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Preclinical Characterization of CK-4021586, a New Class of Cardiac Myosin Inhibitors for the Treatment of Hypertrophic Cardiomyopathy

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is the most common monogenetic heart disease, with an estimated carrier prevalence of ~1 in 500. Hypercontractility of the cardiac sarcomere is a common driver of the pathological hypertrophic cardiac remodeling that is a hallmark of the disease. Clinical trials of the selective cardiac myosin inhibitors mavacamten and *aficamten* have demonstrated that inhibition of the biochemical activity of cardiac myosin can induce cardiac remodeling and improve both patient symptoms and exercise capacity in HCM with left ventricular outflow tract obstruction (oHCM). The diverse genetic underpinnings of HCM suggest that it is possible that additional classes of cardiac myosin inhibitors with distinct biochemical mechanisms of action may also have a salutary impact on disease expression. While both mavacamten and *aficamten* bind to the single-headed motor domain of myosin (subfragment-1) and inhibit its ATPase activity, CK-4021586 (CK-586) is a new class of cardiac myosin inhibitor that inhibits the ATPase activity of two-headed heavy meromyosin (HMM) but not single-headed subfragment-1. CK-586 is a partial inhibitor of cardiac myofibrillar ATPase activity (EC_{50} 2.9 µM, maximal inhibition ~50%) that requires the regulatory light chain. Notably, fractional shortening of electrically paced adult rat ventricular cardiomyocytes was inhibited almost completely (>80% at 5 µM) without alterations in the calcium (Ca^{2+}) transient. In normal Sprague Dawley rats, CK-586 reduced cardiac fractional shortening in a dose-dependent manner. In conclusion, CK-586 is a novel, small molecule, cardiac myosin inhibitor that reduces cardiac contractility in vitro and in vivo. CK-586 has a biochemical mechanism of action distinct from both mavacamten and *aficamten*, providing an additional tool to decrease the number of functionally available myosin heads and treat the cardiac hypercontractility that forms the pathologic basis of HCM.

INTRODUCTION

- In hypertrophic cardiomyopathy (HCM), hypercontractility of the cardiac sarcomere leads to pathological hypertrophy and fibrosis.
- Reducing cardiac contractility with selective cardiac myosin inhibitors has demonstrated clinical benefits in patients with HCM with left outflow tract obstruction (that is, obstructive HCM or oHCM).
- Here we describe in vitro and in vivo data for CK-4021586 (CK-586), a novel compound representing a new class of cardiac myosin inhibitors.
- The mechanism of action of CK-586 is distinct from the previously described inhibitors mavacamten and *aficamten*, in that CK-586 inhibits ATPase activity only in two-headed forms of myosin whereas mavacamten and *aficamten* inhibit both single-headed and two-headed forms.
- The inhibitory effect of CK-586 requires the presence of the myosin regulatory light chain (RLC), further supporting a unique mechanism and binding site for CK-586.

METHODS

Preparation of Reagents

- Myofibrils were prepared from flash-frozen bovine cardiac, bovine masseter, and rabbit psoas tissue as described in Hwee et al.[1] Bovine cardiac myosin and subfragment-1 were prepared as described in Malik et al.[2] Bovine cardiac heavy meromyosin (HMM) was prepared based on the method of Rohde et al.[3] Chicken gizzard myosin and HMM were prepared as described in Sellers et al[4] and Onishi and Watanabe.[5]

ATPase Assays

- Steady-state ATPase activity was measured using a pyruvate kinase and lactate dehydrogenase–coupled enzyme system as described in Hwee et al[1] and Malik et al.[2] 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 nonselective myosin II inhibitor (–)-blebbistatin.

Measurement of Cardiomyocyte Contractility and Calcium Transients

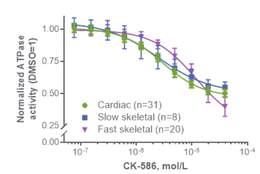
- Adult rat ventricular cardiomyocytes were isolated and loaded with Fura-2 as described in Malik et al.[2] Cardiomyocyte contractility and calcium transients were measured by edge-detection video microscopy and fluorescence photometery (IonOptix, Milton, MA) as described in Malik et al.[2]

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 CK-586 treatment. Animals were orally dosed with vehicle (0.5% hydroxypropyl-methylcellulose (HMPC)/ 0.1% Tween-80) or CK-586 (3, 10, 30, or 60 mg/kg), and measures of left ventricular contractility were assessed 1, 4, 7, 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

Fig 1. CK-586 selectively inhibits the ATPase activity of cardiac and slow skeletal myofibrils compared with fast skeletal myofibrils

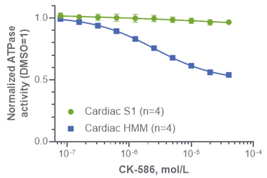


	Myofibrils		
	Cardiac	Slow skeletal	Fast skeletal
EC_{50} (µM)	2.94 (2.74, 3.16)	2.51 (1.65, 4.64)	11.90 (9.30, 17.80)
Top	1.01 (1.00, 1.02)	1.05 (1.01, 1.16)	0.99 (0.98, 1.01)
Bottom	0.47 (0.46, 0.49)	0.50 (0.33, 0.57)	0.24 (0.09, 0.33)

Values shown are mean (95% CI).

