

ORIGINAL RESEARCH



Danicamtiv Increases Myosin Recruitment and Alters Cross-Bridge Cycling in Cardiac Muscle

Kristina B. Kooiker¹*, Saffie Mohran¹*, Kyrah L. Turner¹, Weikang Ma¹, Amy Martinson, Galina Flint, Lin Qi¹, Chengqian Gao, Yahan Zheng¹, Timothy S. McMillen¹, Christian Mandrycky¹, Max Mahoney-Schaefer, Jeremy C. Freeman, Elijah Gabriela Costales Arenas¹, An-Yu Tu, Thomas C. Irving¹, Michael A. Geeves¹, Bertrand C.W. Tanner¹, Michael Regnier¹, Jennifer Davis¹, Farid Moussavi-Harami¹

BACKGROUND: Modulating myosin function is a novel therapeutic approach in patients with cardiomyopathy. Danicamtiv is a novel myosin activator with promising preclinical data that is currently in clinical trials. While it is known that danicamtiv increases force and cardiomyocyte contractility without affecting calcium levels, detailed mechanistic studies regarding its mode of action are lacking.

METHODS: Permeabilized porcine cardiac tissue and myofibrils were used for X-ray diffraction and mechanical measurements. A mouse model of genetic dilated cardiomyopathy was used to evaluate the ability of danicamtiv to correct the contractile deficient.

RESULTS: Danicamtiv increased force and calcium sensitivity via increasing the number of myosins in the on state and slowing cross-bridge turnover. Our detailed analysis showed that inhibition of ADP release results in decreased cross-bridge turnover with cross bridges staying attached longer and prolonging myofibril relaxation. Danicamtiv corrected decreased calcium sensitivity in demembranated tissue, abnormal twitch magnitude and kinetics in intact cardiac tissue, and reduced ejection fraction in the whole organ.

CONCLUSIONS: As demonstrated by the detailed studies of Danicamtiv, increasing myosin recruitment and altering cross-bridge cycling are 2 mechanisms to increase force and calcium sensitivity in cardiac muscle. Myosin activators such as Danicamtiv can treat the causative hypocontractile phenotype in genetic dilated cardiomyopathy.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: animal ■ calcium ■ cardiomyopathy ■ myofibril ■ myosin

The prevalence of heart failure (HF) continues to rise and despite new and improved treatments, there is still significant morbidity and cost.¹ More than half of the patients with HF can be categorized by depressed systolic function or contractility.² Traditional inotropic agents improve cardiomyocyte contractility via increased intracellular cAMP and calcium levels but do not improve patient survival.^{3,4} Directly targeting sarcomeric proteins can overcome adverse effects of

traditional inotropes such as arrhythmias, increased myocardial oxygen demand, and activation of cell death pathways.³ Small molecules targeting myosin and modulating its activity have been developed over the last decade for the treatment of HF. The strategy for identifying these compounds has been to identify molecules that selectively bind to cardiac myosin and increase or inhibit the myosin ATPase activity. Omecamtiv mecarbil (OM) is a first-in-class myosin activator that was originally thought

Correspondence to: Farid Moussavi-Harami, MD, 850 Republican St, D353, Seattle WA 98109. Email moussavi@uw.edu

*K.B. Kooiker and S. Mohran contributed equally.

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Novelty and Significance

What Is Known?

- Myosin-specific small molecules are an emerging therapeutic modality for cardiomyopathies.
- Danicamtiv is a novel myosin modulator being considered for treatment of dilated cardiomyopathy.
- Previous studies showed that Danicamtiv increased left ventricular systolic function in preclinical animal models and humans.

What New Information Does This Article Contribute?

- Danicamtiv increases myosin recruitment and alters cross-bridge cycling by decreasing ADP release resulting in prolonged actomyosin interaction.
- Danicamtiv recovers both tissue and organ level hypocontractility in a rodent genetic dilated cardiomyopathy model.

Novel myosin-specific small molecules have been developed for treatment of hypocontractility in heart

failure. A better understating of the mechanisms of actions of these agents may help to elucidate subsets of patients that will most benefit from them. The work in this study demonstrates how a combination of structural and biomechanical studies informs on how Danicamtiv, a small molecule targeting myosin, increases cardiac contractility. Danicamtiv increases the structural recruitment of myosin and alters the biochemical cross-bridge cycling by decreasing ADP release, resulting in prolonged actomyosin interaction. Danicamtiv shares some of these mechanisms with Omecamtiv Mecarbil, which is the first myosin-specific molecule for increasing contractility. This detailed methodological framework can be used to connect how changes in myosin recruitment and cross-bridge cycling kinetics by small molecules affect cardiac myofibril activation and relaxation.

Nonstandard Abbreviations and Acronyms

cTnC	cardiac troponin C
dATP	2-deoxy ATP
DCM	dilated cardiomyopathy
HF	heart failure
HMM	heavy meromyosin
MYH6	myosin heavy chain 6
MYH7	myosin heavy chain 7
OM	Omecamtiv Mecarbil
pCa	negative log of calcium concentration
P_i	inorganic phosphate

to work via increased phosphate release and priming myosin for binding to actin.^{5,6} More detailed biophysical measurements showed that OM inhibits the myosin working stroke and prolongs the detachment of a small population of nonforce generating myosin heads.^{7,8} The OM-bound myosin heads activate the thin filament and recruit more myosin heads with the net effect of having more myosin heads that are available for force generation. OM recently completed a phase III clinical trial in HF and was found to result in a modest decrease in HF events or death from cardiovascular causes.^{9,10} The successes and challenges of OM show the importance of a better understanding of the molecular mechanisms of new sarcomere modulators.

Danicamtiv or MYK-491 is a promising novel myosin activator that increases myofibril ATPase activity and calcium sensitivity.¹¹ *in vivo* studies demonstrated improved atrial and ventricular function in a canine HF model as well as patients with HF with reduced ejection fraction.^{11,12} There is an ongoing clinical trial enrolling patients with genetic dilated cardiomyopathy (DCM) with sarcomeric variants for treatment with Danicamtiv. We and others have shown that a reduction in the force and kinetics of the cardiac twitch, the tension developed as a function of time, is predictive of the diagnosis and severity of DCM in rodent myocytes and humans.^{13–15} It is logical, therefore, that patients with DCM may benefit from treatment with myosin activators. A recent study in a human-engineered heart tissue platform showed that administration of Danicamtiv resulted in a larger increase in systolic contraction at a smaller lusitropic cost compared with OM.¹⁶ While this suggests possible differences in mechanisms, there is limited information available regarding Danicamtiv's mechanisms of action other than that Danicamtiv increases the number of myosin heads available for force production without altering passive stiffness.¹⁷ Here, we present the first detailed mechanistic study of how Danicamtiv affects the cross-bridge cycle, cardiac muscle contractile kinetics, and myosin structure. We show that Danicamtiv increased calcium sensitivity and elevated force mostly at low calcium levels. Using X-ray diffraction, we demonstrate that treatment with Danicamtiv under resting conditions repositioned myosin heads closer to the thin filament. In myofibrils under load, Danicamtiv slowed the myosin cross-bridge cycle

and prolonged relaxation kinetics by inhibiting the rate of ADP product release. Last, we demonstrate the ability of Danicamtiv to recover hypocontractility at the tissue and organ levels in a rodent DCM model.

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal Use and Ethics

All experiments followed protocols approved by both the University of Washington and the Illinois Institute of Technology Institutional Animal Care and Use Committees according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Farm pig hearts were obtained immediately after the animal was euthanized and rinsed in cold oxygenated Tyrode's buffer.

DCM Mouse Model

We used a previously published rodent genetic DCM model. The I61Q cTnC (cardiac troponin C) is an engineered cTnC variant that desensitizes the myofilament to calcium via altering cTnC calcium binding.^{18,19} Transgenic mice show progressive ventricular dilation and reduced contractility as they age.¹³ Experimental mice were between 3 and 5 months of age.

