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Solution Structure of the Regulatory Domain of Human Cardiac Troponin C in Complex with the Switch Region of Cardiac Troponin I and W7: The Basis of W7 as an Inhibitor of Cardiac Muscle Contraction

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Abstract

The solution structure of Ca^{2+} -bound regulatory domain of cardiac troponin C (cTnTnC) in complex with the switch region of troponin I (cTnI₁₄₇₋₁₆₃) and the calmodulin antagonist, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide (W7), has been determined by NMR spectroscopy. The structure reveals that the W7 naphthalene ring interacts with the terminal methyl groups of M47, M60, and M81 as well as aliphatic and aromatic side-chains of several other residues in the hydrophobic pocket of cTnTnC. The H3 ring proton of W7 also contacts the methyl groups of I148 and M153 of cTnI₁₄₇₋₁₆₃. The N-(6-aminoethyl) tail interacts primarily with the methyl groups of V64 and M81, which are located on the C- and D-helices of cTnTnC. Compared to the structure of the cTnTnC• Ca^{2+} •W7 complex (Hoffman, R. M. B. and Sykes, B. D. (2009) *Biochemistry* 48, 5541-5552), the tail of W7 reorients slightly towards the surface of cTnTnC while the ring remains in the hydrophobic pocket. The positively charged $-\text{NH}_3^+$ group from the tail of W7 repels the positively charged R147 of cTnI₁₄₇₋₁₆₃. As a result, the N-terminus of the peptide moves away from cTnTnC and the helical content of cTnI₁₄₇₋₁₆₃ is diminished, when compared to the structure of cTnTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃ (Li, M. X., Spyropoulos, L., and Sykes B. D. (1999) *Biochemistry* 38, 8289-8298). Thus the ternary structure cTnTnC• Ca^{2+} •W7•cTnI₁₄₇₋₁₆₃ reported in this study offers an explanation for the ~13-fold affinity reduction of cTnI₁₄₇₋₁₆₃ for cTnTnC• Ca^{2+} in the presence of W7, and provides a structural basis for the inhibitory effect of W7 in cardiac muscle contraction. This generates molecular insight into structural features that are useful for the design of cTnC-specific Ca^{2+} -desensitizing drugs.

Keywords

troponin; structure; drugs; mechanism; inhibition

A healthy human heart generates ~3 billion contractile cycles over an average life span. Each contractile cycle involves systolic activation and diastolic relaxation, regulated by Ca^{2+} association and dissociation from troponin in the cardiac myofilaments. Troponin is a

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heterotrimeric complex composed of the Ca^{2+} -binding subunit, troponin C (cTnC), inhibitory subunit, troponin I (cTnI), and the tropomyosin binding subunit, troponin T (cTnT). During diastole, troponin holds tropomyosin in a conformational state that blocks the interaction between myosin and actin. When Ca^{2+} binds cTnC during systole, the switch region of cTnI associates with the N-domain of cTnC (cNTnC). This is coupled with the removal of the inhibitory region of cTnI from actin so that the troponin-tropomyosin complex no longer inhibits the actin-myosin interaction. Subsequently, tension producing actin-myosin cross bridges form, which potentiates actomyosin ATPase activity, and ultimately force generation (for reviews, see [1,2]).

In diseased heart, the contractile cycle is compromised by either Ca^{2+} -desensitization with diminished systolic contractility (*e.g.* in cases of congestive heart failure) or Ca^{2+} -oversensitization accompanied with insufficient diastolic relaxation (*e.g.* in cases of hypertrophic cardiomyopathy). The ability to sensitize or desensitize cardiac muscle to Ca^{2+} has therapeutic potential for the treatment of cardiac dysfunction. Ideally, this mechanism would avoid altering Ca^{2+} transients in myocardial cells, which would perturb the regulation of other Ca^{2+} -based signaling pathways; but rather involve modulating the altered Ca^{2+} response of the myofilaments (for a review, see [3]). The essential role of troponin in the regulation of the contractile cycle makes it an attractive and logical target for the design of cardiostonic drugs. Toward this goal, a group of Ca^{2+} -sensitizing drugs have been developed. One example is levosimendan, a novel Ca^{2+} -sensitizer discovered by using cTnC as a target protein (for a recent review, see [4]). This drug has been proved to be a well-tolerated, effective treatment for patients with severe decompensated heart failure. A recent study has shown that a myosin inhibitor, blebbistatin (1-phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo(2,3-b)-7-methylquinolin-4-one) functions as an effective Ca^{2+} -desensitizer in cardiac muscle contraction without causing arrhythmia, suggesting that Ca^{2+} -desensitization might be beneficial to individuals with hypertrophic cardiomyopathy [5].