Dose-response analysis was performed with cardiac (bovine, n=31), slow skeletal (bovine, n=8), and fast skeletal (rabbit, n=20) detergent-skinned myofibrils as described in Hwee et al.[1] Calcium concentrations were fixed at approximately the pCa_{75} for each myofibril type. The ATPase activity of Triton X-100-skinned myofibrils was measured using a pyruvate kinase/lactate dehydrogenase–coupled assay. Raw ATPase rates were normalized to reactions containing an equivalent concentration of DMSO. 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 were fitted using a four-parameter dose-response equation.
Data shown in the figure are mean values ± SD.

Fig 2. CK-586 inhibits the actin-activated ATPase activity of bovine cardiac heavy meromyosin (HMM) but not subfragment-1 (S1)



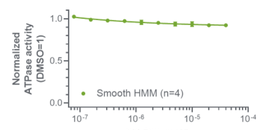
	HMM	S1
EC_{50} (µM)	2.54 (2.32, 2.79)	NA
Top	1.01 (1.00, 1.03)	NA
Bottom	0.49 (0.47, 0.51)	NA

Values shown are mean (95% CI).

- In contrast, previous studies with mavacamten and *aficamten* showed inhibitory effects in both S1 and HMM.[6,7]

The actin-activated ATPase activity of bovine cardiac myosin subfragment-1 (S1) and heavy meromyosin (HMM) was measured in the presence of 14 µM bovine cardiac actin using a pyruvate kinase/lactate dehydrogenase–coupled assay. ATPase activity was normalized to control reactions containing 2% DMSO. ATPase rates in the presence of the nonselective myosin inhibitor blebbistatin were subtracted to eliminate the effects of non-myosin ATPases. Data were fitted using a four-parameter dose-response equation.
Data shown in the figure are mean values ± SD.

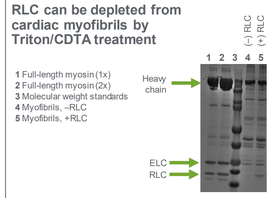
Fig 3. CK-586 does not appreciably inhibit actin-activated ATPase activity of chicken gizzard smooth muscle myosin heavy meromyosin (HMM)



The actin-activated ATPase activity of chicken gizzard smooth muscle myosin heavy meromyosin (HMM) was measured in the presence of 14 µM bovine cardiac actin using a pyruvate kinase/lactate dehydrogenase–coupled assay. ATPase activity was normalized to control reactions containing 2% DMSO. ATPase rates in the presence of the nonselective myosin inhibitor blebbistatin were subtracted to eliminate the effects of non-myosin ATPases. Data were fitted using a four-parameter dose-response equation; however, parameters could not be determined reliably due to the low amount of inhibition observed (<10%).
Data shown are mean values ± SD (n=4 reactions).

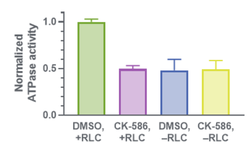
Fig 4. CK-586 inhibits the ATPase activity of bovine cardiac myofibrils only in the presence of the myosin regulatory light chain (RLC)

RLC can be depleted from cardiac myofibrils by Triton/CDTA treatment



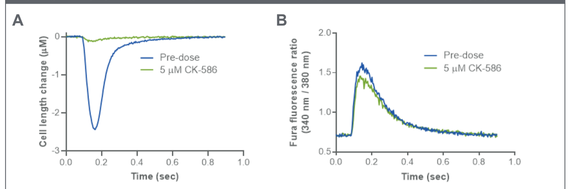
1 Full-length myosin (1x)
2 Full-length myosin (2x)
3 Molecular weight standards
4 Myofibrils, –RLC
5 Myofibrils, +RLC

Inhibition by CK-586 requires RLC



ATPase activity was measured with detergent-skinned bovine cardiac myofibrils in the presence and absence of RLC. RLC depletion was performed by treating myofibrils with Triton/CDTA as described in Szczesna-Cordary et al.[8] Depletion of RLC in myofibrils was confirmed by SDS PAGE. The ATPase activity of myofibrils was measured using a pyruvate kinase/lactate dehydrogenase–coupled assay. Effect of 50 µM CK-586 (CK-586, –RLC [n=32]) was compared with an equivalent concentration of DMSO (DMSO, –RLC [n=31]) in RLC-depleted myofibrils. Raw ATPase rates were normalized to reactions containing an equivalent concentration of DMSO in myofibrils with RLC (DMSO,+RLC [n=24]). ATPase activity of myofibrils with RLC in the presence of 50 µM CK-586 (CK-586, +RLC [n=31]) was measured as well. 1 mM $CaCl_2$ was added. ATPase rates in the presence of the nonselective myosin inhibitor blebbistatin were subtracted to eliminate the effects of non-myosin ATPases.
Data shown in the figure are mean values ± SD. ELC, essential light chain.

