Aficamten, a Selective Small-Molecule Cardiac Myosin Inhibitor for the Potential Treatment of Hypertrophic Cardiomyopathy

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ABSTRACT

Mutations in the cardiac sarcomere increase its contractility and appear to drive the pathogenesis of most forms of hypertrophic cardiomyopathy (HCM). Directly targeting this pathophysiology improves symptoms and exercise capacity in patients with obstructive HCM, as demonstrated by the first-in-class cardiac myosin inhibitor, *mavacamten*. Here we describe the next-in-class selective small molecule inhibitor of cardiac myosin, *aficamten* (CK-3773274). *Aficamten* inhibits the myosin ATPase by slowing phosphate release and stabilizing a weak actin-binding state of myosin. Binding to an allosteric site on the myosin catalytic domain distinct from that of *mavacamten*, *aficamten* prevents the conformational changes necessary to enter the strongly actin-bound force-generating state. In single ATP turnover experiments, *aficamten* slowed ATP turnover to rates to <1/3 of the super-relaxed state (SRX) rate. The expected consequence of this mechanism of action is to reduce the number of functional myosin heads that drive sarcomere shortening. *Aficamten* reduced cardiac contractility in cardiac myocytes and in preclinical models of cardiac function. Cardiac myosin inhibition with *aficamten* may offer an alternative approach to treat the underlying hypercontractility of the cardiac sarcomere in HCM.

INTRODUCTION

- Hypercontractility of the cardiac sarcomere appears to underlie pathological hypertrophy and fibrosis in select genetic hypertrophic cardiomyopathies.
- Direct modulation of the sarcomere is an emerging approach to potentially treat conditions with maladaptive changes in cardiac contractility (Malik et al. 2011, Green et al. 2016).
- Clinically, in phase 2 studies, the next-in-class small-molecule inhibitor of cardiac myosin, *aficamten*, has been shown to reduce gradients, improve functional capacity, and lead to improved cardiac remodeling (Maron et al. 2021).
- The objective of this study was to characterize the biochemical mechanism of action of the small molecule *aficamten*, and the ability to modulate cardiac contractility in vitro and in vivo in preclinical models of cardiac function.

METHODS

Preparation of Reagents

- Myofibrils were prepared from flash-frozen bovine cardiac, bovine masseter, and rabbit psoas tissue as described in Hwee et al. (2015). Bovine cardiac myosin subfragment-1 (S1) was prepared as described in Malik et al. (2011). Bovine cardiac HMM was prepared based on the method of Rohde et al. (2018). Chicken gizzard myosin and subfragment-1 were prepared as described in Sellers et al. (1981).

ATPase Assays

- Steady-state ATPase activity was measured using a pyruvate kinase and lactate dehydrogenase-coupled enzyme system as described in Hwee et al. (2015) and Malik et al. (2011). Non-myosin ATPase activity was subtracted from cardiac and slow skeletal myofibril assays (where indicated) by subtracting the ATPase activity in the presence of a saturating concentration of the non-selective myosin II inhibitor (-)-blebbistatin.

Blebbistatin Binding Assays

- Binding of (-)-blebbistatin to bovine cardiac myosin subfragment-1 was measured in a buffer consisting of 12 mM K-PIPES pH 6.8, 2 mM $MgCl_2$, 1 mM DTT, and 2 mM ADP-vanadate. Fluorescence emission spectra were recorded using a PTI QM-6 (λ_{ex} 426 nm). Compound titrations were performed using a SpectraMax Gemini XS spectrofluorimeter (λ_{ex} 426 nm, λ_{em} 575 nm).

Transient Kinetics

- Transient kinetics were measured at 25°C using an SF-61DX stopped-flow instrument (TgK Scientific) in a buffer consisting of 12 mM K-PIPES pH 6.8, 2 mM $MgCl_2$, and 1 mM DTT using the methods of De La Cruz and Ostap (2009) and Malik et al. (2011). ATP binding and hydrolysis were monitored by intrinsic tryptophan fluorescence (λ_{ex} 295 nm) using a 320 nm longpass filter. Actin-activated phosphate release was monitored using MDCC-labeled phosphate binding protein (λ_{ex} 434 nm) using a 455 nm longpass filter. Single ATP turnover measurements were performed by mixing mantATP-loaded myosin with 1 mM ATP either in a stopped-flow instrument (Hi-Tech Scientific SF-61 DX2, λ_{ex} 374 nm/ λ_{em} >455 nm) or plate-based fluorescence reader (Molecular Devices SpectraMax Gemini XS, λ_{ex} 405 nm/ λ_{em} 455 nm) (Adhikari et al. 2019; Sarkar et al. 2020) in the above buffer supplemented with 60 mM KCl.