In Vivo Mouse Echocardiography

Transthoracic echocardiography was performed using Vevo 3100 high-frequency, high-resolution imaging system (VisualSonics) equipped with MS400 MicroScan Transducer. After initial m-mode measurement, a 27G butterfly needle was inserted into the tail vein and total 125 μ L of N,N-dimethylacetamide: polyethylene glycol 400 (PEG-400): 30% 2-hydroxypropyl- β -cyclodextrin (5:25:70) containing 2 mg/kg of Danicamtiv or DMSO (vehicle) was delivered. This was followed by a 500 μ L of normal saline and removal of the catheter. The mouse continued under sedation and a repeat measurement was performed 10 minutes after the injection.

Demembrated Tissue Mechanics

Tissue preparation and solutions are described in [Supplemental Methods](#).

Isometric Force and Rate of Tension Redevelopment

Permeabilized trabeculae (mouse) or thin strips (pig) were dissected and mounted between a force transducer (Aurora Scientific, model 400A) and a motor (Aurora Scientific, model 315C) using aluminum T-clips (Aurora Scientific).¹⁴ Sarcomere length was set to \approx 2.3 μ m for the experiments. Experiments were conducted in physiological solution (pH 7.0) at 15 $^{\circ}$ C (mouse) or 21 $^{\circ}$ C (pig) containing a range of negative log of calcium concentration (pCa; $-\log_{10}[\text{Ca}^{2+}]$) values from 9.0 to 4.0 with and without 1 μ M Danicamtiv (MedChemExpress). Tissue was allowed to reach steady-state force (F) at each pCa. F-pCa curves were collected and analyzed with custom code

using LabView software and fit to the Hill equation. The rate of tension redevelopment (k_{tr}) and high-frequency stiffness was measured at each pCa (details in the [Supplemental Material](#)).

Quick Stretch Analysis

Permeabilized porcine myocardial strips (\approx 180 \times 700 μ m) were mounted between a piezoelectric motor (P841.40, Physik Instrumente, Auburn, MA) and a strain gauge (AE801, Kronex, Walnut Creek, CA) using aluminum T-clips. They were lowered into a 30 μ L droplet of relaxing solution (pCa 8.0) maintained at 28 $^{\circ}$ C and set at 2.3 μ m sarcomere length. Stress (force per cross-sectional area) was recorded following a step-length change of 0.5% muscle length to assess cross-bridge kinetics as a function of [MgATP] as previously described and repeated with varying [MgATP].²⁰

Myofibril Mechanics

Myofibril activation and relaxation measurements were performed on a custom set-up as previously described.^{21,22} Briefly, myofibrils were mounted between 2 glass needles; one which acted as a force transducer and the other as an inflexible motor arm. A dual diode system measures force based on needle deflection, with force transducer needle stiffness measured at 7.98 mm/ μ N. A double-barreled glass pipette delivered relaxing (pCa=9.0) and activating (pCa=5.8 or 4.0) solutions to the mounted myofibril. Activation and relaxation data were collected at 21 $^{\circ}$ C and fitted as previously described.²²

Intact Twitch Assay

Unbranched, intact trabeculae or papillary muscles were dissected from the right ventricular wall and mounted between a force transducer (Cambridge Technology, Inc, model 400A) and a rigid post. The tissue was submerged in a chamber continuously perfused with oxygenated modified Krebs buffer (1.8 mmol/L CaCl_2) at 33 $^{\circ}$ C. Continuous twitch tension traces were recorded using LabView software at a sampling rate of 1 kHz and analyzed with custom code in MATLAB software (version 2021a, The MathWorks).¹⁴

In Vitro Motility Assay

Myosin and HMM (heavy meromyosin) were purified from pig left ventricular samples as previously described.²² In vitro motility assays were performed at 30 $^{\circ}$ C using unregulated Rhodamine Phalloidin labeled F-actin in the presence of 2 mmol/L ATP and either DMSO or 0.5 μ M Danicamtiv.^{21,22} In a subset of experiments, the nucleotide composition was changed to 1 mmol/L ATP and 1 mmol/L ADP. Custom-built software analyzed images of the moving filaments as in our previous publications.^{21,22}

X-Ray Diffraction

Wild-type Yucatan mini-pig hearts were provided by Exemplar Genetics LLC. Permeabilized tissue preparation and beamline specifications are described in [Supplemental Methods](#). X-ray diffraction experiments were performed at the BioCAT beamline 18ID at the Advanced Photon Source, Argonne National Laboratory.²³ Skinned muscle preparations were mounted in a custom rig allowing for simultaneous X-ray diffraction and force measurements as monitored by an ASI 610A data acquisition

and control system (Aurora Scientific). The muscle was incubated in a customized chamber with the solution temperature between 28 °C and 30 °C. The sarcomere length of the muscles was set to 2.3 μm by monitoring the helium-neon laser (633 nm) diffraction pattern on a screen. X-ray fiber diffraction patterns were collected in pCa 8 solution in the absence or presence of 50 μM of Danicamtiv. Analysis of x-ray diffraction images was done as described previously^{24,25} and provided in the [Supplemental Methods](#).^{24,25}

Please see the Major Resources Table in the [Supplemental Material](#).

Statistical Analysis

We used GraphPad Prism 9 for data presentation and statistical analysis. The data are presented as mean±SEM. We used Shapiro-Wilk test for normality. For data not normally distributed, we used Kruskal-Wallis with Dunn multiple comparison test or Wilcoxon matched-pairs signed rank test. For porcine demembranated tissue mechanics, we used a mixed-effect model with Šídák multiple comparisons test for pCa curves and paired 2-tailed *t* test for the remaining measures. For myofibrils, we used Welch unpaired 2-tailed *t* test (pCa, 4.0), or 2-way ANOVA followed by Tukey multiple comparisons test (pCa, 5.8±ADP). For X-ray diffraction, we used paired 2-tailed *t* tests. For in vitro motility and murine demembranated tissue mechanics, we used 2-way ANOVA with Šídák multiple comparisons. For quick-step analysis, we used 2-way ANOVA with Šídák multiple comparison for k_{rel} and 1-way ANOVA with Tukey multiple comparisons for the remaining measures. For sinusoidal length-perturbation assays, we used multiple unpaired *t* tests. For murine intact mechanics, we used mixed-effect analysis with Dunnett multiple comparisons.

