In Vitro and *In Vivo* Characterization CK-1827452, A Selective Cardiac Myosin Activator

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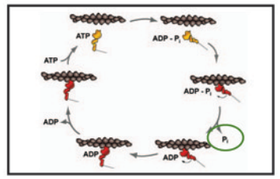
Introduction

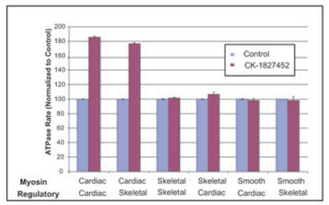


Traditional inotropic agents used in treating acute decompensated heart failure increase cellular contractility by increasing the calcium transient. β-adrenergic agonists activate β-adrenergic receptors resulting in an increase in cAMP and activation of the PKA signaling cascade. Numerous proteins are phosphorylated including phospholamban that results in an increase of the calcium transient and thus contractility. Phosphodiesterase (PDE) inhibitors increase the cAMP concentrations by slowing cAMP degradation also resulting in a calcium transient increase. PDE inhibiting compounds result in increased mortality in clinical trials possibly due to altering the calcium transient (Packer *et al*, 1991; Cohn *et al*, 1998).

A novel approach to improving cardiac contractility is to directly activate the force-generating enzyme cardiac myosin without altering the calcium transient. We have previously reported on a class of small molecule agents directly stimulating activity of the myosin ATPase in the cardiac sarcomere (Rodriguez *et al*, 2004). *In vitro* enzymatic assays demonstrate that myosin activators accelerate the rate limiting transition into the strongly bound state of the actin-myosin enzymatic cycle reported by phosphate release (green circle - right figure above). This portion of the cycle constitutes transition from the weakly to the strongly filament-bound state of myosin. Thus, by reducing the time spent in the weakly bound state, myosin activators shift the myosin enzymatic cycle in favor of the strongly bound, force producing state.

The objective in this study was to determine the *in vitro* mechanism of action of CK-1827452 and *in vivo* efficacy in Sprague Dawley rats, in rats with heart failure and in beagle dogs.

CK-1827452 is a selective cardiac myosin activator



In biochemical mix and match preparations, the myosin ATPase rate is increased only in the presence of cardiac myosin demonstrating that the activity of CK-1827452 is:

1) cardiac myosin specific and
2) not acting via the regulatory apparatus.

Experiments shown performed at pCa^{2+} = 6.75.

CK-1827452 lacks PDE3 activity

	% Inhibition
10uM CK-1827452	-1
30uM CK-1827452	8
8uM IBMX	50

In contrast to many inotropic agents, CK-1827452 lacks PDE3 activity.

Methods

Biochemical assays: Cardiac S1 myosin and thin filament proteins (actin, troponin complex, and tropomyosin) were prepared from bovine heart or rabbit skeletal muscle. Smooth muscle myosin was prepared from chicken gizzard. ATPase assays were performed in a kinetic fashion using NADH coupled enzyme system at pCa^{2+} = 6.75. Rates were normalized to a DMSO control.

PDE activity: Human PDE3 activity assays were performed on human platelets by MDS Pharma Services.

Myocyte contractility experiments: Ventricular myocytes were isolated from adult male SD rats (275- 325g) using a collagenase digestion procedure and used within 5 hrs of isolation. Myocytes were warmed in perfusion chambers, perfused with Tyrode buffer and field stimulated at 1 Hz. To determine contractility, myocytes were imaged through a 40x objective and the images were digitized at a sampling speed of 240 Hz.

Frame grabber, myopacer, acquisition, and analysis software were obtained from IonOptix (Milton, MA). After an initial 1 min basal contractility period, compounds were perfused for 5 minutes before a 2 min washout period.

Calcium transient analysis: Myocytes were loaded with 2 uM fura-2 AM and simultaneous contractility and fura-2 ratios determined using an IonOptix system modified for fluorescence analysis.

