PRX-2904 is a Small Molecule Selective Inhibitor of $K_{Na}1.1$ (KCNT1) that Reverses the ECoG Phenotype of a KCNT1 Gain-of-Function Mouse Model

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Background

- Gain-of-function (GoF) mutations in KCNT1 (encoding $K_{Na}1.1$, Slack, Slo2.2) cause drug-resistance and severe forms of infantile epilepsy including devastating epilepsy of infancy with migrating focal seizures (EIMFS),[1, 2-4] and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE).[5-7]
- Treatment options for KCNT1-related disease are extremely limited, and the seizures and comorbidities are intractable to conventional antiepileptic drugs.
- KCNT1 encodes the neuronal potassium channel $K_{Na}1.1$ (Slack, Slo2.2) which is highly expressed throughout the central nervous system.[8]
- Like other voltage-gated potassium channels, functional $K_{Na}1.1$ channels are tetramers composed of four subunits; each containing a voltage sensing (S1–S4) and a pore-forming (S5-pore loop-S6) domain.
- $K_{Na}1.1$ is weakly gated by voltage and is activated by alterations in cytoplasmic signaling cascades, changes in energy state (ATP, NAD+), and increases in intracellular sodium.
- These features allow the channel to open in response to short-term increases in neuronal activity, whereby increases in potassium efflux is thought to reduce neuronal activity.
- Paradoxically, disease-causing variants in KCNT1 have invariably been found to increase the activity of the channel in a GoF manner.
- As such, orally active inhibitors of $K_{Na}1.1$ would be significant as potential therapeutics and as tools to advance the knowledge of $K_{Na}1.1$ in neurophysiology.
- We recently discovered the first small molecule orally-active $K_{Na}1.1$ inhibitor (compound **31**, also known as PRX-2904).[9]
- A radiolabeled binding panel and off-target ion channels assays show PRX-2904 is selective for $K_{Na}1.1$.
- Here we elaborate on the *in vitro* and *in vivo* profiling of PRX-2904, including its efficacy and tolerability in a Kcnt1-P905L (*Kcnt1*$^{L/L}$) mouse model of KCNT1 GoF.

Methods

- A high throughput screen (HTS) using a rubidium (86Rb) flux assay in HEK-TREX cells stably expressing the human EIMFS variant P924L (hKNa1.1-P924L) was developed.
- Cells were preloaded with Rb and incubated for 10 min with 10 µM of test compound in the presence of elevated KCl (5.4 mM) to depolarize the membrane potential and activate $K_{Na}1.1$ mediated Rb efflux.
- The amount of Rb efflux was quantified and expressed as percent efflux.
- Approximately 72,000 compounds were screened using a custom-built library designed to maximize chemical diversity.
- Hit rate was defined as greater than 55% inhibition. These hits were reconfirmed for activity; 270 of which were found to have a half maximal inhibitory concentration (IC_{50}) of 15 µM or less.
- These 270 compounds were subsequently tested in an automated SyncroPatch patch clamp assay.

***In vitro* profiling**

- PRX-2904 was profiled in automated patch clamp using HEK cells stably expressing human or mouse $K_{Na}1.1$ (WT or a panel of GoF mutants) with 70 mM internal sodium to activate the channel.
- Whole-cell patch clamp recordings from hippocampal CA1 pyramidal neurons in acute brain slices (P16-30) from WT or *Kcnt1*$^{L/L}$ mice were used to evaluate $K_{Na}1.1$ contribution to intrinsic neuronal excitability.
- A current injection protocol (-60 to +340 pA) was used to determine effects on action potential (AP) firing frequency.

***In vivo* profiling**

- PRX-2904 was tested in *Kcnt1*$^{L/L}$ mice (P32-40) implanted with ECoG electrodes to monitor interictal spike and seizure frequencies.
- After establishing a 24-h baseline, *Kcnt1*$^{L/L}$ mice were dosed with PRX-2904 (30-75 mg/kg) or vehicle, and ECoG recorded for an additional 24 h.
- The effects of PRX-2904 (10-150 mg/kg) on spontaneous locomotion were tested in CD-1 mice to determine any nonspecific sedative effects.
- Brain concentrations of PRX-2904 in satellite mice were measured using mass spectrometry.

