The calcium sensitizer drug MCI-154 binds the structural C-terminal domain of cardiac troponin C

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**ABSTRACT**

The compound MCI-154 was previously shown to increase the calcium sensitivity of cardiac muscle contraction. Using solution NMR spectroscopy, we demonstrate that MCI-154 interacts with the calcium-sensing subunit of the cardiac troponin complex, cardiac troponin C (cTnC). Surprisingly, however, it binds only to the structural C-terminal domain of cTnC (cCTnC), and not to the regulatory N-terminal domain (cNTnC) that determines the calcium sensitivity of cardiac muscle.

Physiologically, cTnC is always bound to cardiac troponin I (cTnI), so we examined its interaction with MCI-154 in the presence of two soluble constructs, cTnI\textsubscript{1–77} and cTnI\textsubscript{135–209}, which contain all of the segments of cTnI known to interact with cTnC. Neither the cTnC-cTnI\textsubscript{1–77} complex nor the cTnC-cTnI\textsubscript{135–209} complex binds to MCI-154. Since residues 39–60 of cTnI are known to bind tightly to the cCTnC domain to form a structured core that is invariant throughout the cardiac cycle, we conclude that MCI-154 does not bind to cTnC when it is part of the intact cardiac troponin complex. Thus, MCI-154 likely exerts its calcium sensitizing effect by interacting with a target other than cardiac troponin.

1. **Introduction**

In heart failure, cardiac output is insufficient to satisfy the metabolic needs of the body. In acute decompensated heart failure, cardiac output becomes so compromised that, unless something is done imminently, clinical deterioration and death will ensue. Positive inotropes such as dobutamine and milrinone can increase cardiac output, but these are also associated with systemic hypotension, arrhythmias, and increased mortality \cite{1}.

Dobutamine and milrinone activate β\textsubscript{1}-adrenergic signaling pathways via direct binding to G-protein-coupled receptors in the case of dobutamine or dual phosphodiesterase-3 and -4 inhibition by milrinone \cite{2,3}. This leads to increased cyclic AMP concentrations and activation of protein kinase A, which phosphorylates many different targets within the cardiac myocyte including L-type calcium channels and phospholamban, which regulates sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) \cite{4}. Increased calcium fluxes enhance cardiac output, but at the expense of increased myocardial oxygen consumption and increased risk of arrhythmias. An alternative approach is to use “calcium sensitizers”, drugs that impact muscle function not by altering calcium fluxes, but by enhancing the contractile response to calcium. The best known cardiac calcium sensitizer is levosimendan, which has undergone many trials for the treatment of acute decompensated heart failure \cite{5}.

Many calcium sensitizers are purported to act on the cardiac troponin complex (cTn), the key calcium dependent switch that turns muscle contraction on and off with every heartbeat. It consists of three protein subunits: C, I, and T. The calcium dependence of the complex derives from troponin C (cTnC), which has two calcium-binding EF-hand domains. The N-terminal regulatory domain (cNTnC) determines the contractile state of the heart. Its calcium affinity is tuned to calcium fluctuations in the cardiac myocyte, so that it becomes significantly occupied only during systole, when the cytoplasmic free calcium concentration rises to about 1 μM (for a review, see \cite{6}). Calcium binding stabilizes the open conformation of cTnC, allowing it to bind the switch region of cardiac troponin I (cTnI\textsubscript{47–163}) \cite{7}. This removes adjacent inhibitory cTnI segments from actin, favoring activation of actin-myosin ATPase and cardiac muscle contraction.

