), and their purities were verified by thin-layer chromatography [4]. The specific activities of labeled T_4 and T_3 were 1250 μCi/μg and 2200 Ci/mmol respectively. Purified human TBPA was obtained from Behring Diagnostics (La Jolla, CA) or purified from outdated human plasma in our laboratory by NaCl/phenol extraction and DEAE cellulose chromatography as described by Tritsch [5]. A final purification step involved preparative isoelectric focusing.‡

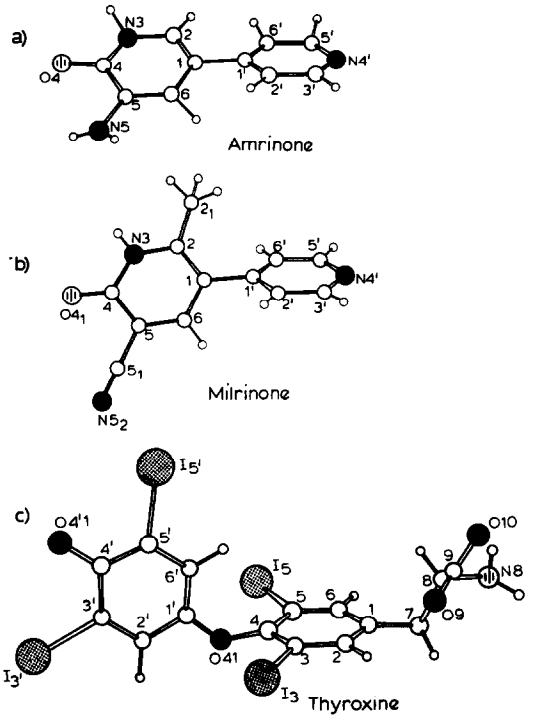

Fig. 1. Molecular conformation and numbering of amrinone (a), milrinone (b) and thyroxine (c).

Human sera. Sera were obtained from healthy volunteers who were verified to be euthyroid by serum thyroid function tests. Sera were studied as pooled samples in various experiments.

Serum protein electrophoresis. Polyacrylamide gel electrophoresis was carried out in single-dimension slab gels at pH 9.0 and at pH 7.4. The electrophoresis buffers were 0.20 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) titrated to pH 7.4 with Na_3PO_4, and 0.20 M Tris titrated to pH 9.0 with NaH_2PO_4, as previously described [6]. The slabs were dried after electrophoresis, and bands containing labeled iodothyronines were identified by radioautography (Kodak XO Mat RP 5) of the dried slabs. Hormone-containing bands corresponding to TBG, albumin and TBPA were excised from the slabs, and radioactivity was quantitated in a well-type scintillation counter.

In separate studies, purified TBPA was subjected to electrophoresis in the presence of [^{14}C]milrinone; radioautography of the gel slab was then carried out to determine whether milrinone co-migrated with TBPA.

Hormone radioimmunoassays. Radioimmunoassay of T_4 and T_3 was conducted by our previously reported method [7] using commercially available first antibodies (Radioassay Systems Laboratories, Carson, CA). Milrinone was added to buffer aliquots for the standard curves of these assays to determine if the bipyridine affected the hormone–antibody interaction.

Equilibrium dialysis of serum or purified TBPA with labeled T_4 and T_3. Serum dialyzable fraction T_4 (DFT_4) and T_3 (DFT_3) were measured by a standard equilibrium dialysis method [8] to which $MgCl_2$ precipitation of dialyzed T_4 [9] was appended. In these studies, various concentrations of milrinone were added to pooled sera and to the dialysis medium to determine if DFT_4 and DFT_3 were altered by the compound.

Determination of association constants of T_4 and milrinone for purified TBPA was by Scatchard analysis of bound/free hormone partition in equilibrium dialysis, as described by Pages *et al.* [10]. For these studies, milrinone concentrations of 10^{-8} to 4×10^{-4} M and T_4 concentrations of 4×10^{-12} to 4×10^{-7} M were used.

