A comprehensive approach to identifying repurposed drugs to treat SCN8A epilepsy

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Summary

Objective: Many previous studies of drug repurposing have relied on literature review followed by evaluation of a limited number of candidate compounds. Here, we demonstrate the feasibility of a more comprehensive approach using high-throughput screening to identify inhibitors of a gain-of-function mutation in the SCN8A gene associated with severe pediatric epilepsy.

Methods: We developed cellular models expressing wild-type or an R1872Q mutation in the Nav1.6 sodium channel encoded by SCN8A. Voltage clamp experiments in HEK-293 cells expressing the SCN8A R1872Q mutation demonstrated a leftward shift in sodium channel activation as well as delayed inactivation; both changes are consistent with a gain-of-function mutation. We next developed a fluorescence-based, sodium flux assay and used it to assess an extensive library of approved drugs, including a panel of antiepileptic drugs, for inhibitory activity in the mutated cell line. Lead candidates were evaluated in follow-on studies to generate concentration-response curves for inhibiting sodium influx. Select compounds of clinical interest were evaluated by electrophysiology to further characterize drug effects on wild-type and mutant sodium channel functions.

Results: The screen identified 90 drugs that significantly inhibited sodium influx in the R1872Q cell line. Four drugs of potential clinical interest—amitriptyline, carvedilol, nilvadipine, and carbamazepine—were further investigated and demonstrated concentration-dependent inhibition of sodium channel currents.

Significance: A comprehensive drug repurposing screen identified potential new candidates for the treatment of epilepsy caused by the R1872Q mutation in the SCN8A gene.

KEYWORDS  
drug library, epilepsy, precision medicine, repurposed drugs, SCN8A
1 | INTRODUCTION

A rare disease is defined by the U.S. Food and Drug Administration (FDA) Orphan Drug Act of 1983 as one affecting <200,000 individuals in the United States. Collectively, however, rare diseases affect >20 million people in the United States, reflecting 6%-7% of the population. The majority of these diseases are genetic, and only about 5% have an FDA-approved treatment. Advances in sequencing technologies have helped to identify a growing number of genes linked to rare diseases, providing insight into the pathoetiologies of these disorders. Recently, there has been interest in developing treatments for genetic epilepsies by targeting underlying mechanisms. Here, we adopt a precision medicine approach to identify potential candidate treatments for a patient with an epileptic encephalopathy caused by a mutation in SCN8A.

SCN8A encodes the alpha subunit of the voltage-gated sodium channel, Na1.6, abundant in the peripheral and central nervous system (CNS), and found predominantly at the nodes of Ranvier. Na1.6 helps regulate neuronal excitability through its unique location in the distal part of the axon initial segment, its low voltage threshold for activation, and its contribution to resurgent and persistent sodium-derived currents. Mutations in Na1.6 are estimated to account for 1% of epileptic encephalopathies. The R1872 residue (GenBank NM_014191.3) is the most frequently reported site for disease-causing mutations in SCN8A, and cellular models have shown a gain-of-function phenotype caused by variants at this site. We addressed the functional effects of an SCN8A de novo mutation where arginine is substituted for glutamine, R1872Q (NM_014191.3:c.5615G>A;p.R1872Q). This mutation was reported to cause a gain-of-function molecular phenotype involving excess sodium influx upon neuronal activation, resulting in hyperexcitability that may contribute to the epileptic encephalopathy clinical phenotype. Therefore, inhibiting excess sodium influx into the cell could have specific therapeutic value by reducing neuronal hyperexcitability caused by this mutation.

We characterized the SCN8A R1872Q mutant channel in a stably transfected HEK293 cell line, confirmed the gain-of-function phenotype, and carried out high-throughput drug screening using a fluorescent imaging plate reader (FLIPR) assay. We identified 90 compounds that significantly inhibited sodium influx into the cell from a chemical library of 1320 compounds that included clinically approved drugs and nutritional supplements. Three compounds appear to be promising therapeutic candidates based on their inhibitory activity, brain uptake, and safety profile: carvedilol (beta-blocker used to treat congestive heart failure), amitriptyline (serotonin-norepinephrine reuptake inhibitor antidepressant), and nilvadipine (calcium channel blocker used for hypertension). We confirmed, and further characterized, their inhibitory effects using electrophysiology in an HEK293 cellular model. These findings demonstrate the value of using personalized cellular models of a clinically relevant patient mutation to generate a clinically relevant platform for comprehensive drug repurposing and effectively screening. This approach is conducive to identifying candidate treatments for patient-specific mutations manifesting as rare diseases, such as the infantile epileptic encephalopathies.