Discovery of PRX-2904: a Potent and Selective Inhibitor of $K_{Na}1.1$ (KCNT1)

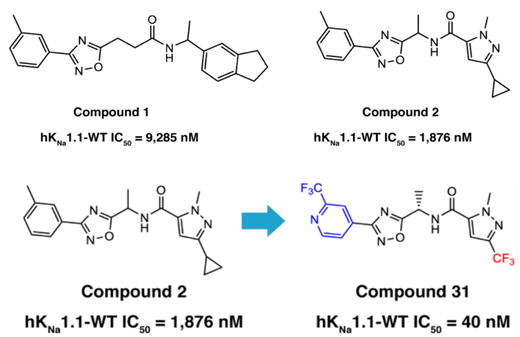


Figure 1. Rubidium (86Rb) flux high throughput screening assay (HTS) yielded compound cluster containing phenyl oxadiazole scaffold as a hit.

Top panel: Structure of Compound **1** and Compound **2**, containing a phenyl oxadiazole scaffold, was selected for structure-activity relationship (SAR) studies.

Bottom panel: Compound **2** was of particular interest, and initial development of the scaffold helped to understand the basic SAR. Subsequent structural modification of Compound **2** lead to the discovery of Compound **31** (PRX-2904).

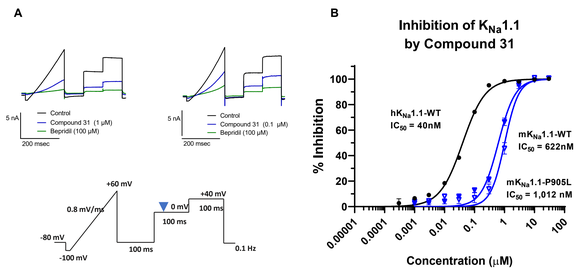
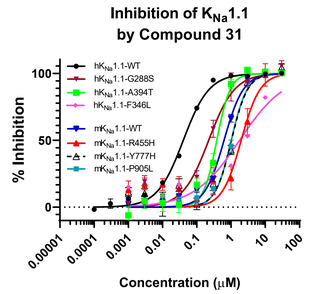


Figure 2. In in-vitro assays, PRX-2904 inhibited hK$_{Na}$1.1-WT with high potency (40 nM) and the activity was retained at mK$_{Na}$1.1-WT (622 nM) and mK$_{Na}$1.1-P905L (1,012 nM).

(A) Top panel shows representative whole cell current-voltage recording from HEK-293 cells expressing hK$_{Na}$1.1-WT and mK$_{Na}$1.1-P905L in the presence of 1 µM PRX-2904 (*blue*) and 100 µM Bepridil (*green*). *Bottom panel* shows voltage protocol used for whole cell recording, cells were voltage clamped at −80 mV, and inhibition was measured using a voltage step to 0 mV marked as blue downward arrow. *(B)* Mean±SEM concentration-inhibition for hK$_{Na}$1.1-WT, mK$_{Na}$1.1-WT and mutant K$_{Na}$1.1-P905L channels in response to 0.003–30 µM of PRX-2904 (n≥5).

PRX-2904 Reduces Evoked Action Potential Firing in $K_{Na}1.1$ GoF CA1 Neurons



Target	IC_{50} (nM) / Hill Slope
$hK_{Na}1.1$- WT	40 / 1.1
$hK_{Na}1.1$- A934T	383 / 2.2
$hK_{Na}1.1$- F346L	1,768 / 0.7
$hK_{Na}1.1$- G288S	222 / 1.1
$cK_{Na}1.1$- WT	49 / 0.7
$mK_{Na}1.1$-WT	622 / 1.5
$mK_{Na}1.1$-P905L	1,012 / 1.9
$mK_{Na}1.1$-R455H	1,975 / 1.6
$mK_{Na}1.1$-Y777H	1,025 / 1.8
$rK_{Na}1.1$-WT	545 / 1.7

Figure 3. In in-vitro assay, PRX-2904 inhibits hK$_{Na}$1.1 and mK$_{Na}$1.1 variants representing recurrent variants.