Computer graphic analysis of the interaction of TBPA and cardiac bipyridines. Crystallographic coordinates of milrinone, amrinone [2] and TBPA [11] were used to model the interactions of these bipyridine cardiotonic agents in the T_4 binding site of TBPA, using an MMS-X computer graphics system.* The orientation of the bipyridines with respect to that of T_4 was determined by making the best overall fit of the two molecules using both a skewed T_4 and an antiskewed T_3 conformation [2].

RESULTS

Effects of milrinone and amrinone on electrophoretically-determined distribution of labeled T_4 and T_3 among serum transport proteins. As shown in Fig. 2 and Table 1, studies at pH 9.0 indicated that milrinone displaced [^{125}I]T_4 from human serum TBPA to serum TBG and albumin. At a 10 μM concentration, milrinone reduced TBPA-binding of T_4 by 61% ($P < 0.01$, paired *t*-test); at 100 μM there was a 73% decrease in TBPA-binding ($P < 0.01$). The radioactive T_4 not bound by TBG, albumin and TBPA migrated between TBG and the electrophoretic origin or anodal to TBPA ("free hormone"), as previously described [6]. The fraction of T_4 migrating in these two zones was unaffected by the addition to serum of milrinone or amrinone. The amount of T_3 bound by TBPA is small, as we [12] and others [13] have shown previously, but milrinone at 10 and 100 μM was shown to decrease TBPA-binding of T_3 by 31 and 42% respectively ($P < 0.05$, paired *t*-test). Amrinone had less than 5% of the capacity of milrinone to displace T_4 from TBPA (Table 1). This estimate is based on a near-maximal T_4-displacing effect of milrinone at 10 μM, no effect of amrinone at 10 μM, and an amrinone effect which was less than one-third of that of milrinone at 100 μM.

Electrophoresis at pH 7.4 also showed that milrinone significantly reduced binding of T_4 and T_3 by TBPA (Table 2). The resolution of TBG and albumin at this pH is imprecise, but the majority of tracer displaced from TBPA was associated with TBG.

[^{14}C]Milrinone was shown by electrophoresis at pH 9.0 to migrate with purified TBPA (Fig. 3, Lane 2). In this figure, binding of tracer T_4 by serum TBPA is also shown for comparison (Lane 1).

*The MMS-X graphics display system was designed and fabricated at Washington University, St. Louis, MO. This system was made possible by a grant (RR-00396) from the Division of Research Resources, NIH.

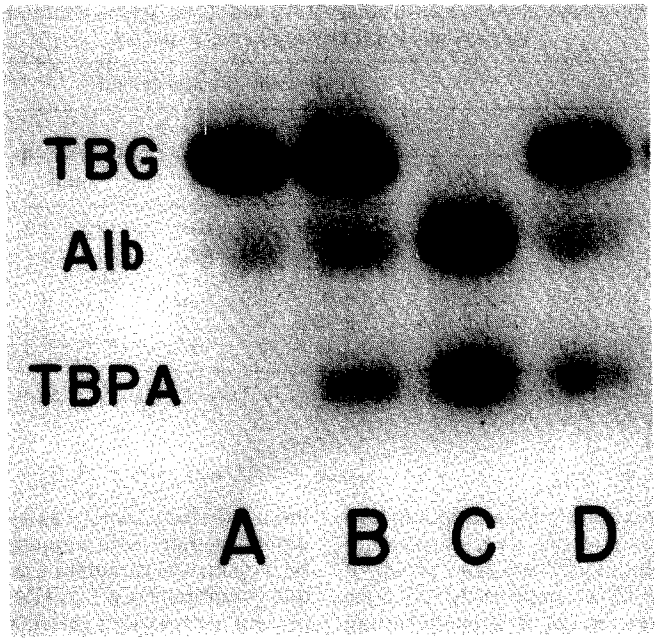

Fig. 2. Radioautograph of gel slab electrophoresis at pH 9.0 of serum samples containing [^{125}I]T_4 and milrinone, amrinone or unlabeled T_4. Anode is at the base of the figure. Lane A, tracer T_4 and milrinone (100 μM) showing displacement of [^{125}I]T_4 from TBPA in the presence of milrinone; Lane B, tracer T_4 only; Lane C, tracer T_4 and unlabeled T_4 (8×10^{-6} M) showing displacement of [^{125}I]T_4 from TBG to albumin and TBPA; Lane D, tracer T_4 and amrinone (100 μM).

