# Supplementary Information

## Discovery of Novel Cardiac Troponin Activators using Fluorescence Polarization-based High Throughput Screening Assays

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**Supplementary Information Figures and Figure Legends**

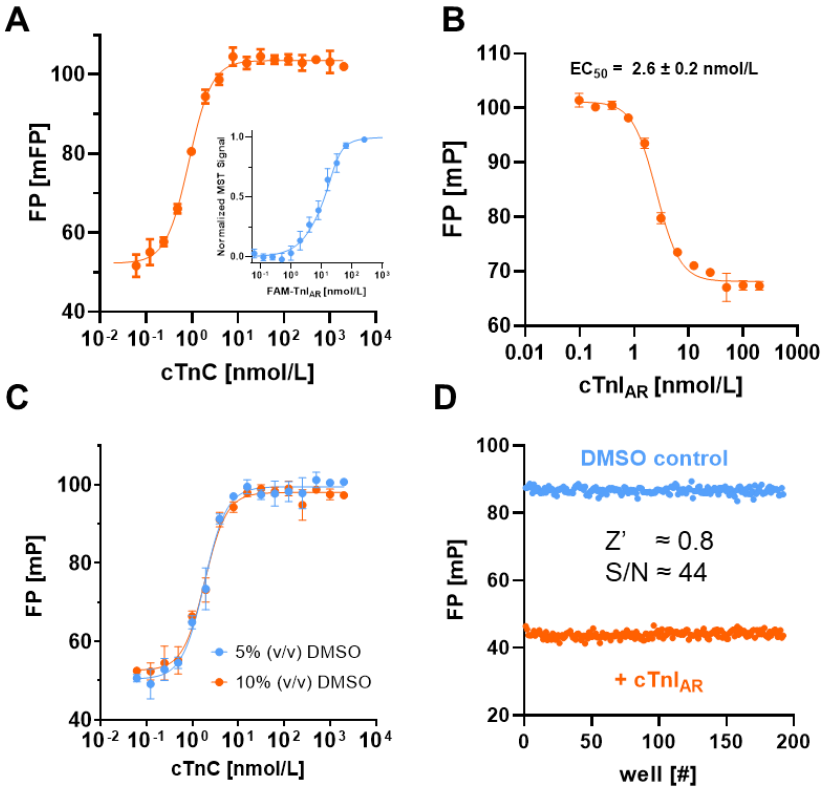


**Figure S1. Characterization and Validation of the cTnI$_{AR}$ HTS assay** (A) Interaction of FAM-cTnI$_{AR}$ with cTnC monitored by fluorescence polarization (FP). FAM-cTnI$_{AR}$ was titrated against increasing concentrations of cTnC (orange). Inset: Interaction of Alexa647-labelled cTnC and FAM-cTnI$_{AR}$ measured by Microscale Thermophoresis (blue). (B) Competitive titration of a mixture of 2 nmol/L FAM-cTnI$_{AR}$ and 5 nmol/L cTnC against increasing concentrations of unlabelled cTnI$_{AR}$ peptide monitored by FP. (C) Interaction of FAM-cTnI$_{AR}$ with cTnC monitored by fluorescence polarization (FP) in the presence of 5% (v/v) (blue) and 10% (v/v) orange dimethyl sulfoxide (DMSO). (D) FP measured from a mixture of FAM- cTnI$_{AR}$ and cTnC in the presence of either vehicle control (DMSO, blue) or 50 nmol/L unlabelled cTnI$_{AR}$ peptide (orange) in 384-well format. Means SEM, n=3-5.