RESULTS

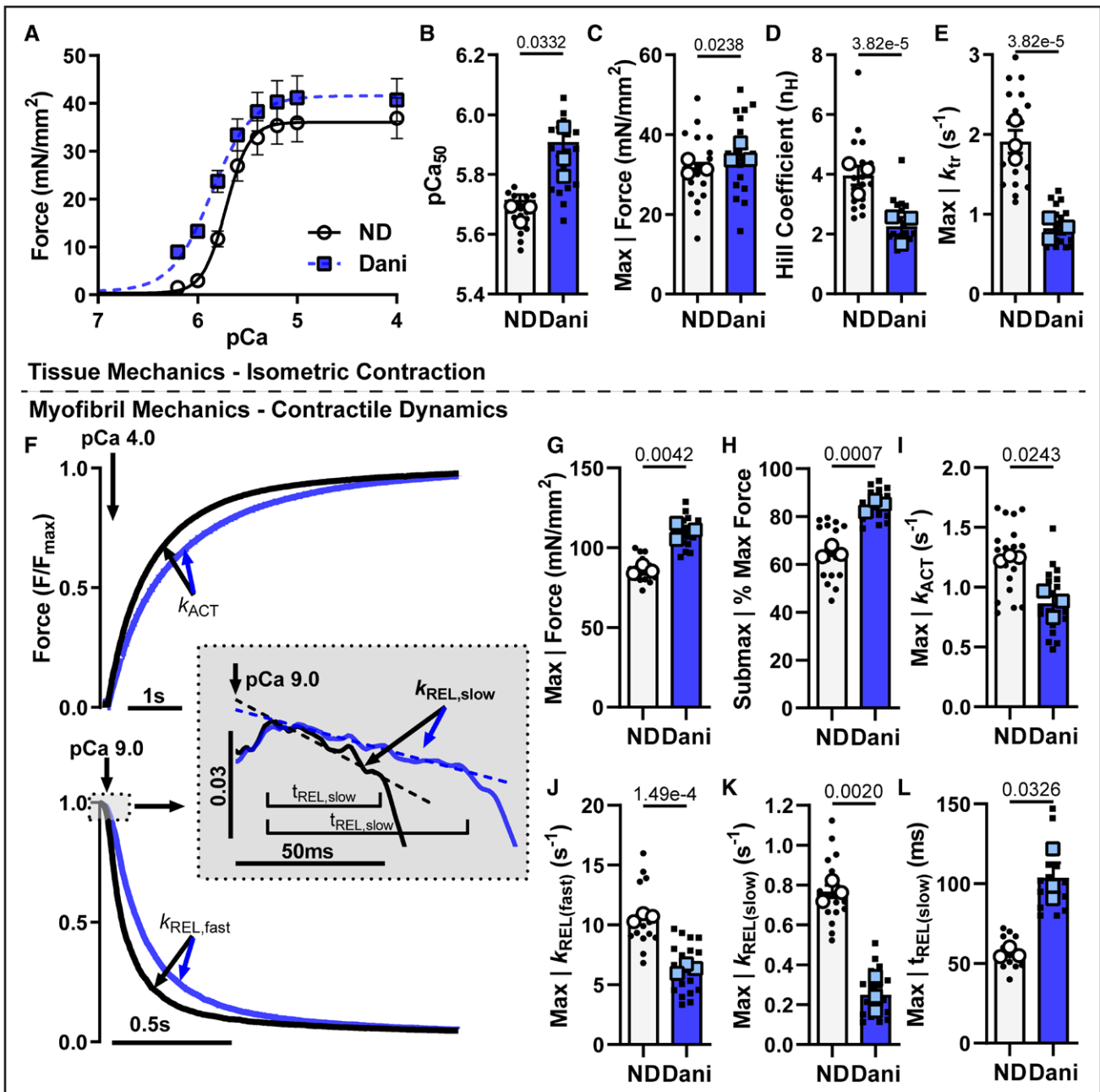
Danicamtiv Increased Submaximal Force and Calcium Sensitivity While Prolonging Myofibril Relaxation

Permeabilized porcine ventricular muscle preparations showed a leftward shift in the force versus calcium (pCa) relationship in the presence of 1 μM Danicamtiv (Figure 1A; [Table S1](#)). This was quantified by an increase in pCa₅₀ (Figure 1B; 5.72±0.02 versus 5.91±0.05) indicating an increase in the sensitivity of the myofilament to calcium. While there was a slight increase in maximally activated force (Figure 1C), there was a larger increase in force at submaximal calcium levels (Figure 1A). This leads to a reduction in the Hill coefficient (Figure 1D; 3.96±0.31 versus 2.27±0.29), suggesting an effect of Danicamtiv on myofilament cooperativity or recruitment. Force redevelopment rate constants (k_r) were significantly slower at each level of force ([Figure S1B](#)) suggesting that Danicamtiv reduced cross-bridge cycling kinetics. Incubation in Danicamtiv resulted in more than a 50% decrease in maximal k_r (Figure 1E; 1.91±0.14 versus 0.83±0.08). High-frequency small amplitude oscillation measurements of steady-state stiffness showed that Danicamtiv increased

stiffness to the greatest degree at lower calcium levels ([Figure S1C](#)). The increased stiffness is proportional to the increase in force, suggesting that the increased stiffness is due to more myosin heads producing force rather than an increase in force per myosin head.

Isolation of subcellular myofibrils allows for measurements of force and kinetics of both activation and relaxation.^{21,22} Figure 1F shows representative normalized force traces of porcine myofibrils transitioning between maximally activated (high Ca²⁺) and relaxed (low Ca²⁺) conditions by rapid solution switching via a double-barreled pipette with and without Danicamtiv. Force is developed exponentially with a rate constant (k_{ACT}) that depends on thin filament activation, myosin recruitment, and myosin cross-bridge cycling. The rapid switch back to low calcium solution results in a biphasic relaxation relationship with an initial linear phase followed by a rapid exponential phase back to baseline. The rate constant during the linear slow phase of relaxation ($k_{REL(slow)}$) reflects the rate of cross-bridge detachment, which is independent of the Ca²⁺ dissociation from troponin C. The duration of the slow phase ($t_{REL(slow)}$) reflects the time it takes for the thin filament to deactivate and has been shown to be influenced by the properties of the thin filament proteins and the level of calcium.^{19,26} The subsequent fast exponential phase of relaxation ($k_{REL(fast)}$) reflects inter-sarcomere dynamics and involves both active and passive elements that are influenced by multiple factors.

As in the demembranated tissue preparations, Danicamtiv increased myofibril force at maximal (pCa=4) and submaximal calcium (pCa=5.8). The ratio of submaximal to maximal activation was also significantly increased with Danicamtiv (Figure 1G and 1H). Treatment with Danicamtiv resulted in a slight decrease in k_{ACT} (Figure 1I). Relaxation kinetics analysis (Figure 1J) revealed that Danicamtiv decreased the fast rate of myofibril relaxation ($k_{REL(fast)}$; 10.64±0.20 versus 6.34±0.22 s⁻¹), which is consistent with the slowed cellular and organ level kinetics in preclinical models.¹² Our analysis also revealed that the initial linear slope ($k_{REL(slow)}$; 0.77±0.03 versus 0.25±0.05 s⁻¹) of myofibril relaxation was decreased while the thin filament deactivation duration ($t_{REL(slow)}$; 56.5±1.9 versus 103.7±9.4 ms) increased (Figure 1K and 1L). These results showing prolonged relaxation kinetics suggest that Danicamtiv impacts cross-bridge turnover kinetics by inhibiting product release, either inorganic phosphate (P) or ADP. Previously reported mechanisms of the myosin activator OM showed that slower cross-bridge turnover can result in increased force generation and prolonged myosin head activation, requiring more time for the thin filament to transition from an active to an inactive state.^{7,27} The rate of cross-bridge turnover has been previously identified as a critical regulator for transitioning the thin filament from an active to an inactive state.^{28,29}



While it is known that Danicamtiv increases myofibril ATPase rate,¹¹ a detailed understanding of the exact mechanisms is lacking. Together, our tissue mechanics and myofibril measurements suggest that Danicamtiv impacts cardiac function through increased myosin head recruitment and altered cross-bridge cycling kinetics (Figure 2). We will investigate this further through the following reductionist assays.

