Reporting Sodium Channel Activity Using Calcium Flux: Pharmacological Promiscuity of Cardiac Nav1.5[§]

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ABSTRACT

Voltage-gated sodium (Nav) channels are essential for membrane excitability and represent therapeutic targets for treating human diseases. Recent reports suggest that these channels, e.g., Nav1.3 and Nav1.5, are inhibited by multiple structurally distinctive small molecule drugs. These studies give reason to wonder whether these drugs collectively target a single site or multiple sites in manifesting such pharmacological promiscuity. We thus investigate the pharmacological profile of Nav1.5 through systemic analysis of its sensitivity to diverse compound collections. Here, we report a dual-color fluorescent method that exploits a customized Nav1.5 [calcium permeable Nav channel, subtype 5 (SoCal5)] with engineered-enhanced calcium permeability. SoCal5 retains wild-type (WT) Nav1.5 pharmacological profiles. WT SoCal5 and SoCal5 with the local anesthetics binding site mutated (F1760A)

could be expressed in separate cells, each with a different-colored genetically encoded calcium sensor, which allows a simultaneous report of compound activity and site dependence. The pharmacological profile of SoCal5 reveals a hit rate (>50% inhibition) of around 13% at 10 μ M, comparable to that of hERG. The channel activity is susceptible to blockage by known drugs and structurally diverse compounds. The broad inhibition profile is highly dependent on the F1760 residue in the inner cavity, which is a residue conserved among all nine subtypes of Nav channels. Both promiscuity and dependence on F1760 seen in Nav1.5 were replicated in Nav1.4. Our evidence of a broad inhibition profile of Nav channels suggests a need to consider off-target effects on Nav channels. The site-dependent promiscuity forms a foundation to better understand Nav channels and compound interactions.

Introduction

Voltage-gated sodium (Nav) channels play critical roles in controlling membrane excitability (Catterall, 2012). They are activated in response to membrane depolarization and mediate rapid influx of sodium ions during the rising phase of action potentials. There are nine highly homologous subtypes: Nav1.1 to Nav1.9, expressed in neuronal, cardiac, skeletal muscle, and endocrine cells. Both genetic evidence and pharmacological studies have demonstrated their validity as therapeutic targets for human diseases.

Recently, several reports have suggested Nav channels are promiscuous drug targets when evaluated with a subset of known drugs (Huang et al., 2006; Harmer et al., 2011). For example, Lounkine et al. (2012) reported that 70 out of 126 tested drugs (56%) showed at least 50% inhibition at 30 μ M on Nav1.5. It is suggested that Nav1.5 may exhibit more promiscuous interactions with compounds than hERG (hit rate as 30%), which is a widely appreciated promiscuous drug

target (Lounkine et al., 2012). The hERG promiscuity is attributed primarily to its wide inner cavity and two aromatic residues (Y652 and F656) that directly interact with diverse compounds (Mitcheson et al., 2000; Sanguinetti and Tristani-Firouzi, 2006). However, very limited information is available regarding the promiscuity of Nav channels when evaluated with a large chemical library consisting of structurally diverse compounds. Also, the fundamental principles of the potential Nav channel promiscuity remain largely unanswered. For instance, the number of drug binding sites in Nav channels is still controversial (Mike and Lukacs, 2010). Furthermore, the most well recognized residue for drug binding, F1760 in Nav1.5, was reported to be more important for an inhibitory effect of class Ib antiarrhythmics but not as important for class Ia and Ic antiarrhythmics (Pless et al., 2011). Therefore, it would be of great interest to systemically evaluate the promiscuity of Nav channels and examine the site dependence using structurally diverse libraries in a high-throughput manner.

Traditional methods to examine compound effects on Nav channels may be divided into two major classes depending on whether the method directly reports the channel activity (e.g., ion flux) or indirectly reports a derivative signal (e.g., membrane potential change). As a direct approach, electrophysiological methods measure ionic currents with high resolution; however, they are often difficult to implement in a high-throughput

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ABBREVIATIONS: DMSO, dimethylsulfoxide; GECCO, genetically encoded calcium sensor; HTS, high-throughput screening; LOPAC, Library of Pharmacologically Active Compounds; Nav, voltage-gated sodium; TP, test pulse; TTX, tetrodotoxin; WT, wild type; XE991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone.

format and are less cost effective. Indirect approaches commonly take advantage of membrane potential changes caused by ionic flux through activated channels; however, they may suffer from high false-positive rates because off-target effects may directly affect membrane potential in the system (Trivedi et al., 2008). In addition, these methods are not designed to specifically examine compound site dependence.

To circumvent the potential issues with existing methods, we engineered Nav channels with elevated sustained calcium conductance, which permitted development of a high-throughput, dual-color calcium sensor-based fluorescent assay to evaluate modulators of Nav function. Unlike conventional electrophysiological methods that can distinguish between tonic and use-dependent inhibitory effects of compounds on Nav channels, the newly developed calcium flux assay measures the combinatorial inhibitory effect for each compound on one target, which parallels compound capability to inhibit Nav persistent current. However, because calcium flux was used as the readout, wild-type (WT), and mutant Nav channels could be expressed in separate cells, each with a different-colored calcium sensor. Thus, by intermixing such cells within assays, simultaneous reporting of compound activity and its site dependence could be performed on WT and mutant Nav channels. Therefore, the assay is more sensitive to the differential effects on two target channels, in particular on WT channels versus channels that harbor mutations at presumed interaction sites for drug action. This strategy provides more rigorous evidence for the site dependence of identified hits. Moreover, differential effects, if observed, strongly support that these compounds act directly on the channel, rather than through off-target effects.

Materials and Methods

Stable Cell Lines. Nav1.5, hERG, and KCNQ2 stable cell lines were generated prior to experimentation. HEK stable cell lines expressing Nav1.5CCW [WT calcium permeable Nav channel, subtype 5 (SoCal5-WT)], Nav1.5CCW-F1760A (SoCal5-F1760A), Nav1.4CCW [WT calcium permeable Nav channel, subtype 4 (SoCal4-WT)], or Nav1.4CCW-F1759A (SoCal4-F1759A) were generated to express channels under the cytomegalovirus promoter on pIRES-puromycin.