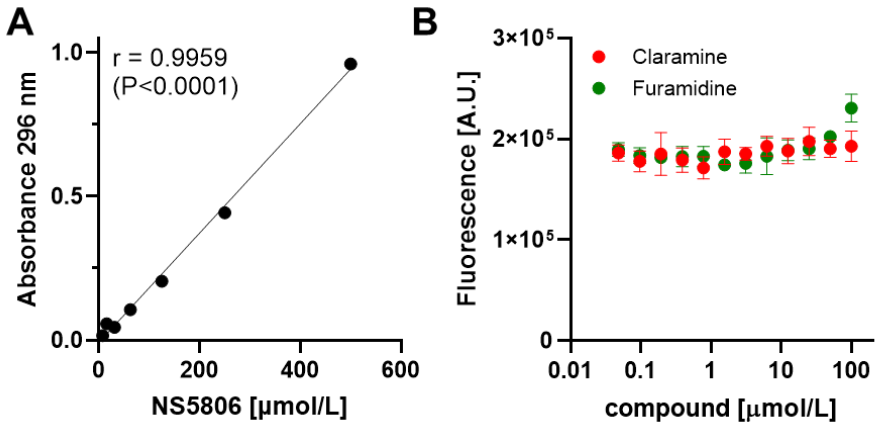


**Figure S2.** (A) Water solubility of NS5806. DMSO stocks of NS5806 were diluted with assay buffer to the indicated concentrations (fixed DMSO concentration of 0.1% (v/v)), potential aggregates removed by ultra-centrifugation and the absorbance at 296 nm plotted against the compound concentrations. The linear increase in absorbance suggests that NS5806 is soluble in aqueous buffers up to a concentration of at least 500 µmol/L. (B) Total fluorescent intensity dose-response curves for Claramine (red) and Furamidine (green) in the presence of 2 nmol/L FAM-cTnI$_{AR}$ and 5 nmol/L cTnC.

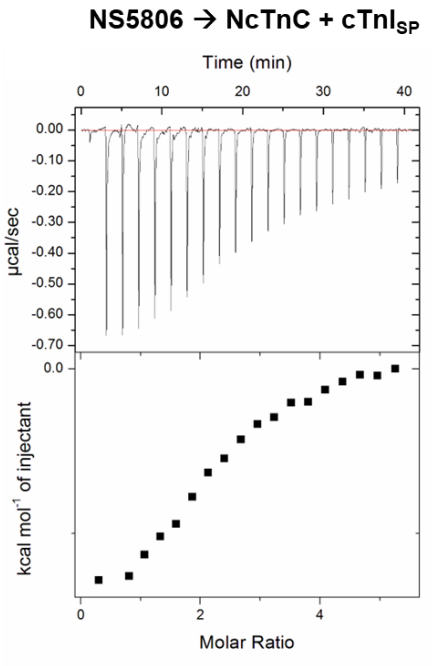


**Figure S3.** Thermogram of NS5806 titration into a mixture of the $Ca^{2+}$-saturated N-lobe of cTnC (NcTnC) and the cTnI switch peptide (SP).

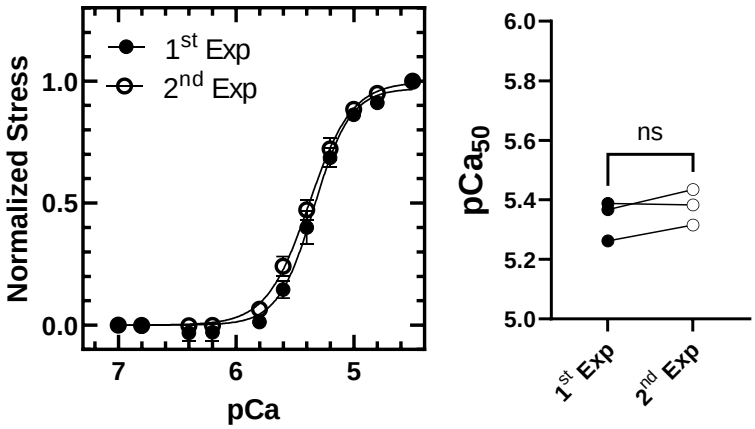


**Figure S4.** Force-pCa relation of demembranated human donor heart muscle strips for two consecutive experiments on the same preparation. Means ± SEM, n=3. Statistical significance of differences was assessed with a two-tailed student's t-test: ns-not significant.

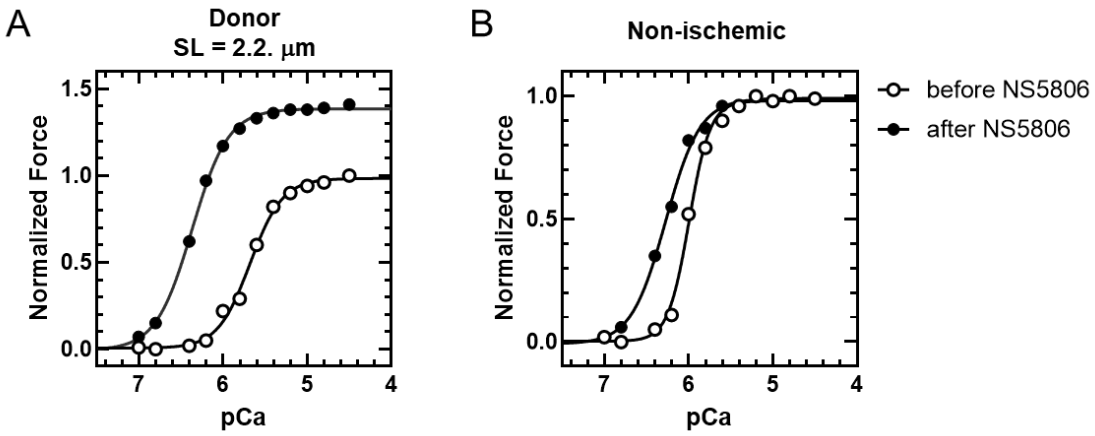


**Figure S5.** (A) Force-pCa relation of demembranated human donor heart muscle strips before (open circles) and after NS5806 incubation (closed circles) at long sarcomere length (2.2 μm). (B) Force-pCa relation of demembranated human heart muscle strips from ischemic heart failure patients before and after NS5806 incubation.

