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## Identification of small molecule inhibitors of hyperpolarization-activated cyclic nucleotide gated channels

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### Abstract

Hyperpolarization-activated cyclic nucleotide gated (HCN) channels function in the brain to limit neuronal excitability. Limiting the activity of these channels has been proposed as a therapy for Major Depressive Disorder, but the critical role of HCN channels in cardiac pacemaking has limited efforts to develop therapies directed at the channel. Previous studies indicated that the function of HCN is tightly regulated by its auxiliary subunit, TRIP8b (tetratricopeptide repeat-containing Rab8b interacting protein), which is not expressed in the heart. To target the function of the HCN channel in the brain without affecting channels' function in the heart, we propose disrupting the interaction between HCN and TRIP8b. We developed a high throughput fluorescence polarization (FP) assay to identify small molecules capable of disrupting this interaction. We used this FP assay to screen a 20,000-compound library and identified a number of active compounds. The active compounds were validated using an orthogonal AlphaScreen assay to identify one compound (0.005%) as the first confirmed hit for inhibiting the HCN-TRIP8b interaction. Identifying small molecules capable of disrupting the interaction between HCN and TRIP8b should enable the development of new research tools and small molecule therapies that could benefit patients with depression.

### Keywords

High throughput screening; protein-protein interactions; fluorescence methods; HCN; TRIP8b

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## Introduction

Major Depressive Disorder (MDD) is a highly prevalent public health problem, affecting 1 in 5 people worldwide. Despite the enormous burden of MDD, antidepressant treatment options – primarily drugs targeting monoaminergic neurotransmitters – have not changed over the past half century<sup>1</sup>. Thus, development of new therapies targeting mechanisms other than monoaminergic pathways could address a major unmet clinical need.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are encoded by the HCN1–4 gene family and mediate a hyperpolarization-activated nonspecific cationic current<sup>2</sup>. HCN channels play an important role in governing the excitability of neurons in the brain and in regulating the rhythm of the heart<sup>2–4</sup>. Importantly, the expression of HCN channels in the brain is tightly regulated by an auxiliary subunit, tetratricopeptide repeat-containing Rab8b interacting protein (TRIP8b)<sup>5–9</sup>. Recent studies using knockout mice lacking HCN channel subunits and shRNA knockdown of HCN1 have linked reduced HCN channel function to antidepressant-like behavior<sup>9,10</sup>. Although these data suggest inhibiting HCN channels holds promise as a novel treatment for MDD<sup>11</sup>, HCN channels are also expressed at high levels in the heart and are critical for cardiac function<sup>12</sup>. As such, the challenge for anti-depressant therapies based on inhibition of HCN channel function is to identify a pharmacological approach that selectively targets HCN channels expressed in the brain while sparing HCN channels expressed in the heart.

TRIP8b is a brain-specific HCN channel auxiliary subunit that controls channel function and subcellular trafficking<sup>9</sup>. Genetic deletion of TRIP8b disrupts normal HCN channel trafficking within neurons and markedly reduces HCN channel function<sup>9</sup>. Consistent with an important role for HCN channel function in MDD, our lab previously noted that genetic deletion of TRIP8b also leads to antidepressant-like behavior<sup>9</sup>. As such, we reasoned that pharmacologically blocking the interaction between TRIP8b and HCN could disrupt HCN channel trafficking and function in the brain without affecting the function of HCN channels in the heart. Important from a drug-development standpoint, the brain specific expression of TRIP8b<sup>7</sup> would preclude off target effects of this pharmacological approach on HCN channels expressed in the heart. Consistent with this, we have observed that TRIP8b knockout mice have normal cardiac function (unpublished data) indicating that cardiac HCN channels remain unaffected.

TRIP8b is expressed as several distinct splice isoforms which differ only at the N-terminus<sup>8,9</sup>. All TRIP8b splice isoforms share a conserved C-terminus that interacts with the intracellular C-terminus of pore-forming HCN channel subunits (HCN1–4) at two distinct sites (Figure 1)<sup>13,14</sup>. The first site is an interaction between the cyclic nucleotide binding domain (CNBD) of HCN and an acidic amino acid sequence located N-terminal to the tetratricopeptide (TPR) domains of TRIP8b<sup>7,13</sup>. The second interaction occurs between the C-terminal tripeptide of HCN (-SNL in HCN1, 2 and 4; -ANM in HCN3) and the TPR domains of TRIP8b<sup>15</sup>.

Our lab has previously shown that disrupting either interaction site impairs the ability of TRIP8b to interact with HCN channels and limits HCN channel function<sup>13</sup>. Furthermore,

others recently demonstrated that disruption of just the interaction between the TRIP8b TPR domains and the HCN tripeptide is sufficient to impair HCN trafficking *in vivo*<sup>5</sup>. Given that blocking the TPR-tripeptide interaction is sufficient to inhibit HCN channel function and that the CNBD of HCN has significant homology with many other mammalian proteins<sup>16</sup>, we reasoned that targeting the TPR-tripeptide interaction would be optimal for inhibiting HCN channel function while minimizing the risk of developing molecules with off-target activity against other proteins containing CNBDs.

In this study, we examined the terminal tripeptide interaction of TRIP8b with each HCN isoform using fluorescence polarization (FP). We also developed an FP-based high throughput screening assay to identify small molecules capable of disrupting the TRIP8b-HCN1 interaction, and we employed the assay to screen a library of 20,000 small molecules. The screen identified one compound that was validated using an orthogonal AlphaScreen assay. To our knowledge, this compound represents the first described inhibitor of the TRIP8b-HCN1 interaction.

## Materials and Methods

### TRIP8b constructs

All restriction enzymes were purchased from New England Biolabs. All oligonucleotides for use in PCR amplification were synthesized by Integrated DNA Technologies (Coralville, IA).

A TRIP8b protein with a C terminal His tag was generated by PCR using forward primer 5'-ata gcg cca tgg aat tcg aaa ggg caa aag cgg cag, and reverse primer 5'-ctt tca att tgg atc ctt gac ccg ggc tcg agg cgg cg followed by subcloning into a modified pGS21-a vector (Genscript, Piscataway, NJ) with the deletion of GST tag at *NcoI/XhoI* sites. A protein expression vector containing His tagged full length TRIP8b (1b-2) isoform was generated by PCR using forward primer 5'-ata gcg cca tgg ctg aca gtg aaa tgg atg gaa g, and reverse primer 5'-ctt tca att tgg atc ctt gac ccg ggc tcg agg cgg cg followed by subcloning into the modified pGS21-a vector (Genscript, Piscataway, NJ). GST-HCN1<sub>C40</sub> containing the last 40 C terminal amino acids was generated by PCR using forward primer 5'-gtg cga att cat ccc ccc caa ccg agg and reverse primer 5'-gtg cct cga gtc ata aat tcg aag caa aac gg followed by subcloning into pGEX-4T-1 (GE healthcare) using *EcoRI/XhoI* sites.

