Video Article

Method for Identifying Small Molecule Inhibitors of the Protein-protein Interaction Between HCN1 and TRIP8b

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URL: http://www.jove.com/video/54540
DOI: doi:10.3791/54540

Keywords: Biochemistry, Issue 117, High-throughput screening, drug discovery, protein-protein interactions, fluorescence polarization, HCN, TRIP8b

Date Published: 11/11/2016

Citation: Han, Y., Lyman, K.A., Clutter, M., Schiltz, G.E., Ismail, Q.A., Cheng, X., Luan, C.H., Chetkovich, D.M. Method for Identifying Small Molecule Inhibitors of the Protein-protein Interaction Between HCN1 and TRIP8b. *J. Vis. Exp.* (117), e54540, doi:10.3791/54540 (2016).

Abstract

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed ubiquitously throughout the brain, where they function to regulate the excitability of neurons. The subcellular distribution of these channels in pyramidal neurons of hippocampal area CA1 is regulated by tetratricopeptide repeat-containing Rab8b interacting protein (TRIP8b), an auxiliary subunit. Genetic knockout of HCN pore forming subunits or TRIP8b, both lead to an increase in antidepressant-like behavior, suggesting that limiting the function of HCN channels may be useful as a treatment for Major Depressive Disorder (MDD). Despite significant therapeutic interest, HCN channels are also expressed in the heart, where they regulate rhythmicity. To circumvent off-target issues associated with blocking cardiac HCN channels, our lab has recently proposed targeting the protein-protein interaction between HCN and TRIP8b in order to specifically disrupt HCN channel function in the brain. TRIP8b binds to HCN pore forming subunits at two distinct interaction sites, although here the focus is on the interaction between the tetratricopeptide repeat (TPR) domains of TRIP8b and the C terminal tail of HCN1. In this protocol, an expanded description of a method for purifying TRIP8b and executing a high throughput screen to identify small molecule inhibitors of the interaction between HCN and TRIP8b, is described. The method for high throughput screening utilizes a Fluorescence Polarization (FP) -based assay to monitor the binding of a large TRIP8b fragment to a fluorophore-tagged eleven amino acid peptide corresponding to the HCN1 C terminal tail. This method allows 'hit' compounds to be identified based on the change in the polarization of emitted light. Validation assays are then performed to ensure that 'hit' compounds are not artifactual.

Video Link

The video component of this article can be found at http://www.jove.com/video/54540/

Introduction

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in the heart and central nervous system where they play an important role in regulating membrane excitability[1]. HCN channels have been implicated in the pathogenesis of Major Depressive Disorder (MDD)[2], which has led several groups to propose that limiting HCN channel function pharmacologically may be effective as a novel treatment for MDD[3]. However, directly targeting HCN channels is not viable because of their important role in the cardiac action potential[4]. Ivabradine, the only FDA approved HCN channel antagonist, is used for the treatment of heart failure to produce a bradycardic effect[5]. As such, there is a need for pharmacologic agents that limit HCN channel function exclusively in the central nervous system.

Tetratricopeptide repeat-containing Rab8b interacting protein (TRIP8b) is a brain specific auxiliary subunit of HCN channels that controls the surface expression and localization of HCN channels[6,7]. Genetic knockout of TRIP8b causes a reduction of brain HCN channels[7] without affecting HCN expression in the heart[8]. Interestingly, TRIP8b knockout mice spend less time immobile on the forced swim task and tail suspension task[7], two commonly used screening tests for antidepressant efficacy[9-11]. These results suggest that rather than directly targeting HCN channels with a small molecule antagonist of HCN channel function, disrupting the interaction between TRIP8b and HCN may be sufficient to produce antidepressant-like behavior.

TRIP8b binds to HCN at two distinct binding sites. The cyclic nucleotide binding domain (CNBD) of HCN interacts with a conserved domain of TRIP8b located N terminal to the TPR domains of TRIP8b[12,13]. Although the residues of the CNBD that are involved in this interaction have been mapped[14], the region of TRIP8b that is involved has not been narrowed down beyond an-80-amino acid fragment[13]. A second interaction occurs between the tetratricopeptide repeat (TPR) domains of TRIP8b and the C terminal tripeptide of HCN ('SNL' in HCN1, HCN2, and HCN4, but 'ANM' in HCN3)[3,12]. The recently solved crystal structure[15] of this C tail interaction revealed substantial structural similarity to the interaction

between the peroxisomal import receptor, peroxin 5 (PEX5), and its interacting partners, containing type 1 peroxisomal targeting sequences (PTS1)[16].