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In theory, calcium sensitizers could act by stabilizing the calcium-bound activated form of cNTnC. This has been convincingly demonstrated for bepridil, a drug originally designed to treat angina by blocking calcium channels, but serendipitously shown to also act as a calcium sensitizer [8]. It was later demonstrated that bepridil binds cNTnC to stabilize its calcium-bound open conformation [9]. Bepridil, however, is not an effective troponin activator because it displaces cTnI switch peptide from cNTnC [10]. We thus searched for compounds that bind to cNTnC without displacing cTnI switch peptide and discovered that derivatives of diphenylamine are effective for this purpose [11]. A structurally similar compound is MCI-154 (Fig. 1), previously developed by Mitsubishi Chemical Corporation as a calcium sensitizing agent. It was shown to increase the calcium sensitivity of cardiac myofibrils as well as isolated cardiac troponin C [12,13]. Thus, out of all the calcium sensitizers developed to date, MCI-154 had some of the strongest evidence for a mechanism of calcium sensitization through direct binding to cardiac troponin C [14]. We thus set out to more thoroughly investigate the interaction of MCI-154 with cTnC.

2. Materials and methods

2.1. Synthesis of MCI-154 (Scheme 1)

Synthesis of 6-(4-(pyridin-4-ylamino)phenyl)-4,5-dihydropyridazin-3(2H)-one (MCI-154) was performed by following the synthetic route outlined on Scheme 1. Intermediate 4-(4-acetamidophenyl)-4-oxobutanoic acid 3 was obtained by aluminum chloride mediated Friedel-Crafts acylation of acetanilide 1 with succinic anhydride 2. Next, acidic hydrolysis of acamide functionality in compound 3 with concentrated hydrogen chloride afforded 4-(aminophenyl)-4,5-dihydropyridazin-3(2H)-one 5 which was coupled with 4-chloropyridine hydrochloride in the presence of triethylamine to give access to desired 6-(4-(pyridin-4-ylamino)phenyl)-4,5-dihydropyridazin-3(2H)-one (MCI-154).

Reactions were carried out in flame-dried glassware. Transfer of anhydrous solvents and reagents was accomplished with oven-dried syringes. Dimethylformamide (DMF) was distilled before use from calcium hydride; N-methylpyrrolidone (NMP) was dried over 4Å molecular sieves. Thin layer chromatography was performed on glass plates pre-coated with 0.25 mm Kieselgel 60 F254 (Merck). Flash chromatography columns were packed with 230–400 mesh silica gel (Silicycle). 1H NMR were measured on Agilent/Varian DD2 MR two channel 400 MHz spectrometer and are reported in ppm relative to tetramethylsilane (0.00 ppm) standard; coupling constants (J) are reported in Hertz (Hz). Standard notation is used to describe the multiplicity of signals observed in 1H NMR spectra: singlet (s), doublet (d), triplet (t), etc. Please see the Supplementary Materials for the NMR spectra of MCI-154 and compounds 3-5.

2.1.1. 4-(4-acetamidophenyl)-4-oxobutanoic acid (3) [15]

Flame-dried round bottom flask was charged with anhydrous aluminum chloride (13.350 g, 100 mmol) under nitrogen atmosphere and DMF (2 mL, 30 mmol) was added dropwise at 0 °C. To the stirred reaction mixture heated at 70 °C was added the mixture of acetanilide 1 (1.352 g, 10 mmol) and succinic anhydride 2 (1 g, 10 mmol) in little portions. The stirring was continued for 1 h at 70 °C. The warm reaction mixture was poured into ice (90 g) and conc. hydrochloric acid (6 mL) was added. After 15 min the solid precipitate was collected by filtration, washed with water and dried. Recrystallization from DMF/H2O mixture afforded the product as beige crystals (1.291 g, 5.48 mmol, 55% yield).

1H NMR (400 MHz, DMSO-d6) δ = 10.26 (s, 1H), 7.94 (d, J = 8.8 Hz, 2H), 7.71 (d, J = 8.8 Hz, 2H), 3.19 (t, J = 6.3 Hz, 2H), 2.56 (t, J = 6.3 Hz, 2H), 2.08 (s, 3H) ppm.

2.1.2. 4-(4-aminophenyl)-4-oxobutanoic acid (4)

Into the round bottom flask charged with 4-(4-acetamidophenyl)-4-oxobutanoic acid 3 (3.102 g, 13.19 mmol) was added conc. hydrochloric acid (6 mL) was added. After 15 min the solid precipitate was collected by filtration, washed with water and dried. Recrystallization from DMF/H2O mixture afforded the product as beige crystals (1.291 g, 5.48 mmol, 55% yield).