Many hydrophobic compounds are known to bind to CaM and perturb CaM-target interactions. Because of the structural homology between cTnC and CaM, these agents may also interact with cTnC and be good candidates as cardiostonic drugs. An earlier study has shown that some CaM antagonists (calmidazolium, bepridil, trifluoperazine, chlorpromazine, pimozide) stimulate myofibrillar ATPase activity while others (W7, haloperidol, mastoparan) inhibit ATPase activity [6]. This suggests that CaM antagonists differentially affect the properties of troponin, probably via different modes of action on the cTnC-cTnI interface. Structural studies have identified multiple binding sites of TFP and bepridil on cTnC [7]. In the X-ray structure of the cTnC• 3Ca^{2+} •3bepridil complex [8], two bepridil molecules pull the N- and C-domains close together to result in a compact structure for cTnC, while a third bepridil appears to stabilize an open regulatory domain conformation by binding to the hydrophobic pocket much like the switch region of cTnI (cTnI₁₄₇₋₁₆₃) as shown in the structures of cTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃ [9] and cTnC• 3Ca^{2+} •cTnI₃₁₋₂₁₀•cTnT₁₈₃₋₂₈₈ [10] complexes. The NMR structure of the cTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃•bepridil complex shows that bepridil and cTnI₁₄₇₋₁₆₃ bind to the hydrophobic pocket of cTnC• Ca^{2+} concurrently [11]. In the X-ray structure of cTnC• Ca^{2+} •2TFP (PDB: 1WRK and 1WRL), two TFP molecules fit in the hydrophobic pocket of cTnC• Ca^{2+} with the $-\text{CF}_3$ group of each TFP pointed towards the hydrophobic cleft. When compared to the structure of cTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃•bepridil, it appears that one of the TFP molecules would be replaced by cTnI₁₄₇₋₁₆₃ peptide. In the X-ray structure of the sTnC• 4Ca^{2+} •sTnI₁₋₁₈₂•sTnT₁₅₆₋₂₆₂ complex [12], a polyoxyethylene detergent molecule, anapoe, binds specifically to the Ca^{2+} -saturated N-domain of sTnC together with the switch region of sTnI (sTnI₁₁₅₋₁₃₁) and this binding is likely responsible for the increase of the contractile force of muscle fibers in the presence of anapoe. The Ca^{2+} -sensitizing effect of levosimendan has been shown to be due to a network of interactions between the drug, cTnC, and cTnI₁₄₄₋₁₆₃ (a longer version of switch region of cTnI) that creates a binding site

for levosimendan and the net result is the stabilization of the open conformation of cNTnC•Ca²⁺ [13].

W7 is a CaM antagonist that has been used to explore a wide range of physiological processes involving Ca²⁺-signaling in cardiomyocytes. Focusing on delineating the role of W7 in the interplay of troponin- and myosin-based pathways of Ca²⁺-activation in skeletal and cardiac muscle, Adhikari and Wang have shown that in both skeletal and cardiac muscle fibers, W7 inhibits the maximum ATPase activity and Ca²⁺ sensitivity [14]. The W7 inhibition is most likely mediated via specific interactions between W7 and TnC. This notion is supported by the observation that W7 binds specifically to TnC and not to tropomyosin, actin, or myosin [15]. We have shown that W7 binds specifically to cNTnC, and this binding can occur concurrently with the switch region of cTnI [16]. Moreover, we have shown that the affinity of W7 for cNTnC in the cTnC•3Ca²⁺•cTnI₃₄₋₇₁•cTnI₁₂₈₋₁₆₃ complex is ~ 2-fold weaker than for the isolated cNTnC•Ca²⁺ [17]. This is in line with the functional data demonstrating that W7 is an effective inhibitor in cardiac muscle contraction. We have also determined the solution structure of cNTnC•Ca²⁺•W7 that located the binding site of W7 in the hydrophobic pocket of cNTnC•Ca²⁺ [18].

In order to elucidate the mechanism of W7 inhibition, we have determined the solution structure of a ternary cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex. In the structure cTnI₁₄₇₋₁₆₃ binds at the A/B interhelical interface and W7 binds in the hydrophobic cavity formed between cNTnC•Ca²⁺ and cTnI₁₄₇₋₁₆₃. In the structure, the N-terminal R147 of cTnI₁₄₇₋₁₆₃ is electrostatically repelled from the positively charged tail of the W7, and thus moves away from cNTnC•Ca²⁺. This offers an explanation for the ~13-fold affinity reduction of cTnI₁₄₇₋₁₆₃ for cNTnC•Ca²⁺ in the presence of W7. The structure provides insights into the design of cardiotonic drugs that modulate the Ca²⁺-sensitivity of the myofilament via perturbation of the cTnC-cTnI interaction.

Materials and Methods

Sample preparation

The engineering of the cNTnC (1-89) construct and the expression of ¹⁵N- and ¹⁵N/¹³C-labeled cNTnC in *E. coli* were as described previously [11]. The synthetic peptide cTnI₁₄₇₋₁₆₃, Acetyl-RISADAMMQALLGARAK-Amide, was obtained from GL Biochem Ltd. (Shanghai, China). The purity and mass were verified by HPLC and electrospray mass spectrometry, respectively. W7 was purchased from Sigma-Aldrich. Stock solutions of 100 mM W7 in DMSO-d₆ (Cambridge Isotopes Inc.) were prepared and since W7 is sensitive to light, the vial was wrapped in aluminum foil. All NMR samples were 500 µL in volume. The buffer conditions were 100 mM KCl, 10 mM imidazole in 90% H₂O/10% D₂O or 5% H₂O/95% D₂O, 15 mM dithiothreitol (DTT), 0.5 mM 3-(trimethylsilyl)-1-propanesulfonic acid (DSS), and the pH adjusted to 6.8. For structure determination, NMR samples contain 0.7 mM cNTnC, 7 mM Ca²⁺, 3 mM W7, and 3.2 mM cTnI₁₄₇₋₁₆₃.

cTnI₁₄₇₋₁₆₃ titration of ¹⁵N-cNTnC•Ca²⁺•W7

Previously, cTnI₁₄₇₋₁₆₃ was titrated into ¹⁵N-cNTnC•Ca²⁺ in the absence of W7 [9]. The titration was performed by adding solid peptide and amino acid analysis was used to determine the peptide/protein ratio at every titration point. This is because cTnI₁₄₇₋₁₆₃ peptide is only soluble in aqueous solution at low concentrations (~ 1 mM) and tends to form a gel at higher concentrations. A similar procedure was employed in this work for the titration of cTnI₁₄₇₋₁₆₃ to ¹⁵N-cNTnC•Ca²⁺ in the presence of W7. The sample was mixed thoroughly with each addition. Changes in pH associated with cTnI₁₄₇₋₁₆₃ additions were compensated

by adjusting to pH 6.8 at every titration point. 1D ^1H and 2D ^1H , ^{15}N -HSQC NMR spectra of ^{15}N -cNTnC were acquired at every titration point.