### Purification of His-TRIP8b and His-HCN protein

To produce recombinant proteins, BL21 bacterial cultures (Agilent technologies, Santa Clara, CA) transformed with appropriate vectors were grown in LB broth (Invitrogen, Carlsbad, CA, USA) containing 50 mg/ml kanamycin with vigorous agitation at 37 °C. Isopropyl beta-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 1 mM after the OD 600 reached 1–1.2 followed by overnight induction at 18 °C. The bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4 °C and then washed with cold phosphate buffered saline (PBS, 140mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3). Bacteria were re-suspended in buffer A (50mM NaPhosphate, 300mM NaCl, 10% Glycerol, pH 8.0). The suspended cells were disrupted by

sonication on ice and centrifuged at 12,000 g for 20 min. The supernatant was applied to the affinity matrix Ni-NTA agarose column with a bed volume of 2ml, which had previously been washed and equilibrated with buffer A. The column was washed with 500 ml buffer A and 300 ml buffer B (50mM NaPhosphate, 300mM NaCl, 10% Glycerol, pH 6.0) as well as 150 ml buffer A containing 5 mM imidazole. Protein was eluted with buffer A containing 200 mM imidazole and dialyzed overnight at 4 °C with PBS.

### Purification of GST-HCN1<sub>C40</sub> protein

To produce recombinant GST-HCN1<sub>C40</sub> protein, BL21 bacterial cultures transformed with pGEX-HCN1<sub>C40</sub> were grown in LB broth (Invitrogen, Carlsbad, CA, USA) containing 50 mg/ml ampicillin with vigorous agitation at 37 °C. IPTG was added to the medium at a final concentration of 1 mM after the OD 600 reached 0.8 followed by an additional 1h induction. The bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4 °C and then washed with cold 1X PBS (140mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and re-suspended in lysis buffer (PBS containing 1% Triton X-100). The suspended cells were disrupted by sonication on ice and the cell solution was centrifuged at 12,000 g for 20 min. The supernatant was applied to the affinity matrix glutathione sepharose 4B column (GE healthcare) with a bed volume of 2ml, which had previously been washed and equilibrated with PBS. The column was washed three times with 20 ml 1X PBS containing 500 mM NaCl and then the fusion protein was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, containing 2 mM reduced glutathione). The eluted protein was then dialyzed overnight at 4 °C with PBS.

### Fluorescence polarization

Fluorescein isothiocyanate (FITC) labeled HCN peptides (HCN1: DAEKPRFASNL; HCN2: DSARSRLSSNL; HCN3: TPRGPQISANM; HCN4: EPVRSKLPNSL) were purchased from GenScript.

For the  $K_d$  measurements, a series of two-fold dilutions of TRIP8b protein and 100 nM of final concentration of FITC labeled peptide were mixed in protein dilution buffer (50mM NaH<sub>2</sub>PO<sub>4</sub>, 50mM KCl, 1mM DTT, pH 6.5). Before reading, the plate was allowed to incubate for 15 min at room temperature in the dark. All experiments were performed in triplicate in black 384-well microtiter plates (Corning). The polarization measurements were obtained using Tecan microplate reader (Tecan) at the Structural Biology facility at Northwestern University, which is generously supported by NCI CCSG P30 CA060553 of the Robert H. Lurie Comprehensive Cancer Center. All curve fitting and data analysis were performed using Igor Pro (WaveMetrics). As described below, curves were fit by the Hill equation<sup>17</sup> based on a previously described protocol<sup>18</sup>, with  $Y = (X^n) / (K_d + X^n)$  where Y is the fraction of binding sites occupied, X is the concentration of ligand,  $K_d$  is the dissociation constant, and n is the Hill coefficient.

To calculate the affinity of full length TRIP8b and TRIP8b(241-602) for FITC-HCN1, a serial dilution of FITC-HCN1 was added to a fixed concentration of 2  $\mu$ M TRIP8b protein.

For the IC<sub>50</sub> measurement, a serial dilution of unlabeled peptide was titrated into protein dilution buffer containing the fixed concentration of 100 nM FITC-HCN peptide and 2 μM of TRIP8b(241-602).

Z factor<sup>19</sup> was calculated as  $Z = 1 - 3 \times (\sigma_{\text{pos}} + \sigma_{\text{neg}}) / (\mu_{\text{neg}} - \mu_{\text{pos}})$ .

### High throughput screening

The mixture of purified TRIP8b(241-602) protein (2 μM) and FITC-HCN1 (50 nM) were dispensed into 384 well plates using Viafill microplate dispenser (Integra Biosciences). Two compounds were pooled into each well at 40 μM per compound using a Labcyte Echo550 acoustic liquid handler. Each plate contained 16 negative controls with DMSO only (no test compound), and 16 positive controls with FITC-HCN1 probe alone (no TRIP8b). Fluorescence polarization (mP) was measured using an AnalystGT (Molecular Devices) after a 2 hour incubation at room temperature. Values were normalized to percent inhibition using the average positive and negative controls from each plate and plotted using TIBCO Spotfire. For dose response testing, compounds were serially diluted in DMSO and dispensed using the Labcyte Echo550 into a mixture of TRIP8b (2 μM) and carboxytetramethylrhodamine (TAMRA)-conjugated HCN1 probe (50 nM) at a final DMSO concentration of 0.4%. Samples were incubated for 3 hours at room temperature before reading on the AnalystGT. IC<sub>50</sub> values were calculated in Graphpad Prism using a four-parameter nonlinear regression model.

### AlphaScreen

A mixture of GST-tagged HCN1<sub>c40</sub> protein (20 nM) and His-tagged TRIP8b (residues 241-602, as in the FP assay, 200 nM) was incubated for 3 hours in the presence of different concentrations of a test compound (0.2–40 μM) at a final DMSO concentration of 0.25%. Anti-GST AlphaScreen Acceptor beads were then added for 1.5 hours, followed by addition of AlphaScreen Nickel chelate Donor beads for 1.5 hours. All incubations were at room temperature. A PerkinElmer EnSpire multimode plate reader was used to detect the AlphaScreen signal.