Electroporation. Genetically encoded calcium sensors (G-GECO and R-GECO plasmids) were obtained from Addgene (Cambridge, MA). G-GECO or R-GECO was transfected to cells at a final concentration of 100 $\mu\text{g}/\text{ml}$ using the Maxcyte STX system (Maxcyte, Rockville, MD). Following electroporation, cells were incubated at 37°C for 20 minutes, then plated to 15 cm culture dishes and cultured overnight at 37°C. Calcium assays were performed 48 hours after electroporation.

Manual Electrophysiological Recording. Traditional whole-cell voltage clamp recording was performed at room temperature with an Axopatch-200A amplifier (Molecular Devices, Sunnyvale, CA). To record sodium conductance carried by SoCal5 and SoCal5-F1760A, extracellular solution contained (in mM) 148 NaCl, 1 MgCl_2 , 10 HEPES, and 10 glucose (pH 7.4 with NaOH). To record calcium conductance, extracellular solution contained (in mM) 135 choline-Cl, 1 MgCl_2 , 10 CaCl_2 , 10 HEPES, and 10 glucose (pH 7.4 with Tris base). Under both conditions, pipette solution contained (in mM) 140 KCl, 1 MgCl_2 , 10 EGTA, and 10 HEPES (pH 7.3 with potassium hydroxide). An online P/4 leak subtraction protocol was used for most protocols except for the determination of channel slow inactivation properties.

To predict permeability of SoCal5 to Ca^{2+} relative to Na^+ , reversal potential was determined using a bionic recording solution recipe. The extracellular solution was (in mM) 140 *N*-methyl-D-glucamine chloride, 10 CaCl_2 , and 20 HEPES (pH 7.4 with *N*-methyl-D-glucamine), and

the internal pipette solution contained (in mM) 100 mM Na-gluconate, 10 NaCl, 10 EGTA, and 20 HEPES-Na (pH 7.4 with NaOH). The permeation ratio of calcium over sodium, $P_{\text{Ca}^{2+}}/P_{\text{Na}^+}$, was predicted by the Goldman-Hodgkin-Katz equation

$$V_{\text{rev}} = \frac{RT}{F} \ln \frac{[\text{Na}^+]_o + 4 \left(\frac{P_{\text{Ca}^{2+}}}{P_{\text{Na}^+}} \right) [\text{Ca}^{2+}]_o}{[\text{Na}^+]_i + 4 \left(\frac{P_{\text{Ca}^{2+}}}{P_{\text{Na}^+}} \right) [\text{Ca}^{2+}]_i \exp^{FV_{\text{rev}}/RT}} \quad (1)$$

To study the gramicidin-mediated hyperpolarization, whole-cell current clamp recording was used to determine membrane potential in sodium-free buffers with different potassium concentrations. The pipette solution contained (in mM) 140 KCl, 1 MgCl_2 , 10 EGTA, and 10 HEPES (pH 7.3 with potassium hydroxide). The basal extracellular solution contained (in mM) 145 choline-Cl, 1 MgCl_2 , 2 CaCl_2 , 10 HEPES, and 10 glucose (pH 7.4 with Tris base). The extracellular buffers, containing 2, 5, 15, or 50 mM K^+ , were made by replacing choline-Cl with KCl at equivalent concentrations. Electrodes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL). When filled with the pipette solution, the electrodes have typical resistance ranging from 2 to 4 M Ω . During the recording, constant perfusion of extracellular solution was maintained using a BPS perfusion system (ALA Scientific Instruments, Westbury, NY). Electrical signals were filtered at 2 kHz and acquired with pClamp9.2 software via a DigiData-1322A interface (Molecular Devices). Capacitance and 60–80% series resistance were routinely compensated.

Automated Electrophysiology Assays. Automated voltage-clamp recording was performed using the population patch-clamp mode with IonWorks Quattro (Molecular Devices). In brief, cells were dislodged from flasks and dispensed into a 384-well population patch-clamp plate; after dispensing, the seal resistance of the cells was measured for each well and the cells were perforated by incubation with 50 $\mu\text{g}/\text{ml}$ amphotericin B (Sigma-Aldrich, St. Louis, MO). The activity of Nav1.5 and SoCal5 was measured with two multipulse protocols. In the first protocol, cells were held at -110 mV and sodium currents were elicited by 12 voltage pulses. During the first test pulse (TP1), membrane potential was depolarized to -30 mV for 20 milliseconds and hyperpolarized to -110 mV for 100 milliseconds. This was repeated 10 times. Then, membrane potentials were depolarized to -30 mV for 500 milliseconds and hyperpolarized to -110 mV for 20 milliseconds prior to the TP12 at -30 mV for 20 milliseconds. To facilitate inactivation, a second multipulse protocol was used in hit validation, in which cells were held at -70 mV and sodium currents were elicited by 11 voltage pulses. During the first pulse, membrane potential was depolarized to -10 mV for 250 milliseconds and hyperpolarized to -70 mV for 250 milliseconds. This was repeated eight times and followed by TP1 at -10 mV for 2000 milliseconds. Then, membrane potential was hyperpolarized to -70 mV for 10 milliseconds prior to TP2 at -10 mV for 20 milliseconds, followed by a 2000 millisecond pulse at -120 mV prior to TP3 at -10 mV for 20 milliseconds. Multipulse protocols were applied to cells before and following 3 minute incubation with test compounds or assay buffer. Peak current amplitudes at the TPs (TP1 and TP12 in the first protocol; TP1, TP2, and TP3 in the second protocol) were measured before and after compound treatment. Cells with peak current amplitude larger than 0.3 nA, seal resistance >30 M Ω , and seal resistance drop rate lower than 25% were included for data analysis. Positive (1 mM lidocaine) and negative controls [external buffer with 0.02% (v/v) dimethylsulfoxide (DMSO)] were applied within each plate to evaluate data quality. Compound effects were assessed by the percentage change in the peak current as calculated by dividing the difference between pre- and postcompound peak currents by the respective precompound peak current in the same well and normalized to positive and negative controls in the same plate. If a compound decreased the peak current by 50%, it was classified as an inhibitor.

