Mechanism-specific assay design facilitates the discovery of Nav1.7-selective inhibitors

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Many ion channels, including Nav1.7, Cav1.3, and Kv1.3, are linked to human pathologies and are important therapeutic targets. To develop efficacious and safe drugs, subtype-selective modulation is essential, but has been extremely difficult to achieve. We postulate that this challenge is caused by the poor assay design, and investigate the Nav1.7 membrane potential assay, one of the most extensively employed screening assays in modern drug discovery. The assay uses veratridine to activate channels, and compounds are identified based on the inhibition of veratridine-evoked activities. We show that this assay is biased toward nonselective pore blockers and fails to detect the most potent, selective voltage-sensing domain 4 (VSD4) blockers, including PF-05089771 (PF-771) and GX-936. By eliminating a key binding site for pore blockers and replacing veratridine with a VSD-4 binding activator, we directed the assay toward non–pore-blocking mechanisms and discovered Nav1.7-selective chemical scaffolds. Hence, we address a major hurdle in Nav1.7 drug discovery, and this mechanistic approach to assay design is applicable to Cav3.1, Kv1.3, and many other ion channels to facilitate drug discovery.

Significance

Subtype-selective modulation of ion channels is often important, but extremely difficult to achieve for drug development. Using Nav1.7 as an example, we show that this challenge could be attributed to poor design in ion channel assays, which fail to detect most potent and selective compounds and are biased toward nonselective mechanisms. By exploiting different drug binding sites and modes of channel gating, we successfully direct a membrane potential assay toward non–pore-blocking mechanisms and identify Nav1.7-selective compounds. Our mechanistic approach to assay design addresses a significant hurdle in Nav1.7 drug discovery and is applicable to many other ion channels.

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We tested the pharmacology of the N1742K-based membrane potential assay utilizing veratridine, an alkaloid from the plant *Veratrum officinale*, to stimulate channel conductance, and candidate compounds are identified based on their inhibition of veratridine-induced channel activities. The effect of veratridine is accounted for by a “foot-in-the-door” mechanism, whereby veratridine binds to the inner vestibule of the pore domain (25) and causes persistent opening by hindering inactivation and deactivation (26). We show that this assay is biased toward nonselective pore blockers and fails to detect highly potent and Nav1.7-selective compounds that act by different mechanisms. Guided by the knowledge of drug binding sites and mechanisms of action, we designed an assay capable of restricting the nonselective pore blockers and enriching compounds binding outside of the pore domains. We validated this approach in a high-throughput screen and identified unique Nav1.7-selective scaffolds.

**Results**

**Evaluation of the Conventional Membrane Potential Assay.** Membrane potential assays for sodium channels have been reported by various groups (20–22). Consistent with previous findings, veratridine (100 μM) elicited robust fluorescence signals in Nav1.7-expressing cells loaded with the blue fluorescence membrane potential dye (Fig. 1A). To evaluate pharmacology of this assay, we tested several classic sodium channel blockers. TTX is a pore-blocking neurotoxin that binds to the outer vestibule of the channel, and tetracaine is a nonselective local anesthetic that binds to the inner pore domain (27–29). TTX and tetracaine inhibited veratridine-evoked signals in a concentration-dependent manner (Fig. 1A and B). The IC₅₀ was 0.034 ± 0.005 μM for TTX and 3.6 ± 0.4 μM for tetracaine (Fig. 1E; n = 6).

Next, we tested PF-771 and GX-936, two of the most potent and selective Nav1.7 inhibitors belonging to the arylsulfonamide series (15, 16). PF-771 and GX-936 inhibited Nav1.7 with an IC₅₀ of 0.011 and 0.001 μM, respectively, in voltage clamp assays (15, 16). However, in the membrane potential assay, PF-771 and GX-936 showed marginal effects (Fig. 1 C, D, and F). At concentrations that are several orders of magnitude over the electrophysiology IC₅₀ values, PF-771 (5 μM) and GX-936 (3 μM) inhibited veratridine-induced response by less than 20% (Fig. 1F; n = 4).

