The Direct Smooth Muscle Myosin Inhibitor, CK-2125927, Represents a Novel Therapeutic Mechanism for Bronchodilation

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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. We previously showed that a novel smooth muscle myosin inhibitor, CK-2019165, inhibited methacholine-induced bronchoconstriction in rat models with a short duration of action (~2 hours). Here we describe the discovery and characterization of a long acting smooth muscle myosin inhibitor for bronchodilation. Using high throughput screening, we identified and subsequently optimized a class of selective inhibitors of smooth muscle myosin. These compounds potently inhibit the smooth muscle myosin ATPase (IC_{50}≤ 30 nM).

A potent example of this class, CK-2125927, inhibits calcium-induced contraction of skinned rat tail artery in a concentration-dependent manner with an IC_{50} of ~ 1 µM. CK-2125927 induced concentration-dependent relaxation of methacholine-pre-constricted rat tracheal rings with an EC_{50} of ~ 1 µM. Further, CK-2125927 (10 µM) completely inhibited electrical field stimulation-induced constriction of guinea pig tracheal rings with a 50% recovery time of > 6 hours, a duration comparable to the long-acting beta-adrenergic agonist salmeterol. Similarly, CK-2125927 dose-dependently bronchoprotected rats or dogs from methacholine-induced increases in tracheal pressure for at least 8 hours. Together these data indicate that long-acting smooth muscle myosin inhibition is achievable, and may provide a novel therapeutic approach for the treatment of diseases where bronchoconstriction plays a role such as asthma and COPD.

Methods

Biochemical Assay: Assays were performed in low salt PM12 buffer (12 mM K-Pipes, 2 mM $MgCl_2$, 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.

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 incubated with skinning solution containing 1% Triton X-100 for 1 hour at room temperature. CK-2125927 was added to the tissue for 15 minutes, followed by addition of solutions with increasing calcium. Force was recorded at each calcium concentration. Data were presented as a percent change from the baseline values.

Tracheal Ring Assay: The rat trachea was removed and placed in Krebs-Henseleit buffer aerated with 95% O_2 and 5% CO_2. The trachea was cut into 2 mm rings, mounted on a tissue bath apparatus, and maintained at a baseline tension of 0.5 g. CK-2125927-induced relaxation was recorded in preparations pre-contracted with a sub-maximal concentration of methacholine (MCh), 3 µM.

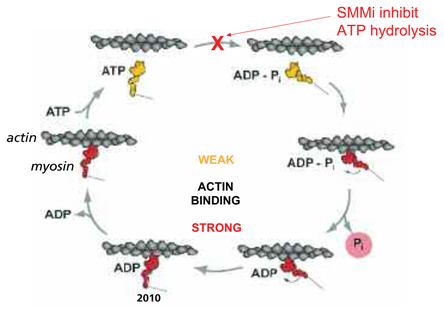
Electrical Field Stimulation Assay: The guinea pig trachea was removed and placed in Krebs-Henseleit buffer aerated with 95% O_2 and 5% CO_2. The trachea was cut into 2 mm rings, mounted on a tissue bath apparatus, and maintained at a baseline tension of 0.5 g. CK-2125927-induced relaxation was recorded in preparations pre-contracted with a sub-maximal electric field stimulation at 10 to 30 Hz, 20 sec/5 min. Tension was monitored for up to ~20 hrs.

Thiophosphorylation Assay: Triton-permeabilized preparations were incubated in rigor solution containing ATPγS (1 mM) for 15 minutes then washed out. CK-2125927 was added 15 minutes before addition of the ATP. ATP induced contraction was measured for 60 minutes and the relaxation was expressed as the percentage of the maximum force.

Anesthetized Rodent Model of Airway Resistance & Compliance: Male Sprague-Dawley rats were anesthetized with Ketamine/Xylazine/Acepromazine (80/10/1 mg/kg) cocktail and tracheotomized with a 14 g tracheal cannula. Rats were paralyzed with Pancuronium Bromide at 2 mg/kg, *i.p.* to prevent spontaneous breathing and placed immediately on a Resistance & Compliance Plethysmograph (Buxco Research Systems). Once rats were stabilized and a baseline was collected, CK-2125927 was intra-tracheally administered by dry powder inhalation via an insufflator device (Penn Century). Following dosing with CK-2125927, rats were subjected to increasing doses of methacholine to induce bronchoconstriction.