Danicamtiv Altered Resting Myosin Thick Filament Structure and Both Passive and Active Elastic and Viscous Moduli

Previous studies have shown that myosin modulators can affect myosin structure.^{25,30} We used small-angle-x-ray diffraction patterns from permeabilized porcine myocardium to study the structural changes

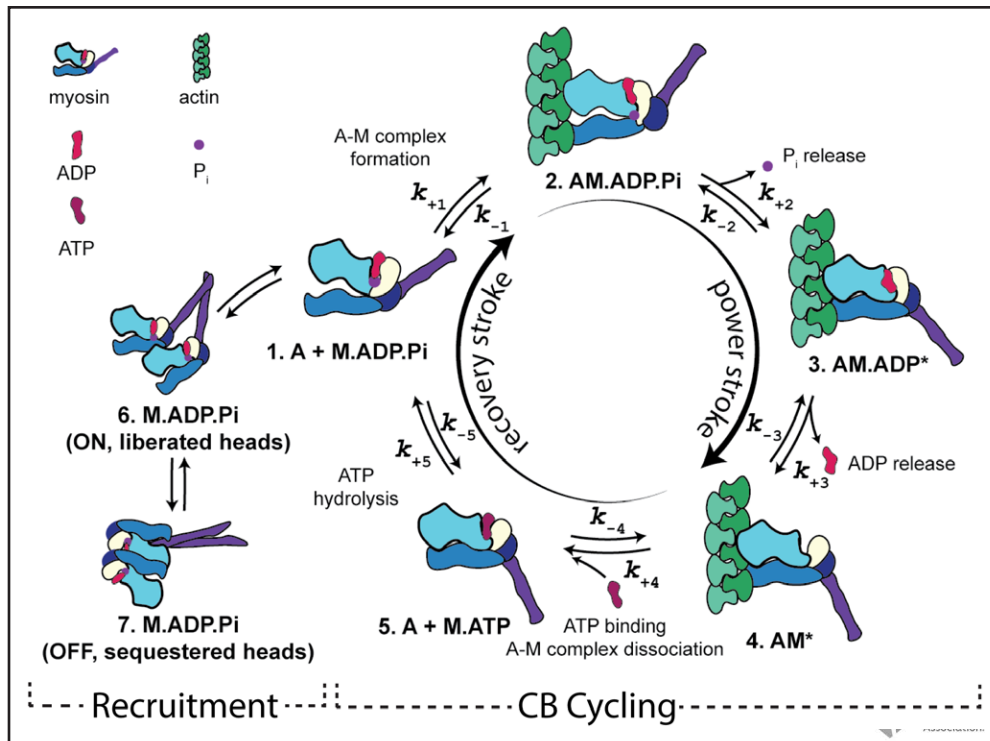


Figure 2. Scheme illustrating a 5-step cross-bridge model of contraction.

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induced by Danicamtiv under relaxed conditions (pCa8, Figure 3A). The lattice spacing ($d_{1,0}$; Figure 3B, top), directly proportional to interfilament spacing,³¹ increased after Danicamtiv treatment (Figure 3C). The increase of $d_{1,0}$ could be a result of increased electrostatic repulsion between the myofilaments when myosin heads move away from the thick filament backbone toward actin filaments. The equatorial intensity ratio ($I_{1,1}/I_{1,0}$) reflects the proximity of myosin heads to the thin filament (Figure 3B, bottom).^{25,30,31} As seen in Figure 3D, Danicamtiv significantly increased $I_{1,1}/I_{1,0}$ under relaxed conditions suggesting that myosin heads moved away from the thick filament backbone and closer to the thin filament.

The meridional reflections arise from axially repeating structures in the myofilaments (Figure 3A).³¹ The distance of these reflections to the beam center are inversely related to the spacing of the axial periodicities along the myofilaments. When cardiac muscle was exposed to Danicamtiv under relaxing conditions, the intensity of the first-order myosin-based layer line (I_{MLL1} ; Figure 3E) and the third-order myosin-based meridional reflection (I_{M3} ; Figure 3F), both of which correlate with the ordering of myosin heads on the thick filament backbone, decreased by 27% and 35%, respectively. The decrease of I_{M3} and I_{MLL1} reflects a reduction in the number of ordered myosin heads. The intensity of the sixth-order myosin-based meridional reflection

(M6) arises primarily from structures within the thick filament backbone.³¹ The spacing of the M6 reflection (S_{M6}) increased by 0.2% with Danicamtiv (Figure S2; Table S2). Both the loss of the helical ordering of the myosin heads and the increase in thick filament backbone periodicity are suggestive of a transition in myosin structure and its position on the thick filament from the OFF to ON state.³² It is well recognized that resting thick filaments can be characterized by different structural and biochemical states that can be affected by both disease and small molecules.^{25,33,34} Here, the increase in the number of ON state thick filaments in the presence of Danicamtiv contributes to increased force generation via more myosin motors available for contraction.³⁰

Our x-ray diffraction findings were supported by performing sinusoidal length-perturbation analysis to porcine cardiac tissue with and without Danicamtiv under resting and activating conditions. The resultant elastic and viscous moduli responses provide insight into the number of bound cross bridges and characteristics such as kinetics of force-generating cross-bridges, respectively. As seen in Figure S3, under relaxed conditions (pCa 8.0, 5 mmol/L [MgATP]), elastic moduli were not significantly changed by Danicamtiv. There was a significant increase in the viscous moduli at a subset of oscillatory frequencies for the Danicamtiv-treated strips. Under maximally activated conditions (pCa, 4.8–5 mmol/L

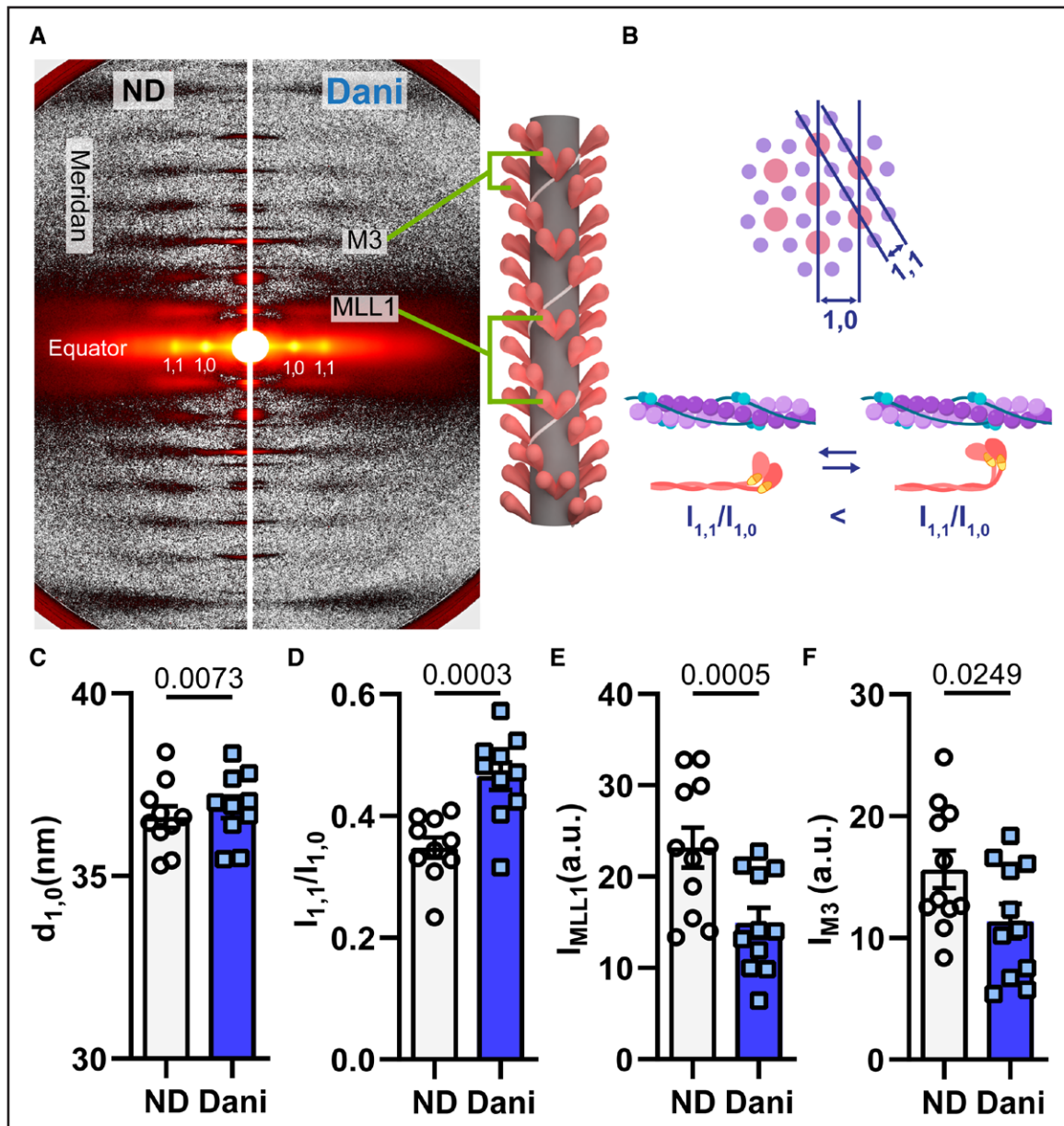


Figure 3. Fifty micrometer Danicamtiv (Dani) partially activated the thick filament in resting cardiac muscle (pCa=8.0).