The utility of membrane potential assay was further evaluated by a pilot screen of a chemical library of 64,000 compounds at 5 μM. The 0.1% DMSO, 1 μM TTX, and four potent blockers that bind to VSD4 domain (electrophysiology IC₅₀ < 0.1 μM) were embedded in the screening sets. The average inhibitory effect of DMSO on veratridine responses was 0.4 ± 13.5% (n = 1,053) and the average inhibition by TTX was 99.7 ± 4.9% (n = 792). The mean inhibition was 6.1% for the 64,000-compound screen, with a SD of 30%. None of the VSD4 blockers showed >10% inhibition, hence these compounds were not identified as active by the screen (Fig. 1G, red dots). Among the 21 most potent Nav1.7 hits, no compound showed greater than twofold selectivity against Nav1.5 channels in patch-clamp experiments (Fig. 1H). In summary, the membrane potential assay fails to detect VSD4 inhibitors and is biased toward the nonselective compounds.

**Characterization of N1742K Pore Mutant Channel.** We explored various ways of enhancing the sensitivity of the membrane potential assay for inhibitors that bind outside of the central cavity. We reasoned that by eliminating key pore binding sites, and by replacing veratridine (a pore binder) with a new activator of a different binding site, we could restrict pore binding and direct the assay toward other mechanisms. In rat Nav1.2 channel, an asparagine residue in the S6 segment of domain 4 (N1769) was critical for local anesthetics binding in the internal pore (30, 31). This asparagine residue is conserved in human Nav1.7 (N1742); therefore, we generated an asparagine-to-lysine mutant channel (N1742K). Whole-cell patch-clamp recordings using SyncroPatch 768 showed that N1742K channels were functional, despite a rightward shift in the voltage dependence of activation and inactivation (Fig. 2 A and B). The half-activation of N1742K was −9.80 ± 0.09 mV (n = 155; Fig. 2B) compared with −29.58 ± 0.14 mV for WT channels (n = 105), and the half inactivation of N1742K was −48.85 ± 0.07 mV (n = 155), compared with −62.85 ± 0.15 mV for WT channel (n = 105).

Next, we evaluated the pharmacology of N1742K channels by testing well-characterized sodium channel blockers using electrophysiology (Fig. 2 C and D). N1742K reduced the potency of tetracaine by ~400-fold, consistent with the role of this aspartagine residue in local anesthetics binding (30). N1742K also reduced the potency of AMG-52 (32) by 243-fold and the potency of CVN1014802 (raxatidine) by 71-fold, suggesting their effects were mediated by interaction with the pore domain. TTX and ProTx-II are neurotoxins that bind to the outer vestibule and voltage sensor domain 2, respectively (25, 28); their potencies were not affected by N1742K. For compounds that bind to the VSD4 domain, N1742K changed the potency of PF-771 by 3.2-fold and the potency of GX-936 by 2.4-fold. Together, these data suggest that N1742K greatly reduces the sensitivity of compounds binding to the inner pore, and has relatively minimal effects on compounds binding outside of that region.

**Identification of 1KoPMTX as an Activator of N1742K Nav1.7 Channels.** Next, we sought to identify an activator alternative to veratridine, to develop an N1742K-based membrane potential assay. We focused on activators binding to the VSD4 domain, because this region exhibits the most sequence divergence among Nav channels and because several peptide toxins and small molecules binding to this region showed subtype selectivity (15, 16, 33–35). 1KoPMTX, a derivative of wasp venom toxin α-pompidilloidotoxin (PMTX) (36, 37), showed the highest TTX-sensitive signal, followed by αPMTX, 1KβPMTX, and βPMTX (Fig. 3 A and B). This group of activators all bind to the VSD4 domain, and remove fast inactivation through immobilizing VSD4 domain (36). N1742K mutant channels had diminished response to veratridine (39 ± 1%, n = 4, relative to 1KoPMTX response). Interestingly, Nav1.7 WT channels did not produce a robust response to 1KoPMTX (Fig. 3B), possibly due to the leftward shift in voltage-dependent inactivation for WT channels (Fig. 2B). Hence, a membrane potential assay using a combination of N1742K and 1KoPMTX was developed.