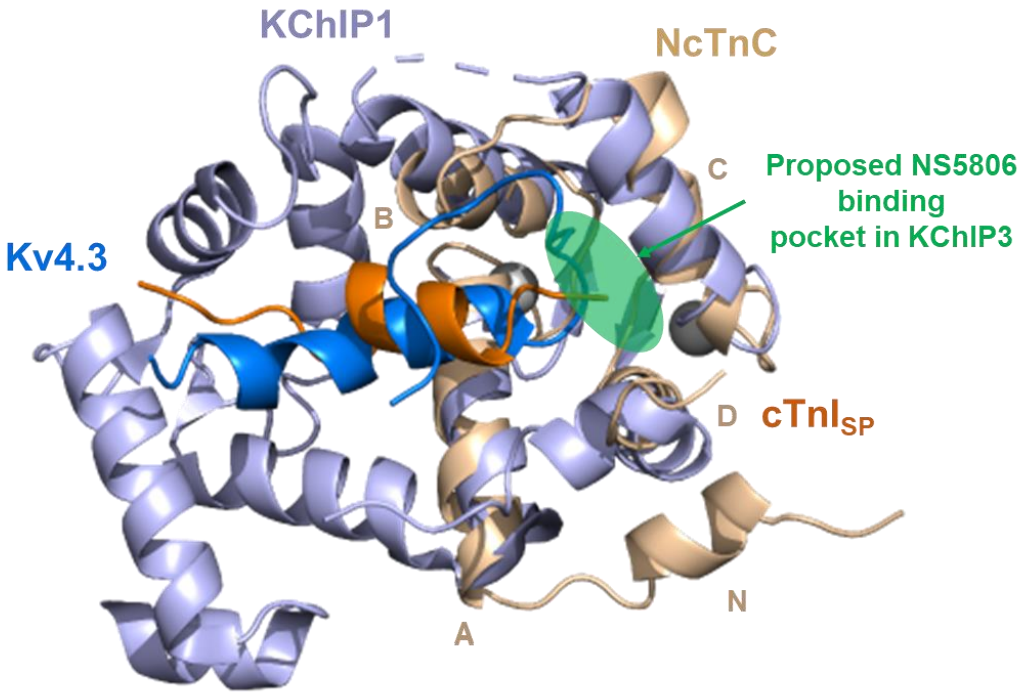


**Figure S6.** Structural alignment of the N-lobe of cTnC (NcTnC, yellow) bound to cTnI switch peptide (cTnI$_{SP}$, orange) (PDB entry 1MXL) with KChIP3 (purple) bound to a fragment of Kv4.3 (blue) (PDB entry 2I2R). NcTnC helices and proposed NS5806 binding site in KChIP are labelled accordingly.