Cell Analysis: For each cell, ten or more contractility and calcium ratio transients at basal and after compound addition, were averaged and compared. Contractility average transients were analyzed using the IonWizard analysis program to determine changes in diastolic length, maximum contraction (CV) and relaxation velocities (RV), time to peak, time to 50% of baseline (T50), and fractional shortening (% decrease in the diastolic length; FS). Data are normalized to basal values (basal equals 100%) and expressed as % of basal. The averaged calcium ratio transients were analyzed to determine changes in fura-2 diastolic and systolic ratios and the 75% time to baseline (T75).

Rat Infusions: Adult male SD rats were anesthetized with isoflurane and infused with 0.25 – 1.2 mg/kg/hr (1.5 ml/kg/hr) CK-1827452 and cardiac function was monitored by echocardiography. Terminal plasmas were taken.

Echocardiography: Using an Aplio ultrasound system (Toshiba), a 10 Hz probe was placed at the level of the papillary muscles and 2-D M-mode images of the left ventricle were taken. Diastolic and systolic wall thickness and dimensions, and fractional shortening (FS) were determined before and after infusion.

Heart failure: Myocardial infarctions (MI) were induced by ligation of the left coronary artery (LCA) in the rat. Only animals with fractional shortening 3 SD lower than the average from the sham animals (surgery without tying off LCA) were utilized in experiments. Experiments were performed 12 weeks post-surgery.

Canine studies: Four beagles were anesthetized with isoflurane to effect and cardiac function monitored by echocardiography prior to and during a 1 hr infusion of vehicle or 0.03 – 1 mg/kg/hr CK-1827452.

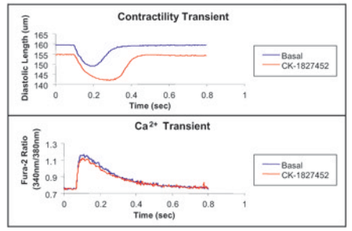
Reagents: CK-1827452 provided by the Cytokinetics Chemistry department. Fura-2 from Molecular Probes and all other reagents from Sigma.

Statistics: Data are mean +/- SEM. Statistics were performed using the Students *t*-test or ANOVA and post-hoc Newman-Keuls as appropriate. P< 0.05 was considered significant.

CK-1827452 increases myocyte contractility *in vitro* – without increasing intracellular calcium

CK-1827452 (μM)	N	FS (%)	Cell Length (% of basal)	CV (μm/sec)	RV (μm/sec)	Time to Peak (% of basal)	T50 (% of basal)
0	8	108.3 ± 4.3	99.6 ± 0.1	-153.9 ± 16.5	102 ± 18.3	112.7 ± 4.3	110.2 ± 2.1
0.1	8	106 ± 3.8	99 ± 0.2	-130 ± 11.5*	76.9 ± 7.9	127.9 ± 4.4*	125.5 ± 3.7*
0.2	8	124.4 ± 4.3*	97.2 ± 0.6	-124 ± 10.4*	94.4 ± 11.4	153.6 ± 7.6*	147.1 ± 7.3*
0.4	4	116.4 ± 14.1	92 ± 0.7*	-74.7 ± 4.2*	73.4 ± 10.9	214.2 ± 20*	199.1 ± 16.5*
0.8	3	63.6 ± 14.5*	81.1 ± 2.6*	-31.2 ± 9.1*	13.2 ± 6.5*	285 ± 16.6*	334.9 ± 14.9*

Increases in fractional shortening (FS) are observed with CK-1827452 in adult rat ventricular myocytes. At the highest concentrations tested, 400 and 800 nM, decreases in cell length and FS are observed due to over-activation of the myosin cross-bridges, an expected on-target effect.



* p= < 0.05

Individual isolated adult rat cardiac myocyte tracings (left) and group data (below) showing contractility and calcium transients after 0.2 μM CK-1827452 treatment. CK-1827452 treatment elicits a significant increase in contractility with no significant change in the fura-2 calcium ratio. Note duration of the contractile transient increases, characteristic of a myosin activator.