Compounds were tested on the hERG channel at 10 μM with a two-step voltage protocol as follows: cells were held at -70 mV, stepped down to -80 mV for 100 milliseconds to estimate leak currents, and depolarized to -30 mV for 100 milliseconds to estimate non-hERG

currents. Then, hERG currents were evoked by two voltage pulses at +40 mV. During the first pulse, membrane potential were depolarized to +40 mV for 2 seconds and hyperpolarized to -30 mV for 2 seconds. Prior to the second pulse, cells were held at -70 mV for 3 seconds. Then, membrane potentials were depolarized to +40 mV for 2 seconds followed by a hyperpolarization to +30 mV for 2 seconds. The train of pulses was applied to cells before and following 3 minute incubation of cells with test compounds or controls. Positive (1 mM dofetilide) and negative controls [external buffer with 0.02% (v/v) DMSO] were applied within each plate to evaluate the data quality. Cells with tail current amplitude larger than 0.2 nA, seal resistance >30 MΩ, and seal resistance drop rate lower than 25% were included for data analysis. Peak amplitudes of tail currents before and after compound treatment were measured. Compound effects were assessed by the percentage change in the hERG tail current as calculated by dividing the difference between pre- and postcompound hERG tail currents by the respective pre-compound tail current in the same well. If a compound decreased the hERG tail current by 50%, it was classified as an inhibitor.

Whole-cell currents of Nav1.5 and hERG were measured in the following recording buffers: 137 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4 adjusted with NaOH (extracellular solution); and 40 mM KCl, 100 mM K-gluconate, 1 mM MgCl₂, and 5 mM HEPES, pH 7.2 adjusted with potassium hydroxide (intracellular solution). The same internal buffer was applied to SoCal5 whereas CaCl₂ was not included in the external buffer in SoCal5 recording since Ca²⁺ may interfere with Na⁺ currents through SoCal5. The voltages listed previously were before correction for liquid junction potential. Current signal was sampled at 10 kHz for the first protocol and 5 kHz for the second protocol in the SoCal5 assay and sampled at 625Hz in the hERG assay.

Calcium-Based Fluorescence Assay. The activity of SoCal5-WT, SoCal5-F1760A, SoCal4-WT, and SoCal4-F1759A was monitored by calcium influx detected with green- or red-GECO proteins. Cells were seeded at 6500 cells per well into poly-D-lysine-coated 384-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) using a Multidrop (Thermo Scientific, Hudson, NH) and incubated overnight at 37°C under 5% CO₂. On the day of assay, the medium was removed. Cells were washed once by 1X HBSS buffer (Hanks balanced salt solution; Invitrogen, Carlsbad, CA) with 10 mM HEPES, 50 μl/well and incubated in sodium-free assay buffer. In assay development, to determine optimal assay conditions, the basal assay buffer consisted of 0, 2, 4, or 8 mM K⁺. Cell plates were loaded to a Hamamatsu FDSS 6000 kinetic imaging plate reader (Hamamatsu Photonics, Amamatsu, Japan). After establishing a fluorescence baseline by 1 Hz scanning for 10 seconds, membrane potentials were hyperpolarized by addition of 10 μg/ml gramicidin (Sigma-Aldrich) and fluorescence measurement was continued at 1 Hz for another 109 seconds; then, channels were activated by addition of stimulus buffer consisting of various K⁺ and Ca²⁺ combinations and fluorescence measurement was continued at 1 Hz for another 160 seconds. Peak fluorescence was compared among different conditions. In the compound screen, cells were incubated with test compounds or controls for 20 minutes and then loaded to FDSS 6000 for signal detection. As previously described, the membrane potential was hyperpolarized using gramicidin and channels were evoked by stimulus buffer. To evaluate the robustness of the high-throughput screening (HTS) GECO-based fluorescence assays, terfenadine and amitriptyline at 10 μM were applied as positive controls, whereas assay buffer was employed as the negative control. Both positive and negative controls were prepared in 0.2% (v/v) DMSO, corresponding to 10 μM test concentrations, respectively. The integrated fluorescence was calculated for each well using the entire 160 second detection window after addition of stimulus and then normalized to positive and negative control wells. Compared with the negative controls, if a compound caused signal decrease by more than 50%, it was classified as an inhibitor.

Thallium Flux-Based Fluorescence Assay. Compounds in the validation set were examined at 10 μM on KCNQ2 using a thallium-based fluorescence assay as previously described (Yu et al., 2010). In

brief, cells were loaded with FluxOR (Invitrogen). FluxOR-containing buffer was replaced by assay buffer and cells were incubated with compounds for 20 minutes before being loaded to FDSS 6000 for signal detection. After establishing the basal fluorescence, thallium influx was evoked by stimulus buffer. 50 μM XE991 [10,10-bis(4-pyridinylmethyl)-9(10*H*)-anthracenone] and assay buffer were applied as positive and negative controls. Peak fluorescence was normalized to positive and negative controls. Compared with the negative controls, if a compound caused signal decrease by more than 50%, it was classified as an inhibitor.

Compound Library. The Library of Pharmacologically Active Compounds (LOPAC) (Sigma-Aldrich) and the validation set of the Molecular Libraries Small Molecule Repository (BioFocus DPI, Saffron Walden, UK) were selected for the calcium and electrophysiological assays. The LOPAC contains 1280 known bioactives dissolved in DMSO at 10 mM. The validation set includes 1999 compounds of various structures. All compounds in the validation set are of >90% purity and were dissolved in DMSO with a concentration of 5 mM.