NMR spectroscopy

NMR data used in this study were collected at 30°C using Varian Inova 800-MHz, Unity 600-MHz, and Varian Inova 500-MHz spectrometers. All spectrometers are equipped with triple resonance probes with Z-pulsed field gradients. The chemical shifts of backbone and side-chain atoms of cNTnC as well as the bound W7 and cTnI₁₄₇₋₁₆₃ were assigned using 2D and 3D NMR experiments outlined in the Supplemental Table S1. 1D ^1H and 2D DQF-COSY spectra of free W7 in DMSO- d_6 were obtained (as described in Hoffman and Sykes [18]) to help in the assignment of the W7 in the ternary complex. 2D DQF-COSY NMR spectra of a NMR sample of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 in D₂O were acquired for the assignment of bound W7. Resonances for aromatic residues were assigned using 2D homonuclear DQF-COSY and NOESY experiments in D₂O. 2D $^{13}\text{C}/^{15}\text{N}$ -filtered TOCSY and 2D $^{13}\text{C}/^{15}\text{N}$ -filtered NOESY [19-21] spectra of a sample of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 in H₂O were acquired for the assignment of bound W7 and bound cTnI₁₄₇₋₁₆₃. The intermolecular NOEs between W7 and cTnI₁₄₇₋₁₆₃ were assigned from 2D $^{13}\text{C}/^{15}\text{N}$ -filtered NOESY. The intermolecular NOEs between $^{13}\text{C}/^{15}\text{N}$ labeled cNTnC and unlabeled cTnI₁₄₇₋₁₆₃ and W7 were obtained using 3D- ^{13}C -edited HMQC-NOESY [22,23] experiment with 150 ms mixing time.

Data processing and peak calibration

All 2D and 3D NMR data were processed using NMRPipe [24] and all 1D NMR data were processed using VNMRJ (Varian Inc.). Assignment of chemical shifts was carried out in NMRViewJ [25] and backbone assignments were aided with the software package SmartNotebook [26]. The chemical shifts that were assigned in NMRView were converted to CYANA nomenclature, for NOE calibration. Initial structures of cNTnC were generated using program CYANA 2.1. Unambiguous restraints were assigned manually and were forced to keep their assignments during the first four runs of CYANA [27] calculations, after which they were open for automatic assignment with the “noeassign” script of CYANA. Distance restraints were calibrated with CYANA standard procedure using upper limits of 6 Å. After the CYANA refinement, the final restraints were converted to XPLOR-NIH [28,29] nomenclature. Intramolecular NOEs of cNTnI₁₄₇₋₁₆₃ and W7 and all intermolecular NOEs were assigned manually and categorized as weak (1.8-6.0 Å).

Coordinates and topology of W7

Representation of W7 was defined using HIC-Up and XPLO2D servers (<http://xray.bmc.uu.se>) and checked manually. Positive charge on W7 tail ($-\text{CH}_2\text{-NH}_3^+$ group) was added manually in analogy to positive charge on $-\text{CH}_2\text{-NH}_3^+$ group in LYS+.

Structural calculations

Dihedral angle restraints from TALOS [30,31] as well as 6 distance restraints from X-ray crystallographic data of chelating oxygen atoms to the Ca²⁺ ion in site II were used for structure calculation in addition to NOE distance restraints. The 20 conformers of cNTnC obtained from CYANA were averaged in XPLOR-NIH, and used as a template structure in the simulated annealing protocol, with 10,000 high temperature steps and 6000 cooling steps. After the structure of cNTnC was well defined, the ternary cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 structure was solved in a similar manner, starting with an extended conformation of cNTnC. The intramolecular and intermolecular NOE restraints used in the calculations are summarized in Table 1. 30 lowest energy conformers of the 300 calculated were refined in explicit solvent by XPLOR-NIH with a water box edge length of 18.8 Å. The final ensemble discussed in this article represent the 20 lowest energy conformers obtained after water refinement (see Table

1 for statistics). The final refined ensemble has been deposited in the protein data bank (www.rcsb.org) with the PDB accession code of 2KRD.