Although both interaction sites are required for HCN channel function, the interaction between the TPR domains of TRIP8b and the C terminal tripeptide of HCN1 serves as the dominant binding site and regulates HCN surface expression. Therefore, this interaction was chosen as the targeting site in this study. For the remainder of the manuscript, when a reference is made to the interaction between TRIP8b and HCN, it is this interaction that is being referred to. This interaction is recapitulated by a highly soluble fragment of TRIP8b corresponding to its conserved C terminal containing the TPR domains required for binding the C terminal tail of HCN (residues 241-602 of the 1a-4 isoforms of TRIP8b)[3].

In order to develop a high throughput screen to identify small molecules capable of disrupting this interaction, a fluorescence polarization (FP)-based assay was employed[17]. Fluorescence polarization is based on the excitation of a fluorophore-tagged ligand with polarized light, and measuring the degree of polarization of the emitted fluorescence[18]. In the presence of a binding partner, the rotational motion of the fluorescent ligand is constrained and polarized light is emitted[19]. In the absence of a binding partner, the rotational motion of the ligand leads to the emission of depolarized light.

In the enclosed protocol, a method for the purification of N terminal-tagged (6xHis) TRIP8b (241-602) using Nickel-Nitrilotriacetic acid (Ni-NTA) beads is presented. A similar protocol was employed to purify the Glutathione-S-Transferase (GST)-tagged C terminal 40 amino acids of HCN1 ($HCN1_{c40}$) used in step 7 of the protocol. For space considerations, a detailed description of that procedure was omitted.

In steps 2 through 7 of the protocol, a high throughput screening workflow is presented (see **Figure 1**). Protein-protein interactions are a notoriously difficult target for high throughput screening and readers are advised to seek out additional resources on the topic[20].

Steps 2 and 3 of the procedure characterize the *in vitro* affinity of the purified TRIP8b (241-602) construct for a fluorescein isothiocyanate (FITC)-tagged eleven amino acid peptide corresponding to the C terminal tail of HCN1 ($HCN1_{FITC}$). Based on the crystal structure of the TRIP8b-HCN complex[15], this eleven amino acid segment is sufficient to produce binding with TRIP8b (241-602). In step 2, the K_d of the interaction is measured by titrating TRIP8b (241-602) into a fixed concentration of $HCN1_{FITC}$. In step 3, an unlabeled version of the HCN peptide used in step 2 is titrated into a fixed concentration of both TRIP8b (241-602) and $HCN1_{FITC}$ to examine if the FITC tag interferes with binding. These experiments are essential to selecting the appropriate concentrations of TRIP8b (241-602) and $HCN1_{FITC}$ used in the high throughput screen.

The premise of the high throughput screen is that a small molecule capable of disrupting the interaction between TRIP8b (241-602) and $HCN1_{FITC}$ will produce a decrease in polarized light. In step 4, the Z factor of the assay is calculated[21] for a given concentration of TRIP8b (241-602) and $HCN1_{FITC}$ to ensure that the assay is appropriate for high throughput screening (step 5). Steps 6 and 7 are validation assays to confirm that the hits identified in the primary high throughput screen are acting by disrupting the interaction between TRIP8b (241-602) and $HCN1_{FITC}$ rather than through a nonspecific mechanism. In step 6, carboxytetramethylrhodamine (TAMRA)-labeled HCN1 peptide ($HCN1_{TAMRA}$) is used in an otherwise identical fluorescence polarization assay to filter fluorescent compounds that compromise the FP assay using the FITC tag. Step 7 utilizes a larger HCN1 C terminal fragment ($HCN1_{C40}$) and employs a bead-based proximity assay, which is based on the 'tunneling' of a singlet oxygen from a donor bead to an acceptor bead brought close to one another by interacting proteins[22].

Protocol

1. Purification of TRIP8b (241-602) Protein

1. Transform the plasmid containing TRIP8b (241-602) in the bacterial protein expression vector pGS21[3] into competent *E. coli* for protein expression according to manufacturer's instructions. Plate 300 µl of the culture on Luria Broth (LB)-agar with 5 µg/ml of Chloramphenicol and Ampicillin. Incubate the plate at 37 °C for 16 h.
2. The next day, pick a single colony to inoculate 50 ml LB with 50 µg/ml of Chloramphenicol and Ampicillin. Incubate the culture at 37 °C for 16 hr (with shaking).
3. Add 50 ml culture to 1 L of LB with 50 µg/ml of Ampicillin. Incubate at 37 °C with shaking.
4. Once the culture has reached an OD_{600} reading of 0.8-1.2, change the temperature of the incubator to 18 °C and add IPTG (Isopropyl-Beta-D-Thiogalactoside) to a final concentration of 1 mM. Allow protein expression to proceed for 16 hr.
5. Spin down the *E. coli* at 6,000 x g for 15 min at 4 °C to pellet the bacteria. After removing the tubes from the centrifuge, keep the bacteria on ice for the remaining of the procedure. Resuspend the bacteria in 36 ml of Buffer A with 0.25 mM of phenylmethanesulfonyl fluoride (PMSF).
6. Sonicate the resuspended bacteria on ice using a flat bolt for 5-10 min, alternating between 30 sec 'on' and 30 sec 'off' at high power.
7. Centrifuge at 12,000 x g for 15 min at 4 °C. Centrifuge for an additional 15 min if the supernatant is not clear yet.
8. Apply the supernatant to a column of 2 ml Ni-NTA beads. Incubate for 60 min at 4 °C with gentle rocking.
9. Allow the unbound material to flow through the column and wash with 500 ml of Buffer A.
10. Wash the column with 250 ml of Buffer B.
11. Wash the column with 125 ml of Buffer A supplemented with 5 mM imidazole.
12. Elute the protein from the column with 20 ml Elution Buffer.
13. Add the eluted protein to a dialysis cassette (Molecular weight cutoff – 10 kDa) using a syringe. Dialyze the protein for 60 min in 4 L of cold Phosphate Buffered Saline (PBS) at 4 °C.
14. Move the dialysis cassette into a new 4 L bucket of cold PBS. Dialyze protein for 16 hr at 4 °C.
15. The next morning, check the protein concentration using a Coomassie protein assay kit, following the manufacturer's instructions. Concentrate the protein using a protein concentrator, following the manufacturer's instructions, if a concentration below 40 µM is observed. Aliquot and freeze at -80 °C for storage.