Modeling Analysis for Multiplex Assay for SoCal5-WT and SoCal5-F1760A.

$$\text{WT response} = \frac{1}{1 + (10/\text{IC}_{50\text{-WT}})^{n1}} \quad (2)$$

$$\text{F1760A response} = \frac{1}{1 + (10/r^* \text{IC}_{50\text{-WT}})^{n2}} \quad (3)$$

For a given compound, when test at 10 μM, its effect on SoCal5 can be predicted by the Hill equation (eq. 2). For the same compound, its effect on SoCal5-F1760A can be predicted by eq. 3 due to the difference in the IC₅₀ value and Hill coefficient. For simplicity and the data from the tool compounds, we assume $n1 = n2 = 1$. We generate different prediction lines by varying the values of r , while setting the IC_{50-WT} values ranging from infinity small to 10 μM to represent compounds with different potencies on SoCal5.

Data Analysis. Calcium assay data were processed in FDSS 6000 and analyzed using the in-house software ichannel. Automated electrophysiological recording data were processed in IonWorks (Molecular Devices) and then analyzed in Excel (Microsoft, Redmond, WA) and Origin 6.0 (OriginLab, Northampton, MA). The dose-response curve was fitted by the Hill equation in Origin 6.0 software as $E = E_{\text{max}}/[1 + (\text{IC}_{50}/C)^P]$, where E_{max} is the maximum response, C is the drug concentration, IC₅₀ is the drug concentration producing half of the maximum response, and P is the Hill coefficient.

Compounds are clustered based on structure similarity. The similarity between two compounds was measured by comparing their respective ECFP₄ fingerprint (using SciTegic Pipeline Pilot from Accelrys Software, Inc., San Diego, CA) with the Tanimoto coefficient. Clustering of five different channel targets was based on their inhibitory profiles by using complete-linkage hierarchical clustering in R statistical software (available at <http://www.r-project.org/>).

Results

Genetically Engineered Nav1.5 (SoCal5) with Persistent Calcium Conductance. Nav channels exhibit fast inactivation upon depolarization. The sodium influx during each depolarization period is very limited, and the increase over basal $[\text{Na}^+]_i$ is negligible when detected by fluorescent dyes. Therefore, traditional optical assays measure indirect readouts such as membrane potential and use pharmacological modulators such as veratridine to attenuate fast inactivation (Felix et al., 2004; Liu et al., 2006; Trivedi et al., 2008). By contrast, calcium ions manifest an approximately 1000-fold difference in concentration across plasma membrane. In addition, calcium reporters, either chemical dyes (Paredes et al., 2008) or genetically

encoded sensors (Behera et al., 2013), are well-developed optical tools and extremely sensitive for detecting small changes in intracellular calcium concentration. To directly detect ionic flux through Nav channels with an enlarged signal window, genetic engineering was applied to generate calcium permeable Nav channels with impaired fast inactivation. The K1237C mutation in the domain III selectivity filter of rNav1.4 dramatically increased the $\text{Ca}^{2+}/\text{Na}^{+}$ permeation ratio (Pérez-García et al., 1997). In addition, rNav1.4 channels with L398C/A399W mutation exhibit significantly impaired fast inactivation and robust expression in HEK293 cells (Wang et al., 2003). The three residues are highly conserved among all the Nav subtypes (Fig. 1A). Therefore, we generated an equivalent triple mutant K1418C/L409C/A410W in hNav1.5 (SoCal5). SoCal5 channels conduct robust sustained calcium currents, which is absent in WT Nav1.5 (Fig. 1, B and D). Under bionic conditions with 10 mM $[\text{Ca}^{2+}]_o$ and 140 mM $[\text{Na}^{+}]_i$, WT hNav1.5 only conducts outward sodium currents, whereas both inward calcium and outward sodium currents flow through SoCal5 (Fig. 1C) with a reversal potential at 19.6 ± 0.4 mV (Fig. 1E), indicating that $P_{\text{Ca}^{2+}}/P_{\text{Na}^{+}}$ is approximately 7.0, comparable to reported values for rNav1.4-K1237C mutant channels (Pérez-García et al., 1997).

SoCal5 Retained Nav1.5 Pharmacology. To test whether the introduced mutations altered the hNav1.5 pharmacology, effects of tool compounds on WT hNav1.5 and SoCal5 channels were compared. Compound effects were examined with a multipulse voltage protocol using automated electrophysiology, IonWorks (Molecular Devices; Fig. 2). TP1 and TP12 were used to evaluate compound effects on closed and inactivated channels, respectively. Amitriptyline shows increased tonic inhibition on SoCal at TP1. However, similar to its effects on hNav1.5 channels, amitriptyline exhibits a more prominent inhibitory effect on SoCal5 at TP12 than at TP1, with complete inhibition at TP12 at 12 μM (Fig. 2A), suggesting that SoCal5 and hNav1.5 may exhibit similar pharmacology at TP12. This was confirmed by similar IC_{50} values for hNav1.5 and SoCal5, when evaluated by known sodium channel inhibitors belonging to different drug classes (Fig. 2, B–D). Except for TTX (tetrodotoxin), the IC_{50} values for the other nine tool compounds were no more than a fivefold difference in hNav1.5 versus SoCal5 at TP12 (Fig. 2D). Therefore, SoCal5 serves as a surrogate to assess compound effects on hNav1.5 in the following studies.

Development of Fluorescence-Based Calcium Assays for SoCal5. Nav channels usually have limited availability for activation in the recombinant expression systems such as HEK293 cells due to their depolarized resting membrane potential (-40 to -20 mV). Although fast inactivation was greatly reduced in SoCal5 channels, slow inactivation may be still present (Wang et al., 2003). The steady-state activation and slow inactivation curves of SoCal5 suggest that most channels are in the inactivated state in HEK293 cells under rest conditions (Fig. 3A). To release SoCal5 from the inactivated state, a reported gramicidin-based method (Belardetti et al., 2009) was used to hyperpolarize membrane potential. In the presence of gramicidin, membrane potential is changed toward the reversal potential of K^{+} when extracellular Na^{+} is replaced by choline and potassium is the primary permeable cation through the membrane (Supplemental Fig. 1). Using such a method, a SoCal5-specific calcium influx was detected by a chemical calcium indicator, fluo-4 (Fig. 3B).