2 | MATERIALS AND METHODS

2.1 HEK293 SCN8A cell line generation

The wild-type hSCN8A gene was polymerase chain reaction (PCR) amplified from human dorsal root ganglion and cloned into a mammalian expression retroviral vector under a cytomegalovirus promoter. The hSCN8A R1872Q variant (g5615a; CGG to CAG) was generated by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis kit; Agilent Technologies, Santa Clara, CA, USA). Plasmids containing wild-type SCN8A were used to establish a pool of control cells. Each stable pool was maintained in growth media containing 400 μg/mL G418 for selection. A clonal cell line expressing either wild-type SCN8A or R1872Q SCN8A was selected from dilution cloning and prioritized by functional sodium current amplitude using the IonWorks...
High-throughput electrophysiology platform (Molecular Devices, Sunnyvale, CA, USA). HEK293 cells expressing either wild-type or R1872Q SCN8A were cultured in Dulbecco modified Eagle medium/high glucose containing 10% fetal bovine serum, 2 mmol/L sodium pyruvate, 10 mmol/L hydroxyethylpiperazine ethane sulfonic acid (HEPES), and 400 μg/mL G418 at 37°C in the presence of 10% CO2. Cells were routinely passaged every 3-5 days to maintain <80% confluency. All studies were completed within cell passages 7-9.

2.2 Quantitative PCR methods

Total RNA was isolated from 1 × 10⁶ cells for each cell line (RNeasy kit 74106; Qiagen, Hilden, Germany). Residual genomic DNA was eliminated by treating RNA with DNase I (Ambion DNA-free kit AM1906; Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed in triplicate using 100 μg total RNA per 20 μL reaction (Taqman One-Step RT-PCR kit 4309169 and ABI Prism 7900HT instrument, Thermo Fisher Scientific). Primer/probe sets used were specific for either human SCN8A or human GAPDH (reference gene). Relative quantification (2^(-ΔΔCT)) was used to compare human SCN8A gene expression among the SCN8A cell lines relative to HEK293 (each cell line was normalized to endogenous GAPDH).

2.3 PatchXpress protocol and analysis to determine voltage-dependent properties

Whole cell patch clamp biophysical experiments were performed using the PatchXpress 7000A automated patch clamp system (Molecular Devices). The biophysical properties of SCN8A R1872Q were compared to wild-type SCN8A. Experiments were conducted across multiple days. Test groups (wild-type or mutant cells) were alternated within a single testing day to help control for assay drift. Upon reaching stable whole cell configuration, cells were held at a membrane potential of −120 mV, then depolarized in 10-mV increments from −120 mV to +60 mV. Each depolarizing step was applied for 500 milliseconds followed by a 20-millisecond step at 0 mV. The extracellular recording solution composition was (in mmol·L⁻¹): 135...
NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 5 glucose, pH 7.4 (300 mOsm). The intracellular solution consisted of (in mmol/L⁻¹): 135 CsF, 10 CsCl, 5 NaCl, 10 HEPES, 5 ethyleneglycoltetraacetic acid, pH 7.3 (290 mOsm).

The peak currents elicited by the Δ10-mV steps (500 milliseconds each) were used to calculate current-voltage relationships. To derive the conductance-voltage (GV) relationship, the calculated reversal potential of +72 mV was used such that conductance = current / (test potential – reversal potential). The peak currents from the second 0-mV (20 milliseconds) step were used to determine voltage-dependent properties of steady-state inactivation (SSI) by plotting as a function of the Δ10-mV (500 milliseconds) prepulse voltage. Both GV and SSI curves were individually fit in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) to the Boltzmann equation to derive the voltage at which 50% of the channels are in the inactivated state (V₁/₂). Individual relationships were normalized such that fractional current = current amplitude/(fitted E max – fitted E min). These normalized data were averaged, plotted as mean ± standard error of the mean values, and fit to the Boltzmann equation to yield 95% confidence intervals in addition to V₁/₂, slope, minimum, and maximum. Nonnormalized amplitudes were comparable between SCN8A wild-type and R1872Q clones.

2.4 | Compounds and reagents for FLIPR high-throughput screening

A library of 1320 small molecules was used for high-throughput screening (HTS). Most of the drugs were taken from the Prestwick Chemical Library (Strasbourg-Ilkirch, France), a collection of 1280 molecules comprised mostly of drugs approved by the FDA, European Medicines Evaluation Agency, and/or other agencies; additional compounds included sodium channel inhibitors and antiepileptic drugs (AEDs). The drug library was prepared as 10 mmol/L⁻¹ stocks in 100% dimethyl sulfoxide (DMSO) and diluted for use at 10 l mol/L⁻¹. Compounds were compressed from 96-well microplates into 384-well Echo LDV plates (Labcyte, San Jose, CA, USA). Lead compounds were obtained from independent suppliers and further characterized with concentration-response curves (CRCs) by adding 15 l L of 10 mmol/L⁻¹ compound stocks to 35 l L of DMSO. Serial dilutions were performed: the final DMSO concentration was 0.3%.