Results and Discussion

Effect of W7 on the stability of cTnTn•Ca²⁺•cTnI₁₄₇₋₁₆₃

We have previously characterized the interaction of cTnI₁₄₇₋₁₆₃ and cTnTn•Ca²⁺ and determined the affinity of cTnI₁₄₇₋₁₆₃ for cTnTn•Ca²⁺ ($K_D = 154 \pm 30 \mu\text{M}$) [9]. In the present study, the assigned 2D ¹H, ¹⁵N-HSQC NMR spectrum of cTnTn•Ca²⁺•W7 was used to monitor the binding of cTnI₁₄₇₋₁₆₃ to cTnTn•Ca²⁺•W7. Using four residues (L29, A31, K39, and E66) as examples, a comparison of the cTnI₁₄₇₋₁₆₃-induced 2D ¹H, ¹⁵N-HSQC NMR peak shifts of cTnTn•Ca²⁺ and of cTnTn•Ca²⁺•W7 is depicted in Figure 1A and 1B, respectively. In both titrations, the chemical shift changes fall into the fast exchange limit on the NMR scale, and the movement of the cross peaks indicates that the binding of cTnI₁₄₇₋₁₆₃ to cTnTn•Ca²⁺ or cTnTn•Ca²⁺•W7 occurs with a 1:1 stoichiometry (Supplemental Figure S1). The cTnI₁₄₇₋₁₆₃-induced chemical shift changes are larger in Figure 1A, corresponding to a closed to open conformational transition in cTnTn as characterized previously [9]. The smaller cTnI₁₄₇₋₁₆₃-induced chemical shift changes in Figure 1B corresponds to the binding of cTnI₁₄₇₋₁₆₃ to an already partially open cTnTn in the cTnTn•Ca²⁺•W7 complex [18]. Plotting the chemical shift changes in Figure 1B as a function of the peptide to protein ratio, a binding dissociation constant (K_D) of $2000 \pm 50 \mu\text{M}$ gives the best fit as shown in Supplemental Figure S1. A global fitting for the titration of cTnI₁₄₇₋₁₆₃ to cTnTn•Ca²⁺ is also shown in Figure S1 for the purpose of a direct comparison. This suggests that the affinity of cTnI₁₄₇₋₁₆₃ for cTnTn•Ca²⁺ is decreased ~ 13-fold (from $154 \pm 30 \mu\text{M}$ to $2000 \pm 50 \mu\text{M}$) by the presence of W7. This helps explain why W7 is effective in inhibiting the activity of actomyosin ATPase and force generation in muscle fibers [6,14].

Overall structure of the ternary complex

The 2D ¹H, ¹⁵N-HSQC NMR spectrum of cTnTn in the ternary cTnTn•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex is well resolved, allowing the chemical shifts of the backbone and the side chain atoms to be assigned using ¹⁵N- and/or ¹⁵N/¹³C-labeled protein. Intramolecular distance restraints for cTnTn in the ternary complex were obtained by analyzing the 3D ¹⁵N- and ¹³C-edited NOESY experiments. Dihedral angle restraints for cTnTn in the ternary complex were obtained from TALOS [30].

The proton NMR chemical shift assignments and intramolecular distance restraints for the bound cTnI₁₄₇₋₁₆₃ and W7 required the collection of ¹⁵N/¹³C filtered 2D-TOCSY and 2D-NOESY experiments using unlabeled cTnI₁₄₇₋₁₆₃ and W7 in complex with ¹⁵N/¹³C labeled cTnTn. These experiments removed all of the resonance peaks arising from ¹⁵N/¹³C labeled cTnTn. 2D DQF-COSY NMR spectra of free (see reference [18]) and bound W7 (see Supplemental Figure S2) were also acquired to aid in the assignment of bound W7. Intermolecular NOEs between cTnTn and W7 (Figure 2A) and between cTnTn and cTnI₁₄₇₋₁₆₃ (Figure 2B) were determined from 3D-¹³C-edited/filtered HMQC-NOESY data. These experiments were optimized for detecting NOEs between protons attached to ¹²C and protons attached to aliphatic ¹³C, thereby editing out all the intramolecular NOEs. Three intermolecular NOEs between cTnI₁₄₇₋₁₆₃ and W7 were obtained from the ¹⁵N/¹³C filtered 2D NOESY experiment (Figure 2C). A total of 1264 NOE distance restraints were used for structure calculations. These include 1184 intramolecular cTnTn distance restraints (~ 13 restraints per residue), 15 intramolecular cTnI₁₄₇₋₁₆₃ distance restraints, 6 intramolecular W7 distance restraints, 24 intermolecular contacts between cTnTn and cTnI₁₄₇₋₁₆₃, 30 intermolecular contacts between cTnTn and W7, and 3 intermolecular contacts between cTnI₁₄₇₋₁₆₃ and W7. 116 dihedral restraints for cTnTn, 10 dihedral restraints for

cTnI₁₄₇₋₁₆₃, and 6 artificial distance restraints to Ca²⁺ were used to calculate the structure of the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex (see Table 1). Figure 3A depicts the ensemble of the 20 lowest energy structures of the ternary complex in stereo view, with overall structural statistics and conformational energies for the ensemble provided in Table 1. The ribbon representations of the structure of the ternary complex are shown in Figure 3B. The structure of cNTnC in the complex is better defined than those of cTnI₁₄₇₋₁₆₃ or W7, as a result of more restraints for the protein than for the ligands.