**Pharmacological Evaluation of the N1742K-Based Membrane Potential Assay.** We tested the pharmacology of the N1742K-based membrane potential assay on TTX, tetracaine, ProTx-II, PF-771, and GX-936. TTX blocked 1KoPMTX activation of N1742K channels, with an IC₅₀ comparable to its inhibition of veratridine-activated WT channels (0.048 ± 0.010 μM for N753K vs. 0.034 ± 0.005 μM for WT, n = 6; Fig. 4A). The potency of tetracaine on N1742K response was reduced dramatically (IC₅₀: 488 ± 153 μM for N1742K vs. 3.6 ± 0.4 for WT, n = 6; Fig. 4B), consistent with the electrophysiology data (Fig. 2D). ProTx-II inhibits Nav1.7 channel by binding to VSD2 (38). ProTx-II blocked 1KoPMTX activation of N1742K and veratridine activation of WT channels with comparable potency (IC₅₀: 0.762 ± 0.066 μM vs. 0.794 ± 0.037 μM, n = 4; Fig. 4 C and D). In contrast to their marginal effect on WT channel responses to veratridine, PF-771 and GX-936 exhibited robust inhibition of the 1KoPMTX-evoked N1742K response, with an IC₅₀ of 0.205 ± 0.005 μM for PF-771 and 0.040 ± 0.007 μM for GX-936, respectively (n = 6; Fig. 4E–H). Therefore, unlike the conventional WT channel-based assay, the N1742K–1KoPMTX assay is capable of detecting VSD4 inhibitors while reducing sensitivity to pore blockers.

**High-Throughput Screen Using N1742K Membrane Potential Assay.** After assay optimization, we developed a fully automated,
1,536-well format membrane potential assay for N1742K channel, and screened a library of 1 million compounds at 5 μM concentration. We used 100 μM 1KαPMTX to activate N1742K mutant channel and 1 μM GX-936 as a control for channel inhibition. Assay robustness was demonstrated by the separation of activity between 0.1% DMSO and GX-936 controls (Fig. 5A). Based on analysis of 103 plates, the signal-to-background ratio was between 2.8 and 4.2 (Fig. 5B); Z’ value, an indication of assay quality, was above 0.5 for 101 of 103 plates; therefore, the assay was suitable for high-throughput screening (Fig. 5C).

Mean inhibition for the screen was 16%, with a SD of 20%. Based on a selection criterion of inhibition greater than three-fold of the SD, the hit rate was 1.3%. Further criteria were applied to remove potential activators, fluorescence quenchers, and toxic compounds, followed by cheminformatics pruning. These analyses generated a set of 9,975 compounds for follow-up studies.

To validate the N1742K-based membrane potential assay, we characterized the 9,975 screening hits and 384 randomly selected compounds by using SyncroPatch 768 electrophysiology (18). All compounds were tested at a concentration of 6.6 μM on WT Nav1.7 channels. Among the 384 randomly selected compounds, only three compounds (0.8%) showed 50–60% inhibition, and no compounds showed >60% inhibition (Fig. 5D). Among the screening hits, 1.7% of the compounds showed inhibition >90%; 5.0% of compounds showed 80–90% inhibition; 5.9% of compounds showed 70–80% inhibition; 7.4% of compounds showed 60–70% inhibition; and 7.4% compounds showed 50–60% inhibition.

Fig. 1. A conventional Nav1.7 membrane potential assay detects robust channel inhibition by TTX and tetracaine but fails to detect PF-771 and GX-936. (A–D) Representative membrane potential traces for TTX, tetracaine, PF-771, and GX-936. A total of 100 μM veratridine was added (indicated by arrows) to evoke channel activity, as represented by an increase in fluorescence signals. Cells were incubated with compounds for 3 min before veratridine application. (E) inhibition concentration–response for TTX and tetracaine. The IC₅₀ was 0.034 ± 0.005 μM for TTX, and 3.6 ± 0.4 μM for tetracaine (n = 6). (F) Inhibition dose–response for PF-771 and GX-936. Only marginal inhibition was observed (n = 4). (G) Activities of 64,000 compounds from a membrane potential assay screen. Each dot represents one compound, numbered from 1 to 64,000. Yellow, 0.1% DMSO as negative control; blue, 1 μM TTX as positive control; red, four potent VSD4 blockers; and gray, library compounds. (H) Fold difference in IC₅₀ between Nav1.5 and Nav1.7 for 21 most potent Nav1.7 hits. No compounds showed greater than twofold selectivity (dotted line).
Identification of Novel Nav1.7-Selective Blockers. The 2,740 compounds with inhibition $>$50% in SyncroPatch electrophysiology assays were further selected for concentration-response studies on Nav1.7 to confirm activity, and on Nav1.5 to determine subtype selectivity. Additionally, compounds were tested against various Nav1.7 mutant channels to reveal drug binding sites and mechanisms of actions. Through this effort, we identified compounds of high interest, as exemplified by GNE-0439 (Fig. 6A).

