

A DIRECT INHIBITOR OF SMOOTH MUSCLE MYOSIN AS A NOVEL THERAPEUTIC APPROACH FOR THE TREATMENT OF SYSTEMIC HYPERTENSION

Xiangping Qian, Xi Wang, James J Hartman, Zhiheng Jia, Sheila Clancy, Bing Yao, Chihyuan Chuang, Pu-Ping Lu, Malar Pannirselvam, David J Morgans Jr, Bradley P Morgan, Fady I Malik

Cytokinetics, Inc., South San Francisco, CA, USA.

INTRODUCTION

Smooth muscle myosin is a mechanochemical enzyme that hydrolyzes ATP to generate mechanical force; ultimately all signaling pathways that modulate smooth muscle tone converge on the regulation of this motor protein. While many drug mechanisms that relax smooth muscle reduce blood pressure, blood pressure still remains inadequately treated in many patients. Given its central role in generating smooth muscle contractility, direct inhibition of smooth muscle myosin should provide a novel and effective means to reduce blood pressure. Using high throughput screening, we identified and subsequently optimized a class of selective inhibitors of smooth muscle myosin. CK-2018509 is a novel, potent, and selective inhibitor of the enzymatic activity of smooth muscle myosin. In addition to the biochemical mechanism of action, we further characterize here its pharmacology in skinned and native isolated blood vessels as well as in rodent models of systemic hypertension.

METHODS

Biochemical Assays: Assays were performed in low salt PM12 buffer (12 mM K-Pipes, 2 mM MgCl₂, pH 6.8) in the presence of actin and 250 μM ATP (>5-10-fold above the K_{M,ATP}). Hydrolysis rates were normalized using reactions containing an equivalent amount of DMSO.

Myosin binding was measured by depletion of soluble myosin from binding reactions using smooth muscle myosin S1 fragment (human, recombinant) and 5 μM bovine cardiac actin. ATP and ADP were included at 1 mM where indicated. Reactions were allowed to equilibrate for 15 minutes prior to centrifugation (540k x g, 30 minutes), and ATP was added just prior to centrifugation to minimize hydrolysis. Supernatants were analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue.

Skinned Ring Assay: Endothelium-denuded rat tail artery segments were cut into 3-mm helical rings, mounted on an isometric force transducer with a resting tension of 0.5 g, and incubated for 30 minutes at room temperature in normal H-T buffer. Tissues were skinned by incubation with solution containing 1% Triton X-100 for 1 hour at room temperature. CK-2018509 was added to the tissue for 15 minutes, followed by addition of solutions with increasing calcium. Force generated at the plateau of each corresponding pCa was recorded. Data were presented as a percent change from the baseline values (Wilson et al., 2002).

Aortic Ring Assay: The thoracic aorta was removed and placed in Krebs-Henseleit buffer aerated with 95% O₂ and 5% CO₂. Arteries were cut into 2-mm rings, mounted on a tissue bath apparatus, and maintained at a baseline tension of 2 g. Endothelium-independent relaxation to CK-2018509 was recorded in preparations pre-contracted with a sub-maximal concentration of phenylephrine (EC₅₀ ~ 0.1 μM).

Thiophosphorylation Assay: Triton-permeabilized preparations were incubated in rigor solution containing ATPγS (1 mM) for 10 minutes. CK-2018509 was added 15 minutes before addition of ATP. ATP induced contraction was measured for 60 minutes and the relaxation was expressed as percentage of the maximum force.

In vivo Assay: Spontaneously hypertensive rats (SHR) and Dahl salt-sensitive rats were purchased from Charles River Laboratory. Dahl salt-sensitive rats were fed 8% NaCl in their diet to maintain hypertension. Rats were placed in restrainers for blood pressure monitoring; blood pressure was measured via the carotid artery using a fluid-filled dome pressure transducer.