To develop a fluorescence-based assay to evaluate Nav1.5 pharmacology by using the SoCal5 calcium influx, we further optimized assay conditions and determined the pharmacology of the tool compounds. An ideal assay condition should possess a robust fluo-4 signal window while the sensor is still within its nonsaturated linear response phase. To achieve that, three major parameters were tuned systematically, including basal $[\text{K}^{+}]_o$, $[\text{K}^{+}]$, and $[\text{Ca}^{2+}]$ in the stimulus buffer (Fig. 4C). After fluo-4 was loaded into cells, the cells were incubated in sodium-free external buffer with either of 0, 2, 4, or 8 mM potassium. As expected, lower $[\text{K}^{+}]_o$ values are more effective in hyperpolarizing membrane potential and give rise to higher peak fluorescence ($\Delta F/F_0$) (Fig. 4A).

To find the best combinations of $[\text{K}^{+}]$ and $[\text{Ca}^{2+}]$ in the stimulus buffer, a two-dimensional matrix was generated, in which $[\text{K}^{+}]$ and $[\text{Ca}^{2+}]$ in the stimulus buffer were tested in a 1:2 gradient with the highest testing concentration at 32 mM, respectively (Fig. 4C). The SoCal5-specific fluo-4 signal was measured by the difference between samples treated with buffer and with lidocaine at 10 mM, a concentration for 100% inhibition (Fig. 4B). An optimal condition was expected to exhibit at least a onefold increase in the SoCal5-specific fluorescent signal. Sensor saturation was estimated by the decay index, which was calculated and normalized based on the ratio of the residual fluorescent signal at 200 seconds (dashed line in Fig. 4A) over the peak signal. More than 50% decay is considered to be a sign indicating the sensor is not saturated. Based on the criterion described previously, a condition of 2 mM basal $[\text{K}^{+}]_o$ in combination with 8 mM $[\text{K}^{+}]$ stimulus buffer was chosen for the fluo-4-based assay. Under the identified optimal condition, the IC_{50} values of the known sodium inhibitors on SoCal5 in the calcium fluorescent assay were similar to their IC_{50} values on WT Nav1.5 at the inactivated state (TP12) (Fig. 4D). Therefore, the SoCal calcium assay can authentically evaluate compound inactivated state inhibition on WT Nav1.5.

High-Throughput Dual-Color Assay Indicates F1760-Dependent Promiscuity of Nav1.5. Several studies have suggested that known drugs may inhibit Nav channels with great promiscuity; however, the number of testing drugs in these studies is small (Huang et al., 2006; Harmer et al., 2011; Lounkine et al., 2012). More importantly, molecular mechanisms for promiscuity are not well understood. The local anesthetic site is the most well studied site responsible for Nav and drug interaction. Also, a conserved phenylalanine (Phe) residue in the domain IV S6 segment appears to be most critical (Wang and Strichartz, 2012). To better understand Nav pharmacology, it is important to investigate whether Nav channels manifest promiscuous interaction with compounds and whether the Phe residue (F1760 in hNav1.5) is responsible for promiscuity. To address these questions systemically, the SoCal5 calcium assay was further optimized toward a multiplex HTS assay by taking advantage of recently developed GECOs (Zhao et al., 2011) with different excitation and emission spectra (e.g., green and red). WT SoCal5 and SoCal5-F1760A channels could be expressed separately in different cells, each with a distinct-colored GECO. By intermixing the two groups of cells, this dual-color approach simultaneously monitors calcium signals in different cell populations under the same experimental conditions, and thus provides more accurate evaluation for compound differential effects on WT and mutant channels.

Fig. 1. SoCal5 conducts sustained calcium currents. (A) Sequence alignment for DI S6 and DIII selectivity filter region among different Nav subtypes. The amino acids for inactivation deficient mutations and calcium permeation mutation are in red. (B) The inward currents of Nav1.5 and SoCal5 under different external buffer conditions. The holding potential was -100 mV; 50 milliseconds of depolarization pulse were used to elicit the step current. (C) Representative traces of Nav1.5 and SoCal5 under bionic conditions. The holding potential was -100 mV and the depolarization pulse ranged from -70 to $+40$ mV in 10 mV increments. (D) The ratio of $I_{\text{sustain}}/I_{\text{peak}}$ for Nav1.5, SoCal5, and SoCal5 calcium conductance. The sustained current amplitude was measured at the end of the 50 millisecond depolarization pulse. (E) The I-V relationship plot of Nav1.5 and SoCal5 under the protocol and condition stated in (C).

To identify an optimal condition, similar strategies used in the fluo-4 assay were applied in GECO-based assays (Supplemental Fig. 2). A condition of 0 mM K^+ external buffer in combination with 16 mM K^+ stimulus buffer was chosen for the GECO-based HTS of SoCal5. The IC_{50} values of the tool compounds, obtained using green-GECO, were nearly identical to those obtained using red-GECO. The compound pharmacology evaluated by GECOs was similar to that using fluo-4 and correlated well with that at TP12 in electrophysiology (Supplemental Fig. 3). Therefore, the different observed responses of green and red fluorescence for the same compound is expected to reflect its differential inhibitory effects on the WT and F1760A mutant channels.

The F1760A mutation in SoCal5 slightly changed the channel biophysical properties in terms of activation and inactivation (Supplemental Fig. 4A). Consistent with the early literature, most tool compounds exhibited lower potency on SoCal5-F1760A in the GECO assay (Supplemental Fig. 4B). To examine the promiscuity of hNav1.5, a dual-color calcium assay was then performed to test the LOPAC compounds using two distinct cell populations, one expressing SoCal5-WT and red-GECO and the other expressing SoCal5-F1760A and green-GECO. The LOPAC compound collection consists of many known drugs. When tested at 10 μ M, different compounds (e.g., terfenadine versus amitriptyline) exhibited different inhibitory patterns (Fig. 5A). Amitriptyline completely inhibited SoCal5-WT but not SoCal5-F1760A, whereas terfenadine