In the N1742K-based membrane potential assay, GNE-0439 (5 μM) nearly completely blocked responses to 1KoPMTX (Fig. 6B). However, in the conventional WT-based membrane potential assay, GNE-0439 did not inhibit the veratridine-evoked responses (Fig. 6B). Therefore, similar to PF-771 and GX-936 (Fig. 1), GNE-0439 would not have been discovered with the conventional membrane potential screen. In electrophysiology experiments (Fig. 6C), GNE-0439 inhibited Nav1.7 with an IC$_{50}$ of 0.34 μM (95% confidence: 0.29–0.73 μM) and inhibited Nav1.5 with an IC$_{50}$ of 38.3 μM (95% confidence: 21.43–70.92 μM).

Therefore, GNE-0439 is highly selective against Nav1.5. GNE-0439 also inhibited N1742K channels (IC$_{50}$ = 0.37 μM, 95% confidence: 0.46–0.76 μM), suggesting this pore residue is not involved in compound binding. R1608A, a mutation of the fourth charged arginine residue in the S4 segment of VSD4 domain, was reported to reduce arylsulfonamide binding by 1,000-fold (15). R1608A greatly reduced the potency of GNE-0439 (41.0 ± 12.8% inhibition at 66 μM, n = 6; Fig. 6C), strongly suggesting that GNE-0439 binds to the VSD4 domain.

The structure of GNE-0439 is unique compared with known selective VSD4 binders such as the arylsulfonamides (GX-936 and PF-771) (15, 16) and acylsulfonamides (39). It is structurally homologous to a class of VSD4 binders with amino acid-like constituents that we discovered through a structure-based drug design approach (40). GNE-0439 and this new class of inhibitor possess a carboxylic acid group that is predicted to form a favorable electrostatic interaction with R1608, similar to the interaction between GX-936 (an acidic arylsulfonamide) and R1608 observed in a cocrystal structure of a Nav1.7 chimeric channel (15). The discovery of GNE-0439 demonstrates that our
assay is capable of identifying Nav1.7-selective inhibitors that bind outside of the channel pore.

**Discussion**

Membrane potential assays have been used to screen tens of millions of compounds for Nav1.7 blockers (20–24). Despite the tremendous efforts, few Nav1.7-selective chemical scaffolds have been identified. This paucity of results reflects the high sequence identity of residues in the pore domain of Nav channels and, particularly, the conservation of several key residues (e.g., F1737, N1742, and Y1744) that afford promiscuous binding to diverse chemicals (14). Here we show that the lack of productivity is further aggravated by poor assay design. In the conventional membrane potential assays, compounds are incubated with Nav1.7-expressing cells first, and veratridine is applied subsequently to stimulate channel activity. As veratridine binds to the pore domain (25), pore blockers (e.g., tetracaine) may preclude veratridine binding, and are therefore preferentially identified in the screening campaign (Figs. 1 and 7).

Besides the bias toward pore binders, the conventional membrane potential assay failed to detect the most potent and selective VSD4 compounds (Fig. 1 C, D, F, and G). At concentrations several orders of magnitude greater than their IC_{50} values, PF-771 and GX-936 had minimal effect on veratridine-induced response. The current data are consistent with a previous report of an assay using voltage-sensor probes (21), where arylsulfonamides failed to inhibit WT response to veratridine. In the assay using blue fluorescence membrane potential dye, arylsulfonamides delayed the onset of fluorescence but failed to inhibit peak signals. This lack of sensitivity to arylsulfonamides could be related to their strong preference for inactivated state block. For example, PF-771 and GX-936 are ∼1,000-fold more potent on the inactivated state compared with the closed state of the channel (15, 16). In the conventional membrane potential

