**Abstract**

We have previously described the use of an absorbance-based kinetic assay for ATPase activity to identify activators of cardiac myosin. One class of these activators has been shown to inhibit the release of inorganic phosphate (Pi) during basal ATP hydrolysis by cardiac myosin (i.e., in the absence of actin or regulatory components). We used a highly sensitive fluorescence-based assay to test whether the ability to inhibit basal ATPase was correlated with activation of the actin-stimulated ATPase for molecules within this structural class. We found a tight correlation between the two effects. Next, we correlated with activation of the actin-stimulated ATPase for molecules within this structural class. We found a tight correlation between the two effects. Next, we correlated with activation of the actin-stimulated ATPase for molecules within this structural class.

**Introduction**

Heart failure affects 5 million people in the United States. The lifetime risk of developing heart failure is 1 in 5. Over 80% of men and 70% of women under 65 years old who develop heart failure, die within eight years of initial diagnosis. At Cytokinetics, we have developed novel agents for improving cardiac contractility by specifically activating cardiac myosin. This approach offers several advantages over current agents such as no activation of second messenger signaling, no increase in cardiac myocyte intracellular calcium or in heart rate and no decrease in blood pressure. In addition, this mechanism of action minimally impacts cardiac oxygen consumption and thus improves myocardial efficiency in a dog model.

Using a biochemical assay composed of thin filaments reconstituted from actin, tropomyosin and troponins, and cardiac myosin, we have previously reported the discovery of activators of the cardiac myosin actin-stimulated ATPase. The most active compounds belonged to a structural series of substituted benzamides.

**Results**

**A highly sensitive assay for ADP detection...**

The ADP-Amplex Red (ADPAR) assay

Conversion of non-fluorescent Amplex Red to fluorescent Resorufin allows continuous fluorimetric detection of ADP generation at wavelengths suitable for high throughput screening of compound libraries.

...allows measurement of the low basal ATPase activity of cardiac myosin

**Basal ATPase Inhibition and Activation of Actin-stimulated ATPase are well correlated**

Activities of substituted benzamides

In the reconstituted thin filament assay (S1 + regulated actin) the concentration of compound required for 40% activation over DMSO control (AC1.4) was measured. In the basal ATPase ADPAR assay, the concentration of compound needed for 50% inhibition (IC50) was measured. Panel A and C show example data from a benzamide compound in each assay and Panel B shows the correlation between the two parameters across a set of benzamide compounds.

**References**

1) S. Malik et al., Meeting Abstract, Heart Failure Society of America, 2005.
3) F. Malik et al., Meeting Abstract, AHA. Update, AHA.

**Acknowledgements**

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**Conclusions**

The basal ATPase activity of cardiac myosin can be measured and utilized for high throughput screening using a highly sensitive fluorimetric readout of ADP generation.

There is a strong correlation between activation of the actin-stimulated ATPase and inhibition of basal ATPase for the benzamide family of sarcomere activators.

This correlation allowed us to identify a new series of cardiac myosin activators using a primary screen for myosin basal ATPase inhibitors.

**A screen for basal ATPase inhibitors yields novel activators of myofibrillar ATPase**

**ADPAR based myosin basal ATPase assay**

The basal ATPase activity of cardiac myosin is very low (Kcat~0.5 s⁻¹), but the continuous fluorimetric assay shown above allows detection of ATPase activity with as little as a few μM of skinned bovine cardiac myosin. 50 μg/ml myosin subfragment-1 (S1) in the presence of 20 μM ATP (~3X Km) was used for the screen (arrow).

Potency results from Cardiac Myosin Basal ATPase screen

A majority of the hits from the screen were inhibitors of cardiac myosin ATPase activity measured in a myofibrillar assay with enough calcium to provide 25% of the maximal calcium activation. Some compounds showed activation of skinned bovine cardiac myofibrillar ATPase. One of these activators was active at a higher calcium concentration corresponding to 50% activation. Hit series are distinguished by color (bluenavichrome).