2.5 | FLIPR experiments

The day prior to screening, cells were harvested in growth media and plated on poly-D-lysine-coated, black-walled 384-well microplates with clear bottoms (Greiner Bio-One, Kremsmünster, Austria); 25 l L of a 0.9 x 10⁶ cells per mL suspension were seeded into the plates. Plates were incubated at 37°C, 10% CO₂ overnight until used. Prior to testing, growth media were removed from the plate and
10 μL of 4 μmol·L⁻¹ Asante NaTRIUM Green-2 (ANG-2; TEFLabs, Austin, TX, USA) was added (mixed with equal volume of 20% Pluronic F127). Cells were protected from light and incubated for 60-90 minutes at room temperature. After incubation, the dye was removed from the plates and replaced with 10 μL (in mM): 135 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 5 Glucose, pH 7.4 (300 mOsm). Cell and assay plates were loaded onto the FLIPR High-Throughput Cellular Screening System (Molecular Devices) and the 5_min_5_min 384 protocol was run, as follows. Ten microliters of a preincubation plate containing EBSS + valinomycin either with or without test compound was added to the cells. Images were taken for 5 minutes to monitor effects on basal Na⁺ fluorescence. After the 5-minute incubation, 20 μL of EBSS + 30 μmol·L⁻¹ veratridine (with or without test compounds) was added and fluorescence responses were monitored for an additional 5 minutes. Data were exported as maximum-minimum over the 5-minute veratridine addition.

2.6 | PatchXpress protocol to assess pharmacological activity

Wild-type and mutant SCN8A cell lines were voltage-clamped at a holding potential of −120 mV to maintain sodium channels in a closed resting state. After current amplitude became stable, the midpoint voltage of SSI was determined for each cell using a series of 5-second conditioning steps to increasingly depolarized voltages (−120 to −40 mV) that preceded a 20-millisecond test pulse to 0 mV to establish magnitude of inactivation. The holding command potential was then set to voltage at which 50% of the channels were in the inactivated state (V₁/₂ - set automatically via PatchXpress scripts). From this holding potential, a 2-millisecond voltage step to holding potential followed by a 20-millisecond depolarizing step to 0 mV and then 2 seconds at the holding potential were applied at a frequency of 0.1 Hz until current amplitude was steady (automatically determined by PatchXpress scripts), at which time a test compound was added. The effect of test reagents on Nav current amplitude was monitored using the voltage protocol described above, and washed out after reaching steady-state as determined by PatchXpress stability scripts.

Data were collected on a PatchXpress platform using Patch Commander Software (Molecular Devices), then processed and analyzed using DataXpress 2.0 (Molecular Devices). Percentage inhibition was normalized to the average of the control and washout currents according to the formula: % Inhibition = \((\frac{(\text{Ctrl} + \text{Wash})}{2} - \text{Drug} \times (\frac{(\text{Ctrl} + \text{Wash})}{2})) \times 100\). Normalized concentration-response relationships were fitted using the XLfit software (ID Business Solutions, Guildford, UK) 4-Parameter Logistic Model. Inhibition = \(A + \left(\frac{B - A}{1 + (\frac{C}{x})^D}\right)\), where \(A = \text{assay}_{\text{min}}\) (fixed at 0%), \(B = \text{assay}_{\text{max}}\) (fixed at 100%), \(C = IC_{50}\), and \(D = \text{slope}\).

3 | RESULTS

3.1 | Clinical description and variant identification

The patient (female, 11 years old) was diagnosed with epilepsy and global developmental delay consistent with type 13 early infantile epileptic encephalopathy (EIEE13). She is nonverbal. The patient can walk <10 feet with assistance and ankle-foot orthoses. Her first afebrile seizure occurred at 3 months. Seizure types in the past have included atypical absence, myoclonic, tonic, and tonic–clonic head drops. The epilepsy was resistant to more than 10 AEDs and several immunomodulatory therapies, but did respond to carbamazepine.

When the child was 8 years old, a medical laboratory with clinical pathology accreditation (London, UK) identified the presence of a heterozygous c.5615G>A; p.R1872Q SCN8A variant in the patient’s DNA through next generation sequencing of 66 genes associated with severe developmental delay; the result was confirmed by Sanger sequence analysis. Parental analysis of lymphocyte DNA did not identify the mutation, consistent with a de novo origin in the child. The R1872Q mutation encoded by the SCN8A gene was classified as pathogenic with reference to a previous report that identified a different mutation in the SCN8A gene (L1290V) associated with epileptic encephalopathy. Subsequently, Wagnon et al. reported on a male patient with the identical R1872Q SCN8A mutation that was associated with EIEE13.