A, Sample X-ray diffraction patterns of demembrated porcine cardiac tissue exposed to X-ray beams while bathed in low calcium solution (pCa=8.0) containing DMSO (left) or Dani (right). **B**, Schematics defining how actin and myosin relationships relate to equatorial measurements. **C**, Summary data for lattice spacing ($d_{1,0}$), **(D)** intensity ratio of the primary equatorial reflections ($I_{1,1}/I_{1,0}$), intensity of **(E)** the first-order myosin-based layer line (I_{MLL1}), and **(F)** the third-order myosin-based meridional reflection (I_{M3}). Data are from 10 paired tissue preparations. The illustrations were created with BioRender.com. a.u. indicates arbitrary units.

[MgATP]), there was an increase in viscoelastic myocardial stiffness for the Danicamtiv-treated strips versus the untreated. Both elastic and viscous moduli values were greater in the presence of Danicamtiv across a wide range of frequencies, suggesting greater cross-bridge binding in the presence of Danicamtiv. The observed leftward shift toward lower frequencies for the elastic and viscous moduli responses also suggests Danicamtiv slowed cross-bridge cycling, consistent with the slowed k_{tr} (Figure 1E).

Prolonged Myofibril Relaxation Induced by Danicamtiv Was due to the Altered ADP Release Rate

Using a combination of reductionistic approaches, we investigated whether Danicamtiv affects the product release rates of P_i and ADP during cross-bridge cycling. Either of these mechanisms could explain the decreased cross-bridge turnover time suggested by the decreased myofibril relaxation kinetics.

It is well accepted that increasing P_i results in decreasing force.^{35,36} As seen in Figure S4, a linear dependence of relative force on $\text{Log } [P_i]$ was found in demembranated porcine cardiac tissue. The difference between the slopes was not significant between the Danicamtiv and no drug group. Hence, the relation between force and $[P_i]$ was unchanged with Danicamtiv. These results are different than what is reported for OM, where 1- μM OM counteracts the inhibitory effects of P_i under similar conditions.³⁷

To test the hypothesis that ADP release is affected by Danicamtiv, we quantified myosin cross-bridge activity in the presence of elevated ADP conditions. Initial experiments utilized the in vitro motility assay, which directly measures cross-bridge activity (Figure 4A). Previous studies have shown that when ADP levels are elevated, filament velocity is decreased because ADP release is inhibited, promoting the strongly bound actin-myosin state.^{38,39} As seen in Figure 4B, Danicamtiv also resulted in a substantial ($\approx 55\%$) decrease in filament velocity (2.65 ± 0.17 versus $1.13 \pm 0.12 \mu\text{m/s}$). There was no further decrease in velocity with nucleotide mixtures containing 50% ATP and 50% ADP in the presence of Danicamtiv ($1.01 \pm 0.05 \mu\text{m/s}$), suggesting no additive effect. These results support the hypothesis that Danicamtiv slows cross-bridge cycling rate through slowed ADP release.

Next, we performed measurements in myofibrils at submaximal calcium ($p\text{Ca}=5.8$). Under isometric conditions, ADP release is the rate-limiting step in the cross-bridge cycle and cardiac muscle relaxation kinetics. Furthermore, by increasing ADP levels, there is an increase in maximal force and calcium sensitivity.^{36,38,39} To answer the specific question of whether Danicamtiv directly affects ADP release from myosin, we made our myofibril measurements with Danicamtiv in the presence of increased ADP levels. As expected, ADP (Figure 4C through 4F; Figure S6) increased force (56.6 ± 3.2 versus $72.6 \pm 2.4 \text{ mN/mm}^2$) and slowed myofibril relaxation rates ($k_{\text{REL(slow)}}$: 1.340 ± 0.101 versus $0.286 \pm 0.001 \text{ s}^{-1}$; $k_{\text{REL(fast)}}$: 12.81 ± 1.05 versus 4.34 ± 0.32), while prolonging the duration of the slow phase of relaxation ($t_{\text{REL(Slow)}}$: 49.1 ± 1.7 versus $155.2 \pm 3.0 \text{ ms}$). Treatment with Danicamtiv at a submaximal calcium level ($p\text{Ca}=5.8$) resulted in a similar increase in force and slowing of activation and relaxation kinetics that was seen at a maximal calcium level (Figure 4C through 4F; Table S1). Combined treatment with Danicamtiv and elevated ADP did not further increase force and minimally decreased cross-bridge detachment $k_{\text{REL(slow)}}$ compared with Danicamtiv and no ADP (Figure 4D and 4E). This suggests that ADP release is the key step in the cross-bridge cycle that is affected by Danicamtiv. Interestingly, the addition of elevated ADP to Danicamtiv-treated myofibrils further prolonged thin filament deactivation duration ($t_{\text{REL(Slow)}}$: 94.9 ± 12.2 versus $222.5 \pm 13.6 \text{ ms}$), likely due to more strongly bound cross bridges.

Nucleotide Handling Rates Were Modified in the Presence of Danicamtiv

To confirm our myofibril results, we measured changes in nucleotide binding and release rates in the presence of Danicamtiv. We calculated ATP binding and ADP release rates in porcine cardiac tissue using a step-length protocol in the presence of increasing $[\text{MgATP}]$ (Figure S7). Cross-bridge detachment rates (k_{rel}) increased as $[\text{MgATP}]$ increased for the untreated and Danicamtiv-treated myocardial strips (Figure 5A; Table S5). k_{rel} was slower for the Danicamtiv-treated strips at $[\text{MgATP}] > 0.5 \text{ mmol/L}$. Fits to Eq. 1 suggests that slowed cross-bridge detachment stems from a combination of slower cross-bridge MgADP release rate ($k_{\text{-ADP}}$) and faster cross-bridge MgATP-binding rate ($k_{\text{+ATP}}$) in the presence of Danicamtiv (Figure 5B and 5C). Because the rate-limiting step of the cross-bridge cycle under load is MgADP dissociation from strongly bound myosin cross bridges in demembranated myocardial strips, it was the slower $k_{\text{-ADP}}$ that primarily drove the differences in cross-bridge kinetics between the untreated and Danicamtiv-treated strips. As seen in Figure 5, $k_{\text{+ATP}}$ was significantly faster than $k_{\text{-ADP}}$ and with $k_{\text{+ATP}}$ being a second-order nucleotide binding process, the $[\text{MgATP}]$ needs to be very low for $k_{\text{+ATP}}$ to affect cross-bridge detachment rates. Figure 5D shows that the $[\text{MgATP}]_{50}$ concentration at half-maximal detachment rate ($[\text{MgATP}]_{50}$) was much lower for the Danicamtiv-treated strips. This suggests that Danicamtiv-treated preparations required less MgATP to reach their maximal cross-bridge detachment rate. Using the same approach, we found a similar relationship between k_{rel} and $[\text{MgATP}]$ in the presence of 1 μM OM. OM resulted in the same magnitude of $k_{\text{-ADP}}$ inhibition and decreased $[\text{MgATP}]_{50}$ as Danicamtiv, suggesting these small molecules affect similar steps of the cross-bridge cycle.