Anesthetized Dog Model of Airway Resistance: Female naïve dogs were anesthetized and tracheotomized. Dogs were stabilized and baseline resistance collected. An abbreviated MCh dose-response (up to 3 doses) was performed to confirm the dose of MCh that induces a 200–300% increase in pulmonary resistance. Fifteen minutes later a single dose of MCh was again given to reconfirm the response. The response to this challenge was used to compare all subsequent MCh challenges following CK-2125927 treatment up to 8 hours. CK-2125927 was delivered as a powder using insufflator devices.

Smooth Muscle Myosin ATPase Cycle

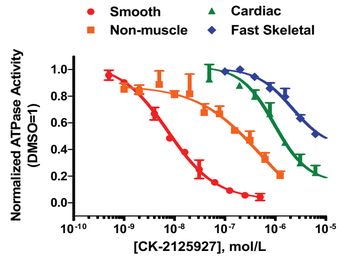


Appropriate compounds need to stabilize at weak actin-binding state, in the presence of cellular (mM) concentrations of ATP.

See Clancy et al. 2010 for additional information.

Results

Figure 1: CK-2125927 Selectively Inhibits the ATPase Activity of Smooth Muscle Myosin



Myosin Isoform	IC_{50}, nM
Smooth	9
Non-muscle IIB	190
Cardiac	1600
Fast skeletal	8600

Figure 1. Inhibition of the Mg^{2+}-ATPase activity of smooth muscle (human recombinant), non-muscle IIB (human recombinant), cardiac (bovine native), and fast skeletal (rabbit native) S1 fragments at varying concentrations of CK-2015927. 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 the graph and table. Data were fit to a four-parameter EC_{50} model with fixed maximal (1) and minimal (0) activity.

Figure 2: CK-2125927 Inhibits Calcium-Induced Contraction of Skinned Caudal Artery Smooth Muscle Preparation

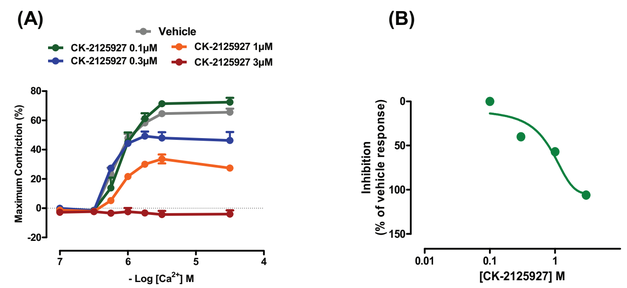


Figure 2A. Mean pCa-response curves in the presence (0.1, 0.3, 1 & 3 µM) or absence of CK-2125927 in skinned caudal artery rings from rat Sprague Dawley rats (n=4-6). Symbols are mean ± standard error values. **B.** Concentration-response curve for CK-2125927 constructed from the maximal inhibition of calcium-induced contraction from figure **A**. CK-2125927 inhibited the calcium induced forced development of the skinned caudal ring in a concentration dependent manner with an EC_{50} of 0.79µM and E_{max} of 110%. Symbols are mean values.

Figure 3: CK-2125927 Relaxes the ATP-Induced Contraction in ATPγS Treated Skinned Caudal Artery

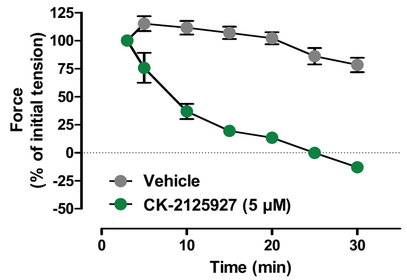


Figure 3: Force development to ATP in the presence or absence of CK-2125927 (5 µM) in thiophosphorylated isolated skinned rings (n=4) from male Sprague Dawley rats. Symbols are mean ± standard error values.