Structure of cNTnC in the ternary complex

cNTnC in the present structure exhibits an overall fold resembling other Ca²⁺-bound domains in the EF-hand family. The secondary structural elements of cNTnC in the ternary cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex are similar to those in the cNTnC•apo (PDB: 1SPY), cNTnC•Ca²⁺ (PDB: 1AP4), cNTnC•Ca²⁺•W7 (PDB: 2KFX), cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ (PDB: 1MXL), and cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil (PDB: 1LXF) complexes. The five helices, N, A, B, C, and D, are well-defined, superimposing with individual backbone rmsds of 0.31±0.12 Å for helix N (residues 5-11), 0.54±0.14 Å for helix A (residues 14-27), 0.20±0.13 Å for helix B (residues 41-46), 0.39±0.13 Å for helix C (residues 54-63), and 0.42±0.11 Å for helix D (residues 74-86). The two EF hands Ca²⁺-binding sites are joined by a short anti-parallel β-sheet. The β-sheet (residues 35-37, 71-73) is well-defined with a backbone rmsd of 0.39±0.09 Å, while the Ca²⁺-binding sites have a slightly more flexible structure (a backbone rmsd of 0.92±0.28 Å). The N- and C-terminal residues (residues 1-4 and 86-89) are less well defined than the helices or the β-sheet. The global fold and the orientations of the five helices of cNTnC in the ternary cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex are similar to the open cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃, cNTnC•Ca²⁺•W7, and cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil structures (see Figure 5). The effects of cTnI₁₄₇₋₁₆₃ and W7 on the structural fold of cNTnC will be discussed below.

Structure of W7 in the ternary complex

W7 consists of a naphthalene group and an N-(6-aminohexyl) tail (Figure 2A). The binding site and conformation of W7 in the ternary complex is similar as in the structure of cNTnC•Ca²⁺•W7. The orientation of W7 was determined by intermolecular NOEs between cNTnC and W7 and between cTnI₁₄₇₋₁₆₃ and W7. Strip plots taken from the 3D ¹⁵N/¹³C F₁-filtered, F₃-edited spectra of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex are shown in Figure 2A and 2B. In the spectra, only NOEs arising from the ¹²C-attached protons in cTnI₁₄₇₋₁₆₃ and W7 and terminating on ¹³C-attached protons in cNTnC are observed. The naphthalene ring of W7 makes a number of contacts with residues that line the hydrophobic pocket of cNTnC, such as A23, L41, V44, M47, M60, I61, M81, I36, V64, and V72. As a result, the naphthalene ring of W7 is nestled deep in the hydrophobic cleft. The contacts between H3 of W7 and the methyl groups of I148 and M153 of cTnI₁₄₇₋₁₆₃ fix the orientation of the bicyclic ring relative to cTnI₁₄₇₋₁₆₃ (Figure 4). The N-(6-aminohexyl) tail of W7 makes contacts to the C/D helices of cNTnC (e.g. H16 to the methyl groups of V64 and M81). The positively charged -NH₃⁺ moiety of W7 and the N-terminal R147 of cTnI₁₄₇₋₁₆₃ repel each other and the two ligands reorient their positions relative to those in the binary cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ or the cNTnC•Ca²⁺•W7 complex to accommodate each other in the hydrophobic pocket of cNTnC•Ca²⁺ (see below). When the heavy atoms of W7 superimpose onto the average structure in the ternary complex, the average rmsd for the naphthalene group is 1.1 Å, while that for the tail is 2.1 Å.

Structure of cTnI₁₄₇₋₁₆₃ in the ternary complex

The structure of cTnI₁₄₇₋₁₆₃ when complexed with cNTnC•Ca²⁺ has been determined in three separate structures. In the structure of the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃, residues 150-157 form a short α-helix. This short helix was defined primarily by the use of chemical shift index and

several intermolecular NOE restraints between cTnNC and the peptide (there were no intramolecular NOEs assigned). In the structure of cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil, 24 intramolecular NOEs including 3 d_{NN} and 6 d_{αN(i,i+3)} (the hallmark of a helical structure) restraints for the 17 residue cTnI₁₄₇₋₁₆₃ peptide were assigned. A combination of these NOEs and the dihedral angle restraints better defined the peptide. In the X-ray structure of the core domain cardiac troponin, cTnC•3Ca²⁺•cTnI₃₄₋₂₁₀•cTnT₁₈₂₋₂₈₈, this fragment of cTnI displays a near identical structure as in the two NMR structures (superimpose to 0.2 Å). In the present structure, cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7, the cTnI₁₄₇₋₁₆₃ conformation is calculated by using both intra- and inter-molecular NOEs and dihedral restraints. The overall conformation and orientation is similar to those in the other three structures but the helical component is diminished. In the ensemble of solution structures (Figure 3), the backbone atoms of the 17 residues superimpose on to the average peptide structure with an rmsd of 1.9±0.3 Å. The assigned intermolecular NOEs are summarized in Figure 2. These NOE contacts dictate a similar orientation for the cTnI₁₄₇₋₁₆₃ in the ternary cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex as compared to that in the cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃ or cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil complexes. The peptide inserts between the A/B helices in cTnNC with the N-terminus (residues 147-149) and the helical region interacts with the protein, while the C-terminus does not interact with the protein and remains disordered in the ensemble of the solution structures (Figure 3). Specifically, the helical portion of the peptide (residues 152-157) sits between the A/B helical interface, contacting the methyl group of residues A22, A23, and I26 in the A-helix and L41, K43, M47 and L48 in the B-helix. The N-terminus of the peptide extends into the hydrophobic pocket of cTnNC, making contacts to the methyl groups of M60, V82 and M85 in cTnNC. As discussed above, the N-terminal residue R147 of the peptide moves away from the tail of W7 because of the electrostatic repulsion.