Determination of T_4 and milrinone association constants for TBPA. The high and low affinity binding sites for T_4 had association constants of $6.1 \times 10^7\ M^{-1}$ and $1.6 \times 10^6\ M^{-1}$, respectively, whereas the association constants for milrinone-TBPA were $1.7 \times 10^6\ M^{-1}$ and $8.9 \times 10^2\ M^{-1}$, respectively. Scatchard analysis of milrinone binding to TBPA, corrected for nonspecific binding, is shown in Fig. 4.

Studies of displacement of tracer T_4 by unlabeled T_4 and milrinone indicated that milrinone was 1000 times less potent than T_4 (results not shown).

Effect of milrinone on DFT_4 and DFT_3. Serum DFT_4 and DFT_3 determinations were unaffected by the addition of milrinone in concentrations up to 100 μM (Table 3).

Effect of milrinone on T_4 and T_3 radioimmunoassays. As shown in Fig. 5A, milrinone (up to 100 μM) caused minimal upward displacement of the T_4 radioimmunoassay curve at high T_4 concentrations. There was no significant displacement by milrinone of the T_3 radioimmunoassay standard curve (Fig. 5B).

Computer graphic analysis of the interaction of TBPA and milrinone. Previous studies of the comparison of milrinone with thyroid hormone indicate that the best fit of these two structures is achieved

Table 1. Effects of milrinone and amrinone on binding of T_4 and T_3 to serum transport proteins, as determined by polyacrylamide gel electrophoresis at pH 9.0

		Percent of tracer bound						
			Milrinone			Amrinone		
Hormone	Protein	Control	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
T_4	TBPA	27.0 ± 0.9*	24.9 ± 1.2†	10.5 ± 1.4‡	7.3 ± 1.1‡	26.6 ± 1.0	25.5 ± 1.1	20.7 ± 1.7‡
	TBG	42.7 ± 1.9	44.0 ± 1.8	55.5 ± 3.9‡	56.7 ± 4.3‡	40.7 ± 1.6	43.3 ± 1.3	45.7 ± 1.7
	Albumin	14.9 ± 1.3	15.7 ± 1.5†	20.1 ± 2.1‡	22.1 ± 2.8‡	15.5 ± 1.5	15.5 ± 1.4†	16.3 ± 1.6
T_3	TBPA	16.6 ± 1.6	13.1 ± 0.7†	11.5 ± 0.3†	9.7 ± 1.1†	14.1 ± 0.9	12.7 ± 0.7†	12.1 ± 1.0†
	TBG	45.0 ± 0.8	45.6 ± 2.9	47.0 ± 1.4	47.8 ± 2.2	46.7 ± 2.9	45.4 ± 2.4	45.9 ± 2.1
	Albumin	28.3 ± 0.9	29.5 ± 1.2†	31.3 ± 0.8‡	31.9 ± 0.8‡	28.5 ± 1.9	31.5 ± 2.2†	31.1 ± 1.5†

*Values represent the mean ± SE of four electrophoreses performed with pooled sera. Tracer concentration was 10^{-10} M. Control samples contained the diluent for milrinone and amrinone: 1% DMSO. Tracer not bound to these proteins was found in the post-TBG region, or as unbound hormone [6].

†$P < 0.05$, paired t-test, comparing binding with and without bipyridine.

‡$P < 0.01$, paired t-test.