2. Small Scale Fluorescence Polarization Assay to Characterize the Interaction of the Two Protein Fragments

1. Thaw 200 µl of 40 µM TRIP8b (241-602) and add it to a 1.5 ml tube. Add 100 µl of PBS to 11 additional 1.5 ml tubes.
2. Perform serial dilutions by transferring 100 µl of TRIP8b (241-602) from the original tube to the next tube in the series, pipetting up and down, and repeating the process. This will produce a 12-point series of 2-fold dilutions of TRIP8b (241-602) ranging from 0.01-40 µM.
3. Prepare a 650 µl master mix of 0.1 µM $HCN1_{FITC}$ (6.5 µl of a 10 µM aliquot) and 2 mM Dithiothreitol (DTT) in PBS.
   Note: The master mix is 2x.
4. Set up 12 new 1.5 ml tubes. Add 50 µl of the master mix containing $HCN1_{FITC}$ to each tube, and then add 50 µl from each of the 12 serial dilutions.
   Note: This will generate 12 tubes, each containing 0.05 µM $HCN1_{FITC}$ and half of the concentration of TRIP8b (241-602) from the original serial dilution.
5. Add the dilution series to an assay plate, 30 µl per well, in triplicate. Use a low binding solid black 384-well plate.
6. Spin the plate for 2 min at 900 x g at RT.
7. Read the plate using a microplate reader, following the manufacturer's instructions. Measure fluorescence polarization using the following measurement parameters: 485 nm excitation, 530 nm emission, 505 nm dichroic mirror, 100 msec integration time.
8. Plot the polarization (mP) values versus the TRIP8b (241-602) concentration on a logarithmic scale. Fit the data with the Hill equation to determine the affinity (K_d) of TRIP8b (241-602) for the labeled HCN1 peptide.
   Note: For preparing the serial dilutions, a multi-well plate can also be used instead of tubes.

3. Examine the Protein-protein Interaction Using a Positive Control

1. Add 200 µl of unlabeled HCN1 peptide (200 µM) to a 1.5 ml tube. Add 100 µl of PBS to 11 additional 1.5 ml tubes. Perform serial dilutions by transferring 100 µl from the tube containing 200 µl of peptide to the first tube of 100 µl PBS. Repeat the process to generate 12 tubes with 2 fold dilutions of unlabeled HCN1 peptide.
2. Prepare a 650 µl master mix of 0.2 µM $HCN1_{FITC}$ and 4 µM TRIP8b(241-602).
   Note: This is a 2x solution.
3. Add 50 µl of master mix to 12 new 1.5 ml tubes.
4. Add 50 µl of each serial dilution to a 1.5 ml tube.
5. Load the black 384-well microtiter plate in triplicate, adding 30 µl per well.
6. Centrifuge the plate for 2 min at 900 x g at RT.
7. Read the plate using a microplate reader capable of FP. Use the same settings as described in step 2.7.
8. Calculate the affinity of the unlabeled peptide for TRIP8b(241-602) using the Cheng-Prusoff equation: $K_d^1 = IC_{50} / (1 + [L]/K_d^2)$, where K_d^1 represents the affinity of the unlabeled peptide for TRIP8b(241-602), IC_{50} is determined in the preceding step and represents the ability of the unlabeled ligand to displace the labeled ligand, [L] is the concentration of the labeled ligand in step 3.2, and K_d^2 is the affinity of TRIPb(241-602) for the labeled ligand.