## Results

### Interaction of TRIP8b and HCN by fluorescence polarization

Fluorescence polarization (FP) is a sensitive, robust, and quantitative method for the study of protein interactions and for high throughput screening in drug discovery<sup>20</sup>. The technique involves exciting a ligand-tagged fluorophore with polarized light and measuring the polarity of emitted fluorescence. In the presence of a binding partner, the rotational motion of the ligand is decreased and the emitted light is polarized to a greater degree than in the absence of the binding partner.

Because we are interested in identifying inhibitors of the interaction between the TPR domains of TRIP8b and the C terminal tripeptide of HCN, we first sought to generate stable and soluble TRIP8b protein capable of mediating this interaction. We cloned and purified both full length TRIP8b and TRIP8b(241-602) (see *Methods* above), a fragment lacking the

structurally disorganized N-terminus of the molecule that is not involved in binding HCN1-4<sup>13</sup>. Additionally, we synthesized a peptide consisting of the C-terminal 11 amino acids of HCN1 conjugated to FITC. We then titrated increasing concentrations of the FITC labeled HCN1 peptide into a fixed concentration of each of the two TRIP8b proteins. As can be seen in Figure 1, the  $K_d$  was similar for the two proteins (Full length TRIP8b  $K_d=1.03\pm 0.02\mu\text{M}$ , TRIP8b(241-602)  $K_d=0.8\pm 0.04\mu\text{M}$ ) suggesting that the TRIP8b C-terminal fragment is capable of reproducing the interaction between the TPR domain and the tripeptide tail of HCN, which is consistent with recent findings<sup>21</sup>. Because the smaller TRIP8b(241-602) peptide was more easily purified and was more stable under the conditions of our assays, we proceeded using this version.

We next sought to examine the differences in interactions between the TRIP8b TPR domain and the C-termini of the distinct HCN subunits. Towards this end, we synthesized peptides consisting of the 11 C-terminal amino acids from each HCN subunit conjugated to fluorescein (see *Methods*, referred to with a subscripted 'P' for 'Peptide' as in HCN1<sub>P</sub>). In Figure 2, we titrated increasing concentrations of TRIP8b(241-602) in the presence of a fixed quantity of the labeled peptides. We noted that HCN1<sub>P</sub>, HCN2<sub>P</sub>, and HCN4<sub>P</sub> all had similar affinities near 1 $\mu\text{M}$  (HCN1<sub>P</sub>:  $K_d=1.23\pm 0.09\mu\text{M}$ , HCN2<sub>P</sub>:  $K_d=0.91\pm 0.03\mu\text{M}$ , HCN4<sub>P</sub>:  $K_d=0.81\pm 0.02\mu\text{M}$ ). In contrast, the affinity of TRIP8b(241-602) for HCN3<sub>P</sub> was lower (HCN3<sub>P</sub>:  $K_d=10.25\pm 1.08\mu\text{M}$ ). Given that HCN3 is expressed at low levels throughout the brain<sup>22</sup> and has a lower affinity for TRIP8b<sup>6</sup>, we focused our subsequent efforts on HCN1, HCN2, and HCN4.

We next investigated if the interaction between the labeled HCN tripeptide and TRIP8b(241-602) could be disrupted. In addition to our fluorescein labeled peptides, we synthesized unlabeled peptides of HCN1, HCN2, and HCN4 (referred to as HCN1<sub>U</sub> for unlabeled). In Figure 3, we titrated increasing concentrations of unlabeled peptide into a fixed concentration of TRIP8b(241-602) and the corresponding labeled peptide (i.e. HCN1<sub>U</sub> against HCN1<sub>P</sub>). For all three peptides, we found roughly similar  $IC_{50}$  values for disrupting the interaction of TRIP8b and the corresponding HCN<sub>P</sub> (HCN1<sub>P</sub>:  $IC_{50}=0.89\pm 0.04\mu\text{M}$ , HCN2<sub>P</sub>:  $IC_{50}=0.76\pm 0.02\mu\text{M}$ , HCN4<sub>P</sub>:  $IC_{50}=1.93\pm 0.17\mu\text{M}$ ). These results indicate that the interaction between the SNL tripeptide and TRIP8b can be disrupted for HCN1, 2, and 4.

In order to identify small molecules capable of disrupting the interaction between TRIP8b and HCN, we developed an assay that could be used for high throughput compound screening. Our strategy was to use the fluorescence polarization assay with labeled HCN1 peptide and TRIP8b(241-602). To examine the utility of this approach, we first examined HCN1<sub>U</sub> as a positive control to displace HCN1<sub>P</sub>, HCN2<sub>P</sub>, and HCN4<sub>P</sub>. In Figure 4, we titrated increasing concentrations of HCN1<sub>U</sub> into fixed concentrations of TRIP8b(241-602) with the labeled HCN peptide indicated. As can be seen in Figure 4, HCN1<sub>U</sub> displaced each labeled HCN peptide with similar affinities (HCN1<sub>P</sub>:  $IC_{50}=0.70\pm 0.05\mu\text{M}$ , HCN2<sub>P</sub>:  $IC_{50}=1.77\pm 0.02\mu\text{M}$ , HCN4<sub>P</sub>:  $IC_{50}=1.60\pm 0.08\mu\text{M}$ ).

We subsequently tested the assay in 384-well format either in the presence or absence of HCN1<sub>U</sub> (to serve as a positive control for inhibition), and calculated a Z factor of 0.896 (mean  $\pm$  standard deviation: no competitor  $224.25 \pm 3.07$ , with competitor  $44.61 \pm 3.15$  mP,

n=384). Together, these data indicate that the assay is quantitative, robust, and well suited for primary high throughput screening (Figure 5).