Table 2. Effects of milrinone and amrinone on binding of T_4 and T_3 to serum transport proteins, determined by polyacrylamide gel electrophoresis at pH 7.4

		Percent of tracer bound						
			Milrinone			Amrinone		
Hormone	Protein	Control	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
T_4	TBPA	17.0 ± 3.0*	14.5 ± 2.5	7.8 ± 0.3†	5.3 ± 0.6†	16.5 ± 2.3	16.8 ± 2.3	14.3 ± 1.6
	TBG	41.3 ± 3.0	42.8 ± 3.0	50.0 ± 7.7	48.7 ± 10.2	50.3 ± 5.2	47.8 ± 6.9	45.2 ± 7.0
	Albumin	25.4 ± 5.0	29.7 ± 7.5	29.9 ± 9.8	31.9 ± 11.6	19.6 ± 5.3	22.5 ± 7.5	26.2 ± 8.7
T_3	TBPA	4.8 ± 1.0	4.7 ± 0.8	3.4 ± 0.1	2.9 ± 0.5†	4.3 ± 0.7	4.1 ± 0.2	3.8 ± 0.4
	TBG	56.1 ± 10.6	53.7 ± 13.4	61.7 ± 10.6	55.7 ± 13.8	56.7 ± 9.9	60.2 ± 10.5	65.4 ± 6.3
	Albumin	27.8 ± 10.0	29.3 ± 12.7	23.5 ± 11.0	28.7 ± 13.7	27.6 ± 9.8	24.1 ± 10.8	19.7 ± 6.7†

*Values indicate percent of tracer bound with controls as in Table 1. Tracer concentration was 10^{-10} M. Mean values ± SE of four electrophoreses are shown.
†$P < 0.05$, paired t-test.

when the substituted pyridine ring is superimposed on the thyroid hormone phenolic ring, as shown in Fig. 6 [2]. In this orientation, the cyano group of milrinone occupies the same space as the phenolic ring 5′-iodine atom; even though the 2-methyl group of milrinone has no counterpart in the hormone structure, these modeling studies show that the binding channel of TBPA has a pocket available for binding of milrinone (Fig. 7). Similar comparisons with amrinone illustrate the lack of conformation homology between hormone structures and that of the bipyridine.

DISCUSSION

Recent studies from our laboratories [2] have defined structural homologies between milrinone, a modified bipyridine, and thyroid hormone, a diphenyl ether. Both compounds have been shown to be bioactive as stimulators *in vitro* of rabbit myocardial membrane Ca^{2+}-ATPase activity [2, 14]. The present observations extend the homologous activity of these two molecules with respect to human serum prealbumin (TBPA), a transport protein for iodothyronines [15] and for retinol-binding globulin [16]. In the electrophoretic studies at pH 9.0 and pH 7.4 reported here, milrinone competed with T_4 and T_3 for binding sites on TBPA. In separate studies, Scatchard analysis of the interaction of T_4 and milrinone with purified TBPA revealed the presence of two classes of binding sites on prealbumin for both T_4 and milrinone. The association constant of the high affinity site for T_4 ($6.1 \times 10^7\ M^{-1}$) is similar to that reported previously by Pages *et al.* [10]. The binding constant of the high affinity site for milrinone was an order of magnitude less than that for T_4. The concentrations of milrinone used in electrophoretic studies to displace labeled iodothyronine from TBPA sites reflect interactions with both classes of sites. Clinical studies indicate that 10^{-6} to 10^{-5} M concentrations of milrinone are achieved during parenteral administration of the