3.2 | Generation and characterization of the wild-type and Na⁺,1.6 R1872Q variant cell lines

To assess the mutation in a cellular model that would be amenable to HTS, we used the HEK293 cell line, an immortalized human cell line utilized extensively for ion channel physiology experiments and HTS studies. We used site-directed mutagenesis to introduce the R1872Q variant (g5615a; CGG to CAG) into the SCN8A cDNA, and then transfected a plasmid containing the mutated cDNA into HEK293 cells and generated a stable clonal Na⁺,1.6 R1872Q cell line. Sequence integrity of the expressed construct in each cell line was confirmed using reverse transcriptase (RT)-PCR and sequencing of the resulting cDNA. A wild-type SCN8A stable clonal cell line was also generated for control purposes.
We then functionally characterized and compared the Nav1.6 R1872Q variant and Na\textsubscript{v}1.6 wild-type cell lines using electrophysiology. Current-voltage plots were obtained by applying test pulses at membrane potentials between $-100$ mV and $+60$ mV (Figure 1A,B). The fractional GV relationship for Na\textsubscript{v}1.6 R1872Q channel activation revealed a significant 4.7 mV leftward shift in $V_{1/2}$ compared to wild-type channels ($P < 0.05$; Figure 1C). These data confirm findings from prior studies on the same mutation.\textsuperscript{9} We also investigated the voltage dependence of steady-state inactivation, finding no significant difference between cells bearing mutant or wild-type channels (Figure 1C). There was a small increase in the window current largely due to the leftward shift in voltage dependent activation as noted above (Figure 1C).

Inactivation kinetics were studied at a test potential of 0 mV (Figure 1D). The decay phase was best fit by a single exponential. A significantly delayed rate of inactivation was found in Na\textsubscript{v}1.6 R1872Q channels compared with Na\textsubscript{v}1.6 wild-type channels ($P < .05$; Figure 1D). The measured time constant for inactivation was significantly slower for the Na\textsubscript{v}1.6 R1872Q variant than the wild-type ($\tau$ inactivation: $0.7 \pm 0.1$ milliseconds, $n = 15$ for R1872Q and $0.4 \pm 0.05$ milliseconds, $n = 11$ for wild-type, $P < .05$; Figure 1E). We did not see an elevated persistent

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**FIGURE 3** Screening results from the 1320-compound library for inhibitory activity against the R1872Q SCN8A sodium channel. The histogram shows the number of screened drugs at each given level of inhibition (5%/bin). The bins in light blue represent compounds with inhibitory activity > 2 standard deviations (SD; $\geq 63.0\%$) above the group mean (10.8%). A total of 90 compounds met or exceeded this level of inhibitory activity and were defined as hits in the assay. See Table 1 for a complete list of compounds and activity.

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**TABLE 1** High-throughput screening results: compounds with significant inhibitory activity against SCN8A sodium channels at 10 $\mu$molL$^{-1}$

| Chemical name                   | Inhibition, % |
|---------------------------------|---------------|
| Dibucaine                       | 97.8          |
| Methyl benzethonium chloride     | 97.7          |
| Darifenacin hydrobromide         | 95.3          |
| Dimethisoquin hydrochloride      | 93.5          |
| Cloperastine hydrochloride       | 93.4          |
| Prenylamine lactate              | 92.6          |
| Fendiline hydrochloride          | 92.5          |
| Bromperidol                     | 90.6          |
| Carvedilol                      | 89.0          |
| Bepridil hydrochloride           | 88.9          |
| Naftopidil dihydrochloride       | 88.6          |
| DO 897/99/BP897                  | 85.6          |
| Proparacaine hydrochloride       | 85.5          |
| Loperamide hydrochloride         | 85.4          |
| Nilvadipine                     | 84.9          |
| Dyclonine hydrochloride          | 83.5          |
| Trihexyphenidyl-D,L hydrochloride| 83.3          |
| GBR 12909 dihydrochloride        | 82.7          |
| Drofenine hydrochloride          | 81.5          |
| Racemicotril                    | 81.5          |
| Lidofozine                      | 81.4          |
| Deptropine citrate               | 78.8          |
| Clomipramine hydrochloride       | 77.8          |
| Flavoxate hydrochloride          | 77.5          |
| Alverine citrate salt            | 77.5          |
| Propafenone hydrochloride        | 76.8          |
| Trimipramine maleate salt        | 76.6          |
| Trimebutine                     | 76.5          |
| Droperidol                      | 76.3          |
| Nefazodone hydrochloride         | 76.2          |
| Ethaverine hydrochloride         | 75.9          |
| Clemastine fumarate              | 75.8          |
| Terfenadine                     | 75.6          |
| Mebeverine hydrochloride         | 75.5          |
| Azelastine hydrochloride         | 75.4          |
| Metergoline                     | 75.2          |
| Eniconazole                     | 74.7          |
| Progestrone                      | 74.4          |
| Promazine hydrochloride          | 74.1          |
| Pradifen hydrochloride           | 74.0          |
| Oxethazaine                     | 73.9          |
current at a test potential of 0 mV in the Na\textsubscript{v}1.6 R1872Q line. These data also confirm findings from previous studies.\textsuperscript{9}