Fig. 2. Comparisons of the IC_{50} values for tool compounds using electrophysiology. (A) Representative traces of Nav1.5 and SoCal5 in the absence and presence of 12 μ M amitriptyline. Test protocol is shown in the left panel. (B) To evaluate the compound pharmacology of Nav1.5 and SoCal5, the IC_{50} values of the tool compounds were tested using automated electrophysiology. Changes in peak current amplitude were normalized to maximum (100% inhibition) and minimum (0% inhibition) effects. Concentration response curves at TP1 and TP12 were generated by sigmoid fitting using the Hill equation. (C) Comparisons of the IC_{50} values of the tool compounds at TP1. The solid lines represent identical and the dashed lines refer to fivefold difference in the IC_{50} values. (D) Comparisons of the IC_{50} values of the tool compounds at TP12.

inhibited both. If a hit was defined as a compound causing at least 50% reduction in activity at 10 μ M ($IC_{50} < 10 \mu$ M), then 176 of 1280 LOPAC compounds were identified as inhibitors for SoCal5-WT with a hit rate of 13.8%, which is slightly higher than a reported hit rate of hERG (11.9%) for the LOPAC compounds (Titus et al., 2009), suggesting Nav1.5 is a promiscuous target. However, only 42 of these identified hits inhibited SoCal5-F1760A (Fig. 5B), suggesting that the high hit

rate is indeed a channel-specific effect and F1760 is important for the observed promiscuity (Fig. 5B). Then, a Hill equation-based model was used to give quantitative assessments of the site dependence of these identified hits. The model suggests that a greater deviation from the identity line reflects a greater dependence on the F1760 site, and most hits show estimated 3–30-fold changes in the IC_{50} values (see *Materials and Methods*; Fig. 5C). The identified hits were further validated

Fig. 3. Voltage change-induced calcium flux through SoCal5 was detected in a fluorescent assay. (A) Steady-state activation and slow inactivation curves for SoCal5 sodium and calcium conductance, respectively. For voltage-dependent steady-state activation, the inset protocols were used and $G/G_{\max}$ was determined by the normalized tail current amplitude at -100 mV after 40 millisecond step depolarization at various voltages. For voltage-dependent slow inactivation, the G-V relationship for SoCal5 was determined by the normalized step current at -40 mV after a 10 second long pulse at voltages ranging from -120 to $+10$ mV in 10 mV increments and a 100 millisecond pulse at -100 mV aimed at removing residual fast inactivation. (B) Calcium flux triggered by the addition of 4 mM potassium (arrow) was detected in SoCal5 cells by the fluorescent intensity change of fluo-4, only after introducing the gramicidin-mediated hyperpolarization step in the 2 mM potassium and sodium-free external buffer for 2 minutes. The detected calcium signal was sensitive to Nav channel inhibitors such as lidocaine. The same assay protocol failed to elicit a calcium signal in the parental HEK293 cells and WT Nav1.5 stable cell line.

by automated electrophysiology. Half of the hits showed more than 50% inhibition on WT Nav1.5 when using a protocol facilitating compound binding to the inactivated state and most hits exhibited strong state dependence (Fig. 5D).

Phe-Dependent Promiscuity of Nav Channels. Considering that the LOPAC is composed of known bioactives that might present a potential bias for interactions with sodium channels, the validation set (Fig. 6A), a representative collection of the Molecular Libraries Small Molecule Repository diverse compound library (Zou et al., 2010), was selected for further studies. Compared with the LOPAC, the validation set contains more distinct novel structures (Fig. 6A). Compounds were tested in duplicate at $10 \mu\text{M}$, and two repeats exhibited good correlation with $R = 0.99$ for both targets (Supplemental Fig. 5). The average of two repeats was used to reflect compound effects. Consistent with the observation in the LOPAC screen, 12.3% (245/1999) compounds reduced more than 50% SoCal5 channel activity and most hits showed less inhibition on F1760A (Fig. 6B). This finding confirmed Phe-dependent promiscuous interaction of hNav1.5 with diverse chemical structures.

The hERG potassium channel is well known as a promiscuous drug target. The effects of compounds on the validation set were compared between Nav1.5, hERG, and a nonpromiscuous channel, KCNQ2. By a hit criterion of 50% reduction in channel activity ($\text{IC}_{50} < 10 \mu\text{M}$), SoCal5 and hERG exhibited remarkably higher hit rates (Fig. 7A). Moreover, similar to hERG inhibitors, the SoCal5 inhibitors differed dramatically in the chemical structures (Fig. 7D), demonstrating that Nav1.5 is a promiscuous target susceptible to blockage by various structurally diverse compounds.

F1760 residue in hNav1.5 is highly conserved among the mammalian Nav channel family (Fig. 7B). If the promiscuity is dependent on this residue, all the Nav subtypes are expected to exhibit promiscuous interactions with diverse chemical structures. To test this hypothesis, compounds in the validation set were tested on SoCal4 (rNav1.4-CCW) at $10 \mu\text{M}$ using a fluo-4 assay. Supporting our hypothesis, the hit rate

for SoCal4 was 12.2 and 68% of the identified SoCal4 hits also inhibited SoCal5 by more than 50% (Fig. 7C). In addition, the SoCal4 hits also exhibited great dependence on the homologous F1759 residue, which further supports the importance of Phe in mediating compound interactions (Supplemental Fig. 6).

Discussion

Several fluorescence-based approaches have been established for identification and characterization of Nav modulators, including membrane potential dye-based indirect assays and sodium dye-based direct assays. Although direct assay is reported to show better correlation with electrophysiological assays (Trivedi et al., 2008), both the direct and indirect assays described previously apply ligands (e.g., veratridine) to activate channels, which may interact with test compounds and thus increase false-positive and false-negative rates. Additionally, both assays evaluate compound effects on one target at one time. Compared with these methods, the dual-color optical assay detects Nav channel activity by measuring persistent calcium flux through two genetically engineered SoCal channels simultaneously. This assay has several attractive features. First, it directly detects ionic flux, which may reduce the false-positive rate associated with indirect readout. Second, channels are activated by depolarization, a more physiologic stimulus, which avoids the risk from application of ligands. Indeed, among the 68 unbiased selected internal controls in the LOPAC with reported IC_{50} values by electrophysiology (Harmer et al., 2011), compounds with $\text{IC}_{50} < 10 \mu\text{M}$ all exhibited at least 50% inhibition, and those with $\text{IC}_{50} > 10 \mu\text{M}$ all exhibited less than 50% inhibition in our assay (Supplemental Fig. 7; Supplemental Table 1), strongly supporting the low false-positive and false-negative rates. Third, in principle the assay strategy can be applied to the entire Nav channel family since the CCW residues are highly conserved (Fig. 1A). Finally, the assay was performed in multiplex format, which may offer a unique