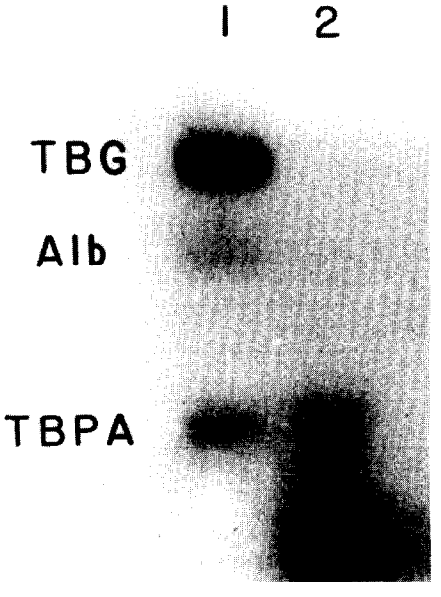

Fig. 3. Radioautograph of gel slab electrophoresis at pH 9.0 of serum containing tracer [^{125}I]T_4 (Lane 1) and of purified human TBPA (1.7×10^{-5} M) equilibrated with [^{14}C]milrinone (4×10^{-7} M] (Lane 2). Anode is at the base of the figure. Unbound milrinone is anodal to TBPA in Lane 2.

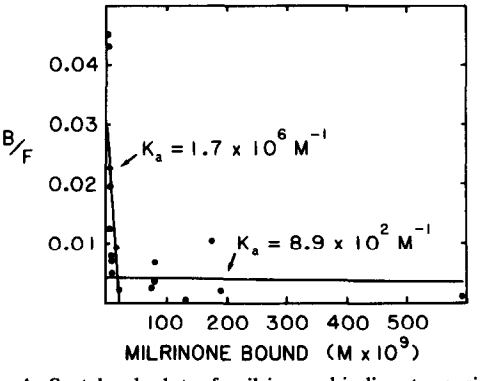

Fig. 4. Scatchard plot of milrinone binding to purified TBPA, corrected for nonspecific binding. The slope defining the low affinity K_a was calculated from milrinone concentrations up to 1200 nM. The concentration of TBPA was 3.7 μM.

Table 3. Effect of milrinone on equilibrium dialysis of T_4 and T_3 in pooled human serum

Milrinone (μM)	DFT_4 (%)	DFT_3 (%)
0	0.037 ± 0.005	0.109 ± 0.003
1	0.035 ± 0.002	0.113 ± 0.003
10	0.035 ± 0.001	0.123 ± 0.005
100	0.034 ± 0.001	0.122 ± 0.007

Results are means ± SE from two dialyses of three pooled sera, in triplicate. Analysis of variance showed no statistically significant changes among dialyzable fractions determined at various milrinone concentrations.

agent [1]. These levels were clearly sufficient *in vitro* to affect the interaction of iodothyronines with TBPA (Table 1).

Computer graphic modeling of milrinone-binding to the T_4 site in the crystal structure of TBPA shows that milrinone best occupies this site when the substituted bipyridine ring overlaps the phenolic ring

