Pharmacologic Characterization of the Cardiac Myosin Inhibitor, CK-3773274: A Potential Therapeutic Approach for Hypertrophic Cardiomyopathy

Darren T. Hwee, James J. Hartman, Jingying Wang, Yangsong Wu, Julia Schaletzky, Preeti Paliwal, Ken Lee, Khanha D. Taheri, Eddie Wehri, Chihyuan Chuang, Bradley P. Morgan, Fady I. Malik, Eva R. Chin

Cytokinetics, Inc., South San Francisco, CA

ABSTRACT

Hypercontractility of the cardiac sarcomere appears to underlie pathological hypertrophy and fibrosis in select genetic hypertrophic cardiomyopathies. Here, we characterize the small molecule CK-3773274 as a novel cardiac myosin inhibitor that decreases contractility in vitro and in vivo. In bovine cardiac myofibrils, CK-3773274 decreased myosin ATPase activity in a concentration-dependent fashion (IC_{50}:1.26 μM). CK-3773274 specifically inhibited myosin activity, as it reduced myosin ATPase activity in a concentration-dependent manner in the absence of other sarcomere proteins, including actin, troponin, and tropomyosin. CK-3773274 (10 μM) reduced fractional shortening (FS) by 84% in electrically paced, isolated adult rat cardiomyocytes relative to control without any effect on the calcium (Ca^{2+}) transient. The effect of CK-3773274 on cardiac contractility in vivo was assessed in healthy male Sprague Dawley (SD) rats using single oral doses ranging from 0.5 to 4 mg/kg. FS and left ventricular dimensions were determined by echocardiography at select time points over a 24-hour period. One hour after dose administration, CK-3773274 significantly reduced FS in a dose-related fashion by 20% to 70% relative to vehicle treatment (FS %: vehicle: 47.9 ± 1%; 0.5 mg/kg: 39 ± 2%; 4 mg/kg: 15 ± 4%; mean ± SEM, $p < 0.05$ vehicle vs. all doses) without any changes to heart rate. Lastly, the effect of CK-3773274 was evaluated by echocardiography in healthy beagle dogs. Left ventricular ejection fraction (LVEF) was evaluated following single oral doses ranging from 0.75–3 mg/kg over a 48-hour period. Two hours after dosing, CK-3773274 decreased LVEF in a dose-related fashion by approximately 15% to 50% relative to vehicle treatment (LVEF vehicle: 74.6 ± 3%; 0.75 mg/kg: 62.5 ± 3%; 2 mg/kg: 44.9 ± 3%; 3 mg/kg: 36.8 ± 2%; mean ± SEM, $p < 0.05$ vehicle vs. all doses). In conclusion, CK-3773274 is a novel, small molecule, cardiac myosin inhibitor that reduces cardiac contractility in vitro and in vivo. Cardiac myosin inhibition may be a viable approach to treat the underlying hypercontractility of the cardiac sarcomere in hypertrophic cardiomyopathies.

INTRODUCTION

- Hypercontractility of the cardiac sarcomere may be essential for the underlying pathological hypertrophy and fibrosis seen in many hypertrophic cardiomyopathy (HCM) patients[1]
- Direct modulation of the sarcomere is a novel approach to potentially treat conditions with maladaptive changes in cardiac contractility[1,2]
- The objective of this study was to characterize the small molecule, CK-3773274, for biochemical activity and selectivity, and the ability to modulate cardiac contractility in vitro and in vivo

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).[3] Bovine cardiac myosin subfragment-1 was prepared as described in Malik et al (2011)[2]

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)[3] and Malik et al (2011).[1] 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

Measure of Cardiomyocyte Contractility and Calcium Transients

- Adult rat ventricular cardiomyocytes were isolated and loaded with Fura-2 as described in Malik et al (2011).[1] Cardiomyocyte contractility and calcium transients were measured by edge-detection video microscopy and fluorescence photometery (IonOptix, Milton, MA) as described in Malik et al (2011)[1]

Rat Echocardiographic Assessment

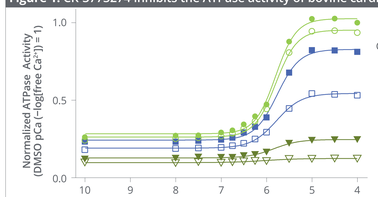
- Adult male Sprague Dawley rats were anesthetized with inhaled isoflurane (1%–5%) throughout the echocardiogram procedure. Baseline contractility was assessed 1 day prior to CK-3773274 treatment. Animals were orally dosed with vehicle (0.5% hydroxypropylmethylcellulose [HPMC]/ 0.1% Tween 80) or CK-3773274 (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 (Milwaukee, Wisconsin), 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 (FS) was determined by analysis of the M-mode images using the GE Vivid7 ultrasound software

