Supplementary Material and Methods

Levosimendan (4-[(4'R)-4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin3-yl]phenyl) hydrazonopropanedinitrile (1) was purchased from Kinbester Co. Ltd. (Hong Kong). 4-(diphenylamino)phenylcyanoacrylic acid (5) was purchased from Dyenamo AB. 9-(2,2-Dicyanovinyl)julolidine (>97%, 4), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (> 98%, 3), and N-acetyl-L-cysteine (99%, NAC) were purchased from Sigma Aldrich.

Synthesis of Compound 2

Scheme S1. Synthesis of ((4-(2,4-difluorophenyl)phenyl)hydrazono)propanedinitrile

![Scheme S1](image)

Compound 2 was synthesized in house using 2',4'-difluoro-[1,1'-biphenyl]-4-amine (97%, DFBPA) from Amatek Chemical Co., Ltd. The procedure for synthesizing 2 was adapted following the protocol in Robertson et al. (1) for 13C-labelling levosimendan. DFBPA (54.6 mg, 0.266 mM) was dissolved in 4 mL of isopropyl alcohol (IPA), resulting in a brownish solution. To acidify the sample, 5 M HCl (aq) (50 µL, 0.25 mM) was added dropwise to the solution while stirring on ice. A pink precipitate formed as the acid was added and the IPA was allowed to evaporate in the fume hood.

The pink precipitate was then dissolved in 30 mL of dH2O with excess HCl (2.16 M) and 1 mL dimethylformamide (DMF). 2 mL of 0.134 mM (0.268 mM) ice cold NaNO2 was added dropwise to the DFBPA solution to form a diazonium compound. The solution went from a brownish-purple to a yellow colour. The diazonium solution was stirred on ice for 5-10 minutes and then was added dropwise to 10 mL of an aqueous solution containing 0.477 mM malononitrile and 10 mM of sodium acetate. Initially, the solution was bright yellow, but as more diazonium was added a precipitate appeared. The reaction was stirred for 30 minutes on ice followed by 30 minutes of stirring at room temperature to evaporate excess NO2.

The resulting slurry was filtered with a Büchner funnel and the precipitate was washed with dH2O. The precipitate was dried under vacuum. The final product collected was a yellow solid and was obtained at an 80% yield. This product was verified by Liquid-Chromatography-Mass Spectrometry (LCMS) and 1H and 13C Nuclear Magnetic Resonance (NMR) (See Figures S1 and S2).
ATPase Activity Measurements

Cardiac myofibril preparation and ATPase measurements were performed as described previously. Cardiomyofibrils (CMFs) were prepared by homogenising freshly frozen bovine ventricular tissue samples in myofibril buffer (composition in mM: 20 Imidazole pH 7.4, 75 KCl, 2 MgCl2, 2 EDTA, 1 DTT, 1% (v/v) Triton X-100, protease inhibitor cocktail (ROCHE), PhosStop cocktail (ROCHE)) followed by centrifugation at 5,000 g for 5 min at 4°C. CMFs were washed and homogenised three more times in the same buffer without Triton X-100.

CMFs were washed three times in ATPase assay buffer (composition in mM: 20 MOPS pH 7.0, 35 NaCl, 5 MgCl2, 1 EGTA, 1 DTT) with varying concentrations of CaCl2 (pCa 9 to pCa 4.3) and the CMF concentration adjusted to 1mg/ml. For ATPase measurements in the presence of drug, CMFs were incubated with varying concentrations of 2 at room temperature for 30 min. Compound 2 was prepared as a stock solution in DMSO and the final DMSO concentration during ATPase measurements was held constant at 1% (w/v).

Reactions were started by the addition of 2.5 mmol/L ATP and quenched with 0.5 volumes ice cold 25% (w/v) TCA solution. Samples were kept on ice at all times, diluted with double-deionized water, precipitation removed by centrifugation at 5000g for 10 min and inorganic phosphate content measured using the malachite green assay according to manufacturer’s instructions (Sigma, MAK030).

Reverse Phase High Performance Liquid Chromatography Mass Spectrometry (RP-HPLC-MS)

To explore if the selectivity for C84 was maintained with other compounds, 2 and 5 were reacted with wild-type (wt) cTnC. A 2 mg/mL stock of wt-cTnC was diluted with aqueous buffer into four samples with a final protein concentration of 25 µM. The aqueous buffer was comprised of 100 mM KCl, 10 mM imidazole and 200 µM Ca2+ at pH 7. Using a 2 mM stock of compound 2 and a 10 mM stock of 5, each small molecule was mixed with the 25 µM cTnC samples to attain a 1:1 and 2:1 concentration of compound to cTnC. The 1mL samples contained 0.25 - 2.5% (v/v) of DMSO and were reacted for 24 hours at room temperature before an aliquot of each sample was analyzed by HPLC/MS.