![Fig. 3. Development of a N1742K-based membrane potential assay using 1KαPMTX as an activator. (A) Activities of 12 Nav activators on N1742K mutant channels tested at 100 μM in the presence and absence of 5 μM TTX (n = 4). The fluorescence signals were normalized to peak fluorescence obtained with 1KαPMTX. (B) Time-course of fluorescence responses to 100 μM 1KαPMTX. Signals were normalized by peak response of 1KαPMTX. (C) 1KαPMTX did not evoked robust response in WT channels. Signals were normalized by peak response of veratridine. Arrows indicate addition of 1KαPMTX.](image-url)
Fig. 4. Characterization of Nav1.7 blockers in N1742K-based membrane potential assay. A total of 100 μM 1KαPMTX and 100 μM veratridine were used to activate N1742K and WT Nav1.7, respectively. (A) Concentration–response relationships of TTX in N1742K (black line) and in WT (dotted line) membrane potential assays. TTX IC_{50} was 0.048 ± 0.010 μM for N1742K, 0.034 ± 0.005 μM for WT (n = 6). (B) Concentration–response relationships for tetracaine for N1742K (black line) and for WT (dotted line) membrane potential assays. IC_{50} was 488 ± 153 μM (n = 4) for N1742K and 3.6 ± 0.4 μM for WT (n = 6). (C) Representative kinetic traces for varying concentrations of ProTx-II in N1742K membrane potential assay. (D) Dose–response of ProTx-II in N1742K and WT membrane potential assays. IC_{50} was 0.762 ± 0.066 (n = 4) for N1742K and 0.794 ± 0.037 μM for WT (n = 4). (E) Representative kinetic traces for PF-771 in N1742K membrane potential assay. (F) Concentration dose–responses of PF-771. IC_{50} for PF-771 was 0.205 ± 0.005 μM for N1742K (n = 6); PF-771 only had marginal effect on WT (n = 4). (G) Representative kinetic traces for GX-936 in N1742K membrane potential assay. (H) Concentration–response relationship for GX-936. IC_{50} for GX-936 was 0.040 ± 0.007 μM for N1742K (n = 6); GX-936 only had marginal effect on WT (n = 4). Dose–responses for WT (dotted lines in A, B, F, and H) appeared in Fig. 1 but were included for comparison purpose. Arrows indicate addition of 1KαPMTX.
assay, veratridine removes inactivation and therefore compromises the effects of these compounds. Regardless of the mechanisms, the conventional membrane potential assay is not capable of detecting VSD4 blockers.

Based on mechanisms of action and drug binding sites (Fig. 7), we modified the conventional assay by replacing WT Nav1.7 with the N1742K mutant, and by replacing veratridine with 1Kα PMTX as an alternative activator. The N1742K mutation disrupted a key binding site, thus reducing sensitivity to pore binders. 1Kα PMTX was derived from a family of wasp toxins known for removing fast inactivation and increasing steady-state currents of several sodium channels (36, 37). PMTXs bind to the extracellular S3–S4 linker of VSD4 domain (41). Among the four VSDs of Nav channels, VSD4 is crucial for mediating fast inactivation (42, 43). Specifically, the intramembrane charge displacement associated with movement of VSD4 is necessary for the development and recovery from fast inactivation (44). Presumably, the binding of 1Kα PMTX impedes VSD4 movement and thereby removes fast inactivation. In addition, due to the low sequence conservation in VSD4 domain, binding to VSD4 confers subtype selectivity for peptide toxins and small molecule inhibitors (15, 16, 33–35). By combining the use of N1742K pore mutant and 1Kα PMTX as an activator, we were able to direct the assay toward non-pore-binding mechanism and facilitate the detection of subtype-selective VSD4 binders, such as PF-771, GX-936, and GNE-0439. Most recently, antillatoxin, a lipopeptide from pantropical marine cyanobacteria, was used to activate Nav1.7 channel in a membrane potential assay (45). Similar to veratridine, antillatoxin activates sodium channels through binding to the inner pore domain (25, 46). The antillatoxin-based assay could detect TTX and local anesthetics, but its sensitivity to VSD4 binders was not reported (45).