### FP-based High throughput screen

To evaluate the FP assay in a high throughput screening (HTS) setting, we conducted a screen of a 20,000-compound diverse drug-like library (see Figure 5F for details) at the Northwestern University High Throughput Analysis (HTA) Lab. The high throughput screen was run in duplicate using pools of two compounds in each well, each at 40  $\mu$ M. Compounds were incubated with a mixture of TRIP8b(241-602) protein (2  $\mu$ M) and FITC-HCN1<sub>P</sub> (50 nM). The addition of DMSO (no compound) served as a negative control, and HCN1<sub>u</sub> peptide served as a positive control. Our HTS identified 32 active pools that showed >50% inhibition of the interaction in both HTS runs (Figure 5). Figure 5B is an alternate visualization of the HTS data plotting the average percent inhibition of the TRIP8b-HCN interaction from both HTS runs against all compound pools from the screen. Positive and negative controls are plotted to illustrate data consistency. Active compounds (pink) were selected from the plot in Figure 5A, and therefore not every compound with average percent inhibition >50% was considered a hit. To determine which individual compound had activity in a given active pool, the compounds were re-tested in duplicate at a single concentration using a TAMRA-conjugated HCN1 probe. The TAMRA probe is an alternate readout that emits in a distinct spectral region compared to the FITC probe, and was used to eliminate artifact hit compounds that interact with fluorescein or emitted in an overlapping spectral region. Using the TAMRA HCN1 probe, we found that five of our original hits exhibited >50% inhibition of binding in both replicates (Figure 5A). Thus, we observed an overall validated hit rate of 0.16% (in the pooled format) and a validated hit rate (compounds showing >50% inhibition at 40  $\mu$ M with both FITC and TAMRA conjugated probes) of 0.025%. From this set of hits, we identified one compound with a favorable structure for downstream medicinal chemistry optimization, NUCC-5953. We therefore characterized the potency of NUCC-5953 by performing a dose response titration of this compound using the TAMRA-conjugated HCN1 probe and obtained an IC<sub>50</sub> value of 24  $\mu$ M (Figure 5D).

### AlphaScreen assay

To confirm that our active compound, NUCC-5953, disrupts the TRIP8b-HCN1 interaction (i.e., its activity in the FP assay is not due to fluorescence interference), we developed an orthogonal assay utilizing AlphaScreen technology. In an AlphaScreen assay, signal is generated when Donor and Acceptor beads are brought within proximity of each other (~200 nm); red laser excitation causes singlet oxygen to “tunnel” from the Donor to the Acceptor, generating a luminescent signal that is proportional to the number of proximal bead pairs in solution<sup>23</sup>. Since AlphaScreen enables monitoring of larger protein complexes than FP, we employed a larger more physiologically relevant HCN1-based peptide in this assay that contained 40 residues from the C-terminal end (including the -SNL), called HCN1<sub>C40</sub> (Figure 6A). For detection, a mixture of GST-tagged HCN1<sub>C40</sub> protein and TRIP8b(241-602) was incubated at room temperature for 3 hours in the presence of a range of test compound concentrations (0.2–40  $\mu$ M). In this assay, NUCC-5953 yielded an IC<sub>50</sub> value of 2.5  $\mu$ M (Figure 6B), thus confirming it as an inhibitor of the TRIP8b-HCN interaction.

## Discussion

In this study, we developed an FP assay for identifying molecules that inhibit the interaction between TRIP8b and HCN and developed a secondary AlphaScreen assay to confirm the validity of FP-identified hits..

In the course of developing the FP screening assay, we made several observations regarding the structure of the interaction between HCN and TRIP8b. When examining the differences in binding the C-terminal peptides of distinct HCN subunits to TRIP8b, we found that HCN1, 2, and 4 C-termini all had nearly equal affinities for TRIP8b while HCN3 had a significantly lower affinity. This is consistent with predictions from the crystal structure for the interaction between the terminal tripeptide of HCN2 and TRIP8b<sup>15</sup>. In particular, HCN3 has an 'ANM' tripeptide instead of the 'SNL' found in HCN1, 2, and 4. The serine to alanine change is predicted to cause a loss of a stabilizing interaction with Tyr-472 of TRIP8b. The terminal leucine residue of HCN2 ordinarily rests in a hydrophobic pocket formed by Val-343, Thr-346, Phe-348, and Thr-458 of TRIP8b. The presence of a hydrophobic methionine at this position in HCN3 likely preserves this interaction albeit with lower affinity given the change from a branched residue (leucine) to a linear one (methionine). Consistent with these results, we did not notice any substantial differences when using an unlabeled HCN1 peptide to displace the labeled HCN peptides from TRIP8b (Figure 4).

We subsequently developed a high throughput assay based on the FP screen to identify compounds that are capable of disrupting the tripeptide interaction between TRIP8b and HCN1. Our assay has significant utility for screening given its high Z factor value (0.896). After screening 20,000 compounds, we identified 1 hit (0.005%) with favorable chemical properties that we validated using our novel AlphaScreen binding assay. Although our hit molecule has limited potency (2.5 $\mu$ M, in our AlphaScreen assay), it provides proof of concept that the TRIP8b-HCN interaction can be pharmacologically modulated, and it will serve as a starting point for subsequent hit-to-lead optimization to generate more potent probe molecules.

The utility of identifying an inhibitor of the TRIP8b-HCN interaction is as a novel antidepressant. We previously demonstrated that that genetic deletion of TRIP8b, HCN1 or HCN2 (the predominant channel subunits in the brain<sup>22</sup>) leads to an increase in antidepressant-like behavior in mice<sup>9</sup>. Consistent with our results, others found that shRNA knock down of HCN1 in the dorsal hippocampus produced a similar antidepressant-like phenotype in rats<sup>10</sup>. These results strongly suggest that limiting HCN channel function in the brain produces antidepressant behavior<sup>11</sup>.

With respect to treating depression, the benefit of developing an inhibitor of the TRIP8b-HCN interaction as opposed to targeting the HCN channel pore is in limiting potential cardiac effects. Given that HCN channels are expressed in the heart without TRIP8b, inhibitors of this interaction should not produce cardiac effects. In contrast, subcellular trafficking of HCN by TRIP8b is critical for channel function in the brain, and the C-terminal tripeptide interaction with TRIP8b is required for normal channel trafficking<sup>5</sup>. In



the CA1 region of the hippocampus, where inhibition of HCN channels leads to antidepressant-like effects<sup>10</sup>, TRIP8b creates a gradient of HCN1 in the pyramidal neuron dendrites that requires the terminal tripeptide interaction with TRIP8b<sup>6</sup>. We thus reasoned that inhibitors of the interaction between the terminal tripeptide of HCN and the TPR domains of TRIP8b will block brain HCN channel function without affecting cardiac HCN channels that lack TRIP8b.

Our work has identified the first small molecule inhibitor of the interaction between HCN and TRIP8b, an interaction that has been shown to be a potential drug target for MDD. In addition to structure optimization of our identified hit, future work will focus on screening additional compound libraries using the workflow described here to identify more potent inhibitors with novel chemotypes. These efforts will aid in the development of new research tools as well as possible therapies for the treatment of MDD.