Comparison of the cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 structure with the cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃, cTnNC•Ca²⁺•W7, and cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil structures

The conformational change in cTnNC•Ca²⁺ that occurs upon binding cTnI₁₄₇₋₁₆₃, bepridil, and W7 involves the straightening of the B-helix and the BC unit moving away from the NAD unit, consisting of a 'closed' to 'open' structural transition. This peptide or drug induced structural change in cTnNC•Ca²⁺ is similar to that observed for the apo to Ca²⁺-saturated transition in sTnNC [32]. An interesting finding from the current study is that while the cTnNC in this structure exhibits an open conformation, the A/B and C/D EF hands behave as separate units. When the NAD unit of cTnNC in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 is superimposed onto the NAD unit of cTnNC in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃, the B helix moved up along with cTnI₁₄₇₋₁₆₃ (up 4±1 Å toward the A-helix) while the C-helix moved down (Figure 5A). As a result, the A/B pair is more closed but the C/D pair is more open. This change is also reflected in the A/B and C/D interhelical angle changes (see Table 2). It seems the C/D pair needs to be more open to accommodate W7 and the repulsion between W7 and cTnI₁₄₇₋₁₆₃ shifts the peptide away from the hydrophobic pocket with a concomitant destabilization of the helix. As a result, the NOE contacts between cTnNC and cTnI₁₄₇₋₁₆₃ from cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃ to cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 are altered. For instance, in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃, A23 but not A22 on the A-helix makes a strong contact to L157 of cTnI₁₄₇₋₁₆₃ peptide, while both A22 and A23 make strong contacts to L157 in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7. When the NAD unit of cTnNC in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 superimpose onto the NAD unit of cTnNC in cTnNC•Ca²⁺•W7, the A/B unit did not change but the C-helix moved down (Figure 5B, see the A/B and C/D interhelical angle changes listed Table 2). In both the W7 bound structures, the strong NOE contacts between the hydrophobic residues in cTnNC and the naphthalene ring of W7 serve as the primary anchoring site for this molecule in the hydrophobic core of the protein. In the presence of cTnI₁₄₇₋₁₆₃, W7 needs to rearrange its ring and makes more contacts with the C/D interhelical interface. As a result, the C/D pair is more open. When the NAD unit of cTnNC in cTnNC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 superimpose onto the NAD unit of cTnNC in

cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil, both the A/B and C/D pairs exhibit more open interhelical angles (Figure 5C). As a result, in cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7, the BC unit is farther away from the NAD unit than that in cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil. This could be attributed to the different effects of W7 and bepridil on the interaction of cNTnC and cTnI₁₄₇₋₁₆₃.

The docking of W7 and cTnI₁₄₇₋₁₆₃ on cNTnC is represented by comparing the molecular surfaces of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃, cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7, and cNTnC•Ca²⁺•W7 in Figure 6. In cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃, the helical region fit nicely between the A/B interhelical interface and the positive charged N-terminal R147 forms salt bridges with the acidic residues on the C/D helices of cNTnC (Figure 6A). To accommodate W7, the helical region of the peptide unwinds slightly and moves up toward the A helix while the N-terminal R147 moves away from the tail of W7 as a result of electrostatic repulsion (Figure 6B). In the absence of cTnI₁₄₇₋₁₆₃, W7 finds its place in the hydrophobic pocket of cNTnC•Ca²⁺ (Figure 6C) and it reorients its position slightly to allow the concurrent binding of cTnI₁₄₇₋₁₆₃ (see Figure 5B).

In summary, the interaction between the cTnI₁₄₇₋₁₆₃ peptide and W7 in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 ternary complex makes the two ligands reorient their positions relative to those in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ or cNTnC•Ca²⁺•W7 complexes. The consequence is a rearrangement of interhelices of both AB and CD EF hands in the ternary complex than in the binary complexes. The interference between W7 and cTnI₁₄₇₋₁₆₃ explains the weaker affinity of cTnI₁₄₇₋₁₆₃ for cNTnC•Ca²⁺ in the presence of W7 than in cNTnC•Ca²⁺ alone.

Comparison of the structures of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 and cNTnC•Ca²⁺•W7 with the structure of CaM•4Ca²⁺•2W7

The NMR structure of CaM•4Ca²⁺ in complex with two W7 molecules was determined [33]. In this structure, each domain of CaM interacts with one W7 molecule. W7 binds CaM via many Van der Waals interactions between the naphthalene ring of the W7 and the hydrophobic residues of the CaM binding pocket. Osawa et al. compared the CaM binding mode between W7 and TFP and between W7 and tryptophan of CaM target peptide and concluded that the naphthalene ring is the largest that can fit into the hydrophobic pocket of CaM domain. As the N-terminal and C-terminal domains of CaM are similar, the orientation of W7 within the binding pocket is similar as well. The tail of W7 in the CaM domains is flexible but more defined in the structures of cNTnC•Ca²⁺•W7 and cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7. Thus, W7 appears to inhibit the CaM-mediated activation of target proteins by blocking the hydrophobic pocket of each domain with its naphthalene ring that normally plays a role in anchoring targets. However, in cNTnC, the tail of W7 is playing a major role in interfering with cTnC's major target, cTnI.