4. Evaluate Assay Performance (Calculate Z Factor)

1. Prepare a master mix by making a 12 ml solution of FP buffer with 2 µM TRIP8b (241-602), 50 nM $HCN1_{FITC}$, and 1 mM DTT in FP Buffer.
   Note: This will require 60 µl of 40 µM TRIP8b (241-602), 60 µl of 10 µM $HCN1_{FITC}$, and 1,080 µl of FP Buffer.
2. Add 30 µl of the master mix to each well of a black 384-well microtiter plate.
3. Add 1 µl of 1 mM unlabeled HCN1 peptide to 192 wells to serve as a positive control.
4. Add 1 µl of FP buffer to 192 wells to serve as a negative control.
5. Centrifuge the plate for 2 min at 900 x g at RT.
6. Read the plate using a microplate reader.
7. Calculate the Z factor for the assay using the following formula: $Z = 1 - 3*(\sigma_{pos}-\sigma_{neg})/(\mu_{neg}-\mu_{pos})$ where σ_{pos} and σ_{neg} are the standard deviations of the positive and negative control wells, and μ_{pos} and μ_{neg} represent the average signals in the positive and negative control wells.

5. High Throughput Screen

1. Thaw aliquots of TRIP8b (241-602) and $HCN1_{FITC}$ and keep them on ice.
2. Prepare 10 ml of TRIP8b (2 µM) and $HCN1_{FITC}$ (50 nM) in FP buffer.
3. Dispense 25 µl of the mixture into each well of a low binding 384-well black microtiter plate using a pipette.
4. For library screening, add compounds into each well of columns 3 to 22 (40 µM final concentration for each) using an acoustic liquid handler.
   Note: Due to the relatively low hit rate observed with this assay, two different compounds were pooled into a single well. The two compounds from each active well were subsequently tested individually to identify which conferred the activity.
5. Add 100 nl of Dimethyl sulfoxide (DMSO) into each well of columns 1 and 23 of each plate as negative controls.
6. Add unlabeled HCN1 peptide into each well of columns 2 and 24 of each plate as positive controls.
7. Incubate the plates at RT for 2 h. Read plates or incubate at 4 °C for 16 h before reading.
8. Measure fluorescence polarization on a plate reader. Use the same settings as described in step 2.7.
9. Calculate percent inhibition by normalizing the signals using the average positive and negative controls from each plate as 100%. Use the equation $XN = 100\% * ((X_{neg} - X)/(X_{neg} – X_{pos}))$ where X_N is the normalized percent inhibition corresponding to the signal X, X_{pos} is the average signal from the positive control wells, and X_{neg} is the signal from the negative control wells.

6. Confirmation of Hits Using TAMRA-tagged HCN Peptide

1. Validate hits with over 50% inhibition using TAMRA-labeled HCN1 peptide ($HCN1_{TAMRA}$).
    1. Prepare a master mix of 12 ml of 2 µM TRIP8b (241-602) and 50 nM $HCN1_{TAMRA}$ in FP buffer.
    2. Dispense 25 µl of the master mix into each well of a black 384-well microtiter plate.
    3. Transfer serial dilutions of each active compounds into the respective wells. Make two fold dilutions such that the concentration of each hit ranges from 0.2 µM to 200 µM.
    4. For the negative control reactions, transfer 100 nl of DMSO into each well. For the positive control add 100 nl of serially diluted unlabeled HCN1 peptide with final concentration ranging from 200µM to 0.1µM.
    5. Incubate the plate at RT for 2 hr. Read plates or incubate for 16 h at 4 °C before reading.
    6. Measure fluorescence polarization (mP) using the following measurement parameters: 535 nm Excitation; 580 nm emission; 535 nm dichroic mirror, 20 msec integration time.
    7. Calculate IC_{50} values by fitting the concentration dependent FP data of each compound using a four-parameter nonlinear regression model[23].