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## Abbreviations

|               |   |
|---------------|---|
| <b>HCN</b>    | Hyperpolarization-activated cyclic nucleotide-gated           |
| <b>CNBD</b>   | cyclic nucleotide binding domain                              |
| <b>TPR</b>    | tetratricopeptide repeat                                      |
| <b>TRIP8b</b> | tetratricopeptide repeat-containing Rab8b interacting protein |
| <b>FP</b>     | fluorescence polarization                                     |
| <b>cAMP</b>   | 3',5'-cyclic adenosine monophosphate                          |
| <b>FITC</b>   | fluorescein isothiocyanate                                    |
| <b>TAMRA</b>  | tetramethylrhodamine  |
| <b>GST</b>    | glutathione-S-transferase                                     |
| <b>His</b>    | histidine   |
| <b>PBS</b>    | phosphate buffered saline                                     |
| <b>IPTG</b>   | isopropyl beta-D-1-thiogalactopyranoside                      |

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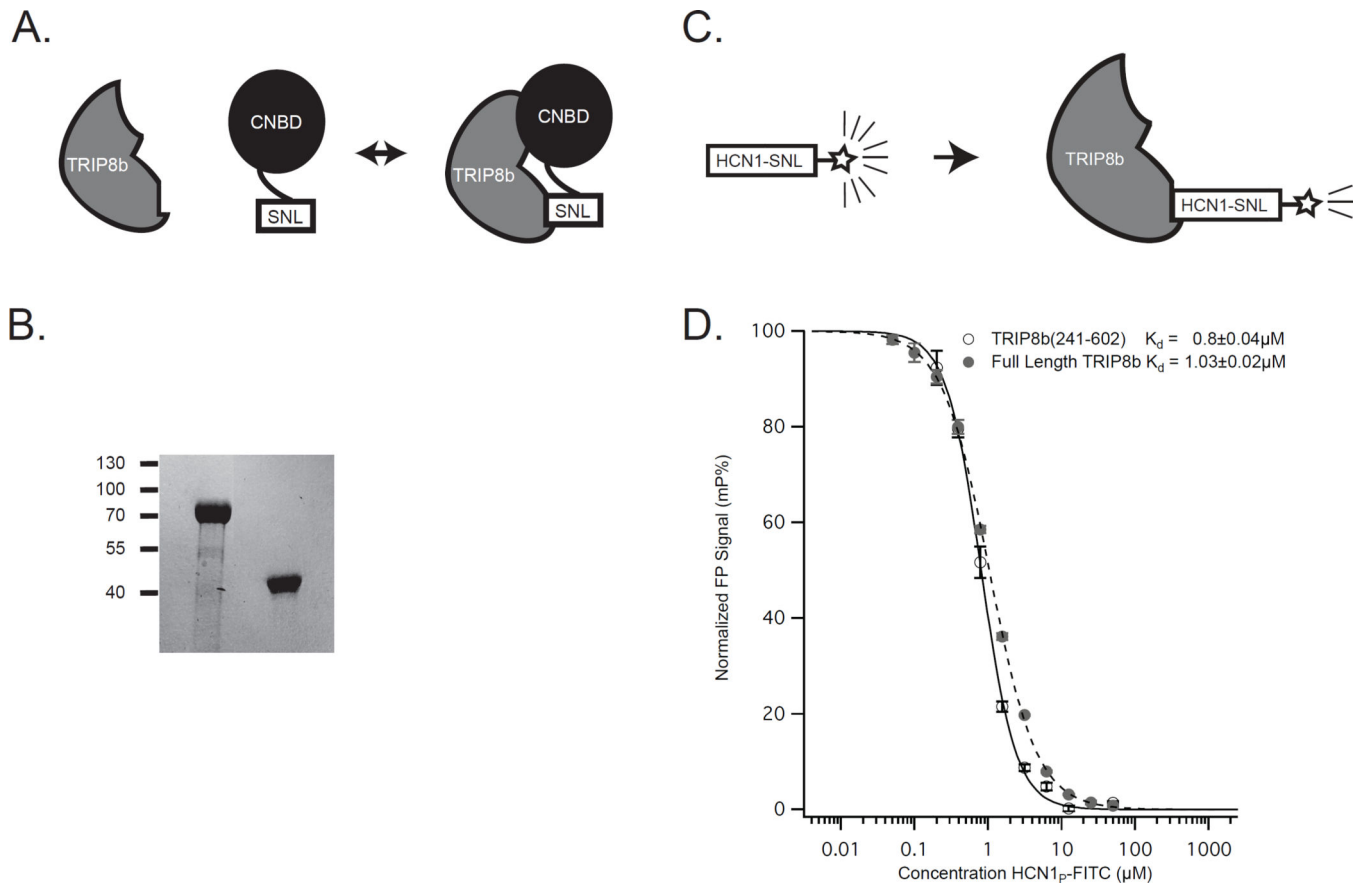