### 3.3 Screening of a library of approved drugs

We utilized a stimulus-activated, fluorescence-based Na\textsuperscript{+} flux assay with an FLIPR high-throughput cellular screening system.\textsuperscript{11} Fluorescence-based screening assays are frequently used for assessing ion channel function.\textsuperscript{12,13} After preincubation with the Na\textsuperscript{+} indicator dye, ANG-2, clonal cell lines were tested for their abilities to generate a fluorescent readout in response to veratridine, a neurotoxin that causes persistent opening of sodium channels. Veratridine CRCs were generated using the Nav1.6 R1872Q clonal cell line, revealing a robust dynamic range (Figure 2A) consistent with historical data generated on wild-type Na\textsubscript{v}1.6 channels and suitable for screening.\textsuperscript{14} The ability to detect a concentration-dependent reduction in sodium flux was demonstrated using known sodium channel inhibitors including tetracaine, flecainide, and mexiletine (Figure 2B). The pharmacology of expressed Nav1.6 sodium channels toward channel-specific inhibitors was also evaluated. Cells demonstrated sensitivity to tetrodotoxin, which inhibits voltage-gated sodium channels except Na\textsubscript{v}1.5, Na\textsubscript{v}1.8, and Na\textsubscript{v}1.9, and lack of activity of the tarantula venom peptide, ProTx-II, which is a selective inhibitor of Nav1.7 channels (Figure 2C).\textsuperscript{15}

We then initiated screening of a library of 1320 pharmacologically diverse drugs (including the Prestwick Chemical Library of 1280 compounds) at 10-\mu mol\textpercm{L} concentration; compounds were evaluated in duplicate. A set concentration of 10 \mu mol\textpercm{L} was considered to be suitable to identify compounds with a level of potency and effectiveness in the in vitro screen that might translate to pharmacological activity in vivo. Z-prime values were >0.5, signifying a robust,

| Chemical name | Inhibition, % |
|---------------|--------------|
| Tetracaine hydrochloride | 73.6 |
| Thioridazine hydrochloride | 73.3 |
| Clemizole hydrochloride | 73.2 |
| Ritonavir | 72.8 |
| Dicyclomine hydrochloride | 71.8 |
| Penbutolol sulfate | 71.6 |
| Ethopropazine hydrochloride | 71.4 |
| Amitriptyline hydrochloride | 71.2 |
| Benfluorex | 70.9 |
| (R)-Duloxetine hydrochloride | 70.7 |
| Verapamil hydrochloride | 70.3 |
| Fluvoxamine maleate | 70.0 |
| Homochlorcyclizine dihydrochloride | 69.8 |
| Cyclobenzaprine hydrochloride | 69.7 |
| Triflupromazine hydrochloride | 69.6 |
| Butacaine | 69.4 |
| Fluoxetine hydrochloride | 69.0 |
| Ciproheptadine hydrochloride | 68.8 |
| Spiperone | 68.3 |
| Metixene hydrochloride | 68.2 |
| Perhexiline maleate | 68.1 |
| Benoxinate hydrochloride | 67.8 |
| Dilazep dihydrochloride | 67.7 |
| Imipramine hydrochloride | 67.6 |
| Pergolide mesylate | 67.4 |
| Benperidol | 67.1 |
| Indatraline hydrochloride | 67.0 |
| Econazole nitrate | 66.7 |
| Chloropyramine hydrochloride | 66.6 |
| Benzonatate | 66.6 |
| Dydrogesterone | 66.2 |
| Perphenazine | 66.1 |
| Mebhydrolin 1,5-naphtalenedisulfonate | 65.8 |
| Nafinyl oxalate | 65.7 |
| Trimiprazine tartrate | 65.6 |
| Flunarizine dihydrochloride | 65.5 |
| Nicergoline | 65.3 |
| Melengestrol acetate | 65.0 |
| Perospirone | 64.8 |
| Pizotifen maleate | 64.8 |
| Moricizine hydrochloride | 64.3 |
| Biperiden hydrochloride | 64.2 |
| Atomoxetine hydrochloride | 64.0 |

(Continues)
A high-throughput assay suitable for screening of the Na$_{1.6}$ R1872Q cell line to identify inhibitors of sodium influx. Screening was conducted against a background of 30 µmol·L$^{-1}$ veratridine and normalized to 30 µmol·L$^{-1}$ tetracaine (100%) and DMSO (0%) controls. The average inhibition for the entire library was 10.8%. Ninety compounds showed >2 standard deviation inhibition (≥63.0%) from the mean (Figure 3A); the majority of these have not been previously linked to sodium channels, in particular with activity against Na$_{1.6}$, or other ion channel activity in general. This group of 90 compounds was then screened a second time using independently sourced drugs at 10 µmol·L$^{-1}$ to confirm activity in the assay. The complete list of 90 hit compounds along with inhibitory activity is shown in Table 1.