7. Dose-response Validation of Bead-based Proximity Assay

1. Thaw 1 aliquot of His-tagged TRIP8b (241-602) (His-TRIP8b) and GST-tagged HCN1 (GST- $HCN1_{C40}$) fusion proteins and keep them on ice.
2. Combine His-TRIP8b with GST-$HCN1_{C40}$ in assay buffer at concentrations of 400 nM His-TRIP8b and 40 nM GST $HCN1_{C40}$. Prepare 300 µl of the mixture for each compound to be tested.
   Note: Compounds are run in triplicate at 11 different concentrations plus a DMSO control (no compound).
3. For control wells, prepare 30 µl of His-TRIP8b (200 nM) without GST-HCN1C40 and then separately prepare 30 µl of GST-HCN1C40 (20 nM) without His-TRIP8b in assay buffer.
4. For each compound to be tested, add 7 µl of the His-TRIP8b:GST-$HCN1_{C40}$ mixture (prepared in step 7.2 above) to 36 wells of a plate using a 16-channel pipette. For testing a single compound, arrange the 12 different concentrations in rows A-L and the three replicates in columns 1-3. Add 7 µl of the His-TRIP8b solution (prepared in 7.3) to columns 1-3 of row M and 7 µl of GST-$HCN1_{C40}$ solution (prepared in 7.3) to columns 1-3 of row N. Briefly centrifuge the plate to ensure the liquids are at the bottom of each well and to remove any bubbles.
5. Dispense the hit compounds to be tested into the plate such that the concentration of each hit ranges from 200 µM (in row A) down to 0.1 µM (in row K). Dispense a volume of DMSO in rows L-N that matches the amount of compound dispensed in rows A-K.
6. Shake the plate for 2 min and incubate for 2.5 h at RT.
7. Dilute the anti-GST Acceptor beads 1:50 with assay buffer.
8. Add 3.5 µl of the diluted Acceptor beads to each well of the plate with a 16-channel pipette and mix by gently pipetting to avoid creating bubbles.
9. Incubate the plate for 1 hr at RT in the dark.
10. Dilute the Nickel Chelate Donor beads 1:50 with assay buffer. Do not expose the mixture to light.
11. Add 3.5 µl of the diluted Donor beads to each well of the plate with a 16-channel pipette and mix by gently pipetting to avoid creating bubbles.
12. Incubate the plate for 1.5 hr at RT in the dark.
13. Read on a plate reader. Use a plate reader with the following measurement parameters: 40 msec excitation time, 100 msec emission time, 0.1 mm detection height.
14. Calculate IC_{50} values by fitting data for each compound using a four-parameter nonlinear regression model[23].

Representative Results

To avoid copyright issues with our previous publication, a TAMRA-tagged probe $HCN1_{TAMRA}$ was used to generate **Figures 2** and **3**. Note that this substitution did not make an appreciable difference in the results, and the protocols are identical to those outlined above with $HCN1_{FITC}$. To assess the interaction with $HCN1_{TAMRA}$, TRIP8b (241-602) was titrated into a fixed concentration of $HCN1_{TAMRA}$ using the protocol outlined in step 2 (**Figure 2**). Next, the experiment outlined in step 3 was performed and unlabeled HCN1 peptide was titrated into a fixed concentration of TRIP8b (241-602) and $HCN1_{TAMRA}$ (**Figure 3**).

To verify that the assay was suitable for high throughput screening, its performance was next examined by the protocol in step 4; and a Z factor of 0.89 was obtained, indicating that the assay was ready for high throughput screening (**Figure 4**). Then, a 20,000 compound small molecule library was screened by the procedure outlined in step 5. All compounds that have percentage inhibition above 50% were then tested in the second FP assay with $HCN1_{TAMRA}$ (step 6). Finally, the confirmed hits were tested in the bead-based proximity assay (step 7, **Figure 5**). One hit compound, NUCC-5953, was identified as a result of these experiments.

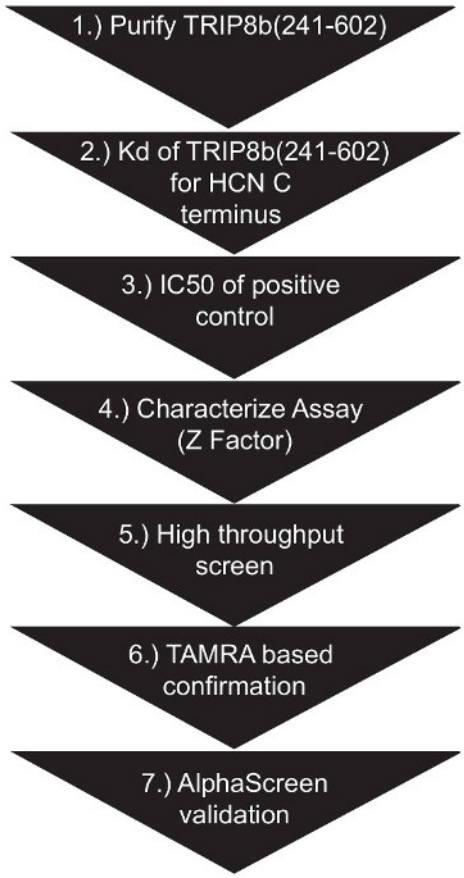

Figure 1: HTS Workflow. Schematic describing the workflow for high throughput screening. Diagram proceeds from the top to bottom, with each triangle representing a step in the protocol. Please click here to view a larger version of this figure.