For protein molecular weight determination reverse phase high performance liquid chromatography followed by detection using mass spectrometry (RP-HPLC-MS) was performed using an Agilent 1200 SL HPLC System with a Phenomenex Aeris 3.6um, WIDEPORE XB-C8, 200Å, 2.1x50mm (Phenomenex, Torrance, USA) with guard thermostated at 40°C. A buffer gradient system composed 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile (ACN) as mobile phase B was used.
An aliquot of 5 µL of sample was loaded onto the column at a flow rate of 0.5 mLmin\(^{-1}\) and an initial buffer composition of 95% mobile phase A and 5% mobile phase B. After injection, the column was washed using the initial loading conditions for 1 minute to effectively remove salts. Elution of the proteins was done by using a linear gradient from 5% to 15% mobile phase B over a period of 0.5 minutes, 15% to 45% mobile phase B over a period of 5.5 minutes, 45% to 98% mobile phase B over a period of 1 minute, kept at 98% mobile phase B over a period of 1 minute and back to 5% mobile phase B over a period of 1 minute. Mass spectra were acquired in positive mode of ionization using an Agilent 6220 Accurate-Mass TOF HPLC/MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source with the second sprayer providing a reference mass solution. Mass correction was performed for every individual spectrum using peaks at \(m/z\) 121.0509 and 922.0098 from the reference solution. Mass spectrometric conditions were drying gas 10 L/min at 325°C, nebulizer 20 psi, mass range 100-3200 Da, acquisition rate of ~1.03 spectra/sec, fragmentor 200V, skimmer 65V, capillary 4000V, instrument state 4GHz High Resolution. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.03.01 SP3.

NMR

NMR experiments were performed at 30 °C on a 600 MHz Varian Inova spectrometer using z-axis pulsed field gradient triple resonance probes unless stated otherwise. Samples were made up in 5 mm NMR tubes with a starting sample volume of 550 µL. All aqueous NMR samples contained 10% (v/v) 2,2-dimethyl-2-silapentane-5-sulfonate-d6 sodium salt (DSS\(_{d6}\)) with 0.01% NaN\(_3\) in 90%H\(_2\)O/10% D\(_2\)O as an internal standard purchased from Chenomx Inc. Stocks of the small molecules investigated by NMR were dissolved in either deuterated dimethyl sulfoxide (DMSO\(_{d6}\)) or deuterated N,N-dimethylformamide (DMF\(_{d7}\)) purchased from Cambridge Isotopes Inc.

Solubility curve for compound 2 monitored using 1D \(^1\)H NMR

A titration of compound 2 was conducted into a sample containing an aqueous buffer (100 mM KCl, 10 mM Tris and 200 µM Ca\(^{2+}\)) with 10% (v/v) Chenomx at a pH of 7.6. The pH of the sample was measured using the position of the Tris peak\(^3\). Initially, additions (5.5 µL) from a 5 mM stock of 2 in DMF\(_{d7}\) were made. After 100 µM of 2 was added to the sample, very little change in the intensity of the proton peaks of 2 were observed (data not shown).

A second titration of 2 was conducted into a sample prepared as described above. To assist in compound 2 solubility, an additional 5% (v/v) DMF\(_{d7}\) was added to the sample. Deviations from the expected concentration of 2 started after 250 µM was added to the sample. This was determined from the integration of the proton peaks of 2 against DSS\(_{d6}\). The final sample contained 14% (v/v) DMF\(_{d7}\) and approximately 453 µM of 2 and had a pH of 7.6 (Fig.S5a). To see if the sample’s pH could be brought into the optimal working range for 2D- \(^1\)H,\(^{15}\)N-HSQC experiments, 0.1 M HCl\(_{aq}\) was added to the sample (Fig.S5b). The addition of acid caused further precipitation of compound 2 and additional/broadening to the peaks in the \(^1\)H spectrum.
**Supplementary Figures**

**Scheme S2:** Michaelis-Menten equation used to describe reversible covalent bond formation between a protein (P) and a drug (D).