RESULTS

CK-2018509 selectively inhibits the ATPase activity of smooth muscle myosin

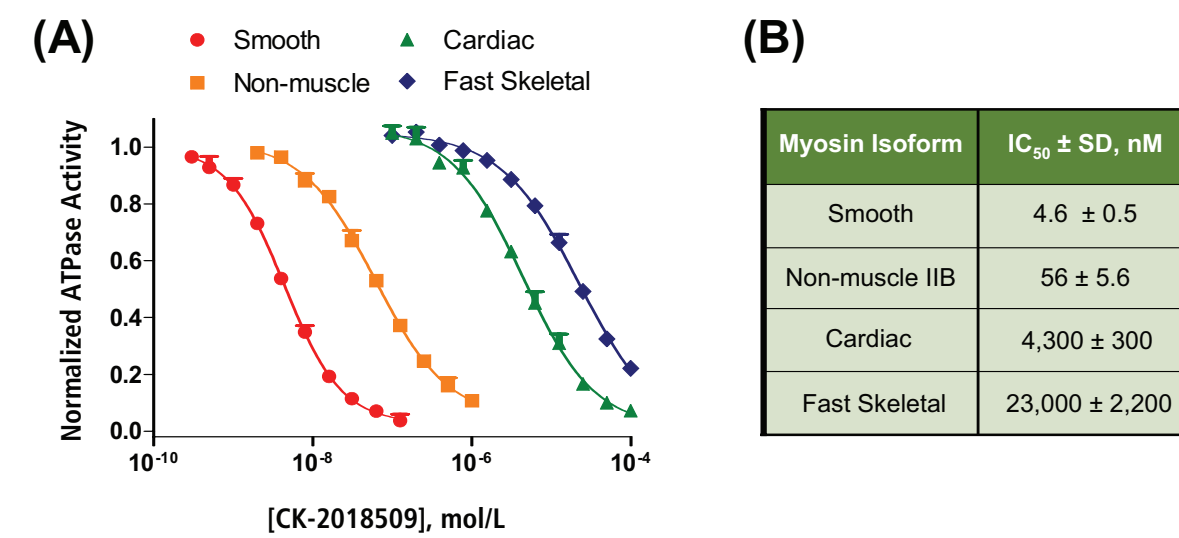


Figure 1: Inhibition of the Mg²⁺-ATPase activity of smooth muscle (human recombinant, circles), non-muscle IIB (human recombinant, squares), cardiac (bovine native, triangles), and fast skeletal (rabbit native, diamonds) S1 fragments at varying concentrations of CK-2018509. ATPase activity was measured in the presence of actin and 250 μM ATP (~5-10-fold above the K_{M,ATP}). ATPase rates were normalized to reactions containing an equivalent amount of DMSO. Representative curves from duplicate reactions are shown in panel (A). Pooled results from three experiments are shown in (B).