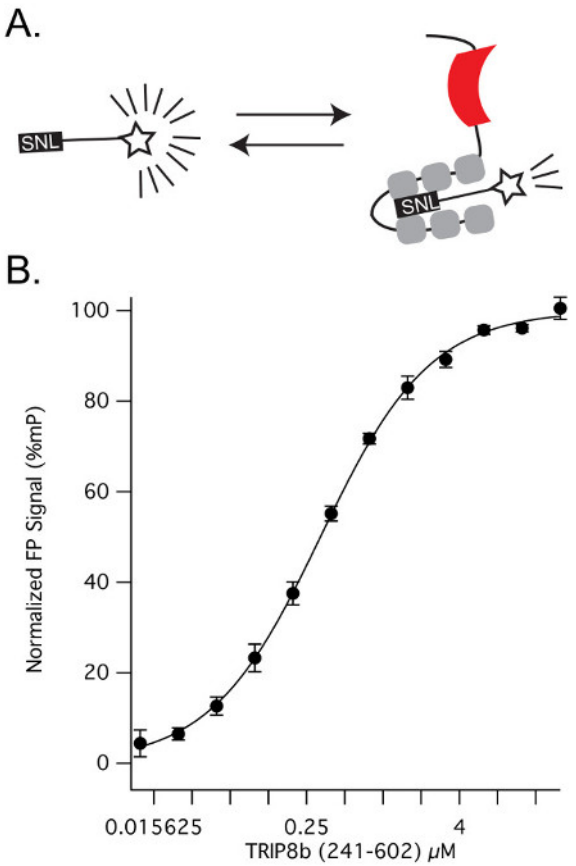

Figure 2: K_d of Protein-protein Interaction. (**A**) Schematic showing the experiment described in step 2. As TRIP8b (241-602) is titrated into a fixed concentration of $HCN1_{TAMRA}$, the TPR domains of TRIP8b (in gray) bind to the 11 amino acid peptide (in black, with terminal 'SNL' highlighted). (**B**) As the concentration of TRIP8b (241-602) increases, more $HCN1_{TAMRA}$ molecules become bound and polarization increases (K_D = 0.320.01μM). Error bars denote standard deviation. Please click here to view a larger version of this figure.

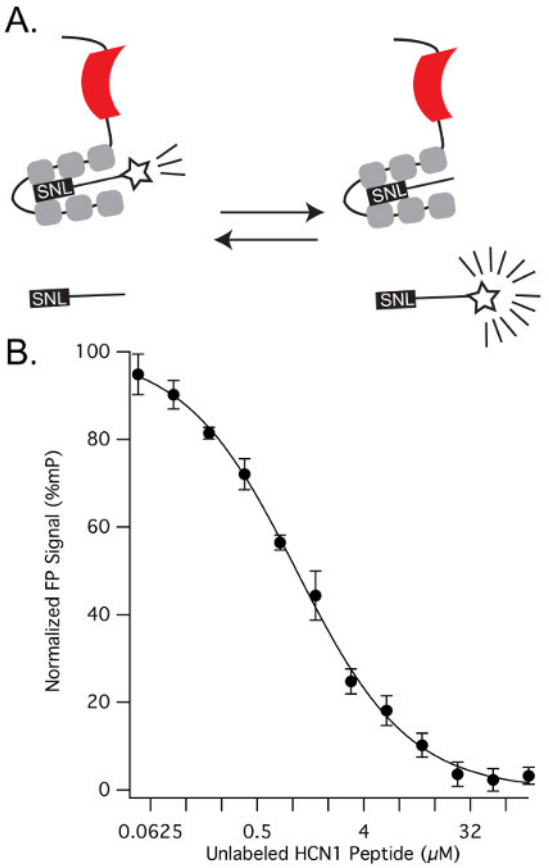

Figure 3: IC_{50} of Positive Control. (**A**) Schematic demonstrating experimental paradigm for step 3. As unlabeled HCN1 peptide is titrated into a fixed concentration of TRIP8b (241-602) and $HCN1_{TAMRA}$, the labeled peptide is displaced. (**B**) Note that the signal decreases as the concentration of unlabeled HCN1 increases, indicating displacement of $HCN1_{TAMRA}$ (IC_{50} = 1.070.08μM) . Error bars denote standard deviation. Please click here to view a larger version of this figure.

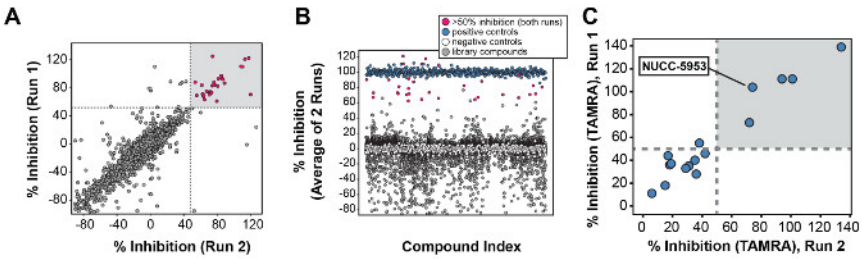

Figure 4: Representative Results from HTS. (**A**) Results from the high throughput screen performed using the protocol outlined in step 5. Each point on the graph represents a single compound. The X and Y coordinates are determined by the percent inhibition in each run. (**B**) Results from the screen plotted with positive and negative controls (see legend). Each compound's average percent inhibition (across two runs of the assay) is plotted on the Y axis. (**C**) Results from confirmatory FP experiments using a TAMRA-labeled HCN1 peptide. Compounds showing greater than 50% inhibition in step 5 were then used in step 6. As in (**A**), the X and Y coordinates are determined by the percent inhibition in two runs of the assay. Reproduced from Han *et al.*[3]. Please click here to view a larger version of this figure.