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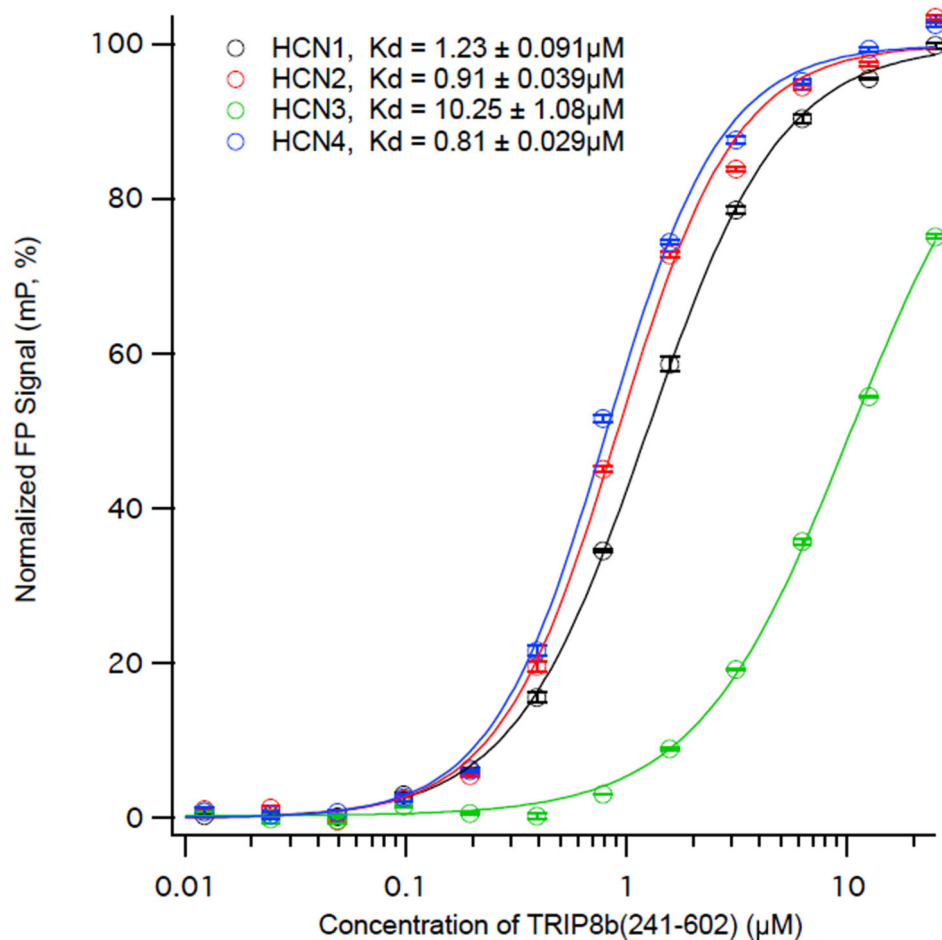
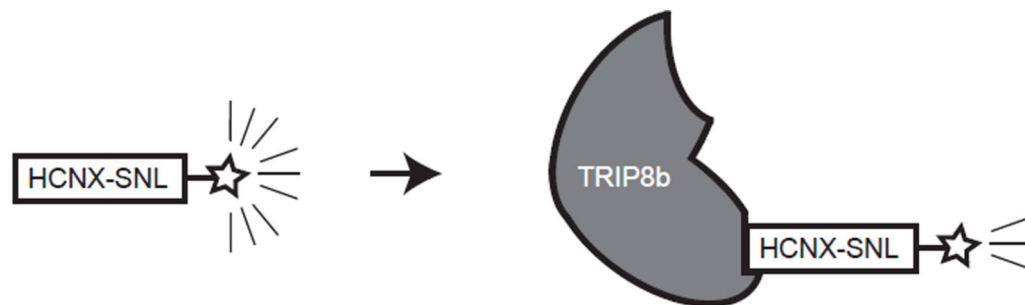
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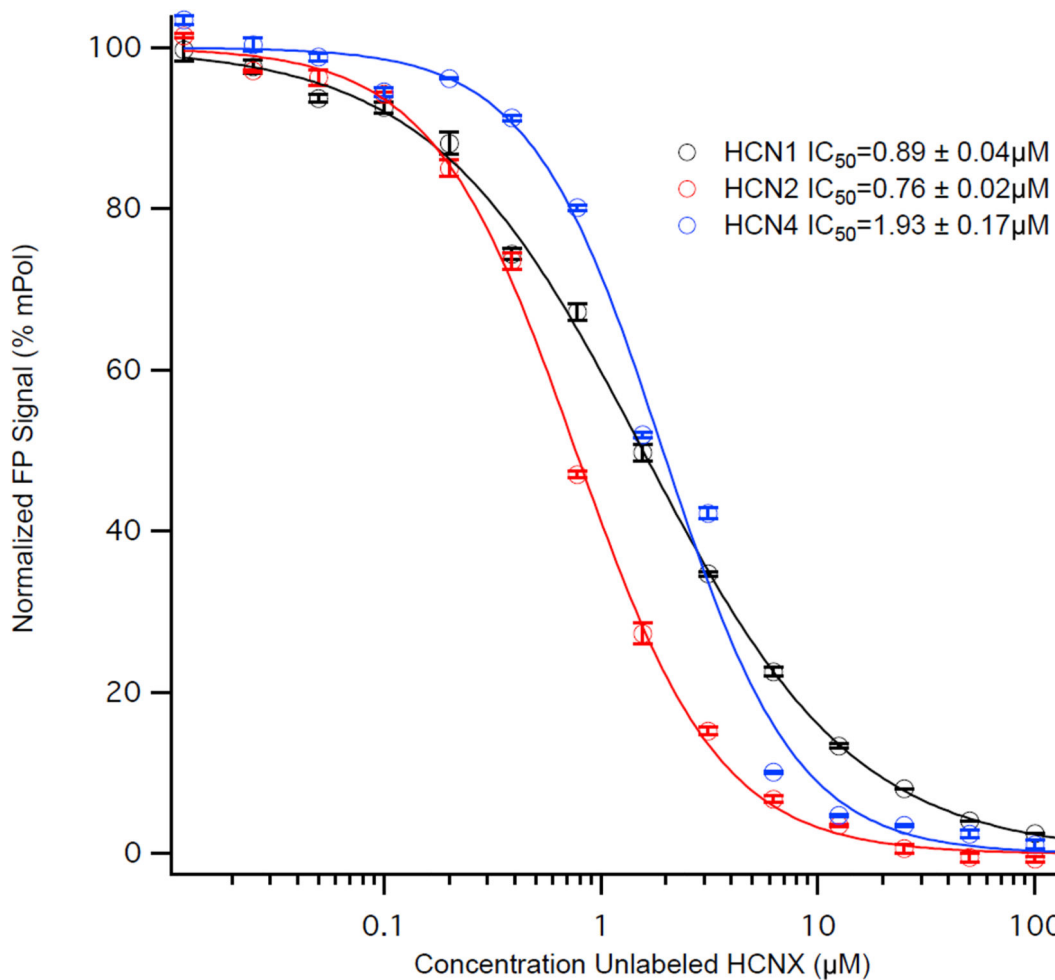
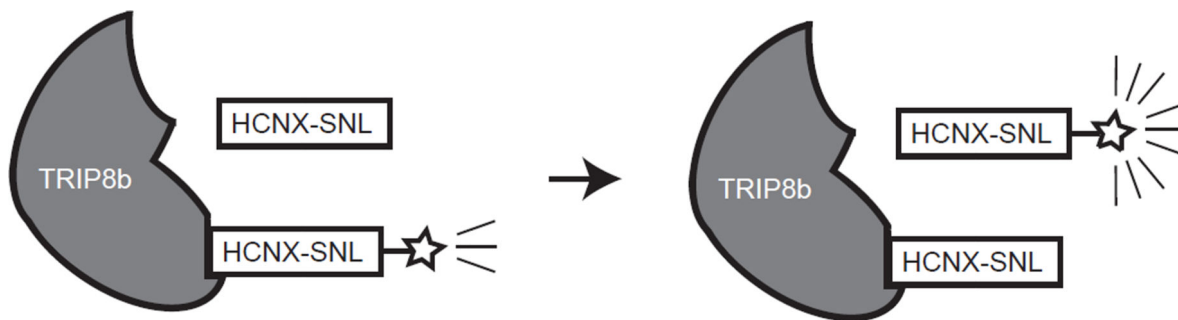


**Figure 1. The interaction of TRIP8b and HCN**

**A.** Schematic showing the interaction between TRIP8b and HCN. Although two interaction sites exist, the downstream interaction between the TPR domains of TRIP8b and the SNL tripeptide of HCN channels is crucial for surface trafficking and function of the channel. **B.** Coomassie stain showing the full length TRIP8b at left and the shorter TRIP8b(241-602) on the right. Molecular weight markers are indicated on the left. **C.** Schematic showing the fluorescence polarization assay displayed in **(D)**. **D.** The fluorescence polarization signal for each concentration of HCN1 peptide is shown. By titrating in increasing quantities of HCN1 peptide labeled with a FITC tag, we observed similar affinities of the two TRIP8b peptides for HCN1.