Relevance to the future design of cTnC-specific pharmacological agents for the treatment of heart disease

There is a need for the development of novel approaches in the therapy of heart disease. The current therapeutic paradigm for decreased systolic function is the employment of drugs that increase systolic Ca²⁺-levels; however, prolonged use leads to increased heart rate, hypertension, arrhythmias, and mortality. Presently, there is no drug available for the treatment of familial hypertrophic cardiomyopathy that is associated with hypertrophy and fibrosis contributing to the risk of ventricular arrhythmias and sudden cardiac death. Mutations in myofilament proteins or altered myofilament structures lead to changes in the myofilament response to Ca²⁺ and thus represent good targets for the design of novel inotropic agents. A group of Ca²⁺-sensitizers has been developed, which have been shown to increase the myocardial contractility by generating more force for a given amount of cytosolic Ca²⁺. Levosimendan and EMD 57033 are typical examples. Recently, a new class of pharmacological

agents, cardiac myosin activators (*e.g.* CK-1827452), have been shown to directly target the kinetics of the myosin head (for a review see [34]. *In vitro* studies have demonstrated that these agents increase the rate of effective myosin-actin cross-bridge formation and improve myocyte energy utilization with no effect on intracellular Ca^{2+} or cAMP. Animal model and in human studies have demonstrated significant increases in systolic ejection time and cardiac output, suggesting that myosin activators offer the promise of a safe and effective treatment for heart failure.

The aforementioned compounds all belong to a class of compounds known as Ca^{2+} -sensitizers, and are effective in treating symptoms of decreased cardiac output; however, there are less treatment options for patients suffering from impaired diastolic relaxation or increased contractility. The therapeutic advantage of compounds that target the contractile machinery was illustrated in a recent study that showed arrhythmias susceptibility caused by the Ca^{2+} -sensitizing agent EMD 57033 in animal models is prevented by the myosin inhibitor, blebbistatin [5,35]. The protective effect of blebbistatin provides the first direct evidence that Ca^{2+} -desensitization in myofilaments is antiarrhythmic and might be beneficial to individuals with hypertrophic cardiomyopathy.

The data presented in this work provide the structural basis of W7 inhibition of the regulatory cTnC-cTnI interaction. W7 functions as a Ca^{2+} -desensitizer by decreasing the binding affinity of cTnI₁₄₇₋₁₆₃ for cNTnC• Ca^{2+} . The NMR solution structure of the cNTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃•W7 ternary complex furthers understanding of the molecular mechanism underlying the effects of cardiotonic drugs on the cTnC-cTnI interface. Together with a series of structures determined previously in our laboratory, it provides a molecular model important for the design of cTnC-specific cardiotonic drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TnC	troponin C
cTnC	cardiac troponin C
cNTnC	N-domain (residues 1-89) of cTnC
cCTnC	C-domain (residues 91-161) of cTnC
sTnC	skeletal troponin C
CaM	calmodulin
TnI	troponin I

cTnI	cardiac troponin I
cTnI ₁₄₇₋₁₆₃	cTnI peptide Acetyl-RISADAMMQALLGARAK-Amide
sTnI	skeletal troponin I
sTnI ₁₁₅₋₁₃₁	sTnI residues 115-131
TFP	trifluoperazine

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Figure 1.

Titration of (A) cNTnC•Ca²⁺ and (B) cNTnC•Ca²⁺•W7, respectively, with cTnI₁₄₇₋₁₆₃. 2D ^1H , ^{15}N -HSQC NMR spectra for residues L29, A31, K39, and E66 of cNTnC at various peptide additions are superimposed, showing the progressive shift of peaks with increasing cTnI₁₄₇₋₁₆₃ concentrations.

Figure 2.

Intermolecular NOE contacts between cTnTnC•Ca²⁺ and W7 (A), between cTnTnC•Ca²⁺ and cTnI₁₄₇₋₆₃ (B), and between cTnI₁₄₇₋₆₃ and W7 (C). The NMR sample of cTnTnC•Ca²⁺•cTnI₁₄₇₋₆₃•W7 contains 0.7mM cTnTnC, 7mM Ca²⁺, 3.4mM cTnI₁₄₇₋₆₃, and 3mM W7: (A) The chemical structure of W7. The number designation and the chemical shift assignments of W7 are indicated. Strips from the 3D-¹⁵N/¹³C-edited HMQC-NOESY NMR experiments showing the intermolecular NOE contacts between the ring of W7 and cTnTnC and between the tail of W7 and cTnTnC. The circled peaks are intermolecular NOE contacts between the cTnI₁₄₇₋₆₃ peptide and cTnTnC (left panel) and are unassigned (right panel); (B) The sequence of cTnI₁₄₇₋₆₃ peptide. Example strips from the 3D-¹⁵N/¹³C-edited HMQC-

NOESY NMR experiments showing the intermolecular NOE contacts between cNTnC and cTnI₁₄₇₋₁₆₃; (C) Intermolecular NOE contacts between the methyl groups of I148 and M153 of cTnI₁₄₇₋₁₆₃ to H3 from the naphthalene ring of W7, obtained from 2D-¹⁵N/¹³C-filtered-NOESY NMR spectra.

Figure 3.

A) The solution structure of the cTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex in stereo view. The backbones (N, Cα, C') of a family of 20 structures are shown in gray. The assembly of the cTnI₁₄₇₋₁₆₃ structures is shown in gold. The assembly of W7 is shown in blue. The Ca²⁺ ions are shown as green spheres.

B) The ribbon representation of the lowest energy structure in stereo view. The protein is shown in green and Ca²⁺ ions are shown as gray spheres. Heavy atom skeleton of W7 is shown in blue. The cTnI₁₄₇₋₁₆₃ peptide is shown in purple.

Figure 4.
A close up view of the skeleton representation of the peptide backbone and W7, showing the interaction between cTnI₁₄₇₋₁₆₃ and W7 with (A) and without (B) the protein surface present.