Fig. 4. Optimized fluo-4-based SoCal assay gives rise to the right pharmacology for tool compounds. (A) Representative traces in the fluo-4 calcium assay. Traces for cells exposed to various basal potassium concentrations (left panel) or stimulated by various potassium concentrations while fixed potassium at 8 mM (middle panel) in stimulus buffer or stimulated by various potassium concentrations with fixed calcium (right panel) in stimulus buffer. (B) Traces in the absence and presence of a known channel inhibitor, lidocaine at an optimal condition of 2 mM basal $[K^+]_o$ in combination with 8 mM $[K^+]_o$. Fluorescence signals were then triggered by a stimulus matrix with a series of K^+/Ca^{2+} combinations. In each matrix, K^+ (from top to bottom) and Ca^{2+} (from left to right) were both titrated to 0, 2, 4, 8, 16, and 32 mM. For each K^+/Ca^{2+} combination, $\Delta F/F_0$ was measured in the absence and presence of lidocaine at 10 mM. The signal window was evaluated by the difference between these two populations ($\Delta \Delta F/F_0$). To assess whether fluo-4 is saturated, the decay index was calculated and normalized based on a ratio of the fluorescence signal at 200 seconds over that at peak: decay index = $(1 - \Delta F_{200s}/\Delta F_{peak}) \times 2$. Combinations with bigger signal windows (>1.0) and higher decay index (>1.0) are optimal conditions. (D) Comparison of the IC_{50} values of known inhibitors between the fluo-4 assay and IonWorks (Molecular Devices) assay (TP12). Testing inhibitors included amitriptyline, bupivacaine, flecainide, lidocaine, propafenone, quinidine, terfenadine, and verapamil.

advantage that allows evaluation of compound effects on two targets simultaneously. It can be adaptable to scenarios where a differential effect is of the greatest interest. For example, in addition to examining the site dependence of compound effects, the platform may be applied to screening for subtype-selective compounds in one assay. Different types of SoCal channels may be coupled with calcium sensors of different optical properties in different cells and monitored in the same optical field. This can minimize the variability from separate measurements, and thus be more sensitive to the differential effects. Admittedly, only one IC_{50} value is reported for each compound on one target in our assay and the assay cannot distinguish between tonic and use-dependent blockers based on our current screening protocol. An electrophysiological approach is required to characterize the state dependence of hit compounds. Nevertheless, our multiplexed HTS assay can serve as a powerful platform to systemically determine the site dependence of compounds and isolate subtype-specific compounds.

The SoCal5 fluorescent assay measures very similar pharmacology when compared with the SoCal5 and WT Nav1.5 channel in TP12 (inactivation state) in electrophysiology (Figs.

2D and 4D). This similarity can be interpreted in the following three aspects. First, in the SoCal fluorescent assay, channels are activated by high potassium triggering a single sustained depolarization. The increase in integrated fluorescent intensity should correlate with the cumulative total ionic flux ($\int Idt$) during this depolarization. Based on a previously published kinetic model (Edrich et al., 2005) of inactivation deficient Nav1.5, the compound effect on total ionic flux ($\int Idt$) is closer to its effect on persistent current than on peak current (Supplemental Fig. 8). Although ionic conductivities differ between open and inactivation states, the persistent open state of Nav1.5 may share similar conformation with its inactivation state in terms of the architecture of critical residues mediating compound binding. Traditional inactivated state blockers (e.g., lidocaine, benzocaine, and amitriptyline) were reported to inhibit persistent open states with similar potency (Wang et al., 2004a,b). Second, three residues mutated in SoCal5 may not be molecular determinants in the inactivated channels for most compounds. The potency of TTX is reduced since its interaction site may be physically close to the selectivity filter. However, our data suggest that the vast majority of Nav inhibitors appear to differ from the TTX site by acting within the channel

Fig. 5. F1760- and state-dependent promiscuous interactions of SoCal5 with the LOPAC compounds. (A) Representative image and traces in dual-color calcium assay. Open bar, 20 μ m. (B) Differential effects of the LOPAC compounds on SoCal5-WT and SoCal5-F1760A. Effects of compounds in the LOPAC library were tested on SoCal5-WT and SoCal5-F1760A using a dual-GECO calcium assay. Compounds were tested at 10 μ M. (C) Modeling of F1760A-dependent IC_{50} shift of identified hits. (D) Validation of the SoCal5 hits in automated electrophysiology assay, IonWorks (Molecular Devices). Hit compounds were tested at 10 μ M using the voltage protocol shown in (D). Effects at TP1, TP2, and TP3 were used to evaluate use-dependent, state-dependent, and state-independent compound inhibitory effects.

inner cavity via the conserved phenylalanine residue. We observed an increased tonic inhibition on SoCal by compounds such as lidocaine or amitriptyline, consistent with an earlier report that mutations at K1237 in Nav1.4 selectivity filter (K1418 in SoCal5) will increase lidocaine block at the resting state because neutralization of the positive charge in the lysine residue can reduce electrostatic repulsion from the positively charged amine group of lidocaine (Sunami et al., 1997). The

similar mechanism may also apply to amitriptyline, which also has an amine group on the side chain. However, state-dependent Nav inhibitors might interact with different residues during tonic and activity-dependent blocks. In our electrophysiological experiments amitriptyline had very similar IC_{50} values at TP12 on SoCal5 ($1.74 \pm 0.34 \mu$ M) and Nav1.5 ($2.82 \pm 0.31 \mu$ M), suggesting the K1418 residue is not a key determinant for activity-dependent inhibition.