Danicamtiv Recovered Abnormal Tension in a Rodent DCM Model

Since Danicamtiv is under investigation for the treatment of patients with genetic cardiomyopathy, we tested its ability to recover the force deficit in a thin filament DCM mouse model with the characteristic progressive dilation and hypocontractility of human genetic DCM. Permeabilized muscle preparations showed a rightward shift in the force versus $p\text{Ca}$ relationship in the I61Q cTnC DCM model.¹³ Additionally, the I61Q cTnC mutant decreased F_{max} and the Hill coefficient. As seen in Figure S8, 1 μM Danicamtiv was able to shift the force versus $p\text{Ca}$ curves to the left for both control and I61Q cTnC mice with an increase in $p\text{Ca}_{50}$ (5.58 ± 0.01 versus 5.72 ± 0.02 for control, 5.35 ± 0.04 versus 5.47 ± 0.03 for I61Q cTnC). This increase in $p\text{Ca}_{50}$ was significant versus no drug regardless of genotype. Similar to the results in pig cardiac tissue, we found a decrease in the Hill coefficient

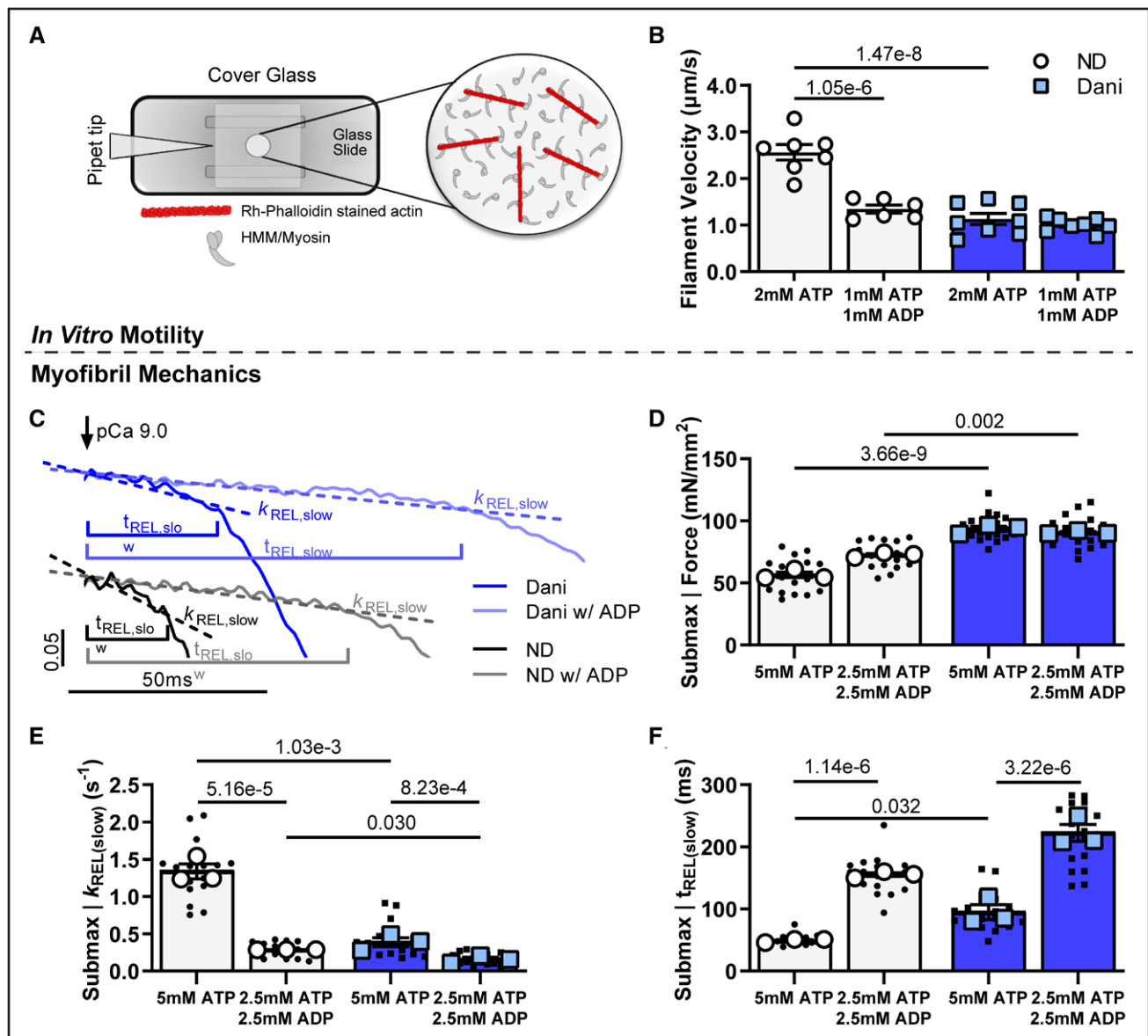


Figure 4. Effect of Danicamtiv (Dani) and ADP on cross-bridge cycling.

A, The in vitro motility assay was used to look at cross-bridge cycling in isolated myosin and actin. **B**, Filament sliding velocity with 0.5 μM Dani-treated ATP-bound myosin (left columns), and with (50:50 ADP:ATP)-bound myosin (right columns). **C**, Example tracings of the slow phase of myofibril relaxation with and without 1 μM Dani and with ATP or 50:50 ADP:ATP mixture at pCa=5.8. Example tracings are offset for visual clarity. Summary data of the effect of Dani and ADP on **(D)** submaximal force, and the **(E)** rate constant and **(F)** duration for the slow phase of relaxation. Data are from 7 to 8 slides per condition for in vitro motility and 18 DMSO or 21 Dani-treated myofibrils (small symbols) from 3 biological replicates (large symbols).

and maximal k_r but with no increase in maximal force (Figure S8; Table S3).

Our intact trabecula measurements showed a decrease in peak tension (T_p) and tension index in the I61Q cTnC mice (Figure 6; Table S4) as previously reported.^{13,40} In the presence of Danicamtiv, T_p increased in both control and DCM. In control mice, 1 and 3 $\mu\text{mol/L}$ Danicamtiv resulted in $\approx 63\%$ and 131% increase in T_p , while the increase is $\approx 87\%$ and $\approx 148\%$, respectively in I61Q cTnC trabecula. While the time to peak is unchanged in the presence of Danicamtiv, the

time to 50% and 90% relaxation is increased in the presence of Danicamtiv (Figure S9; Table S4). We have previously shown that the tension index, the area under the twitch curve that is subtracted from a healthy twitch, is predictive of cardiomyopathy phenotype.^{13,14} While the tension index increased with Danicamtiv in a dose-dependent manner, the increase was greater in the control mice. This is mostly due to the more pronounced relaxation change in the control tissue. Using mixed-effects analysis, the interaction between genotype and Danicamtiv is statistically significant, suggesting

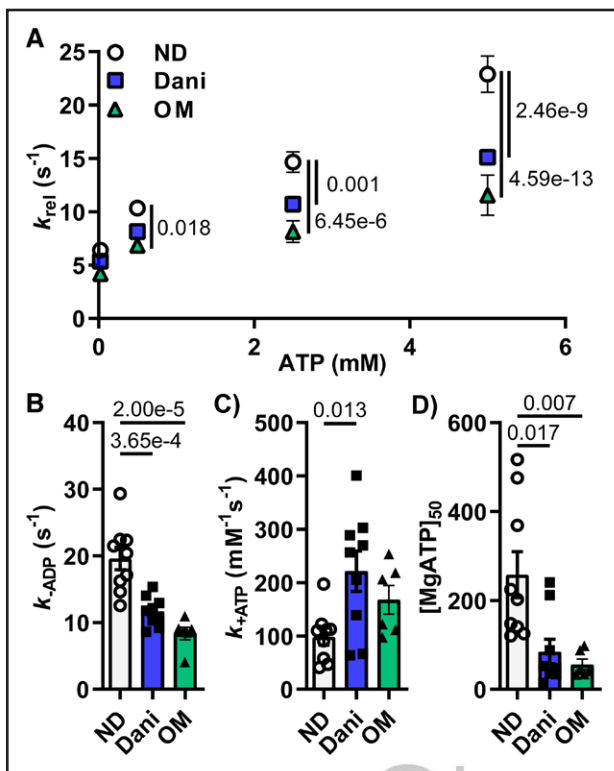


Figure 5. One-micrometer Danicamtiv (Dani) and Omecamtiv Mecarbil (OM) increased ATP binding and decreased ADP release in activated porcine cardiac muscle.