### 3.4 | Comparison to traditional pharmacological agents

We sought to further assess the response of both the Na$_{1.6}$ R1872Q and Na$_{1.6}$ wild-type lines to 24 AEDs using CRCs (10 points, 10 nmol·L$^{-1}$ to 300 µmol·L$^{-1}$, n = 4 across 2 separate trials). Drugs were tested in both the Na$_{1.6}$ R1872Q and wild-type Na$_{1.6}$ cellular models. Across a full CRC, several AEDs showed strong inhibition of both channels (eg, clonazepam), whereas the majority were far less effective, even at relatively high drug concentrations of 300 µmol·L$^{-1}$ (Table 2). We also generated CRCs for 10 known sodium channel inhibitors in wild-type and Na$_{1.6}$ R1872Q cell lines. Most compounds demonstrated a moderate level of activity with a trend for greater

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**Table 2** Characterization of known antiepileptic drugs against wild-type and R1872Q SCN8A cell lines

| Compound name | SCN8A R1872Q maximum inhibition, % | Wild-type SCN8A maximum inhibition, % | SCN8A R1872Q average IC$_{50}$, µmol·L$^{-1}$ | Wild-type SCN8A average IC$_{50}$, µmol·L$^{-1}$ |
|---------------|-------------------------------------|---------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Clonazepam    | 96.6                                | 102.2                                 | 39.8                                          | 22.6                                          |
| Tiagabine     | 80.6                                | 94.2                                  | 71.8                                          | 31.1                                          |
| Clobazam      | 73.1                                | 83.7                                  | 137.7                                         | 89.2                                          |
| Retigabine    | 67.0                                | 82.4                                  | 133.2                                         | 62.3                                          |
| Phenytoin     | 60.6                                | 80.7                                  | 119.0                                         | 55.9                                          |
| Carbamazepine | 60.0                                | 70.4                                  | 228.4                                         | 153.2                                         |
| Lamotrigine   | 36.8                                | 69.8                                  | >300                                          | 179.8                                         |
| Esticarbazepine acetate | 28.8 | 41.7 | >300 | >300 |
| Oxcarbazepine | 26.7                                | 46.7                                  | >300                                          | 261.2                                         |
| Ethotoin      | 20.1                                | 20.5                                  | >300                                          | 300.0                                         |
| Felbamate     | 19.8                                | 30.2                                  | >300                                          | 247.0                                         |
| Ethosuximide  | 18.8                                | 12.1                                  | >300                                          | >300                                          |
| Methsuximide  | 18.1                                | 31.5                                  | >300                                          | >300                                          |
| Phenobarbital | 18.0                                | 31.1                                  | >300                                          | >300                                          |
| Vigabatrin    | 16.2                                | 10.4                                  | >300                                          | >300                                          |
| Levetiracetam | 15.2                                | 13.4                                  | >300                                          | >300                                          |
| Lacosamide    | 14.3                                | 8.3                                   | >300                                          | >300                                          |
| Topiramat     | 11.9                                | 12.9                                  | >300                                          | >300                                          |
| Divalproex sodium | 11.8 | 14.4 | >300 | >300 |
| Gabapentin    | 11.5                                | 7.2                                   | >300                                          | >300                                          |
| Rufinamide    | 10.5                                | 3.0                                   | >300                                          | >300                                          |
| Primidone     | 8.3                                 | 8.0                                   | >300                                          | >300                                          |
| Valproic acid | 5.7                                 | 6.2                                   | >300                                          | >300                                          |
| Zonisamide    | −0.4                                | 6.8                                   | >300                                          | >300                                          |