Dog Echocardiographic Assessment

- Non-naïve male beagle dogs (approximately 6 to 33 months old) were sedated with butorphanol (0.05 to 0.06 mL) and ketamine (0.05 to 0.06 mL) for echocardiographic assessments. Baseline contractility assessment was performed weekly prior to CK-3773274 treatment. Animals were orally dosed with vehicle (0.5% HPMC/0.1% Tween 80) and CK-3773274 at doses of 0.75, 2, and 3 mg/kg. All animals received all treatments, with at least 1 week between treatments. Measures of left ventricular contractility were assessed 2, 6, 24, and 48 hours post-dose. Echocardiographic images were obtained in 2D parasternal long axis view with a GE Vivid E95 machine. Echocardiographic parameters including left ventricular (LV) ejection fraction (EF) were determined by image analysis GE EchoPAC software

RESULTS

Figure 1. CK-3773274 inhibits the ATPase activity of bovine cardiac myofibrils

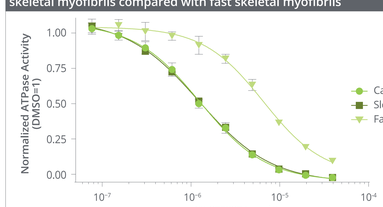


Best-Fit Values	DMSO	CK-3773274				
		0.15 μM	0.46 μM	1.4 μM	4.1 μM	12 μM
pCa_{50}	5.80 (5.77 to 5.83)	5.80 (5.77 to 5.82)	5.76 (0.573 to 5.79)	5.78 (5.75 to 5.81)	5.83 (5.77 to 5.90)	6.03 (5.72 to 6.33)
Hill slope	−1.91 (−2.09 to −1.73)	−1.85 (−2.00 to −1.71)	−1.75 (−1.90 to −1.59)	−1.58 (−1.72 to −1.45)	−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)

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

- 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).[3] The table shows the results of fitting to a four-parameter dose-response equation along with the 95% confidence interval of the fitted parameters

Figure 2. CK-3773274 selectively inhibits the ATPase activity of cardiac and slow skeletal myofibrils compared with fast skeletal myofibrils

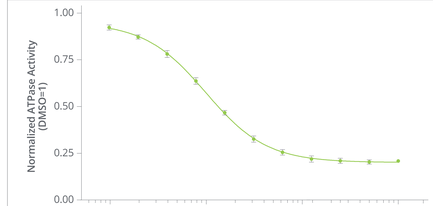


	Cardiac	Slow Skeletal	Fast Skeletal
IC_{50} (μM)	1.26 (1.20 to 1.33)	1.23 (1.17 to 1.29)	6.52 (5.72 to 7.71)
Hill slope	−1.16 (−1.24 to −1.10)	−1.06 (−1.12 to −1.00)	−1.27 (−1.49 to −1.08)
Top	1.07 (1.05 to 1.09)	1.10 (1.08 to 1.12)	1.04 (1.02 to 1.07)
Bottom	−0.04 (−0.06 to −0.03)	−0.05 (−0.07 to −0.04)	−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).[3] 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. The table shows the results of fitting to a four-parameter dose-response equation along with the 95% confidence interval

Data shown are mean values ± SD.

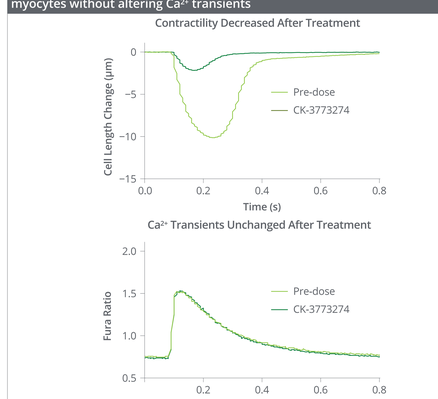
Figure 3. CK-3773274 inhibits the ATPase activity of bovine cardiac myosin subfragment-1



- The ATPase activity of bovine cardiac myosin subfragment-1 (1 μM) was measured using a pyruvate kinase/lactate dehydrogenase-coupled assay. 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 4. CK-3773274 decreases shortening of isolated adult rat ventricular myocytes without altering Ca^{2+} transients



- Shown are an average of 10 cell shortening and calcium transients for a representative adult rat cardiomyocyte before and after exposure to 10 μM CK-3773274. Pooled data are shown in table

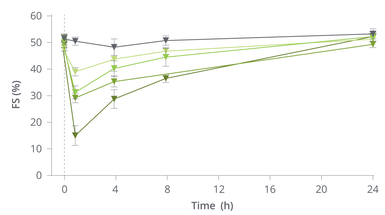
Treatment	N	FS (% of basal)	Diastolic Fura Ratio	Systolic Fura Ratio	T_{75} (seconds)
Basal	5	100	0.83 ± 0.07	1.58 ± 0.13	0.25 ± 0.03
10 μM CK-3773274	5	16.4 ± 3.4*	0.81 ± 0.09	1.56 ± 0.06	0.26 ± 0.02

Data presented as mean ± standard error of the mean (SEM). *$p < 0.05$ vs. basal FS