A, The cross-bridge detachment rates (k_{rel}) increased with increasing $[MgATP]$ for the untreated, Dani-treated and OM-treated myocardial strips. Treatment with both Dani and OM decreased k_{rel} at $[MgATP] > 0.5$ mmol/L (**B**). Dani and OM decreased ADP release rate (**B**, k_{ADP}) while increasing ATP binding (**C**, k_{ATP}). $MgATP$ concentration at half-maximal detachment rate ($[MgATP]_{50}$) was lower for both Dani-treated and OM-treated strips (**D**). Data are from 9 tissue preparations per condition.

a different treatment response in control versus I61Q cTnC tissue. These results suggest that the underlying mechanism of cardiomyopathy might need to be considered when dosing a medication such as Danicamtiv. We tested in vivo efficacy of Danicamtiv by intravenous injection of 2 mg/kg and saw a significant increase in ejection fraction in both controls (58.1 ± 2.5 versus 74.7 ± 2.0) and DCM (36.6 ± 2.9 versus 46.3 ± 2.0) hearts 10 minutes post-treatment (Figure 6; Table S6). There was a decrease in heart rate post-treatment, which was likely due to sedation. Treatment with vehicle (DMSO) did not significantly increase ejection fraction or heart rate (Table S7).

DISCUSSION

We set out to perform a first detailed analysis of the mechanism of action of the myosin activator Danicamtiv. There are several key findings that emerged from this study. First, we showed that treatment with Danicamtiv restructured the thick filament of resting muscle to a

more ON configuration, where myosin heads are positioned closer to actin. This increased the number of myosin heads available to contribute to contraction, a result that is consistent with more myosin motors binding at lower calcium concentrations. It also suggests reduced reliance on calcium-mediated cooperativity of myosin binding, hence an increase in calcium sensitivity with a decreased Hill coefficient. Second, we showed that Danicamtiv slowed cross-bridge turnover and subsequently a decreased rate of myofibril relaxation and prolonged thin filament deactivation. This prolonged relaxation manifested in intact cardiac muscle, which had slower twitch relaxation kinetics. Third, we provided evidence to show that the decrease in myosin ADP release rate is the mechanism for decreased cross-bridge turnover and slower relaxation. Fourth, we showed that Danicamtiv improved the tissue and the organ level hypocontractility in a genetic DCM model.

Danicamtiv Restructures Myosin in an Analogous Manner to Other Myosin Activators

Myosin activation is a novel way to treat HF. OM, the first-in-class myosin activator, recently completed a phase 3 clinical trial for the treatment of systolic HF.⁹ Studies using fluorescent probes have demonstrated that OM stabilizes the ON state of the thick filament resulting in more myosin motors available for binding to the thin filament.¹⁰ Our x-ray diffraction studies also showed partial thick filament activation with Danicamtiv under resting conditions. This activation under resting conditions can be expected to result in more myosin motors binding at lower calcium concentrations with less reliance on cooperativity. Results of our sinusoidal length-perturbation analysis further support that Danicamtiv increases cross-bridge binding and promotes an increase in the ratio of ON to OFF cross-bridge populations. Similar changes in myosin structure under resting conditions are induced by 2-dATP (deoxy ATP), a nucleotide known to increase force and calcium sensitivity in rodent, dog, and human cardiac myocardium,^{22,41,42} which correlate well with the active force.³⁰

Danicamtiv Inhibits Cross-Bridge Cycling Rate, Prolonging Thin Filament Deactivation and Relaxation in Myofibrils

The myofibril kinetics measurements suggest that Danicamtiv prolongs thin filament activation, as indicated by the longer $t_{REL(slow)}$ and that this is due to a decreased rate of cross-bridge detachment as measured by $k_{REL(slow)}$. A similar mechanism of action involving prolonged actomyosin attachment resulting in cooperative thin filament activation has been proposed as a mechanism for OM.^{7,43} The inhibitor-like mechanism is further supported by decreased ATP turnover in HMM with increasing