Characterization of known antiepileptic drugs against wild-type and Na$_{1.6}$ R1872Q SCN8A cell lines. Data are based on 10-point concentration-response curves, 10 nmol·L$^{-1}$ to 300 µmol·L$^{-1}$, n = 4 across 2 separate trials in the presence of 30 µmol·L$^{-1}$ veratridine. Percentage inhibition is normalized to 30 µmol·L$^{-1}$ tetracaine (100%) and dimethyl sulfoxide controls (0%).
TABLE 3  Characterization of known sodium channel inhibitors for selectivity to Na<sub>1.6</sub> R1872Q

| Compound name       | SCN8A R1872Q maximum inhibition, % | Wild-type SCN8A maximum inhibition, % | SCN8A R1872Q average IC<sub>50</sub>, µmol·L<sup>-1</sup> | Wild-type SCN8A average IC<sub>50</sub>, µmol·L<sup>-1</sup> | Indicated therapeutic class |
|---------------------|-------------------------------------|---------------------------------------|------------------------------------------------|-------------------------------------------------|-----------------------------|
| Mexiletine hydrochloride | 68.6                                | 84.9                                  | 119.6                                        | 53.1                                            | Antiarrhythmic              |
| Disopyramide        | 68.0                                | 72.9                                  | 150.0                                        | 84.4                                            | Antiarrhythmic              |
| Carbamazepine       | 60.0                                | 70.4                                  | 228.4                                        | 153.2                                           | Analgesic/anticonvulsant    |
| Prilocaine hydrochloride | 46.9                                | 57.9                                  | 299.7                                        | 174.8                                           | Anesthetic                  |
| Articaine hydrochloride | 40.5                                | 60.2                                  | >300                                         | 153.4                                           | Anesthetic                  |
| Procaine hydrochloride | 28.1                                | 34.6                                  | >300                                         | >300                                            | Anesthetic                  |
| Oxcarbazepine       | 26.7                                | 46.7                                  | >300                                         | 261.2                                           | Anticonvulsant              |
| Benzocaine          | 24.3                                | 42.3                                  | >300                                         | 279.2                                           | Anesthetic                  |
| Topiramate          | 11.9                                | 12.9                                  | >300                                         | >300                                            | Anticonvulsant              |
| Tocainide hydrochloride | 9.4                                 | 15.1                                  | >300                                         | >300                                            | Anesthetic                  |

Data acquired by 10-point concentration-response curves from 10 µmol·L<sup>-1</sup> to 300 µmol·L<sup>-1</sup>, n = 4 across 2 separate trials, in the presence of 30 µmol·L<sup>-1</sup> veratridine. Percentage inhibition is normalized to 30 µmol·L<sup>-1</sup> tetracaine (100%) and dimethyl sulfoxide controls (0%).

FIGURE 4  Electrophysiological characterization of lead compounds. Graphs show concentration-dependent current inhibition (%) of Na<sub>1.6</sub> wild-type (black circles) and Na<sub>1.6</sub> R1872Q (red squares) channels for amitriptyline, carvedilol, nilvadipine, and carbamazepine. Five-point concentration-response curves (n ≥ 4 for each data point) were generated for all 4 test compounds on wild-type and Na<sub>1.6</sub> R1872Q variants. Table shows the fitted mean IC<sub>50</sub> for each compound against both cell lines.

| Compound        | R1872Q IC<sub>50</sub> (µM) | Wild-Type IC<sub>50</sub> (µM) |
|-----------------|-----------------------------|--------------------------------|
| Amitriptyline   | 3.84                        | 1.74                           |
| Carvedilol      | 4.14                        | 3.83                           |
| Nilvadipine     | 5.81                        | 3.34                           |
| Carbamazepine   | 70.24                       | 55.96                          |
inhibitory potency on the Na\textsubscript{\textit{v}}1.6 wild-type cell line compared to the Na\textsubscript{\textit{v}}1.6 R1872Q cell line (Table 3).

### 3.5 | Electrophysiological evaluation of select compounds

To further explore the activity of compounds that demonstrated strong inhibitory effects in the FLIPR screen, we evaluated 3 lead compounds, amitriptyline, carvedilol, and nilvadipine, in an electrophysiological assay, along with carbamazepine; a well-known sodium channel blocker and an AED with demonstrated clinical benefit in the SCN8A R1872Q patient. The inhibitory effect of these compounds was assessed using the PatchXpress automated electrophysiological platform, with Na\textsubscript{\textit{v}}1.6 R1872Q and wild-type cell lines held at the midpoint voltage of SSI. Peak currents in response to a depolarizing pulse were measured across a 5-point CRC, which confirmed a strong inhibitory effect of the test compounds in a concentration-dependent manner (Figure 4). The IC\textsubscript{50} values for Na\textsubscript{\textit{v}}1.6 wild-type and Na\textsubscript{\textit{v}}1.6 R1872Q were determined from the CRCs and showed the same trend for greater potency of effect on the wild-type over mutant channel as was noted above with AEDs (Table 2) and sodium channel inhibitors (Table 3).

### 4 | DISCUSSION

Our study confirms the utility of a high-throughput functional assay to comprehensively evaluate approved medicines to identify potential targeted treatments that could be available for immediate clinical use. This work shows that at least for a sodium channel with a clear gain-of-function mutation, a comprehensive drug repurposing screen is both feasible and effective. The present study suggests that testing only one or a few candidate drugs to identify a potential repurposed treatment for a genetic condition provides too limited a picture of the potential drug opportunities that could be therapeutically evaluated.