Left panel: Mean±SEM concentration-inhibition plots for wild-type and mutant $K_{Na}1.1$ channels (human and mouse) in response to 0.003-30 µM of PRX-2904 (n≥5).

Right panel: Table shows corresponding IC_{50} and Hill slope for PRX-2904.

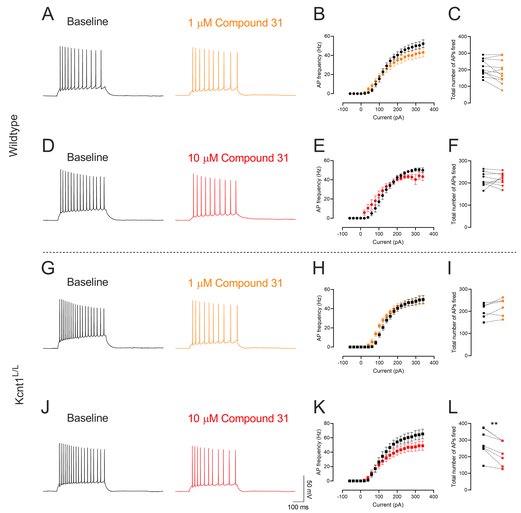


Figure 4. PRX-2904 did not affect evoked neuronal AP frequency from CA1 pyramidal neurons in WT brain slices, but reduced firing by 21% (p=0.0015) in Kcnt1$^{L/L}$ slices.

(A,D,G,J) Representative traces of baseline (*black*) and in the presence of 1 µM (*orange*) and 10 µM (*red*) PRX-2904. *(B,E,H,K)* Quantification of input-frequency relations, and *(C,F,I,L)* total number of action potentials. Data presented as mean±SEM and paired individual data points. **p<0.01.

PRX-2904 Normalizes Abnormal Interictal Spikes Without Affecting Locomotion

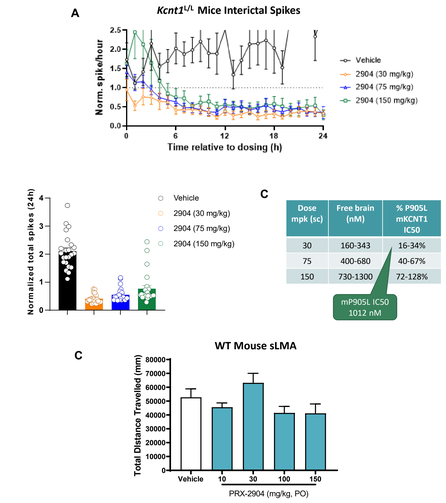


Figure 5. PRX-2904 normalizes EEG phenotype in Kcnt1$^{L/L}$ mice (P32-40) without affecting spontaneous locomotor activity.

(A) Acute administration of PRX-2904 (30, 75 and 150 mg/kg, subcutaneously) decreased interictal spike frequency and *(B)* decreased normalized total spikes in *Kcnt1*$^{L/L}$ mice. (C) Table showing free brain concentration of PRX-2904 with respect of different doses mentioned. (D) PRX-2904 (10–150 mg/kg) did not affect spontaneous locomotor activity in wildtype CD-1 mice. Data represented as mean±SEM, n=4–10; *P<0.05 vs respective baseline; ***P<0.0001 vs. vehicle.

Conclusions

- **PRX-2904, a selective inhibitor of $K_{Na}1.1$, demonstrated activity in human/mouse and in WT/mutant channels.**
- **PRX-2904 normalized AP firing in *Kcnt1*$^{L/L}$ mouse brain slices.**
- **PRX-2904 inhibits interictal spikes and spontaneous seizures in *Kcnt1*$^{L/L}$ mice.**
- **PRX-2904 did not affect locomotion at doses up to 5-fold higher than the lowest effective dose in *Kcnt1*$^{L/L}$ mice.**
- **Our combined *in vitro* and *in vivo* data suggest PRX-2904 may have an acceptable therapeutic window balancing potential efficacy and tolerability.**
- **Future work is needed to evaluate PRX-2904 in additional mouse models of *KCNT1* GoF.**

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