1H NMR (400 MHz, DMSO-d6) δ = 10.26 (s, 1H), 7.94 (d, J = 8.8 Hz, 2H), 7.71 (d, J = 8.8 Hz, 2H), 3.19 (t, J = 6.3 Hz, 2H), 2.56 (t, J = 6.3 Hz, 2H), 2.08 (s, 3H) ppm.

Scheme 1. Synthesis of MCI-154.
aquaase saturated solution of sodium carbonate. The product was collected by filtration, washed with water and dried. The product was obtained as off-white powder (0.869 g, 4.46 mmol, 34% yield). ^1H NMR (400 MHz, DMSO- d 6) δ = 7.68 (d, J = 8.8 Hz, 2H), 6.56 (d, J = 8.8 Hz, 2H), 6.01 (s, 2H), 3.05 (t, J = 6.3 Hz, H), 2.50 (t, J = 6.3 Hz, 2H) ppm.

2.1.3. 6-(4-aminophenyl)-4,5-dihydropyridazin-3(2H)-one (5) [16]

In a round bottom flask, 4-(4-aminophenyl)-4-oxobutanoic acid 4 (0.850 g, 4.46 mmol) was dissolved in ethanol (5 mL) and hydroxylamine monohydrate (0.4 mL, 7 mol%) was added. The reaction mixture was refluxed for 2 h. After cooling down to room temperature, the precipitate was filtered off, washed with ethanol and dried. The product was obtained as brown solid (0.592 g, 3.13 mmol, 70% yield). ^1H NMR (400 MHz, DMSO- d 6) δ = 10.60 (s, 1H), 7.45 (d, J = 8.8 Hz, 2H), 6.56 (d, J = 8.8 Hz, 2H), 5.46 (s, 1H), 2.82 (t, J = 8.1 Hz, 2H), 2.35 (t, J = 8.1 Hz, 2H) ppm.

2.1.4. 6-(4-(pyridin-4-ylamino)phenyl)-4,5-dihydropyridazin-3(2H)-one (MCI-154) [17]

In a round bottom flask, triethylamine (0.22 mL, 156 mmol) was added to the solution of 6-(4-aminophenyl)-4,5-dihydropyridazin-3(2H)-one 5 (0.592 g, 3.13 mmol) in NMP (3.2 mL). The reaction mixture was heated to 90 °C followed by addition of 4-Chloropyridine hydrochloride (0.455 g, 3.13 mmol) and stirred at 90 °C for 2 h. Then the mixture was cooled down to 0 °C in ice bath, followed by addition of acetone (40 mL). The resulting precipitate was filtered off, dissolved in water and alkanilized with aqueous 1 M solution of sodium hydroxide. The formed precipitate was purified by silica gel flash column chromatography (eluent: CHCl3/MeOH, 10:1) which afforded white solid (0.646 g, 2.24 mmol, 78% yield). ^1H NMR (400 MHz, DMSO- d 6) δ = 10.83 (s, 1H), 9.02 (s, 1H), 8.23 (d, J = 5.9 Hz, 2H), 7.72 (d, J = 8.8 Hz, 2H), 7.23 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 5.9 Hz, 2H), 2.92 (t, J = 8.2 Hz, 2H), 2.43 (t, J = 8.2 Hz, 2H) ppm. ^13C NMR (125 MHz, DMSO- d 6) δ = 166.9, 150.1, 149.3, 149.0, 141.6, 129.5, 126.9, 118.7, 110.8, 26.0, 21.7 ppm. HRMS (ESI+) m/z calc.: for C14H14N2O4 [M + H]+: 267.1240, found 267.1241.