\[
\begin{align*}
\text{Scheme S2: } & \quad \text{Michaelis-Menten equation used to describe reversible covalent bond} \\
& \quad \text{formation between a protein (P) and a drug (D).} \\
& \quad \text{\[
\begin{array}{cc}
\mathbf{K}_1 & \mathbf{K}_2 \\
\mathbf{P} + \mathbf{D} & \Leftrightarrow \mathbf{P:D} & \Leftrightarrow \mathbf{P-D} \\
\mathbf{K}_1 & \mathbf{K}_2
\end{array}
\]}
\end{align*}
\]
Figure S1. LC-MS for panel of compounds. The spectra for compounds 1, 2 and 3 were detected in the negative mode. The spectra for compounds 4 and 5 were acquired in the positive mode. Compound 1 (C\textsubscript{14}H\textsubscript{12}N\textsubscript{6}O) has an expected molecular weight (MW) of 280.3 Da, and the major species found had a MW of 279.1 Da (a). Compound 2 (C\textsubscript{15}H\textsubscript{8}F\textsubscript{2}N\textsubscript{4}) has an expected MW of 282.3 Da and the major species detected had a m/z of 281.1 Da (b). Compound 3 (C\textsubscript{10}H\textsubscript{5}F\textsubscript{3}N\textsubscript{4}O) has an expected MW of 254.2 Da, and the major species found had a MW of 253.0 Da. (c). Compound 4 (C\textsubscript{16}H\textsubscript{15}N\textsubscript{3}) has an expected MW of 249.3 Da and the major species detected had a m/z of 250.1 Da (d). Compound 5 (C\textsubscript{22}H\textsubscript{18}N\textsubscript{2}O\textsubscript{2}) had an expected MW of 340.4 Da and the major species detected had a m/z of 341.1 Da (e). The minor series in spectrum e at 358.2 Da is from an NH\textsubscript{4}\textsuperscript{+} coordinated to compound 5.
Figure S2. 1D $^1$H (a) and $^{13}$C NMR (b) of compound 2 collect at 30 °C on a 500 MHz Bruker NMR spectrometer in DMSO$_d$$_6$. Both spectra are referenced using the DMSO peak at 2.5 ppm in a and 39.5 ppm in b. $^{13}$C spectrum of 2 was assigned based on $^{13}$C NMR spectra of levosimendan$^1$ and precursor, DFBP-o (unpublished data). $^1$H NMR (500 MHz, DMSO$_d$$_6$): $\delta$ 7.8 (m, 5H, H$_{6,8,9,11,12}$), 7.4 (t, 1H, H$_3$), 7.2 (t, 1H, H$_1$), 3.4 (H$_2$O), 2.5 (DMSO$_d$$_6$). $^{13}$C NMR (500 MHz, DMSO$_d$$_6$): $\delta$ 163 (d, $-C_2F$), 158 (d, $-C_4F$), 141 (s, $-C_{10}$), 132 (m, $-C_6$), 131.8 (s,$-C_7$), 130 (s, $-C_{8,12}$), 124 (d, $-C_5$), 117 (s, $-C_{9,11}$), 114 (s,$-C_{17}$), 112 (d, $-C_1$), 110 (s, $-C_{16}$), 105 (t, $-C_3$), 85 (s, $-C_{15}$), 39.5 (DMSO$_d$$_6$).
Figure S3. ATPase activity measurements with compound 2. Inorganic phosphate calibration curve monitored by absorption of malachite green at 650 nm (a) and control experiment for the ATPase activity of bovine cardiomyofibrils (CMFs) as a function of calcium (b). Above 250 µM, precipitation of compound 2 becomes apparent (c). At a constant calcium concentration, the addition of compound 2 causes an increase in CMFs ATPase activity (d).
**Figure S4.** $^1$H NMR spectra of the reaction of 2 mM compound 5 and 4 mM NAC at 30 $^\circ$C in DMSO$_{d6}$. Spectrum a contains only solvent and compound 5: $\delta$ 13.6 ppm (s, 1H, -CO$_2$H), 8.18 (s, 1H, -CH), 7.97 (d, 2H, -CH aromatic, J=8.7 Hz), 7.49 (t, 4H, -CH aromatic, J=8.0 Hz), 7.30 (t, 2H, -CH aromatic, J=7.3 Hz), 7.26 (d, 4H, -CH aromatic, J=8.0 Hz) and 6.92 (d, 2H, -CH aromatic, J= 8.9 HZ). Spectrum b was the starting spectrum in the 24 hour time course experiment (Fig.3). The additional peaks in b are attributed to the protons of NAC: $\delta$ 12.82 ppm (s, 1H, -CO$_2$H), 8.21 (d, 1H, -NH, J=7.7 Hz), 4.43 (m, 1H, -CH), 2.89-2.79 (m, 2H, -CH$_2$), 2.48 (t, unidentified), 1.93 (s, 3H, -CH$_3$). Peaks at 3.36 ppm and 2.54 ppm in a and b are from residual water and DMSO, respectively.
Figure S5. $^1$H NMR spectra of compound 2 in DMF$_{d7}$ at 30 °C before (a) and after (b) pH adjustment with 0.1M HCl$_{(aq)}$. A sample containing compound 2 with 14%(v/v) DMF$_{d7}$ in an aqueous buffer at pH 7.6 (a). The pH of the sample was measured from the chemical shift of the Tris peak (not shown). Lowering the pH (out of range of Tris peak) of the sample (b) caused precipitation and degradation of 2.
References

(1) Robertson, I. M.; Pineda-Sanabria, S. E.; Yan, Z.; Kampourakis, T.; Sun, Y.-B.; Sykes, B. D.; Irving, M. Reversible Covalent Binding to Cardiac Troponin C by the Ca$^{2+}$ -Sensitizer Levosimendan. *Biochemistry* **2016**, *55* (43), 6032–6045.

(2) Kampourakis, T.; Zhang, X.; Sun, Y.-B.; Irving, M. Omecamtiv Mercabil and Blebbistatin Modulate Cardiac Contractility by Perturbing the Regulatory State of the Myosin Filament. *J. Physiol.* **2018**, *596* (1), 31–46.

(3) Baryshnikova, O. K.; Williams, T. C.; Sykes, B. D. Internal PH Indicators for Biomolecular NMR. *J. Biomol. NMR* **2008**, *41* (1), 5–7.