Measurement of Cardiomyocyte Contractility and Calcium Transients

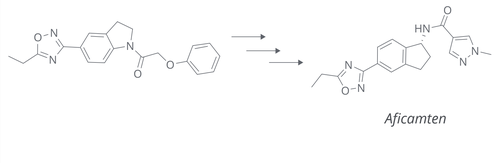
- Adult rat ventricular cardiomyocytes were isolated and loaded with Fura-2 as described in Malik et al. (2011). Cardiomyocyte contractility and calcium transients were measured by edge-detection video microscopy and fluorescence photometry (IonOptix, Milton, MA) as described in Malik et al. (2011).

Rat Echocardiography Assessment

- Adult male Sprague Dawley rats were anesthetized with inhaled isoflurane (1-5%) throughout the echocardiography procedure. Baseline contractility was assessed 1 day prior to *aficamten* treatment. Animals were orally dosed with vehicle (0.5% hydroxypropylmethylcellulose (HPMC)/0.1% Tween-80) or *aficamten* (0.5, 1, 2, or 4 mg/kg) and measures of left ventricular contractility were assessed 1, 4, 8, and 24 hours post-dose. Using a GE Vivid7 machine, a 10 MHz probe was placed at the level of the papillary muscles and 2D M-mode images of the left ventricle were captured. Images and measurements were obtained in parasternal long axis view. In vivo percent fractional shortening was determined by analysis of the M-mode images using the GE Vivid7 ultrasound software.

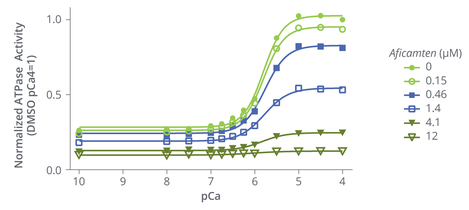
RESULTS

Figure 1. The chemical structures of the initial screening hit (CK-2172010) and *aficamten*



Chuang et al. (2021).

Figure 2. *Aficamten* inhibits the ATPase activity of bovine cardiac myofibrils

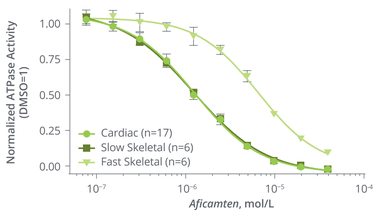


Best-Fit Values	DMSO	*Aficamten* 0.15 μM	0.46 μM	1.4 μM	4.1 μM	12 μM
pCa_{50}	5.80 (5.77 to 5.83)	5.80 (5.77 to 5.82)	5.76 (5.73 to 5.79)	5.78 (5.75 to 5.81)	5.83 (5.77 to 5.90)	6.03 (5.72 to 6.33)
Hill slope	−1.91 (−2.09 to −1.73)	−1.85 (−2.00 to −1.71)	−1.75 (−1.90 to −1.59)	−1.58 (−1.72 to −1.45)	−1.59 (−1.90 to −1.28)	−1.16 (−2.02 to −0.30)
Minimum	0.28 (0.27 to 0.29)	0.26 (0.25 to 0.27)	0.24 (0.24 to 0.25)	0.19 (0.19 to 0.20)	0.13 (0.12 to 0.13)	0.10 (0.09 to 0.10)
Maximum	1.03 (1.01 to 1.04)	0.95 (0.94 to 0.96)	0.83 (0.82 to 0.84)	0.54 (0.54 to 0.55)	0.25 (0.24 to 0.25)	0.12 (0.12 to 0.13)

- The ATPase activity of Triton X-100-skinned bovine cardiac myofibrils was measured using a pyruvate kinase/lactate dehydrogenase-coupled assay as described in Hwee et al. (2015). The table shows the results of fitting to a four-parameter dose–response equation along with the 95% confidence interval (CI) of the fitted parameters.

Data shown are mean values ± standard deviation (SD) (n=4).