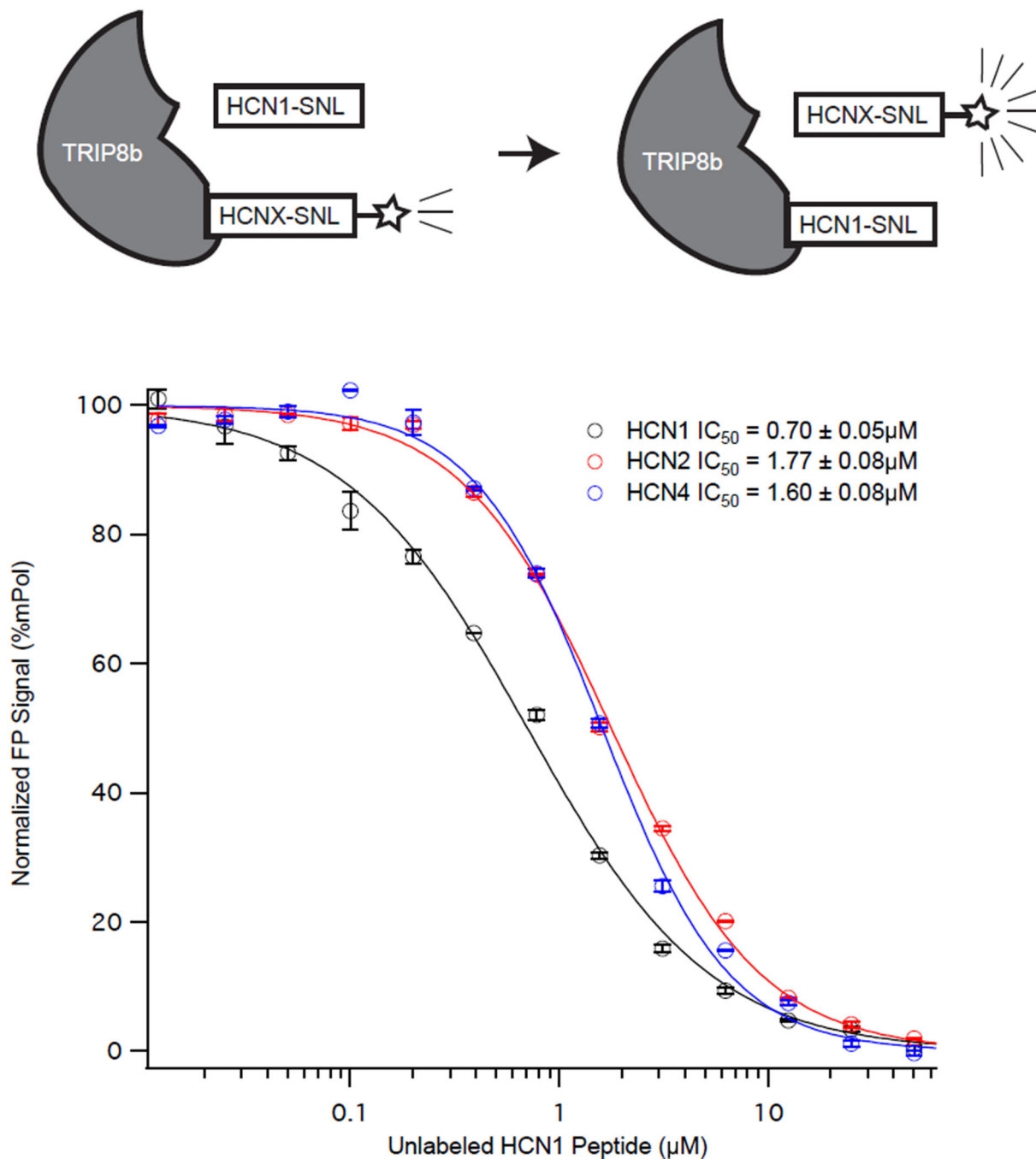


**Figure 2. The interaction of TRIP8b and HCN 1, 2, 3, and 4 by FP assay**  
 TRIP8b(241-602) binds to HCN3 with less affinity than HCN1,2, and 4. We titrated increasing concentrations of TRIP8b(241-602) into a fixed concentration of the indicated FITC labeled HCN isoform (see the schematic above).