Fig. 6. Dual-color screen of SoCal5 channels using structurally diverse compounds. (A) Composition of validation set compounds and comparison of compound similarity between the validation set and LOPAC library; 616 LOPAC compounds have structurally similar neighbors in the validation set using a cutoff of Tanimoto = 0.80. Special set: known bioactives. (B) Comparison of compound effects on SoCal5-WT and SoCal5-F1760A. The effects of compounds in the validation set were tested on SoCal5-WT and SoCal5-F1760A. Compounds were tested at 10 μ M in duplicate. The average of two repeats was used to compare compound effects. Black circle, buffer; gray circle, compounds in validation set; red circle, 10 μ M terfenadine. GPCR, G protein-coupled receptor.

Fig. 7. Phe-dependent promiscuity of Nav channels. (A) Evaluation of promiscuity for various targets. Promiscuity of various targets was determined by the increase of distinct hits (structurally similar hits will only count once) as more compounds are sampled. Each round of 150 (approximately 10%) compounds are randomly sampled from the validation set (without replacement). (B) Alignment of the S6 segment in the fourth transmembrane domain of mammalian Nav channels and S6 of bacteria sodium channels. (C) Effects of compounds in the validation set on SoCal4 using a fluo-4 calcium assay. Compounds were tested at 10 μ M in duplicate. Repeats showed good correlation with $R = 0.92$. Hit rate and overlapped hits between SoCal4 and SoCal5 are shown. (D) Structure-activity relationship of compounds in the validation set. In the vertical dimension, the compounds in the validation set are clustered in terms of the Tanimoto coefficient based on structural similarity. In the horizontal dimension, based on their inhibitory profiles in the validation set, five different channel targets are clustered by the complete-linkage clustering algorithm.

Finally, calcium ions may not have significant impacts on compound pharmacology in the fluorescence assay. Within a certain range of voltages (-90 to -20 mV), calcium ions affect inactivation of SoCal5 channels (Fig. 3A). However, in the imaging experiments, the voltage is not clamped and it is possible that a single long depolarization can drive the membrane potential positive enough, under which the fraction of inactivated/opened channel will not be affected by the type of permeation ion.

The gating of SoCal differs from WT Nav1.5 since it has greatly impaired fast inactivation and enhanced slow inactivation. The $V_{1/2}$ (half maximum voltage) of WT Nav1.5 fast inactivation is -87 mV, whereas the $V_{1/2}$ of SoCal5 slow inactivation is -67 mV (calcium) or -60 mV (sodium). SoCal5 also developed a persistent open state, which is absent in the WT Nav1.5. A potential limitation of our approach would be the following: if a compound could distinguish these states, then its pharmacology based on SoCal5 may not be able to reflect its original pharmacology on Nav1.5. Therefore, although the SoCal fluorescent assay generally shows good approximation with WT

Nav1.5 inactivated state inhibition for the vast majority of the tested compounds, it is still recommended that the hits identified by the SoCal assay should be further validated by electrophysiological testing in WT Nav1.5.

A promiscuous target describes a given target interacting with multiple diverse ligands (Pérez-Nueno and Ritchie, 2012), for example, the hERG channel. The promiscuity underlies off-target side effects, and conversely allows development of new therapeutic applications for old drugs. Similar to hERG, our screens revealed SoCal5 channels were inhibited by substantial structurally diverse compounds in both the LOPAC and validation set collections with a hit rate of approximately 13%. By an automated patch clamp study in WT Nav1.5, at least 50% of the hits have been validated. Extended from earlier observations from known drugs, this finding has demonstrated Nav1.5 is a promiscuous target using extensive experimental evidence from diverse compounds and provides a rationale to include Nav1.5 in the cardiac safety evaluation since Nav1.5 inhibition can lead to QRS complex prolongation (Erdemli et al., 2012). Indeed, a very

recent report from the pharmaceutical industry categorized both hERG and Nav1.5 as being in the same class in terms of their high degree of promiscuity (Bowes et al., 2012).

A second important finding is that the promiscuity is F1760 residue dependent; F1760A mutation slightly changed SoCal5 activation and the inactivation curve, and may contribute to the slower decay in the fluorescent signal (Fig. 5A). However, our assay condition was tuned to set up membrane voltages that can minimize the influence from these differences. In the SoCal fluorescent assay, consistent with the literature (Pless et al., 2011), class Ib antiarrhythmics (such as lidocaine) has more than 10-fold change in IC₅₀ values by F1760A mutation, whereas class Ia and Ic antiarrhythmics (such as flecainide, quinidine, and propafenone) only show five- to sevenfold change (Supplemental Fig. 4B). Therefore, the SoCal5-F1760A fluorescent assay could be used to investigate the site dependence of different classes of drugs. The local anesthetic site in Nav channels is a well established drug binding site for local anesthetics and related compounds. F1760 was reported to be the most important residue within the local anesthetic site for drug interaction (Mike and Lukacs, 2010). In our screens, most compounds deviated from the identity line in the plot of SoCal5-F1760A and SoCal-WT activity (Figs. 5D and 6B), indicating that they rely on F1760 residue, albeit to different extents. The differential dependence at F1760 may reflect a partial overlap of multiple binding sites (Mike and Lukacs, 2010), consistent with recent indications that multiple drug binding sites in Nav may exist (Mike and Lukacs, 2010). Given that the Phe residue is highly conserved among all the Nav subtypes, promiscuity may exist in the entire Nav channel family. This indeed is supported by the high hit rate in SoCal4. Finally, the conservation of this Phe residue also partially explains why most Nav inhibitors lack subtype selectivity.

Taken together, the present study has revealed a single phenylalanine residue and inactivated state-dependent promiscuity of Nav channels. This finding has shed light on designs of sodium channel inhibitor screens. To date, only a few small molecule sodium channel inhibitors exhibit subtype selectivity (Jarvis et al., 2007; Bregman et al., 2011; McCormack et al., 2013). Screens using Phe mutant Nav channels may enrich subtype-selective inhibitors and identify inhibitors acting on novel sites with less conservation, such as the voltage sensor domain.

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Authorship Contributions

Participated in research design: Zhang, Zou, Li.

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