Figure 4: CK-2125927 Causes Concentration Dependent Relaxation of Isolated Tracheal Rings

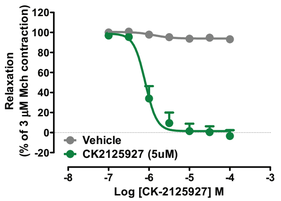
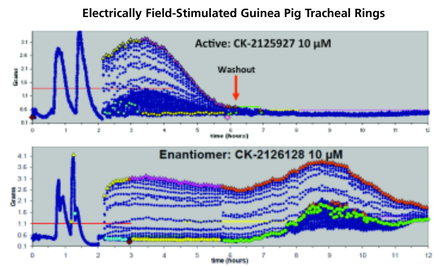


Figure 4. Concentration-response curve to CK-2125927 in isolated tracheal rings (n=4) pre-constricted with 3 µM methacholine from male Sprague Dawley rats. CK-2125927 relaxed the isolated tracheal rings in a concentration-dependent manner with an IC_{50} of 0.81 µM and I_{max} of 97%. Symbols are mean ± standard error values.

Figure 5: CK-2125927 Has a Long Duration of Action *in vitro* Enantiomeric Selectivity: On-Target Activity in Tissue



Biochemical inhibition of ATPase
CK-2125927 IC_{50} = 0.017 µM
CK-2126128 IC_{50} = 0.65 µM

Figure 6: CK-2125927 Inhibited the Methacholine-Induced Bronchoconstriction in Anesthetized Rat Models

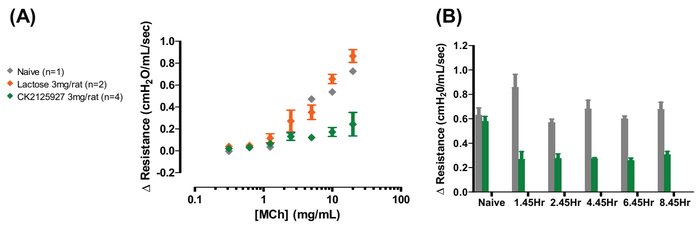


Figure 6 A. Effect of CK-2125927 on methacholine-induced bronchoconstriction in anesthetized, paralyzed and mechanically ventilated rats (n=3-7 rats). Symbols are mean ± standard error values. **B.** CK-2125927 inhibited the methacholine-induced bronchoconstriction in resistance and compliance plethysmography rat model for up to 8hrs. Each bar represents the resistance at [MCh]=20mg/mL ± standard error values.

Figure 7: CK-2125927 has a Long Duration of Action *in vivo* Inhaled Dry Powder – Ventilated Dog Model

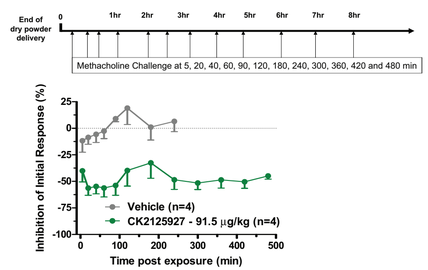
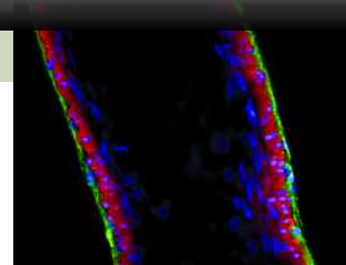


Figure 7. At a sub-maximal inhaled dose (~91.5 µg/kg), CK-2125927 protects against repeated methacholine challenges for at least 8 hours. Repeat-dosing might decrease required dose.



Conclusions

1. CK-2125927 selectively inhibits the ATPase activity of smooth muscle myosin over other myosin II isoforms (non-muscle myosin, cardiac and fast skeletal muscle myosin)
3. CK-2125927 inhibited 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
3. CK-2125927 relaxed methacholine pre-constricted tracheal rings in a concentration dependent manner suggesting its potential use as a bronchodilator
4. CK-2125927 inhibited methacholine-induced bronchoconstriction in two animal models of bronchoconstriction for up to 8hrs indicating the potential for a long duration of action with this novel mechanism

* These data together suggest that direct, long-acting inhibition of smooth muscle myosin could be a novel therapeutic approach for the treatment of chronic obstructive pulmonary disease and asthma

References

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