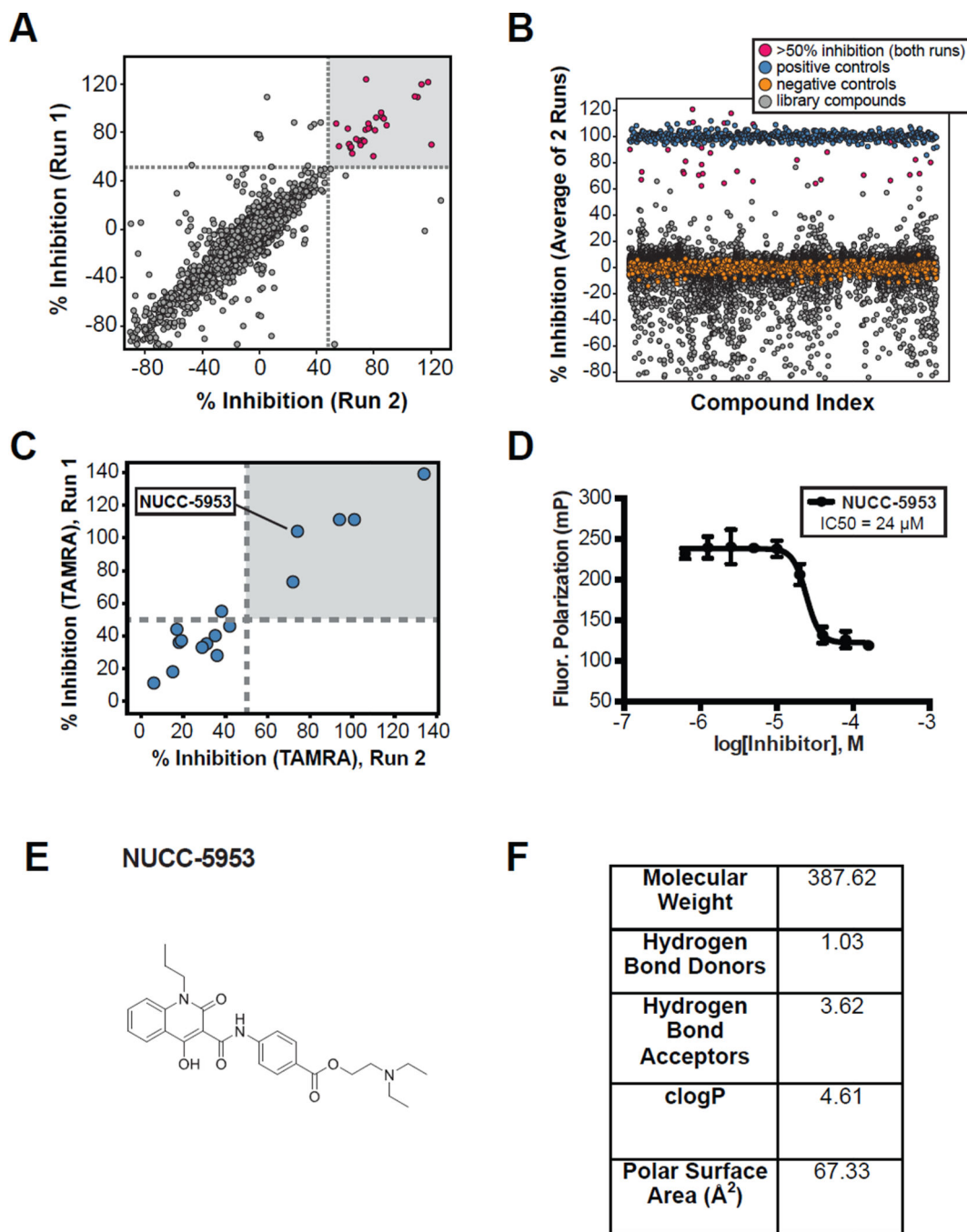


**Figure 3. Unlabeled HCN peptides displace labeled HCN peptides from TRIP8b(241-602)**

In order to examine differences in the ability of the distinct HCN peptides to be displaced from TRIP8b(241-601), we titrated increasing concentrations of each unlabeled HCN peptide into a fixed concentration of labeled HCN peptide and TRIP8b(241-602). For example, unlabeled HCN2 peptide was titrated against FITC labeled HCN2 peptide, as in the schematic above.



**Figure 4. Unlabeled HCN1 peptide displaces labeled HCN peptides from TRIP8b(241-602)** HCN1 binds TRIP8b(241-601) with less affinity than either HCN2 or HCN4. To examine differences in the binding affinity of the different HCN peptides to TRIP8b(241-602) we titrated increasing concentrations of unlabeled HCN1 peptide into a fixed concentration of FITC labeled HCN peptide and TRIP8b(241-602) (as in the schematic).



**Figure 5. Summary of high throughput screening assay performance**

**A.** Comparison of the percent inhibition for compounds in two distinct runs of the assay. Note that linearity indicates a highly consistent assay. **B.** Graph showing the percent inhibition (as an average of the two runs) for the compounds screened. **C.** Hits identified on the FITC screen were subsequently assayed using a TAMRA labeled HCN peptide to rule out compounds that interact with FITC. **D.** NUCC-5953 was identified as an inhibitor in both the FITC screen and follow up TAMRA screen. Subsequent dose response testing



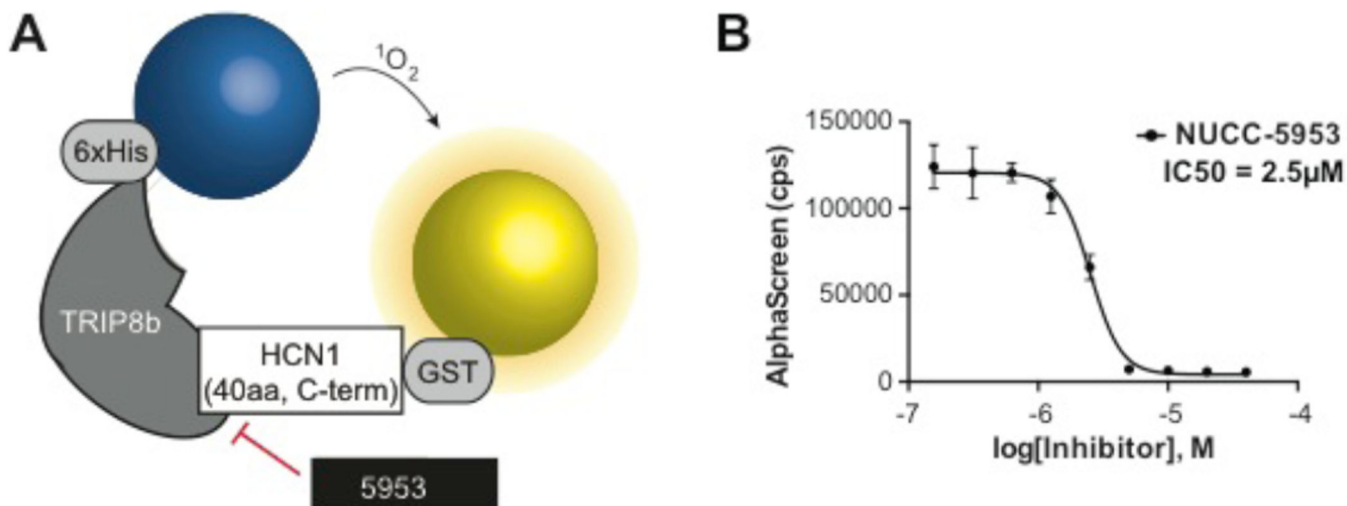
using the TAMRA labeled HCN probe is shown. **E.** Structure for compound NUCC-5953 is shown. **F.** Table with properties of the screening library.

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**Figure 6. AlphaScreen testing**

**A.** Schematic showing the mechanism underlying the AlphaScreen assay sees text for details. **B.** Dose response curve generated from compound NUCC-5953 using the AlphaScreen assay with IC<sub>50</sub> value of 2 μM.