- Basal reference values are diastolic cell length = 137.6 ± 9.7 μm, FS = 6.79 ± 1.05 μm, contraction velocity = 182.1 ± 31.1 μm/sec, relaxation velocity = 124.3 ± 34.3 μm/sec, time to peak = 0.116 ± 0.015 s, and time to baseline (T_{50}) = 0.183 ± 0.015 s

Figure 5. CK-3773274 reduced FS in vivo in Sprague Dawley rats in a dose- (A) and concentration- (B) related manner

A

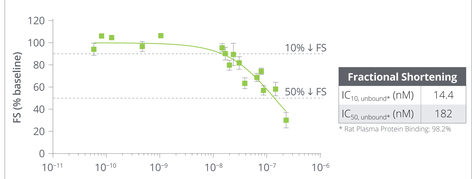


Dose (mg/kg)	FS (%)				
	Pre-dose Baseline	1 h	4 h	8 h	24 h
Vehicle	50.7 ± 1.1	47.9 ± 1.3	47.5 ± 1.8	50.8 ± 1.8	50.4 ± 1.5
0.5	49.0 ± 1.1	39.0 ± 1.6*	43.8 ± 4.6	46.8 ± 1.3	51.3 ± 0.6
1	49.3 ± 1.7	31.3 ± 2.5**	40.3 ± 2.9	44.5 ± 3.4	52.3 ± 1.7
2	51.3 ± 1.6	29.2 ± 1.8**	35.3 ± 2.0	–	49.3 ± 1.2
4	49.3 ±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

- Sprague Dawley rats received vehicle or CK-3773274 (0.5, 1, 2, or 4 mg/kg, PO) and echocardiographic assessments were performed at select time points over 24 hours

B



Fractional Shortening	
$IC_{10,unbound*}$ (nM)	14.4
$IC_{50,unbound*}$ (nM)	182

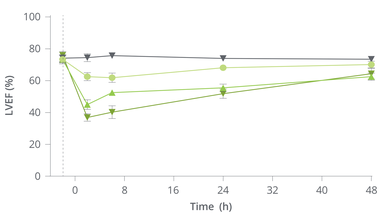
* Rat Plasma Protein Binding: 98.2%

- CK-3773274 concentration-FS response plot with the horizontal dotted lines indicating a 10% and 50% reduction of FS relative to baseline (IC_{10} and IC_{50}).

Values are expressed as mean ± SEM.

Figure 6. CK-3773274 reduced EF in vivo in beagle dogs in a dose- (A) and concentration- (B) related manner

A

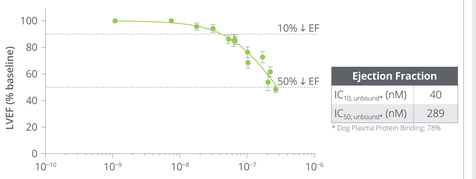


Dose (mg/kg)	EF (%)				
	Pre-dose Baseline	2 h	6 h	24 h	48 h
Vehicle	74.1 ± 2.2	74.5 ± 2.5	75.7 ± 0.9	74 ± 1.7	73.5 ± 1.4
0.75	73.4 ± 1.6	62.5 ± 2.5*	61.9 ± 2.9*	68.1 ± 1.8	70.1 ± 2.1
2	73.3 ± 2.7	45 ± 3.0****	52.5 ± 1.6****	55.5 ± 2.4***	62.5 ± 2.3**
3	76.0 ± 2.3	36.8 ± 2.4****	40.3 ± 4.1****	51.9 ± 3.0***	64.4 ± 3.3

Values are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs. baseline values within each timepoint by 2-way ANOVA

- Beagle dogs received vehicle or CK-3773274 (0.75, 2, or 3 mg/kg, PO) and echocardiography assessments were performed at select time points over 48 hours

B



Ejection Fraction	
$IC_{10,unbound*}$ (nM)	40
$IC_{50,unbound*}$ (nM)	289

* Dog Plasma Protein Binding: 78%

- CK-3773274 concentration-EF response plot with the horizontal dotted lines indicating a 10% and 50% reduction of EF relative to baseline (IC_{10} and IC_{50}).

Values are expressed as mean ± SEM.

References

1. Green, et al. *Science.* 2016;351:617–621.
2. Malik, et al. *Science.* 2011;331:1439–1443.
3. Hwee, et al. *J Pharmacol Exp Ther.* 2015;353:159–168.

Disclosures

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SUMMARY

- CK-3773274 is a novel small molecule that selectively inhibits cardiac myosin ATPase activity and contractility in vitro
- CK-3773274 reduced cardiac contractility in vivo in Sprague Dawley rats and beagle dogs in a dose- and concentration-related manner
  - CK-3773274 achieved a 10% reduction (IC_{10}) in contractility at unbound nanomolar concentrations
  - A >9× difference between the IC_{50} and IC_{10} in both Sprague Dawley rats and beagle dogs may translate into a measured, gradual on-target effect
- Cardiac myosin inhibition may be a viable approach to treat the underlying hypercontractility of the cardiac sarcomere in hypertrophic cardiomyopathies

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