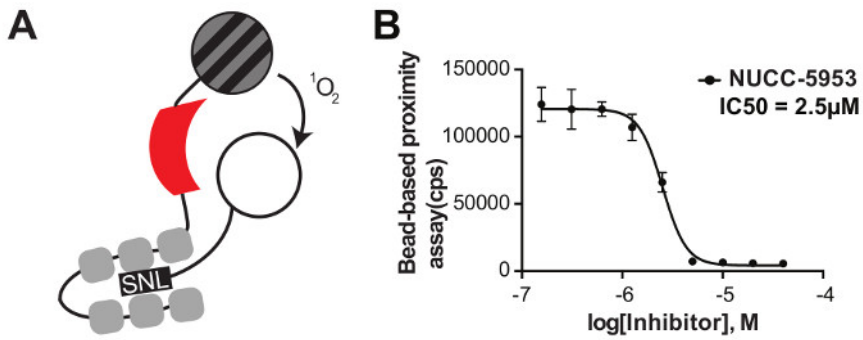

Figure 5: Bead-based Proximity Assay. (**A**) Schematic showing the bead-based proximity assay. TRIP8b (241-602) contains an N terminal hexahistidine tag that binds Nickel Chelate donor beads (circle with stripes). $HCN1_{C40}$ contains an N terminal GST tag that binds acceptor beads. When brought into proximity by the interaction of the TPR domains of TRIP8b and the C terminal tripeptide of HCN1, excitation of the donor bead by 680 nm wavelength light produces singlet oxygen. This singlet oxygen transfers energy to acceptor beads within a defined radius and leads to the emission of light. (**B**) Titration of increasing concentrations of the hit compound (NUCC-5953) into a fixed concentration of TRIP8b (241-602) and $HCN1_{C40}$. Reproduced from Han *et al.*[3]. Error bars denote standard deviation. Please click here to view a larger version of this figure.

Discussion

Because of its potential as a therapeutic target in MDD[24], there has been considerable interest in pharmacological approaches that antagonize HCN channel function in the central nervous system[4]. However, these efforts have been stalled by the important role of HCN channels in cardiac pacemaking and the risk of arrhythmia[25]. We reasoned that disrupting the interaction between HCN and its brain specific auxiliary subunit, TRIP8b[8], might be sufficient to produce antidepressant-like effects without affecting cardiac HCN channels[3]. This hypothesis was bolstered by the observation that mice lacking TRIP8b exhibit antidepressant-like behavior[7]. Targeting this interaction could become a new paradigm for the treatment of MDD.

A detailed protocol for identifying small molecule inhibitors of the interaction between the TPR domains of TRIP8b and the C terminal tripeptide of HCN1 is presented above. To facilitate its general application, we here describe our reasoning that led to the development of this assay. Although TRIP8b binds to HCN subunits at two locations, we focused on the interaction between the TPR domains of TRIP8b and the C terminal tail of HCN. The structural elucidation of this interaction by x-ray crystallography revealed a deep pocket formed by the TPR domains of TRIP8b around the C terminal of HCN[15]. The second TRIP8b-HCN interaction occurs between an 80 amino acid stretch of TRIP8b (located N terminal to the TPR domains) and the cyclic nucleotide binding domain of HCN. This interaction occurs across many different amino acids of each protein and constitutes a diffuse surface with fewer well defined intermolecular interactions[26]. Based on these structural observations, we reasoned that the interaction between the TPR domains of TRIP8b and the C terminal of HCN is more susceptible to disruption by a small molecule inhibitor.

Initially, a larger fragment of TRIP8b was used in the assay based on the assumption that a longer construct may have allosteric regulatory sites and increase the chance for success. Full length TRIP8b was initially used, but this approach was limited by protein aggregation, degradation, and sensitivity to freeze-thaw cycles. Subsequently, two intermediately sized TRIP8b constructs, a longer one (residues 219-602) and a shorter one (residues 259-602), were tested before an intermediate construct (residues 241-602) was selected. Although each of the four truncations described above contain the TPR domains relevant for binding to the C terminal of HCN, only the intermediate length clone (241-602) was sufficiently stable for use in screening assays. In particular, we were able to obtain large quantities of the protein after purification by Ni^{2+} affinity chromatography without additional separation steps.