Theoretically, the N1742K-based assay could lead to the identification of false positives by introducing a potential new binding site residue (K1742). However, such compounds would gain potency on the N1742K mutant channel and could be eliminated by comparing potency on WT and K1742 mutant channels using electrophysiology (as in Figs. 5E and 6C). Of note, the N1742K-based assay reduced but did not completely eliminate detection of pore binders. Although N1742 has been implicated in the binding of many pore blockers (30, 31), N1742 is not the only critical residue, and thus N1742K mutation can reduce, but may not abolish, pore binding. In fact, several other residues, including F1737 and Y1744, are also implicated...
in binding of pore blockers (14). Channels harboring two (e.g., F1737A/Y1744A) or three (F1737A/N1742K/Y1744A) mutations would potentially restrict a larger subset of pore binders. Unfortunately, the response of double- or triple-mutant channels to 1KαPMTX was not robust enough for compound screening. Of note, we observed a significant potency shift between the membrane potential assay and electrophysiology experiments (e.g., IC50: 0.205 μM vs. 0.011 μM for PF-771). There could be several explanations for this shift in potency. First, the membrane potential assay is not a linear readout of channel function. Second, to obtain robust signals for compound screening, a high concentration of 1KαPMTX (100 μM) was used, which may potentially affect IC50 values. Third (15, 16); electrophysiology data were obtained from channels in inactivated states (Fig. 2C). The membrane potential assay did not control voltage and the 1KαPMTX responses might be obtained from different channel states. Interestingly, both the N1742K–1KαPMTX assay and the WT–veratridine assay could detect ProTx-II, a VSD2 binder without state dependency, though its potency (IC50: 0.762 μM for N1742K and 0.794 μM for WT; Fig. 4D) was significantly reduced compared with electrophysiology measurements (IC50: 0.6 nM for N1742K and 0.3 nM for WT) (38). This decrease in potency might be partially caused by the slow kinetics of ProTx-II and by its promiscuous binding to compound plates and pipette tips during membrane potential experiments. It is possible that our assay is biased toward VSD4 ligands, and its utility for detecting small molecules with other binding sites (e.g., VSD2) remains to be determined. Despite these limitations, targeting VSD4 has been the most successful strategy for achieving subtype-specific modulation (15, 16, 33–35), and our assay represents a valuable tool for Nav1.7 ligand discovery. A high-throughput screen using this assay successfully enriched sodium channel blockers, leading to Nav1.7-selective hits (Figs. 5D and 6).

It is conceivable that our current assay could be further improved, or designed toward specific mechanisms or drug binding sites by using various combinations of mutant channels and activators. The mechanism-specific assay design can also be extended to other assay formats (e.g., electrophysiology), other sodium channel isoforms (e.g., Nav1.1), and other ion channel families. For example, we now routinely use electrophysiology to screen compounds using mutant channels for specific mechanisms (e.g., pore and VSD4; Fig. 6C). Besides Nav1.7, achieving subtype selectivity is challenging for many other ion channel targets such as Nav1.1, Cav1.3, and Kv1.3 (10, 35, 47). Nav1.1 possesses the same local anesthetics binding sites in the pore domain. By combining Nav1.1 pore mutations with Hm1α, a selective activator binding to VSD4 (35), assays could be developed to identify Nav1.1-selective compounds. Similarly, the promiscuous drug binding sites for Cav1.3 (dihydropyridine) and Kv1.3 (tetrodathylammonium) reside in the highly conserved pore domains. Point mutations that disrupt these sites could be
exploited to direct compound screening toward non- pore-mediated mechanisms. In summary, we revealed the intrinsic limitations of a conventional membrane potential assay that has confounded Nav1.7 drug discovery. By using a rational assay design, we directed the assay toward desired mechanisms and identified unique Nav1.7-selective scaffolds. The mechanism-specific assay design can be applied to other ion channels to facilitate future drug discovery.

### Materials and Methods

**Cells.** Human Nav1.7, N1742K and R1608A, and Nav1.5 were cloned in the tetacycline (Tet)-inducible system (pcDNA6/TIR), and stably expressed in HEK293 cells. Each cell line was grown in DMEM high-glucose medium supplemented with 10% FBS (Tet-free), 2 mM l-glutamine (Gln), bacitracin at 5 μg/mL, Zocor at 400 μg/mL, in 5% CO2, and 37 °C. When cell cultures reached ~70% confluency, expression was induced with doxycycline (1 μg/mL) in DMEM + 2% FBS + l-Glu, in the absence of antibiotics for 16 h. Cells were harvested with Accutase, spun down at 100 × g (Allegra 6R; Beckman Coulter) for 10 min, and resuspended in DMEM + 2% FBS + l-Glu at a density of 5 × 10^5 cells per milliliter.