Treatment	N	FS (% of basal)	Diastolic Fura-2 Ratio	Systolic Fura-2 Ratio	T_{75} (seconds)
Basal	8	100	0.82 +/- 0.01	1.26 +/- 0.01	0.32 +/- .01
0.2 μM CK1827452		133.1 +/- 6.8*	0.84 +/- 0.01	1.23 +/- 0.01	0.31 +/- .02

CK-1827452 overcomes BDM inhibition with no increase in calcium transient parameters

Treatment	N	FS (% of basal)	Diastolic Fura-2 Ratio	Systolic Fura-2 Ratio	T_{75} (seconds)
Basal	4	100	0.87 ± 0.02	1.23 ± 0.01	0.37 ± 0.02
10 mM BDM		4.2 ± 2.8	0.87 ± 0.01	1.16 ± 0.03	0.42 ± 0.03
BDM + 10 μM CK-1827452		28.0 ± 11.2	0.88 ± .02	1.14 ± 0.02	0.41 ± 0.03

To determine possible effects on the calcium transient at high concentrations of CK-1827452 experiments were conducted in the presence of the myosin inhibitor BDM. BDM inhibits the myosin cross-bridges but doesn't alter the calcium cycling. A large concentration of CK-1827452 (10 μM) reduces the effect of BDM inhibition but does not alter the calcium transient (panel above). In contrast,the β-adrenergic agonist Isoproterenol (ISO) alters the calcium transient parameters (below).

Treatment	N	FS (% of basal)	Diastolic Fura-2 Ratio	Systolic Fura-2 Ratio	T_{75} (seconds)
Basal	5	100	0.96 ± 0.02	1.25 ± 0.01	0.24 ± 0.01
10 mM BDM		5.8 ± 1.7	0.97 ± 0.02	1.24 ± 0.01	0.26 ± 0.01
BDM + 5 nM ISO		47.4 ± 14.7	1.02 ± .0.03	1.45 ± 0.03*	0.14 ± 0.01*

CK-1827452 increases fractional shortening *in vivo* – in SD rats

CK-1827452 (mg/kg/hr)	N	plasma (μM)	FS (% of baseline)	Diastolic Diameter (% of baseline)	Systolic Diameter (% of baseline)
0	6	< 0.01	108.1 +/- 2.3	99.3 +/- 1.4	92.3 +/- 2.5
0.25	5	0.21 +/- 0.02	104.4 +/- 2.1	101.3 +/- 3.1	96.5 +/- 3.7
0.7	6	0.43 +/- 0.06	119.3 +/- 3.5*	96.5 +/- 2.6	76.8 +/- 1.6*
1.2	6	0.72 +/- 0.04	120.4 +/- 4.3*	95.4 +/- 2.2	75.0 +/- 4.8*

Echocardiography parameters after 30 min infusion of vehicle or 0.25 – 1.2 mg/kg/hr CK-1827452. A dose response increase in fractional shortening is observed in SD rats.

CK-1827452 increases fractional shortening *in vivo* – in SD rats with heart failure

Group	N	Body Weight (kg)	Heart Weight (g)	BWt/HWt Ratio	Fractional Shortening
Sham	16	0.503 ± 0.042	1.629 ± 0.153	3.261 ± 0.423	49.6 ± 0.9
MI	28	0.514 ± 0.057	1.946 ± 0.361*	3.816 ± 0.762*	21.5 ± 0.7*

Rats with myocardial infarction (MI) had similar body weights to sham controls but had increased heart weights, HW/BW ratios and decreased FS consistent with heart failure.