When translating findings from cellular assays to therapeutic use, it is essential to consider whether concentrations required for in vitro activity are physiologically relevant to prescribed clinical doses. To that end, we compared the drug concentrations of our lead compounds, amitriptyline, nilvadipine, and carvedilol, as well as carbamazepine, required to inhibit Na\textsubscript{\textit{v}}1.6 R1872Q sodium channel activity in vitro, to published data on CNS drug concentrations from standard oral clinical doses. Carbamazepine has an average brain concentration of 3.52 \textmu mol/L\textsuperscript{-1} over a variety of doses when unbound fraction and brain penetration are taken into account.\textsuperscript{16,17} Relating that CNS level to the data generated with our electrophysiological evaluation (Figure 4) correlates to 4.7% inhibition against the Na\textsubscript{\textit{v}}1.6 R1872Q channel and 9.6% inhibition against the wild-type ion channel with clinical doses. When carvedilol is taken at a 100-mg daily dose (50 mg, twice daily), a steady-state maximum plasma concentration of 205 ng/mL (0.50 \textmu mol/L\textsuperscript{-1}) is reached within 1.5 hours.\textsuperscript{18} Carvedilol penetrates the blood-brain barrier, resulting in an estimated average CNS concentration of 0.15 \textmu mol/L\textsuperscript{-1}.\textsuperscript{19} The steady-state free brain concentrations would therefore correspond to an inhibition of 5.6% and 7.4% against the mutant and the wild-type channels, respectively, which is similar to carbamazepine. Likewise, nilvadipine, a calcium blocker used to treat hypertension, reaches a steady-state maximum plasma concentration of 11.7 ng/mL with 16 mg taken daily.\textsuperscript{20} The concentration that reaches the brain, roughly 0.05 \textmu mol/L\textsuperscript{-1},\textsuperscript{21} would correspond to 1.3% inhibition against the mutant and 0.4% against the wild-type according to the CRCs, which is lower than predicted values for carbamazepine. Amitriptyline is an effective antidepressant at steady-state plasma concentrations at 150-250 ng/mL.\textsuperscript{22} Although data on brain levels of amitriptyline following chronic dosing are somewhat limited, studies in rodents suggests levels can reach 5-7 \textmu mol/L\textsuperscript{-1}.\textsuperscript{23} From the present study, amitriptyline at this concentration would correspond to a 59% to 84% inhibition of Na\textsubscript{\textit{v}}1.6 channels based on results from the electrophysiological assay (Figure 4).

Whereas nilvadipine has not been previously reported to act on sodium channels, both amitriptyline and carvedilol are known to inhibit sodium channels in a use-dependent manner.\textsuperscript{24,25} Amitriptyline has high affinity for both open and inactivated channels, but low affinity for resting state channels.\textsuperscript{26} Binding to the open and inactivated channel states is also a common feature of AEDs,\textsuperscript{27} allowing for preferential inhibition of high-frequency repetitive firing neurons during seizures with more limited impact on normal brain activity.\textsuperscript{28} Precise mechanisms of binding and how these 3 inhibitors affect the Na\textsubscript{\textit{v}}1.6 contribution to resurgent and persistent sodium-derived currents in neurons remain to be investigated.\textsuperscript{6,7} Nevertheless, this work identified interesting drug candidates for further investigation, especially amitriptyline, which was shown to be a potent inhibitor of Na\textsubscript{\textit{v}}1.6. Although the way SCN8A mutations cause disease in vivo cannot be fully captured in an in vitro heterologous expression system, the active compounds we identified through HTS represent important candidates for further characterization in appropriate in vivo models, and for subsequent design of new chemical entities.

Identifying viable candidates from an extensive library of clinically approved drugs that directly inhibit a gain-of-function pathogenic mutation illustrates the feasibility and potential of comprehensive drug repurposing screening to identify new therapeutic options for serious genetic conditions. Comparing estimated CNS exposure at clinically
established doses of a drug with assay inhibitory activity provides an opportunity for more informed selection of therapeutically meaningful treatment decisions in affected patients.

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DISCLOSURE OF CONFLICT OF INTEREST

B.C.G., C.M.M., J.R., M.A.F., G.R.S., S.Petrov., O.D., M.M., S.Petrou, and D.B.G. have financial interests in Painnomix. D.B.G. also has a financial interest in Praxis Pharmaceuticals. S.Petrou also has financial interest in Praxis Pharmaceuticals and receives funding from RogCon. O.D. receives research support from Novartis, PTC Therapeutics, GW Pharmaceuticals, and Zogenix. The remaining authors have no conflicts of interest. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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