CK-2018509 does not promote strong actomyosin binding

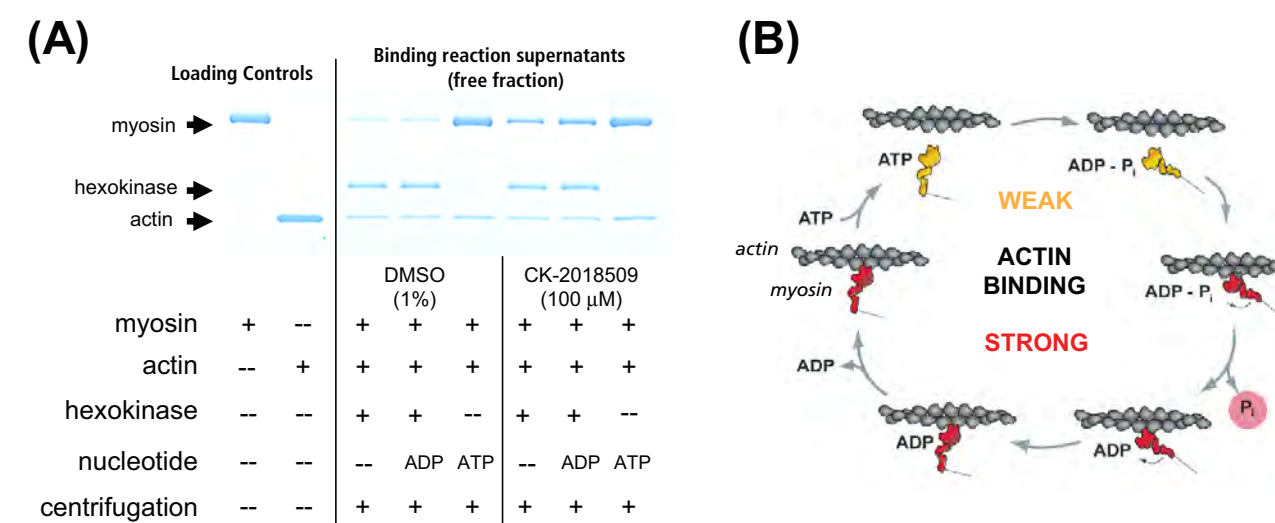


Figure 2: Strong actomyosin binding is revealed by depletion of soluble smooth muscle myosin (recombinant human S1) upon centrifugation in the presence of actin and various nucleotides (A). Total amounts of myosin (3 μM) and actin (5 μM) are indicated by the loading controls (left), while unbound myosin is shown in the binding reaction supernatant fractions (right). Where included, hexokinase is present to deplete residual ATP. (B) Smooth muscle myosin chemomechanical cycle, indicating weak and strong actin binding states.

CK-2018509 inhibits calcium-induced contraction of skinned caudal artery preparation

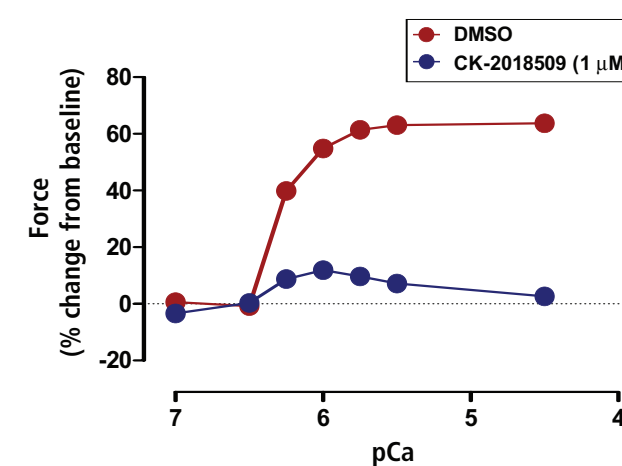


Figure 3: Mean pCa-response curves in the presence or absence of CK-2018509 in skinned caudal artery rings from Sprague Dawley rats (n=2). CK-2018509 inhibits the calcium-induced force development of the skinned caudal ring with complete inhibition observed at 1 μM.

CK-2018509 causes concentration dependent relaxation of isolated aortic rings

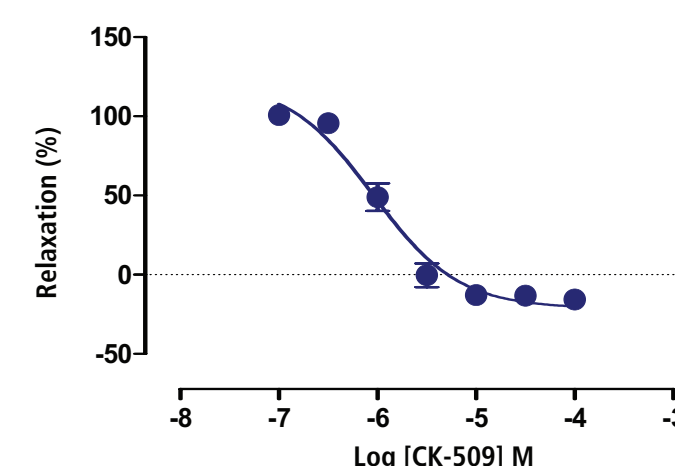


Figure 4: Concentration-response curves of CK-2018509 in isolated aortic rings from Sprague Dawley rats (n=4, mean ± sem). CK-2018509 relaxes isolated aortic rings in a concentration-dependent manner with a pEC₅₀ of 6.05 and E_{max} of 117%.