2.2. Protein sample preparation

Recombinant human cardiac cTnC (residues 1–161) with the mutations C35S and C84S, cTnI (residues 1–77), and cTnI (residues 135–209) were used in this study. The expression and purification of cTnI (residues 1–77) and cTnI (residues 135–209) were performed by using MCI-154 binding to cTnC•3Ca2+ was followed by solution 2D {1H, 15N} HSQC NMR analyses. The pH changes from ligand additions were compensated by 1 M NaOH or 1 M HCl. Both 1D ^1H and 2D (^1H, ^15N)-HSQC NMR spectra were acquired at every titration point.

2.3. NMR Spectroscopy

All NMR experiments were run on a Varian Inova 500 MHz spectrometer at 30 °C. The spectrometer is equipped with a triple resonance ^1H, ^15N, and ^13C probe and z-axis pulsed field gradients. Both 1D ^1H and 2D (^1H, ^15N)-HSQC NMR spectra were acquired at every titration point. The dissociation constant (Kd) for MCI-154 binding to cTnC•3Ca2+ was calculated by plotting chemical shift changes as a function of the ligand-to-protein ratio and then fitting the values to a function using our in-house curve-fitting software, xcrvfli. The function relating the predicted change in chemical shift (∆δ) to total protein (P) and total ligand concentrations (L) is as follows:

\[ \Delta \delta = \Delta \delta_{\text{max}} \left( \frac{P + L + K_D}{(P + L + K_D)^2 + 4P} \right) \]

where \( \Delta \delta_{\text{max}} \) is the change in chemical shift expected at 100% saturation and \( K_D \) is the dissociation constant for the 1:1 protein-ligand complex. A \( \chi^2 \) function measuring the sum of differences between observed and predicted ∆δ values was minimized, using \( K_D \) and \( \Delta \delta_{\text{max}} \) as fitting parameters.

2.4. Titration of MCI-154 to ^15N-cTnC•3Ca2+ ^2+

For NMR sample preparation, 4.8 mg of solid ^15N-cTnC was dissolved into 500 μL MCI-154 buffer containing 100 mM KCl, 10 mM imidazole, 0.5 mM DSS in 90% H2O/10% D2O to generate a 450 μM NMR sample (protein concentration was estimated based on weight and integration of 1D ^1H NMR spectroscopy). 5 μL of 1 M CaCl2 was added to ensure that the protein was Ca2+-saturated and the pH was adjusted by 1 M NaOH and 1 M HCl to ~ 6.7. Aliquots of 1, 1, 1, 0.5, 1.5, 2, 5 μL of 90.2 mM MCI-154 in d6-DMSO were added consecutively to the ^15N-cTnC•3Ca2+ sample. The sample was mixed thoroughly with each addition. The change in protein concentration due to volume increase was taken into account for data analyses. The pH changes from ligand additions were compensated by 1 M NaOH or 1 M HCl. Both 1D ^1H and 2D (^1H, ^15N)-HSQC NMR spectra were acquired at every titration point.

2.5. Titration of cTnI147-163 to ^15N-cTnC•3Ca2+ ^2+

Since cTnI147-163 is insoluble in aqueous solutions, solid cTnI147-163 was dissolved in d6-DMSO to make a 5 mM stock solution, aliquots of 1, 1, 2, 7, 5, 8, 11, 11, 11, 11, 11, 11 μL were added consecutively to a 500 μL NMR sample of 200 μM ^15N-cTnC•3Ca2+ (NMR buffer conditions are 100 mM KCl, 10 mM imidazole, 0.5 mM DSS in 90% H2O/10% D2O, and protein concentration was estimated based on weight and integration of 1D ^1H NMR spectroscopy). The sample was mixed thoroughly with each addition. The change in protein concentration due to volume increase was taken into account for data analyses. The pH changes from ligand additions were compensated by 1 M NaOH or 1 M HCl. Both 1D ^1H and 2D (^1H, ^15N)-HSQC NMR spectra were acquired at every titration point.