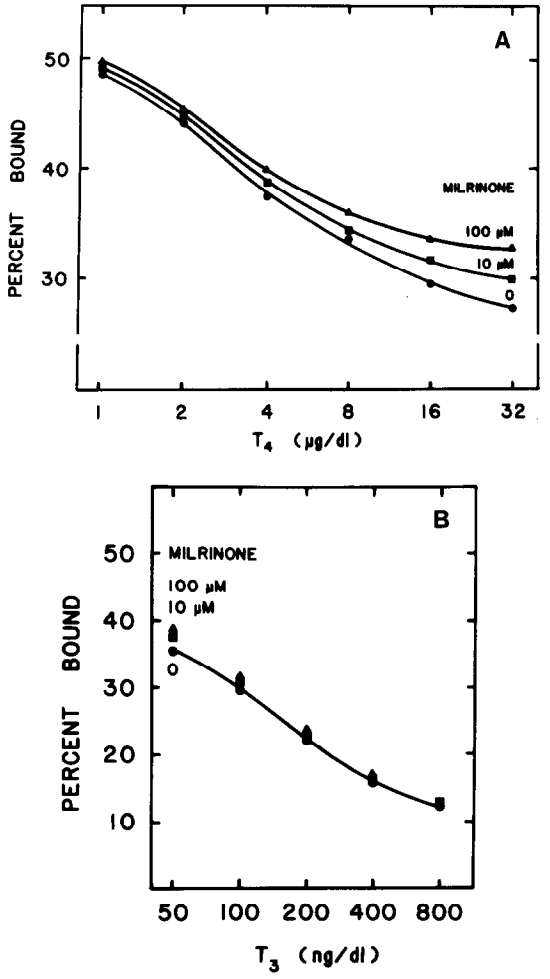

Fig. 5. (A) Radioimmunoassay standard curves for T_4, without (●) and with 10 (■) and 100 μM (▲) milrinone. There was some upward displacement of the curve by 100 μM milrinone at high T_4 concentrations. (B) Radioimmunoassay of T_3, without (●) and with 10 (■) and 100 μM (▲) milrinone. Milrinone did not affect the T_3 RIA in this concentration range.

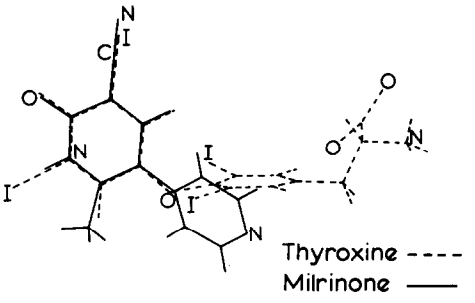

Fig. 6. Comparison of the crystal structure of milrinone (solid lines) with that of thyroxine (dashed lines) oriented with the substituted pyridine ring of milrinone superimposed on the T_4 phenolic ring [2]. Note that in this orientation the keto and cyano groups of milrinone match those of the 4′-hydroxyl and 5′-iodine of thyroxine.

of T_4 [2]. In this orientation, the cyano group occupies a similar volume as the 5′-iodine of T_4 and has electronegativity characteristics similar to iodine. In addition, the keto function overlaps the T_4 4′-hydroxyl and can thus participate in similar intermolecular interactions. Amrinone, the parent bipyridine of milrinone, lacks the 5-cyano and 2-methyl substituents of milrinone and is relatively ineffective as a competitor of T_4 and T_3 for the TBPA hormone-binding site. This suggests that the 5-cyano group of milrinone is recognized by this thyroid hormone-binding protein as structurally homologous to a hormonal iodine; the fit of the 2-methyl into the extra pocket in TBPA [17] enhances this structural similarity to thyroid hormone. On the other hand, the 5-amino group of amrinone is smaller in radius and has different electronegativity characteristics; thus, it will form different intermolecular interactions with the hydrophobic residues on binding proteins.

Because the binding affinity of TBPA for iodothyronines is low relative to TBG, the addition of milrinone to sera prior to DFT_4 or DFT_3 assays displaces hormone to TBG and does not affect measurements of unbound hormone. The small upward shift in the T_4 radioimmunoassay curve induced by milrinone and obtained only at high concentrations of T_4 relates to antibody binding sites, rather than those on TBPA. The basis for this shift is not clear, but it suggests a complex interaction of several independent sites on the antibody(ies) for T_4 and milrinone. For example, milrinone-binding at a non-T_4 site may induce a conformation change at a low affinity T_4 site and heightened T_4 binding at high T_4 concentrations. An analogy is the effect of 2,3-diphosphoglycerate on the hemoglobin-O_2 dissociation curve.

In contrast to salicylate, another non-hormonal competitor for iodothyronine binding to TBPA [13], milrinone does not affect significantly hormone-binding to TBG, as indicated by the lack of milrinone effect on DFT_4 and DFT_3. Thus, milrinone is a useful noniodinated probe of the T_4 site in the TBPA channel.