Figure 3. *Aficamten* selectively inhibits the ATPase activity of cardiac and slow skeletal myofibrils compared with fast skeletal myofibrils

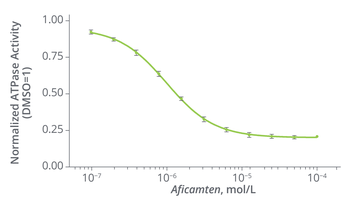


	Cardiac	Slow Skeletal	Fast Skeletal
IC_{50} (μM) (95% CI)	1.26 (1.20 to 1.33)	1.23 (1.17 to 1.29)	6.52 (5.72 to 7.71)
Hill slope	−1.16 (−1.24 to −1.10)	−1.06 (−1.12 to −1.00)	−1.27 (−1.49 to −1.08)
Top	1.07 (1.05 to 1.09)	1.10 (1.08 to 1.12)	1.04 (1.02 to 1.07)
Bottom	−0.04 (−0.06 to −0.03)	−0.05 (−0.07 to −0.04)	−0.005 (−0.09 to 0.06)

- Dose–response analysis was performed with cardiac (bovine, n=17), slow skeletal (bovine, n=6), and fast skeletal (rabbit, n=6) detergent-skinned myofibrils as described in Hwee et al. (2015). Free Ca^{2+} concentrations were fixed at approximately the pCa_{75} for each myofibril type. Raw ATPase rates were normalized to reactions containing an equivalent concentration of DMSO, and for cardiac and slow skeletal reactions, ATPase rates in the presence of the nonselective myosin inhibitor blebbistatin were subtracted to eliminate the effects of non-myosin ATPases.

Data shown are mean values ± SD.

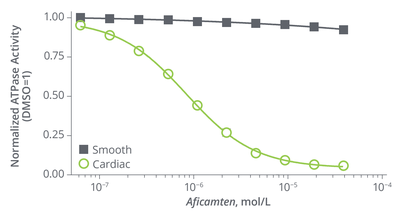
Figure 4. *Aficamten* inhibits the basal ATPase activity of bovine cardiac myosin subfragment-1



- The basal ATPase activity of bovine cardiac myosin subfragment-1 (1 μM) was measured using a pyruvate kinase/lactate dehydrogenase-coupled assay in a buffer consisting of 12 mM K-PIPES pH 6.8, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. ATPase activity was normalized to control reactions containing 2% DMSO. Data were fitted using a four-parameter dose–response equation (95% CI): IC_{50} = 0.99 μM (0.95 to 1.03), Hill slope = −1.37 (−1.44 to −1.30). Top = 0.95 (0.94 to 0.96), Bottom = 0.20 (0.19 to 0.21).

Data shown are mean values ± SD (n=8 reactions).

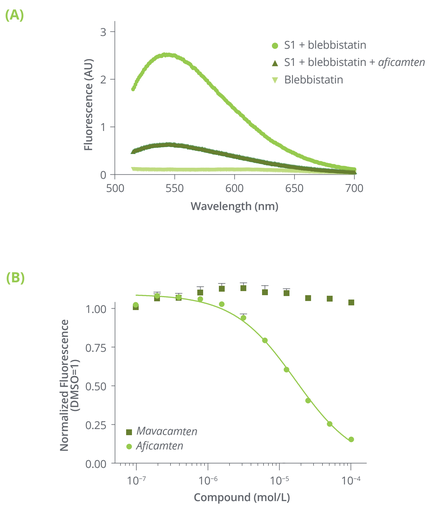
Figure 5. *Aficamten* selectively inhibits the actin-activated ATPase activity of bovine cardiac versus chicken gizzard smooth muscle myosin subfragment-1



- ATPase activity was measured using a pyruvate kinase/lactate dehydrogenase-coupled assay in a buffer consisting of 12 mM K-PIPES pH 6.8, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. ATPase activity was normalized to control reactions containing 2% DMSO.

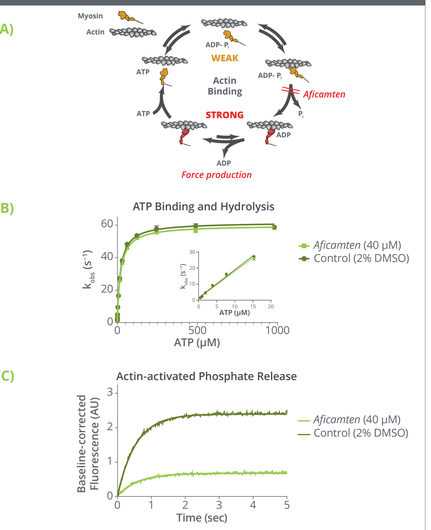
Data shown are mean values ± SD (n=4).