2.6. Isothermal Titration Calorimetry

Isthermal titration calorimetry (ITC) was carried out on a Malvern VP-ITC MicroCalorimeter at 25 °C (note that the NMR experiments were performed at 30 °C), and data were analyzed using the original ITC analysis software package (MicroCal). 2 mM MCI-154 was titrated into 200 μM purified cTnC protein, both in buffer containing 50 mM imidazole at pH 6.8, 100 mM KCl, 10 mM CaCl2, and 1% DMSO. The initial injection volume was 4 μL, followed by 15 μL injections spaced 300 s apart. Data were corrected and fitted to a single binding site model, from which apparent molar reaction enthalpy (∆Hf), entropy (ΔS), and dissociation constant (Kd) were generated.

3. Results

3.1. MCI-154 binds to the structural C-terminal domain of cTnC

MCI-154 was titrated into isolated calcium-saturated ^15N-enriched cTnC, and binding was followed by solution 2D (^1H, ^15N)-HSQC NMR spectra. Chemical shift changes were observed in residues from the...
This is somewhat surprising, since one would expect a calcium sensitizer to interact with the regulatory cNTnC domain, which determines the calcium sensitivity of cardiac muscle. (It should also be noted that titration of cTnC with DMSO yields no chemical shift changes.) With each titration point, cCTnC signals progressively move from the unbound state to an MCI-154-bound state without significant broadening, indicating that MCI-154 binding occurs with fast kinetics relative to the NMR frequency differences between free and bound cCTnC states. The MCI-154-induced total chemical shift changes ($\Delta \delta$) of each affected residue was calculated as follows: $\Delta \delta = \sqrt{\left(\Delta \delta_{1H}\right)^2 + \left(\Delta \delta_{15N}/5\right)^2}$ at every titration point, then averaged for a group of residues (L117, T124, I128, T129, G140, F153, E155, F156, M157, K158, G159, E161) that underwent significant chemical shift changes as shown in Fig. 2. The averaged data were normalized according to $(\delta_{\text{obs}} - \delta_{\text{initial}})/(\delta_{\text{final}} - \delta_{\text{initial}})$. The curve was fit as a function of chemical shift perturbation vs. [MCI-154]_total/[cTnC]_total. A solid line represents the best fit to the data ($K_D \approx 0.5$ mM).

3.2. MCI-154 does not bind to cTnC in the presence of cTnI1–77

cCTnC domain, but none was observed in the cNTnC domain (Fig. 2). This is somewhat surprising, since one would expect a calcium sensitizer to interact with the regulatory cNTnC domain, which determines the calcium sensitivity of cardiac muscle. (It should also be noted that titration of cTnC with DMSO yields no chemical shift changes.) With each titration point, cCTnC signals progressively move from the unbound state to an MCI-154-bound state without significant broadening, indicating that MCI-154 binding occurs with fast kinetics relative to the NMR frequency differences between free and bound cCTnC states. The MCI-154-induced total chemical shift changes ($\Delta \delta$) of each affected residue was calculated as follows: $\Delta \delta = \sqrt{\left(\Delta \delta_{1H}\right)^2 + \left(\Delta \delta_{15N}/5\right)^2}$, then averaged for a group of residues that underwent significant chemical shift changes. MCI-154 appears to bind to the central hydrophobic cavity of the cCTnC domain (where all small molecules have been found to bind, see Discussion section), as shown by chemical shift perturbation mapping (Fig. 1). The linear movement of the signals as the titration progressed is suggestive of 1:1 stoichiometry. The dissociation constant ($K_D$) of MCI-154 for cTnC was calculated as described in the Methods section. Fitting of the titration points yields a binding dissociation constant, $K_D$, of about 0.5 mM (Fig. 3). This affinity is much weaker than the observed calcium sensitizing activity of MCI-154, which was $\sim 50\%$ active at concentrations of 1–10 μM [21], suggesting that the interaction between MCI-154 and cTnC cannot account for its biological activity.