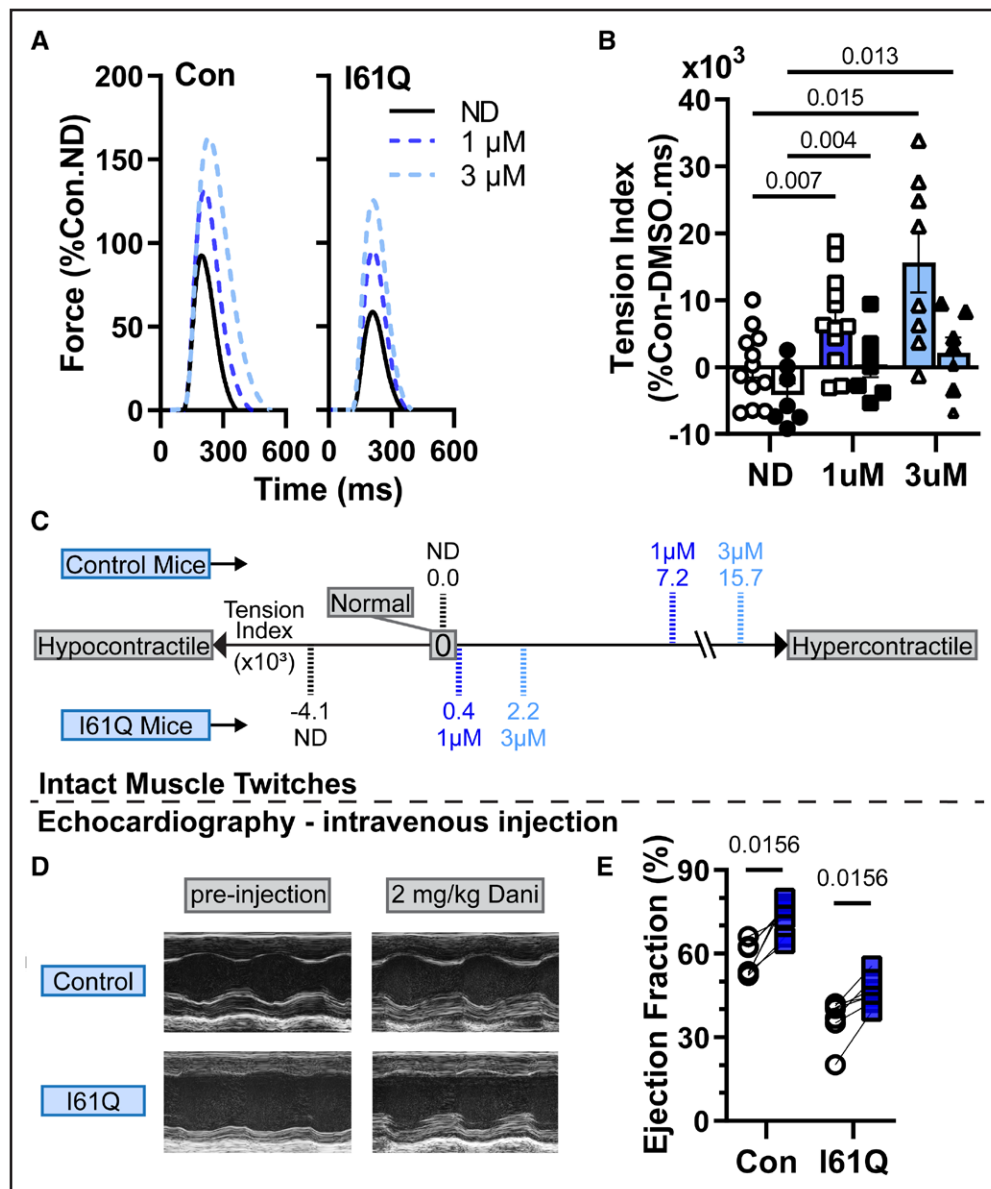


Figure 6. Danicamtiv (Dani) mitigated the contractile abnormalities in a rodent dilated cardiomyopathy model.

A, Average twitch force-time traces (in % control-ND T_p) of intact papillary muscle trabeculae from control (left) and I61Q cTnC (cardiac troponin C; right) mice stimulated at 1 Hz. Dani increased T_p (**A**) and tension index (**B** and **C**) in control and I61Q cTnC twitches in a dose-dependent manner. **C**, Intravenous injection of 2 mg/kg Dani resulted in significant increase in ejection in both control and I61Q cTnC hearts 10-minute post-injection. Data are from 15 control mice and 11 I61Q mice for intact twitches and 7 mice for in each echo group.

concentrations of Danicamtiv, which was also observed for OM (Figure S5). While both Danicamtiv and OM inhibit maximal force at high drug concentrations, OM inhibits to a greater extent and at a lower concentration (Figure S4). The duration of the slow phase of relaxation ($t_{REL(slow)}$) is known to be affected by changes in troponin complex calcium affinity,¹⁹ so we measured the effect of Danicamtiv on troponin function. Danicamtiv did not change Ca^{2+} binding to cTnC as measured by steady-state fluorescence spectroscopy (Figure S10). Therefore, the decreased cross-bridge detachment rate is likely explained by the decreased ADP release rate, and this is supported by myofibril relaxation measurements

in the presence of elevated ADP, which mimic relaxation kinetic changes seen with Danicamtiv under conditions of load.

Danicamtiv Corrects Abnormal Contraction in a Genetic DCM Mouse Model

Using a rodent model of sarcomeric genetic DCM with decreased thin filament activation, we demonstrated that Danicamtiv could normalize the decreased calcium sensitivity of contraction in I61Q cTnC mice. At the lowest calcium levels of our F-pCa curve, there is a full recovery of the force deficit to levels of control myocardium.

However, the maximum force is still significantly lower in the Danicamtiv-treated I61Q cTnC trabeculae. The decreased k_{tr} in mice suggested that slowing of cross-bridge kinetics was present in hearts with both MYH6 (myosin heavy chain 6; mice) and MYH7 (myosin heavy chain 7; pig). Intact trabecula experiments demonstrated that tension increased in both control and I61Q cTnC mice after treatment with Danicamtiv. Control mice consistently achieved a higher peak force and tension index, a summative measure encompassing peak tension and kinetics of both activation and relaxation, compared with I61Q. Control mice also expressed greater sensitivity to Danicamtiv treatment with significantly greater time to 50% and 90% relaxation compared with I61Q. An explanation for the difference in relaxation is that Danicamtiv normalizes the duration of thin filament deactivation in the I61Q cTnC, which are abnormal without treatment. However, in the control mice, the thin filament stays on much longer, resulting in prolonged relaxation. Most of the DCM mice achieved a positive tension index at a dose of 3 μ M, which is higher than the reported maximum serum concentrations.⁴⁴ Our in vivo treatment with Danicamtiv resulted in about a 27% increase in ejection fraction in both control and I61Q hearts. While the cardiac function of the I61Q hearts improved, it did not fully recover to the untreated control levels, which matches the twitch and tension index data.

Our study has some limitations. Our mouse model of genetic DCM is based on an engineered mutation in cTnC and not a known disease-causing mutation. It is also worth noting that MYH6 is the dominant form of cardiac myosin in mice, while humans express the MYH7 variant. It is possible that our genetic DCM results could be different in organisms with MYH7 as the dominant myosin. Intact twitch measurements were only performed in mice, which expresses the fast form of myosin. Our studies have not assessed the relative affinities of Danicamtiv for different cardiac myosin isoforms; however, we found comparable results in mouse and pig, which have different predominant isoforms.

In conclusion, we use a variety of tools to demonstrate the biophysical mechanisms of how Danicamtiv works to increase force and calcium sensitivity. This methodological framework can be used to connect information from mechanical measurements to understand how myosin recruitment or the cross-bridge cycle affect cardiac myofibril activation and relaxation. The initial discovery of Danicamtiv was based on increased myofibril ATPase activity. Our studies support a more complex process than a simple increase in myosin cycling rate. The inhibition of ADP release leads to slower relaxation kinetics of myofibrils and consequently slower relaxation in intact tissue. In addition, myosin heads are more primed to interact with actin at low levels of calcium, resulting in increased calcium sensitivity and higher contractile forces at levels of calcium the sarcomere experiences during the cardiac cycle. This increase in

force production and widening of twitch duration contribute to the increased tension index and augmenting cardiac function. In addition to providing the mechanism of action, our data may help to identify subsets of patients that may derive the most benefit from Danicamtiv. For example, there could be a different response in sarcomeric versus nonsarcomeric DCM or thin filament versus thick filament variants. Genetic variants are being recognized as an increasing cause of HF and DCM. Myosin activators may provide a treatment that addresses the causative hypocontractile phenotype. This motivated a current clinical trial of patients with genetic DCM for treatment with Danicamtiv. However, there is lack of data about the utility of this medication in genetic cardiomyopathy preclinical models. Our study shows that Danicamtiv increased cardiac function in rodent genetic DCM hearts. Future studies will need to look at a diverse group of variants and extend the studies to humans with genetic DCM.

ARTICLE INFORMATION

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Affiliations

Division of Cardiology, Medicine (K.B.K., M.M.-S., J.C.F., E.G.C.A., F.M.-H.), Center of Translational Muscle Research (K.B.K., S.M., G.F., T.S.M., C.M., A.-Y.T., M.R., J.D., F.M.-H.), Center for Cardiovascular Biology (K.B.K., A.M., M.R., J.D., F.M.-H.), Department of Laboratory Medicine and Pathology (A.M., J.D., F.M.-H.), Institute for Stem Cell & Regenerative Medicine (K.B.K., S.M., A.M., T.S.M., A.-Y.T., M.R., J.D., F.M.-H.), Department of Bioengineering (S.M., A.M., G.F., C.M., A.-Y.T., M.R., J.D.), and Department of Anesthesiology and Pain Medicine (T.S.M.), University of Washington. School of Molecular Biosciences, Washington State University (K.L.T.). Department of Biology, Illinois Institute of Technology, Chicago (W.M., L.O., T.C.I.). College of Basic Medical Sciences, Dalian Medical University, Liaoning, China (C.G., Y.Z.). School of Biosciences, Division of Natural Sciences, University of Kent, Canterbury, United Kingdom (M.A.G.). Department of Integrative Physiology and Neuroscience, Washington State University (B.C.W.T.).

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Disclosures

None.

Supplemental Material

Supplemental Methods
Tables S1–S7
Figures S1–S10

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