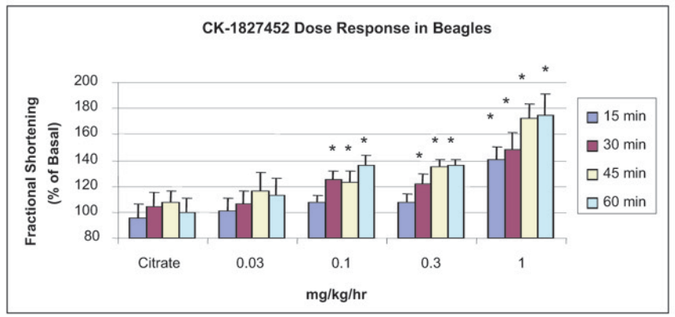
Sham (mg/kg/hr)	N	Plasma (μM)	FS (% of Baseline)	Diastolic Diameter (% of Baseline)	Systolic Diameter (% of Baseline)
0	4	ND	107.8 ± 4.6	96.8 ± 3.6	90.3 ± 5.6
0.25	4	0.26 ± 0.02	108.9 ± 4.7	94.9 ± 3.9	87.3 ± 6.1
0.7	4	0.57 ± 0.06	117.2 ± 3.6*	92.8 ± 3.6	76.9 ± 7.4*
1.2	4	1.17 ± 0.14	120.6 ± 6.3*	90.3 ± 4.3*	70.9 ± 7.7*
MI (mg/kg/hr)	**N**	**Plasma (μM)**	**FS (% of Baseline)**	**Diastolic Diameter (% of Baseline)**	**Systolic Diameter (% of Baseline)**
0	7	ND	95.4 ± 8.0	92.5 ± 8.8	101.6 ± 2.9
0.25	7	0.24 ± 0.03	108.5 ± 4.6	96.5 ± 5.5	94.1 ± 1.6*
0.7	7	0.47 ± 0.03	118.2 ± 3.7*	93.2 ± 9.5	94.8 ± 2.1
1.2	7	0.98 ± 0.13	114.4 ± 6.3*	95.4 ± 1.6	92.0 ± 2.6*

Echocardiography parameters after 30 min infusion of vehicle or 0.25 – 1.2 mg/kg/hr CK-1827452. A dose response increase in fractional shortening (FS) is observed in both groups demonstrating in vivo efficacy of CK-1827452 in sham as well as heart failure animals.

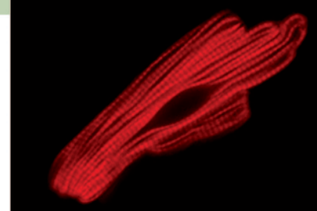
CK-1827452 increases fractional shortening *in vivo* – in anesthetized beagles

Time	Vehicle	0.03 mg/kg/hr	Fractional Shortening 0.1 mg/kg/hr	0.3 mg/kg/hr	1 mg/kg/hr
0	29.6 +/- 3.4	28.8 +/- 3.9	25.4 +/- 2.3	26.2 +/- 2.3	26.1 +/- 1.9
60	29.3 +/- 3.6	32.0 +/- 3.7	34.8 +/- 4.2 *	35.4 +/- 3.3 *	44.9 +/- 3.7 *

Fractional shortening at baseline and after 1 hr infusion of vehicle or 0.03 – 1 mg/kg/hr CK-1827452.



Dose response and time course over a 1 hr infusion of CK-1827452 in beagle dogs. Fractional shortening increases up to 175% of basal were observed at the highest doses and longest time periods. Plasma concentrations were 0.172 +/- 0 .019 and 1.886 +/- 0.142 μM at the 60 min time point for the 0.1 and 1 mg/kg/hr doses, respectively.



Conclusions

The cardiac myosin activator CK-1827452

1) Selectively activates cardiac myosin
2) Increases contractility in cardiac myocytes without increasing intracellular calcium
3) Significantly increases contractility in SD rats and rats with defined heart failure and in normal dogs
4) These data suggest that CK-1827452 may be a useful therapeutic in the treatment of human heart failure.

References

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3) Rodriguez H, Sylvester S, Qian X, Morgan B, Morgans Jr. D, Malik F, Sakowicz R. Activation of Cardiac Sarcomere ATPase by CK-1122534, a Small Molecule Agent that Specifically Targets Cardiac Myosin. American Society of Cell Biology, December 2004

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