Isothermal titration calorimetry (ITC) was used to confirm the binding affinity. A $K_D$ value of 0.5 mM ± 0.1 mM was obtained, in perfect agreement with the value obtained by NMR. In addition, $\Delta H$ of binding was determined to be $-2.5$ kcal/mol, with $\Delta S = +6.6$ cal/mol K (see Fig. 4). The stoichiometry of binding could not be precisely determined using ITC.

cTnC is strongly tethered to the rest of the cardiac troponin complex by a tight interaction with residues 39–60 of cTnI, which form an amphipathic helix that occupies the large hydrophobic patch in the cCTnC domain [22]. We previously demonstrated that residues 19–37 of cTnI interact electrostatically with the regulatory cNTnC domain, and this interaction fixes the positioning of the cNTnC domain relative to cCTnC and the rest of the troponin complex [23]. Full-length cTnI is highly insoluble and does not form a stable soluble complex with cTnC at physiologic pH. We thus prepared a complex of cTnI1–77, which is fully soluble, with cTnC. When MCI-154 compound was titrated into cTnC-cTnI1–77 complex, no chemical shift changes could be observed in either domain of cTnC. Thus, the interaction between cTnI residues 19–37 and the cNTnC domain did not

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create a binding site for MCI-154. As well, binding of cTnI residues 39–60 to the cCTnC domain out-competes MCI-154.

3.3. MCI-154 does not bind to cTnC in the presence of cTnI135–209

cTnC also interacts with cTnI135–209 region (see Fig. 1), which comprises all the inhibitory segments that bind to actin to shut down cardiac muscle contraction. It also contains the switch region, cTnI147–163, that binds to cNTnC to activate cardiac muscle contraction. Titration of MCI-154 into the cTnC-cTnI135–209 complex showed no chemical shift changes. This indicates that MCI-154 does not interact with the cNTnC-cTnI switch region complex. Also, binding of the cTnI135–209 peptide to the cCTnC domain out-competes MCI-154.

3.4. Binding of full-length cTnC to cTnI147–163 switch peptide

It is very well established that the binding of cTnI switch region, cTnI147–163, to cNTnC constitutes the key event that triggers cardiac muscle contraction. It was somewhat surprising that binding of cTnI135–209 to cTnC should eliminate binding of MCI-154 to the cCTnC domain (see Results Section 3.3 above), since this segment of cTnI was not known to bind the cCTnC domain. To gain more insight into this, we titrated a shorter peptide corresponding to the switch region, cTnI147–163, into full-length cTnC. Interestingly, large chemical shift changes were induced in both domains of cTnC (Fig. 5). Given the similar magnitude of chemical shift changes in both domains, one possible explanation is that the cCTnC domain is able to bind the switch region as well as the cNTnC domain. Another possibility is that the cNTnC-cTnI147–163 complex interacts with the cCTnC domain. In either case, we have previously shown that when the cCTnC domain is bound to cTnI37–71, addition of cTnI147–163 into full-length cTnC impacts only the cNTnC domain, and not the cCTnC domain [24]. Apparently, the cCTnC domain is able to bind promiscuously to a variety of peptides and small molecules in the absence of its physiologic binding partner: cTnI residues 39–60.
4. Discussion

The binding of the positive inotropic compound MCI-154 to cTnC has not previously been studied by NMR. Previous studies suggested that it directly increased the calcium binding affinity of free cTnC [25–28]. It is possible that it enhances the affinity of the cTnC domain by binding to its central hydrophobic cavity, thus stabilizing the calcium-bound open form of the domain. The lack of binding to the cTnTnC domain is consistent with our recent study of diphenylamine analogs binding to a cTnTnC-cTnI136–163 chimeric construct [11]. Bulky sub-
cations in the para position of diphenylamine (as is present in MCI-
154, Fig. 1) were not well tolerated. Hence, the positive inotropic effect of MCI-154 in experimental models is most likely related to a me-
chanism distinct from cTnC binding. MCI-154 has potent inhibitory activity

Appendix B. Supplemental material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.012.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

This is a continuing project in the BDS/PMH labs, MXL conducted most of the experiments and analyzed the results, GID titrated cTnI136–163 to cTnC, PBL purified all the cTnC proteins used in this work, and PMH wrote most of the paper. SH synthesized MCI-154 under the supervision of FGW. YW performed the ITC experiments as part of the MJL lab.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.012.
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