CK-2018509 relaxes the ATP-induced contraction in ATPγS treated skinned caudal artery

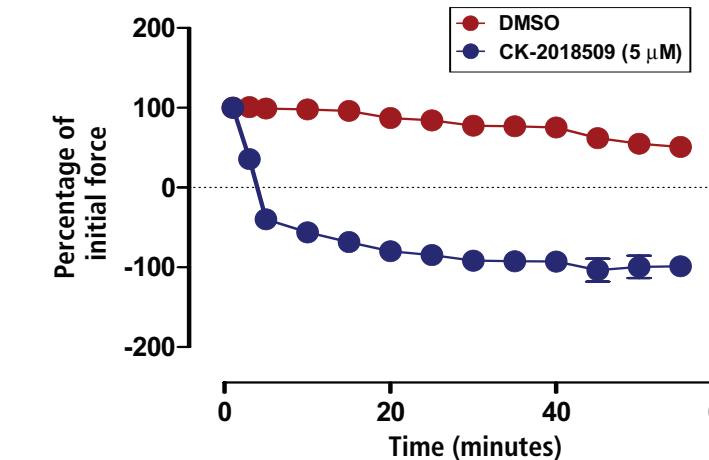


Figure 5: Force development (expressed as a percentage of the initial force) to ATP in the presence or absence of CK-2018509 in isolated skinned and thiophosphorylated aortic rings from Sprague Dawley rats (n=3, mean ± sem).

CK-2018509 causes a drop in mean arterial pressure in spontaneously hypertensive rats following i.v. administration

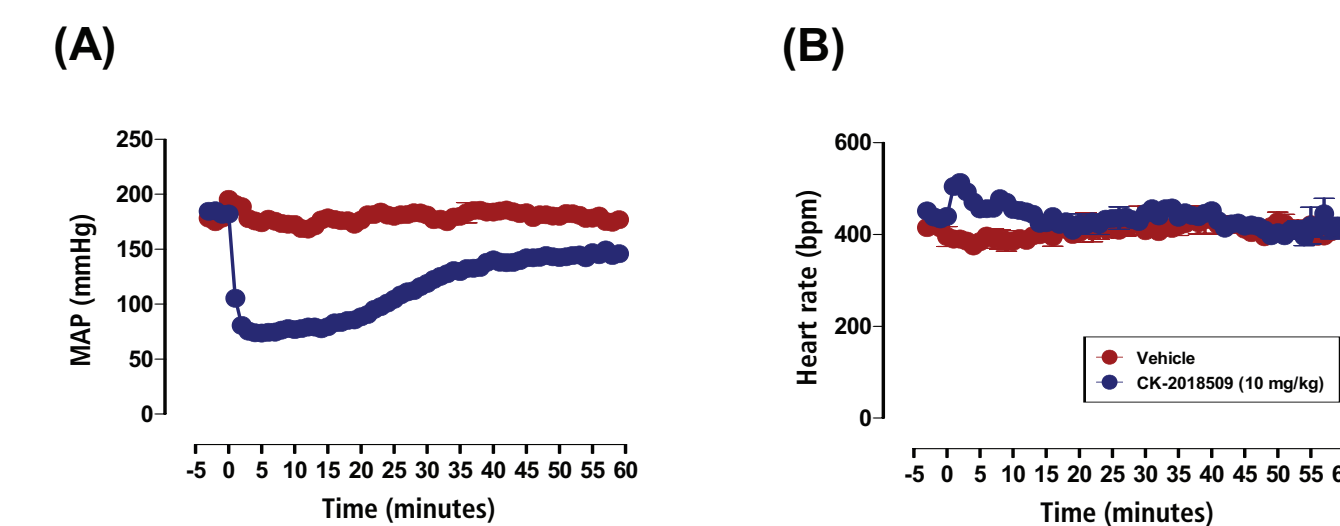


Figure 6: Effect of CK-2018509 (10 mg/kg, i.v.) on mean arterial pressure (MAP) (A) and heart rate (B) in spontaneously hypertensive rats (n=5, mean ± sem).