Figure 5.

A) Backbone overlay of cNTnC in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex (green) and cNTnC in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ complex (blue). Note the position shift and helical content changes of cTnI₁₄₇₋₁₆₃ from yellow to gold in the presence of W7 (pink).

B) Backbone overlay of cNTnC in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex (green) and cNTnC in the cNTnC•Ca²⁺•W7 complex (red). Note the change of position of W7 from cyan to pink when cTnI₁₄₇₋₁₆₃ is present.

C) Backbone overlay of cNTnC in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 complex (cNTnC in green, cTnI₁₄₇₋₁₆₃ in gold, W7 in pink) and cNTnC in the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil complex (cNTnC in brown, cTnI₁₄₇₋₁₆₃ in gray, bepridil in green).

Figure 6. Molecular surface representations of (A) cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ (PDB, 1MXL), (B) cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•W7 (PDB, 2KRD), and (C) cNTnC•Ca²⁺•W7 (PDB, 2KFX). For cNTnC and cTnI₁₄₇₋₁₆₃, positively charged residues are shown in blue, negatively charged residues are shown in red, and other residues are shown as charge gradient. Non-hydrogen atoms of W7 are shown as stick models in yellow and nitrogen is shown in blue.

Table 1

Structural statistics of the family of the 20 structures calculated.

Intramolecular NOE restraints in cNTnC		
Total	1184 (~13/residue)	
Short range ($ i-j \leq 1$)	817	
Medium-range ($2\leq i-j \leq 4$)	211	
Long-range ($ i-j \geq 5$)	156	
Dihedral Restraints in cNTnC		
φ	59	
ψ	57	
Intermolecular NOEs between cNTnC and W7	30	
Intermolecular NOEs between cNTnC and the cTnI ₁₄₇₋₁₆₃ peptide	24	
Intermolecular NOEs between the cTnI ₁₄₇₋₁₆₃ peptide and W7	3	
Intramolecular NOE restraints in W7	6	
Intramolecular restraints in cTnI ₁₄₇₋₁₆₃ peptide		
NOEs	15	
φ angle restraints	5	
ψ angle restraints	5	
Artificial restraints to Ca ²⁺	6	
NOE violations/structure > 0.2 Å		
Before water refinement	0.0±0.0	
After water refinement	8.7±2.1	
Dihedral violations/structure > 1°		
Before water refinement	0.55±0.12	
After water refinement	1.00±0.22	
φ, ψ in core or allowed regions ^c		
Before water refinement	99.5%	
After water refinement	98.9%	
R.M.S.D. from the average structure (Å) ^d		
	Well-defined residues	Helices
Before water refinement	0.80±0.11	0.32±0.05
After water refinement	0.87±0.12	0.37±0.06

^a Well-defined backbone atoms (N, C, CA) with R.M.S.D. smaller than 1 Å. The R.M.S.D. for helices are calculated from the weighted mean average of the R.M.S.D. of individual helices.

^b The final K_{NOE} and K_{dihedral} used were 50 kcalmol⁻¹ and 200 kcalmol⁻¹rad⁻², respectively.

^c As determined using PROCHECK

Table 2

Inter-helical angles of various EF hands.

Calcium Binding Protein	Interhelical angles(°) ^a		Accession Code (PDB)
	A-B	C-D	
cNTnC·apo (NMR)	140±3	128±4	1SPY
cNTnC·Ca ²⁺ (NMR)	132±3	113±4	1AP4
cNTnC·Ca ²⁺ ·cTnI ₁₄₇₋₁₆₃ (NMR)	102±5	96±5	1MXL
cTnC·3Ca ²⁺ ·cTnI ₅₁₋₂₁₀ ·cTnT ₁₈₃₋₂₈₈ (X-RAY)	103	96	1J1E
cNTnC·Ca ²⁺ ·bepridil·cTnI ₁₄₇₋₁₆₃ (NMR)	121±4	91±4	1LXF
cTnC·3Ca ²⁺ ·bepridil (X-RAY)	92	90	1DTL
cNTnC·Ca ²⁺ ·W7 (NMR)	114±3	92±2	2KFX
cNTnC·Ca ²⁺ ·W7·cTnI ₁₄₇₋₁₆₃ (NMR)	113±5	66±4	2KRD
CaM(N-domain)·Ca ²⁺ ·W7 (NMR)	102±4	75±5	1MUX
CaM(C-domain)·Ca ²⁺ ·W7 (NMR)	105±4	85±4	1MUX

^aThe 180° interhelical angle corresponding to a completely closed EF hand domain (antiparallel helices), whereas 0° angle defines a completely open conformation (parallel helices). The extent of openness is characterized by the A/B and C/D interhelical angles. cNTnC in 2KRD is open as compared to the closed 1SPY or 1AP4. The A/B helical pair is more closed than 1MXL, while the C/D pair is more open. As compared to 2KFX, the A/B pair is similar but the C/D pair is more open. Both the A/B and C/D pairs of 2KRD are more open than cNTnC in 1LXF. While the C/D helical pair in this complex is more open than that in all the open cNTnC structures, it is similar to the corresponding helical pair in CaM(N-domain)•2Ca²⁺•W7 and CaM(C-domain)•2Ca²⁺•W7.

The axis for an α -helix is defined by two points, taken as the average coordinates of the first and last 11 backbone atoms of the α -helix. Inter-helical angles were calculated using the program interhelix (K. Yap, University of Toronto).