In light of bioactivity of milrinone in the thyroid hormone-stimulable Ca^{2+}-ATPase model, its structural homologies with T_4 and its binding to TBPA,

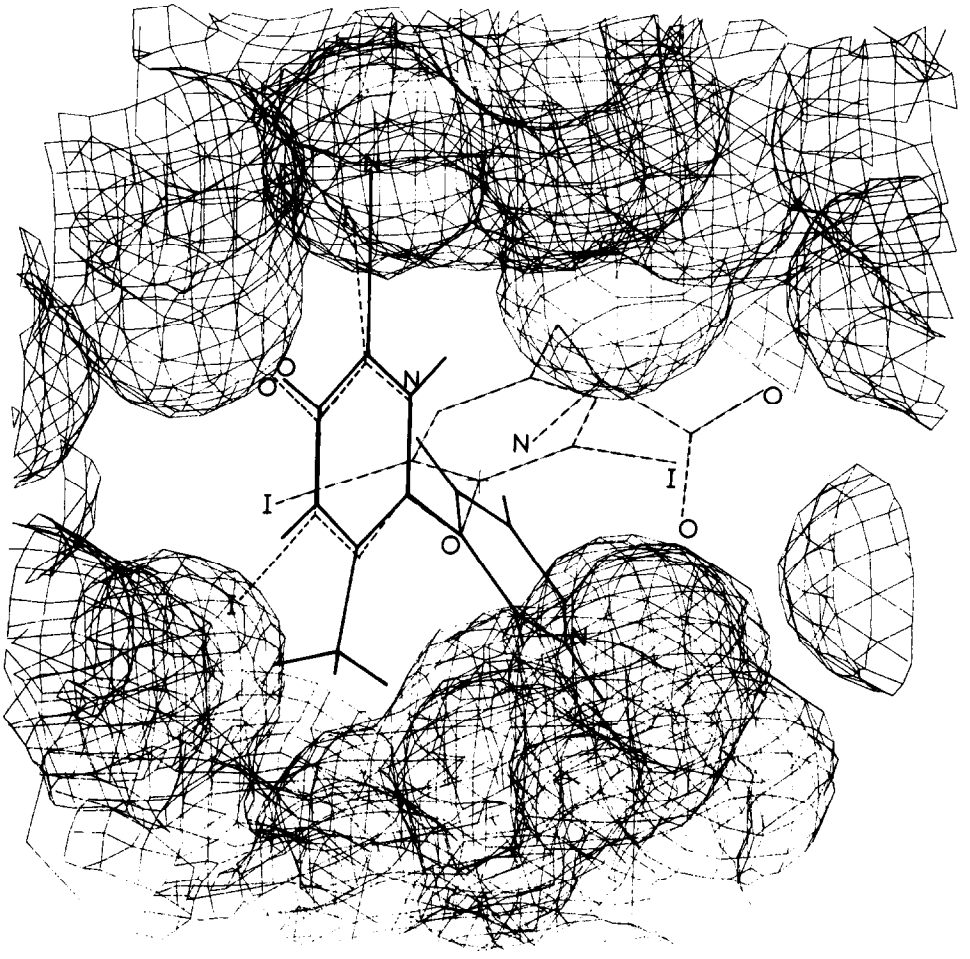

Fig. 7. Computer graphic model of milrinone (solid lines) bound in the thyroxine (dashed lines) site as observed in the crystal structure of the TBPA–T_4 complex. The TBPA binding channel is viewed from the center of the protein toward the channel entrance. Milrinone is oriented with its substituted pyridine ring on the phenolic ring of thyroxine. Note the free pocket for the 2-methyl substituent of milrinone. This site is not occupied by thyroid hormone. The van der Waals surface of the protein is also shown. Only those TBPA amino acid residues which form the binding channel were used to calculate the van der Waals surface. The binding site forms hydrophobic pockets which are specific for the iodine atoms.

milrinone can be described as thyromimetic. Clinically, milrinone enhances myocardial contractility and increases heart rate, actions which are shared with thyroid hormone [1, 18]. The possibility that effects in common of milrinone and iodothyronines will also include alterations of organism metabolic rate remains to be investigated.

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