Figure 6. *Aficamten* and blebbistatin binding to cardiac myosin is mutually exclusive



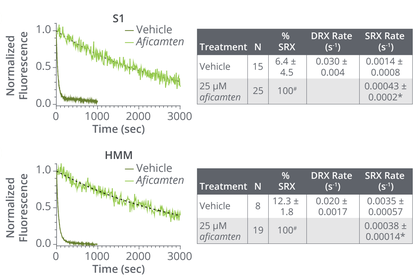
A. Fluorescence emission of (-)-blebbistatin (5 μM) increases upon binding to bovine cardiac myosin subfragment-1 (1 μM) in the ADP-VO_4 state. This increase is reduced when a high concentration of *aficamten* (50 μM) is present. **B.** *Aficamten* (but not *mavacamten*) reduces myosin-enhanced blebbistatin fluorescence in a concentration-dependent manner, consistent with mutually exclusive binding.

Figure 7. *Aficamten* slows actin-activated phosphate release without affecting ATP binding or hydrolysis



A. Myosin chemomechanical cycle. **B.** ATP binding and hydrolysis were measured by following the intrinsic tryptophan fluorescence of bovine cardiac myosin subfragment-1 (1 μM final concentration) after rapid mixing with varying concentrations of ATP. Three to five fluorescence transients were averaged and fit to a single exponential. Data shown are mean ± SD (n=3 separate experiments). DMSO: $k_{+H} + k_{-H}$ = 62 s^{-1}, ATP binding rate = 1.7E6 M^{-1} s^{-1}. *Aficamten*: $k_{+H} + k_{-H}$ = 60 s^{-1}, ATP binding rate = 1.7E6 M^{-1} s^{-1}. **C.** Actin-activated phosphate release was measured by rapidly mixing bovine cardiac myosin subfragment-1 (2 μM) with ATP (1 μM), aging the reaction for 30 seconds, followed by mixing with bovine cardiac actin (28 μM) and MDCC-PBP (10 μM). The graph shows the average of 4-5 fluorescence transients, averaged and fit to a single exponential. DMSO: rate = 1.8 s^{-1}, amplitude = 2.4. *Aficamten*: rate = 1.6 s^{-1}, amplitude = 0.7.

Figure 8. *Aficamten* slows the single ATP turnover rate of cardiac S1 and HMM



Treatment	N	% SRX	DRX Rate (s^{-1})	SRX Rate (s^{-1})
Vehicle	15	6.4 ± 4.5	0.030 ± 0.004	0.0014 ± 0.0008
25 μM *aficamten*	25	100*		0.00043 ± 0.0002*

Treatment	N	% SRX	DRX Rate (s^{-1})	SRX Rate (s^{-1})
Vehicle	8	12.3 ± 1.8	0.020 ± 0.0017	0.0035 ± 0.00057
25 μM *aficamten*	19	100*		0.00038 ± 0.00014*

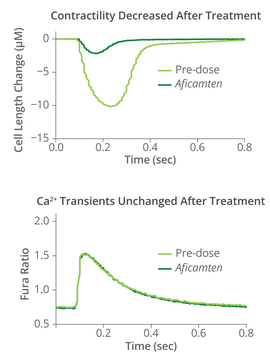
- Bovine cardiac myosin S1 or HMM was preincubated with mantATP followed by rapid mixing with excess ATP. Final concentrations: 0.5 μM myosin, 1.0 μM mantATP, 1 mM ATP. *Aficamten* was included in all solutions at 25 μM. Data shown is a single representative reaction. Aggregated data is shown in the accompanying table.

Data are presented as mean ± standard error of the mean (SEM). #Traces for reactions containing *aficamten* were equally well-fit by single and double exponential equations.
*p<0.0001 vs. vehicle.