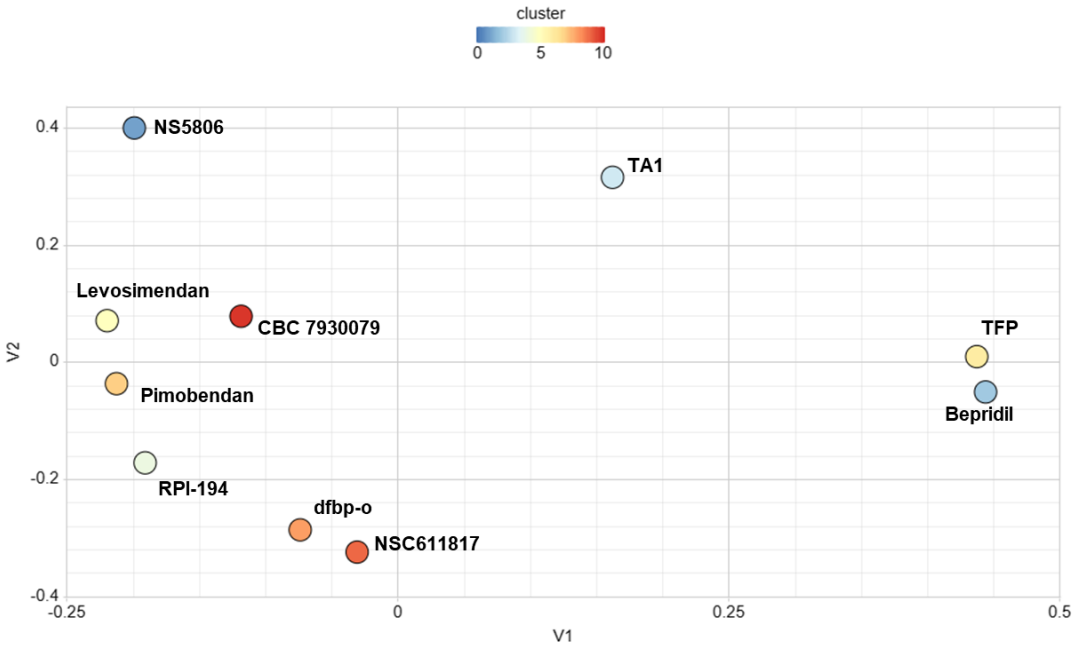


**Figure S7.** Structural clustering of known cardiac troponin C calcium sensitizers using multi-dimensional scaling (TFP – Trifluoperazine, CBC 7930079 – ChemBridge Compound 7930079).

## Supplementary Information Tables and Table Legends

**Table S1.** Hit compounds identified in $cTnI_{SP}$ primary screens.

| Library | Compound | FP (mP) |
|---|---|---|
| **ENZO Screenwell®** | Sertaconazole | 88.2 |
| | Clomiphene Citrate | 63.2 |
| | Gefitinib | 83.7 |
| | Chlorpromazine.HCl | 88.9 |
| | Fluphenazine.HCl | 76.1 |
| | Raloxifene.HCl | 76.9 |
| | Cinacalcet.HCl | 82.4 |
| | Fingolimod | 66.1 |
| | Clofazimine | 85.2 |
| | Toremifene Base | 60.7 |
| | Colistin Sulfate | 78.5 |
| | Thioridazine.HCl | 74.3 |
| | Tamoxifen Citrate | 69.1 |
| | Reserpine | 88.4 |
| | Sulconazole Nitrate | 87.6 |
| | Perphenazine | 73.2 |
| | Levothyroxine·Na | 119.8 |
| | Micafungin | 128.6 |
| **TOCRIScreen** | Tamoxifen citrate | 62.2 |
| | Raloxifene hydrochloride | 87.9 |
| | Raloxifene hydrochloride | 82.1 |
| | (S)-(+)-Niguldipine hydrochloride | 85.7 |
| | SR 33805 oxalate | 79 |
| | Tetrindole mesylate | 65 |
| | JTC 801 | 84.3 |
| | Bax channel blocker | 77.1 |
| | NSC 23766 | 89.5 |
| | PD 166285 dihydrochloride | 78.7 |
| | Dynole 34-2 | 64.1 |
| | Y 134 | 78.9 |
| | C 021 dihydrochloride | 87.1 |
| | NPS 2143 hydrochloride | 87.5 |
| | Bepridil hydrochloride | 87 |
| | GF 109203X | 82.4 |
| | PF 477736 | 64 |
| | CCT 137690 | 83.6 |
| | Ro 31-8220 mesylate | 57.9 |
| | Ro 3280 | 81.8 |
| | IKK 16 | 82 |
| | 10-DEBC hydrochloride | 82.8 |
| | PHA 665752 | 76.4 |
| | CASIN | 80.4 |
| | PQ 401 | 80.2 |
| | DDR1-IN-1 dihydrochloride | 74.4 |
| | BMS 599626 dihydrochloride | 88.2 |