CK-2018509 lowers mean arterial pressure in spontaneously hypertensive rats following oral administration

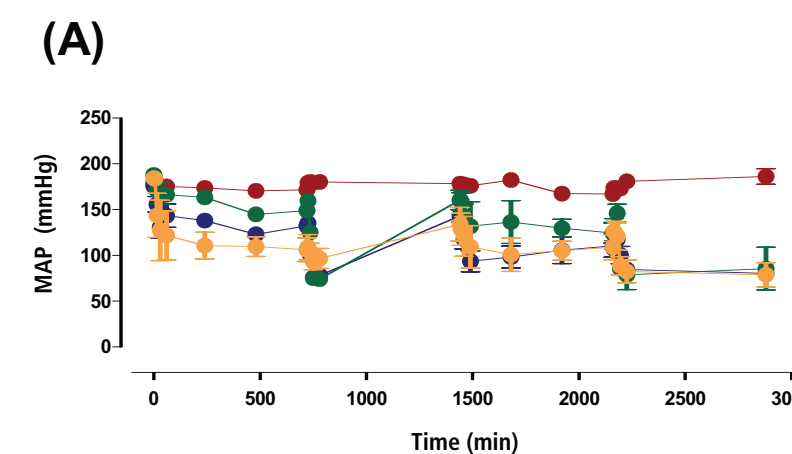


Figure 7A: Effect of CK-2018509 on mean arterial pressure (MAP) in spontaneously hypertensive rats (mean ± sem).

CK-2018509 does not change the heart rate in spontaneously hypertensive rats following oral administration

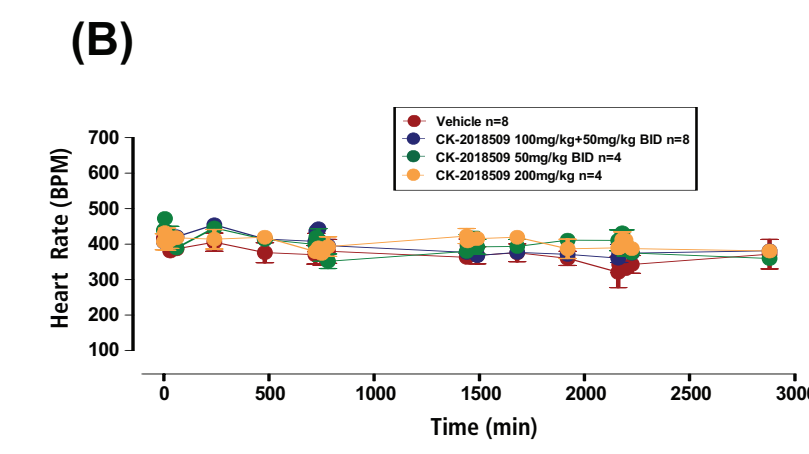


Figure 7B: Effect of CK-2018509 on heart rate in spontaneously hypertensive rats (mean ± sem).

CK-2018509 lowers mean arterial pressure in Dahl salt-sensitive rats following oral administration