**Reagents.** Blue membrane potential dye (RB034) was obtained from Molecular Devices. Tet-free FBS was obtained from Clontech (631101), and other cell culture reagents were from Life Technologies. TTX was obtained from Enzo Life Sciences; 1KpmTMTX and voltage-gated sodium channel activator explorer kit were obtained from Alomone Labs; Veratridine and tetracaine were from Sigma Aldrich; PF-771, GX-936, and GNE-0439 were synthesized at Genentech.

**Membrane Potential Assays for WT and N1742K Mutant Channels.** Assays were run in the 1,536-well format. BioRAPTR (Beckman Coulter) was used to dispense cells and membrane potential dye. ECHO (Labcybe) was used for dispensing of library compounds. Multidrop Combi (Thermo Fisher) was used to dilute compounds in 1,536 plates. FDS7500 (Hamamatsu) was used for compound addition and detection of fluorescent signals. Cells were dispensed at 2,000 cells per well in 4 μL total volume into Aurora Kalyspsy, 1,536 black, clear-bottom plates (CLS3833-100EA; Corning). For Nav1.7 WT cells, a 2-h attachment period at 37 °C preceded membrane potential dye addition. For N1742K cells, membrane potential dye was added at the same time as the cells. Membrane potential dye was diluted into buffer A (157.5 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, 10 mM glucose, pH 7.4) and transferred by BioRAPTR to the plates at 2 μL per well. Cells and dye were incubated for 1 h at 37 °C, then 15 min at room temperature. Plates were then transferred to FDS7500. Compound plates (1,536, 782270-1B; Greiner) were generated on ECHO and diluted with buffer A (see above) on multidrop.

For high-throughput screening, compounds were tested at a concentration of 5 μM. After 3-min incubation, veratridine was added to activate wild-type Nav1.7, and 1KpmTMTX was used to activate N1742K. Veratridine and 1KpmTMTX were each dissolved in buffer B (115 mM NaCl, 45 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Heps, 10 mM glucose, pH 7.4), and 2 μL per well was added to a final 100 μM concentration. Fluorescence signals were collected every second for 4.5 min, starting at 10 s before compound addition. Each compound plate contained 92 wells for negative control (0.1% DMSO) and 36 wells for positive control (1 μM TTX for WT–veratridine assay, or 1 μM GX-936 for N1742K–1KpmTMTX assay).

Membrane potential assay results were processed by evaluating changes in fluorescence from the average fluorescence of initial 10 vs. peak fluorescence upon activator addition. Data were normalized to basal levels. Genedata and Vortex (Dotmatics) software programs were used to process high-throughput screening data. GraphPad Prism was used for generating inhibition concentration responses.

**Electrophysiology.** Automated patch-clamp recordings were performed using the SyncroPatch 768PE (Nanion). Chips with single-hole medium resistance (5–8 MΩ) were used. Pulse generation and data collection were performed with PatchController384 V1.4.1 and DataController384 V1.3.3. Whole-cell recordings were conducted according to Nanion’s procedure. Briefly, cells were stored at 10 °C in a cell holder (shake speed at 60 rpm). Cell catching, sealing, whole-cell formation, liquid application, recording, and data acquisition were performed sequentially. The voltage protocol consists of 220 ms leak pulse to obtain seal resistance, series resistance, and cell capacitance. Series resistance compensation was set to 80% and currents were sampled at 10 kHz. All data were corrected for leak currents. After establishing baseline currents (I_{baseline}), compounds were applied and currents were obtained after 5-min incubation of compounds (I_{comp}), then followed by 2 μM TTX treatment to obtain full block (I_{block}). Compound effect after 5 min was calculated using the following formula:

\[
\text{Block} \% = \frac{1 - \left( \frac{I_{\text{comp}} - I_{\text{block}}}{I_{\text{baseline}} - I_{\text{block}}} \right)}{1} \times 100
\]

IC50 values were calculated by fitting dose–response to four-parameter Hill equation using GraphPad Prism with constrained bottom at 0 and top at 1. For high-throughput screen data analysis, the Genedata screen was used.

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