| | | |
|---|---|---|
| | Mps1-IN-1 dihydrochloride | 84.6 |
| | GSK 1562590 hydrochloride | 89.9 |
| | TC-S 7003 | 73.5 |
| | FRAX 486 | 83.2 |
| | HKI 357 | 73.4 |
| | AZ 191 | 83.4 |
| | Sunitinib malate | 83.4 |
| | NVP ADW 742 | 68.4 |
| | AMG 548 | 90 |
| | XMD 8-87 | 81.2 |
| | AEE 788 | 77.8 |
| | CHR 6494 trifluoroacetate | 81.9 |
| | ERK5-IN-1 | 64.9 |
| | TAK 960 hydrochloride | 88 |
| | SU 11274 | 64.5 |
| | BS 181 dihydrochloride | 73.2 |
| | HTH 01-015 | 87.1 |
| | ZM 447439 | 89.2 |
| | Iressa | 81.8 |
| | UNC 1999 | 80.5 |
| | Furamidine dihydrochloride | 83.1 |
| | AZ 6102 | 83.4 |
| | WH-4-023 | 56.3 |
| | Prochlorperazine dimaleate | 78.2 |
| | FTY 720 | 71.6 |
| | Deltarasin | 87.7 |
| | DG 172 dihydrochloride | 81 |
| | Bazedoxifene acetate | 74.5 |
| | SP 100030 | 70.3 |
| | Aripiprazole | 86 |
| | Calpeptin | 121.8 |
| | CFTRinh 172 | 127.9 |
| | NS 3623 | 121.6 |
| | NS 5806 | 137.6 |
| **LOPAC®1280** | Chlorpromazine hydrochloride | 91.3 |
| | IKK-16 dihydrochloride | 84.3 |
| | Calmidazolium chloride | 83.6 |
| | Benazoline oxalate | 83.7 |
| | TNP | 90.9 |
| | Arbidol hydrochloride | 89.4 |
| | WZ4003 | 86.4 |
| | Enclomiphene hydrochloride | 78.4 |
| | CYM50358 | 84.1 |
| | Fluspirilene | 88.6 |
| | cis-(Z)-Flupenthixol dihydrochloride | 87.6 |
| | Ellipticine | 86.1 |
| | Fluphenazine dihydrochloride | 87.3 |
| | CID2858522 | 89.6 |
| | Methiothepin mesylate | 86.5 |
| | NNC 55-0396 | 88.7 |
| | AZ191 | 88.5 |

| | |
|---|---|
| BMS-189453 | 88.8 |
| Raloxifene hydrochloride | 85.5 |
| Pyridostatin trifluoroacetate salt | 85.6 |
| Tegaserod maleate | 78.4 |
| SID 3712249 | 86.6 |
| Ruthenium red | 83.7 |
| Trifluoperazine dihydrochloride | 84.5 |
| Lasofoxifene tartrate | 86.8 |
| CCT137690 | 87 |
| Thioridazine hydrochloride | 89.4 |
| HTH-01-015 | 87 |
| Furamidine dihydrochloride | 91.8 |
| Prochlorperazine dimaleate | 86.2 |
| Palmitoyl-DL-Carnitine chloride | 109.8 |
| 5HPP-33 | 112 |

**Table S2.** Hit compounds identified in $cTnI_{AR}$ primary screens.

| Library | Compound | FP (mP) |
|---|---|---|
| **ENZO Screenwell®** | Carboplatin | 67.3 |
| | Oxcarbazepine | 70.9 |
| | Cisplatin (Cis-Diamineplatinum(Ii) Dichloride ) | 61.9 |
| | Colistin Sulfate | 71.7 |
| | Chlorhexidine Dihydrochloride | 58.8 |
| | Indapamide | 116.5 |
| | Fomepizole | 118 |
| **TOCRIScreen** | WIN 64338 hydrochloride | 71 |
| | Furamidine dihydrochloride | 73.2 |
| | GSK 269962 | 104.8 |
| | A 425619 | 105.5 |
| | DQP 1105 | 103 |
| | GF 109203X | 104.5 |
| | TC-S 7010 | 108.3 |
| | AZD 3988 | 115.5 |
| | Repaglinide | 103.2 |
| **LOPAC®1280** | Icaritin | 64.6 |
| | KT203 | 71.7 |
| | Claramine | 53.9 |
| | SP600125 | 65 |
| | Cisplatin | 56.6 |
| | Mitoxantrone | 57.1 |
| | Ruthenium red | 44.3 |
| | cDPCP | 67.5 |
| | GW2974 | 117.8 |
| | Leukadherin-1 | 121.9 |
| | U-74389G maleate | 115.3 |
| | Tyrphostin AG 112 | 115.8 |

**Table S3.** Summary of steady-state dissociation constants ($K_d$ in μmol/L) of hit compounds binding to cardiac troponin constructs measured by isothermal titration calorimetry.

| | $Ca^{2+}$/NcTnC | $Ca^{2+}$/cChimera | cChimera | $Ca^{2+}$/NcTnC/cTnI$_{SP}$ | $Ca^{2+}$/cTnC |
|---|---|---|---|---|---|
| **NS5806** | 60.7 | 7.6 ± 0.3 | cnd | 18.7 | |
| **Furamidine** | | | | | 11.9 ± 5.5 |
| **Claramine** | | | | | 21.6 ± 1.8 |

Means ± SEM, n=1-5. cnd-cannot be reliably determined.