Fig 5. CK-586 decreases shortening of isolated adult rat ventricular myocytes (A) without significantly altering calcium transients (B)



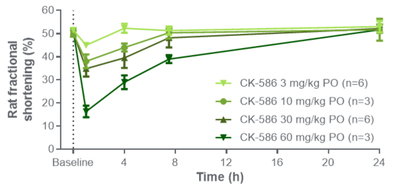
Contractility, mean ± SEM	5 µM CK-586 (n=13)
Fractional shortening (% of basal)	18.6* ± 9.9
Cell length (% of basal)	100.0 ± 2.9
Contraction velocity (% of basal)	24.5 ± 12.2
Relaxation velocity (% of basal)	39.1 ± 30.3
Time to peak (% of basal)	74.6 ± 4.9
Relaxation T_{50} (% of basal)	107.4 ± 9.4

Calcium transient, mean ± SEM	5 µM CK-586 (n=13)
Systolic Fura ratio (% of basal)	96.3 ± 3.4
Diastolic Fura ratio (% of basal)	102.5 ± 2.3
Time to baseline 75% (% of basal)	99.1 ± 5.8

CK-586 (5 µM) significantly decreased fractional shortening (*P<0.05) without increasing or decreasing the diastolic or systolic Fura ratio or time to baseline 75%.

Shown is a representative time course of cell shortening (A) and calcium transients (B) measured using the ratio of Fura-2 fluorescence when excited at 340 and 380 nm. Data shown are an average of >10 contractions for one cell before (blue line) and after (green line) treatment with 5 µM CK-586.
Basal reference values: diastolic cell length = 130.7 ± 3.8 µM, fractional shortening = 4.24 ± 0.54 µM, contraction velocity = 115.1 ± 17.5 µM/sec, relaxation velocity = 65.1 ± 11.0 µM/sec, time to peak = 0.093 ± 0.002 sec, time to baseline (T_{50}) = 0.158 ± 0.005 sec, systolic Fura ratio = 1.450 ± 0.081, diastolic Fura ratio = 0.777 ± 0.026, time to baseline 75% = 0.299 ± 0.022.

Fig 6. CK-586 reduced fractional shortening (FS) in vivo in Sprague Dawley rats in a dose-related manner



Dose (mg/kg)	Fractional shortening (%)				
	Pre-dose baseline	1 h	4 h	8 h	24 h
3	51.5 ± 0.9	45.0 ± 0.9*	52.3 ± 1.9	51.3 ± 1.8	53.0 ± 2.5
10	50.0 ± 1.3	38.0 ± 3.0*	44.0 ± 1.7	50.3 ± 2.5	51.7 ± 4.7
30	50.3 ± 1.1	34.8 ± 3.4*	39.5 ± 4.4*	48.2 ± 4.2	52.2 ± 1.8*
60	51.2 ± 0.3	16.3 ± 2.5*	29.0 ± 3.0*	39.0 ± 1.7*	51.7 ± 0.6

Values shown are mean ± SD.
*P<0.05 vs pre-dose baseline at each respective timepoint by 2-way ANOVA.

Sprague Dawley rats received CK-586 (3, 10, 30, or 60 mg/kg, PO), and echocardiography assessments were performed at select timepoints over 24 hours.

SUMMARY

- CK-586 is a selective, small molecule inhibitor of cardiac myosin.
- CK-586 inhibits the ATPase activity of cardiac and slow skeletal myofibrils 2- to 3-fold more potently than fast skeletal myofibrils, with minimal inhibition of smooth muscle myosin.
- CK-586 inhibits myosin ATPase activity in two-headed HMM but not in single-headed S1, in contrast to mavacamten and *aficamten*, which inhibit both HMM and S1.
- The inhibitory effect of CK-586 requires the regulatory light chain (RLC) of myosin.
- In adult rat cardiomyocytes, CK-586 inhibits contractility in the absence of effects on calcium transients.
- CK-586 reduced fractional shortening in vivo in Sprague Dawley rats in a dose-related manner.

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1. Hwee DT, et al. *J Pharmacol Exp Ther* 2015;353:159-68. **2.** Malik FI, et al. *Science* 2011;331:1439-43. **3.** Rohde JA, et al. *Proc Natl Acad Sci USA* 2018;115:E7486-94. **4.** Sellers JR, et al. *J Biol Chem* 1981;256:13137-42. **5.** Onishi H, Watanabe S. *J Biochem* 1979;85:457-72. **6.** Kawas RF, et al. *J Biol Chem* 2017;292:16571-77. **7.** Sarkar SS, et al. Poster presented at the Gordon Research Conference: Cardiac Regulatory Mechanisms, New London, NH; June 26–July 1, 2022. **8.** Szczesna-Cordary D, et al. *J Biol Chem* 2004;279:3535-42.

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