After choosing a suitable fragment of TRIP8b and HCN, we next determined the affinity of the labeled HCN1 peptide for TRIP8b(241-602). As a general rule, an accurate measurement can only be made if the concentration of the ligand is substantially below the K_D of the binding partner[28]. In our case, we used 50 nM of labeled HCN1 peptide for the experiment in step 2, and obtained a K_D of 0.320.01μM. For more experimental design considerations regarding protein-protein interactions, readers are referred to one of several excellent reviews on the topic[27-30].

Once we determined the K_D of the labeled HCN1 peptide for TRIP8b(241-602), we then determined if the label itself interferes with binding. In step 3, we obtained a IC_{50} value of 1.070.08 μM by titrating an unlabeled HCN1 peptide into a fixed concentration of TRIP8b(241-602) to displace the labeled peptide. Combined with the K_D of the labeled peptide for TRIP8b(241-602), and applying the Cheng-Prusoff equation, we then estimated the affinity of the unlabeled peptide for TRIP8b(241-602) as K_D = 0.93μM. This is in close agreement with the affinity of TRIP8b(241-602) for the labeled peptide, and suggests that labeling the peptide did not substantially affect its affinity to TRIP8b. An important feature of the fluorescence polarization-based screen is its excellent signal to noise ratio as indicated by a Z factor of 0.89. With other parameters the same, the magnitude of the change in FP signal is dictated by the size of the binding partner (TRIP8b (241-602)) and the size of the fluorophore-labeled ligand. A change in polarization (from 44 to 224 mP) observed with the 11 amino acid peptide ligand between its free and bound states with the ~42 kDa TRIP8b (241-602) protein is sufficient to reproduce the targeted interaction and provides a sufficient dynamic range for library screening.

One of the challenges of any high throughput screening assay is distinguishing true 'hit' compounds from artifactual changes in signal intensity. In fluorescence polarization-based screens, it is common for many compounds to either interact directly with the fluorophore or fluoresce on their own. To circumvent these issues, a two-stage screening procedure using two distinct fluorophores is described above. This procedure reduces the likelihood that fluorescent compounds and compounds interacting directly with the fluorophore will advance through the screening process. In addition to binding the fluorophore, some compounds act as 'aggregators' *in vitro* and lead to nonspecific changes in the fluorescence

polarization signal. These compounds are thought to form detergent-sensitive micellar structures and inhibit protein-protein interactions[31,32]. To mitigate these effects, it is important that the fluorescence polarization buffer used in the high throughput screening protocol outlined above includes a detergent (Triton), although additional validation with other detergents should be considered.

It should be noted that there are several important limitations of the screening procedure described above. Although TRIP8b binds to HCN in two locations, the screen described above examines only one interaction site and will not identify small molecules targeting the CNBD interaction. Similarly, compounds that allosterically modulate TRIP8b binding to HCN by interacting with TRIP8b in the N terminal region will not be identified as hits. Both of these considerations are the result of using a smaller TRIP8b fragment (see above), which ensures reproducibility in the screening assays outlined in the procedure. In order to avoid these limitations, future efforts may be directed at using a cell based screen incorporating full length HCN and TRIP8b constructs[33,34]. This sort of screen may rely on high throughput electrophysiological methods in order to identify compounds capable of disrupting the interaction between HCN and TRIP8b, and limiting the expression of HCN channels at the cell surface. Of note, approaches such as these would need to include counter-screens to ensure that small molecules were not directly limiting HCN channel function as antagonists, as this could lead to off target effects *in vivo*.

Although HCN1, HCN2, and HCN4 all have a conserved 'SNL' C terminal peptide, the residues N terminal to this tripeptide vary substantially and likely affect the TRIP8b binding affinity. This raises the possibility that a small molecule hit obtained by screen or designed based on other hit compounds may provide HCN isoform specificity in disrupting the TRIP8b-HCN interaction. In the original screen, NUCC-5953 was identified as the first small molecule capable of disrupting the interaction between TRIP8b and HCN1[3]. Future work with this assay may identify additional small molecule inhibitors with desirable chemical properties for drug development.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by National Institutes of Health Grant R21MH104471 and R01MH106511 (D.M.C.), Brain Research Foundation SG 2012-01 (D.M.C.), Northwestern University Clinical and Translational Sciences Institute 8UL1TR000150 (Y.H.), Chicago Biomedical Consortium HTS-004 (Y.H. and D.M.C.), and National Institutes of Health Grant 2T32MH067564 (K.L.). A part of this work was performed by the Northwestern University Medicinal and Synthetic Chemistry Core (ChemCore) at the Center for Molecular Innovation and Drug Discovery (CMIDD), which is funded by the Chicago Biomedical Consortium with support from the Searle Funds at the Chicago Community Trust and Cancer Center Support Grant P30 CA060553 from the National Cancer Institute awarded to the Robert H. Lurie Comprehensive Cancer Center. The high throughput screen work was performed in the High Throughput Analysis Laboratory which is also a core facility of the Robert H. Lurie Comprehensive Cancer Center.

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