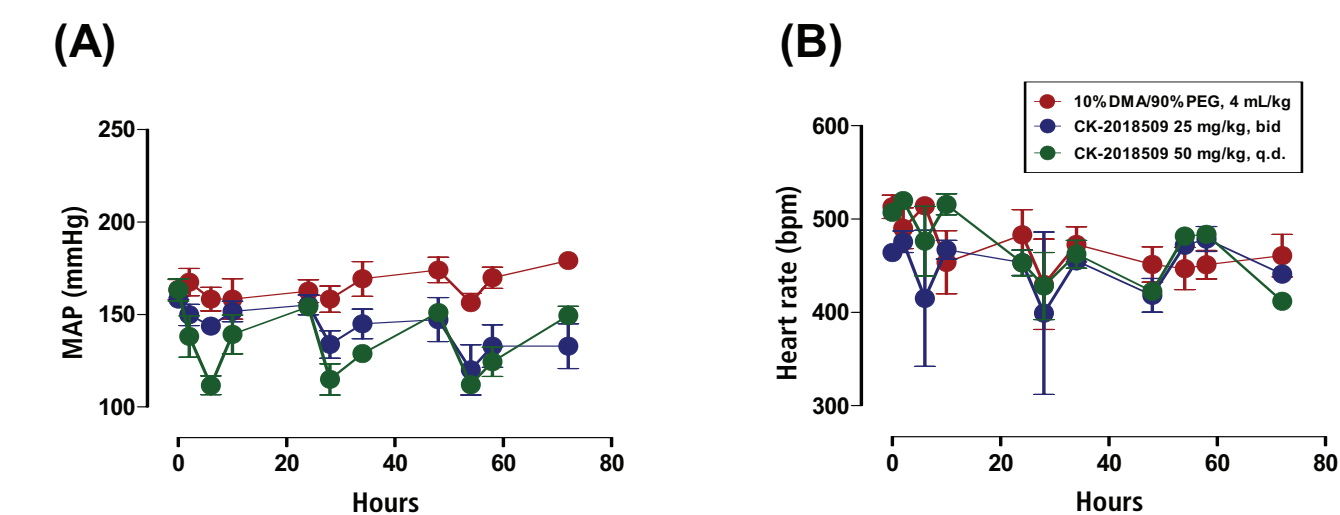
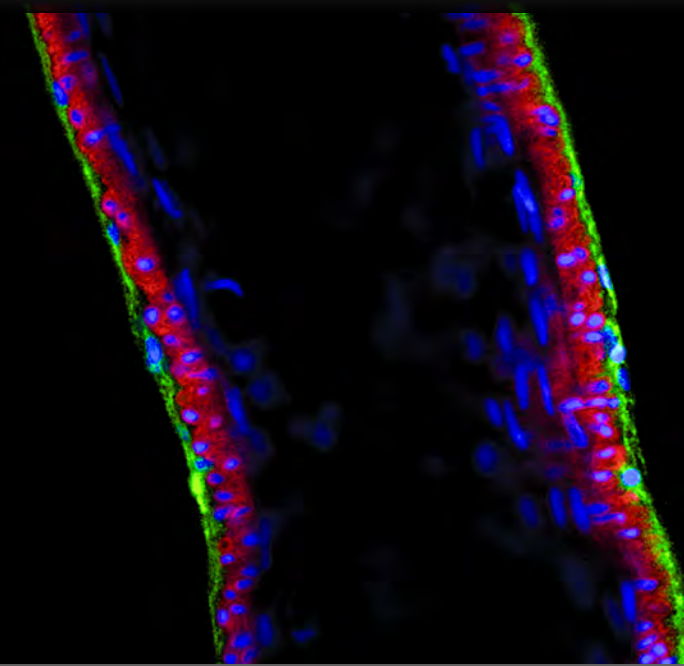


Figure 8: Effect of CK-2018509 on mean arterial pressure (MAP) (A) and heart rate (B) in Dahl salt-sensitive rats (n=3-4) compared with vehicle.



CONCLUSIONS

- CK-2018509 selectively inhibits the ATPase activity of smooth muscle myosin as compared to other myosin II isoforms (non-muscle myosin, cardiac and skeletal muscle myosins).
 - CK-2018509 confers its vasorelaxation activity by locking smooth muscle myosin in a weak actin-binding state.
 - CK-2018509 inhibits calcium-induced force development in skinned caudal artery and relaxes skinned rings activated by thiophosphorylation, consistent with relaxation occurring as a consequence of direct inhibition of smooth muscle myosin.
 - CK-2018509 relaxes phenylephrine pre-constricted aortic rings in a concentration-dependent manner, suggesting its potential use as a vasodilator.
 - CK-2018509 decreases the elevated mean arterial blood pressure with a minimal effect on heart rate in two animal models of hypertension, spontaneously hypertensive rats and Dahl salt-sensitive rats.
- Taken together, these data suggest that direct inhibition of smooth muscle myosin may be a novel therapeutic approach for the treatment of systemic hypertension.

REFERENCES

Wilson DP, Sutherland C, Walsh MP. Ca²⁺ activation of smooth muscle contraction: evidence for the involvement of calmodulin that is bound to the triton insoluble fraction even in the absence of Ca²⁺. *J. Biol. Chem.* 2002, 277(3):2186-92.



CYTOKINETICS