SUMMARY

- ***Aficamten* is a small molecule that selectively inhibits cardiac myosin ATPase activity and contractility in vitro.**
- **Binding of *aficamten* is mutually exclusive with blebbistatin, suggesting they bind to the same or overlapping sites on cardiac myosin. In contrast, *mavacamten* and blebbistatin binding are not mutually exclusive, consistent with *aficamten* and *mavacamten* having distinct binding sites.**
- ***Aficamten* slowed actin-activated phosphate release with little effect on ATP binding and hydrolysis, consistent with a mechanism that stabilizes myosin in weak actin-binding conformations.**
- **In single nucleotide turnover experiments, *aficamten* slowed ATP turnover to rates <1/3 that of the super-relaxed state (SRX) rate.**
- **The expected consequence of this mechanism of action is to reduce the number of functional myosin heads that drive sarcomere shortening.**
- ***Aficamten* reduced cardiac contractility in vivo in Sprague Dawley rats in a dose- and concentration-related manner.**
- **Cardiac myosin inhibition may be a viable approach to treat the underlying hypercontractility of the cardiac sarcomere in hypertrophic cardiomyopathies.**
- **Data from phase 2 studies of *aficamten* in patients with obstructive HCM support the potential impact on clinical endpoints (Maron et al. 2021). Further phase 2 and phase 3 studies are currently enrolling.**

Figure 9. *Aficamten* decreases shortening of isolated adult rat ventricular myocytes without altering Ca^{2+} transients

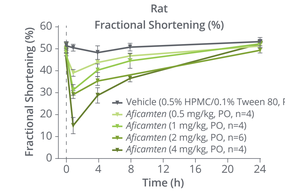


Treatment	N	FS (% of basal)	Diastolic Fura Ratio	Systolic Fura Ratio	T_{75} (seconds)
Basal	5	100	0.83 ± 0.07	1.58 ± 0.13	0.25 ± 0.03
10 μM *aficamten*	5	16.4 ± 3.4*	0.81 ± 0.09	1.56 ± 0.06	0.26 ± 0.02

- Shown are an average of 10 cell shortening (A) and calcium transients (B) for a representative adult rat cardiomyocyte before and after exposure to 10 μM *aficamten*. Pooled data are shown in table. Basal reference values are: diastolic cell length = 137.6 ± 9.7 μM. Fractional shortening (FS) = 6.79 ± 1.05 μM. Contraction velocity (CV) = 182.1 ± 31.1 μM/sec, relaxation velocity (RV) = 124.3 ± 34.3 μM/sec, time to peak = 0.116 ± 0.015 sec, and time to baseline (T_{50}) = 0.183 ± 0.015 sec.

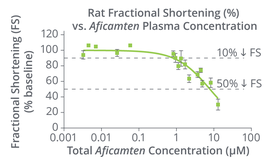
Data presented as mean ± SEM. *p<0.05 vs. basal FS.

Figure 10. *Aficamten* reduced fractional shortening (FS) in vivo in Sprague Dawley rats in a (A) dose- and (B) concentration-related manner



Dose (mg/kg)	Fractional Shortening (%) Pre-dose Baseline	1 h	4 h	8 h	24 h
Vehicle	50.7 ± 1.1	47.9 ± 1.3	47.5 ± 1.8	50.8 ± 1.8	50.4 ± 1.5
0.5	49.0 ± 1.1	39.0 ± 1.6*	43.8 ± 4.6	46.8 ± 1.3	51.3 ± 0.6
1	49.3 ± 1.7	31.3 ± 2.5**	40.3 ± 2.9	44.5 ± 3.4	52.3 ± 1.7
2	51.3 ± 1.6	29.2 ± 1.8**	35.3 ± 2.0	–	49.3 ± 1.2
4	49.3 ± 1.1	15.0 ± 3.7***	28.8 ± 3.5*	36.5 ± 1.6**	52.3 ± 1.9

Values are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. baseline values within each timepoint by 2-way analysis of variance (ANOVA).



Fractional Shortening	
IC_{10} (μM)	0.8
IC_{50} (μM)	7.9

Rat plasma protein binding: 98.2%

A. Sprague Dawley rats received vehicle or *aficamten* (0.5, 1, 2, or 4 mg/kg, PO) and echocardiography assessments were performed at select timepoints over 24 hours. **B.** *Aficamten* concentration-fractional shortening response plot with the horizontal dotted lines indicating a 10% and 50% reduction of FS relative to baseline (IC_{10} and IC_{50}).

Chuang et al. (2021). Values are expressed mean ± SEM.

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Disclosures

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