Synthesis and Evaluation of Potent KCNQ2/3-Specific Channel Activators

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ABSTRACT

KQT-like subfamily (KCNQ) channels are voltage-gated, non-inactivating potassium ion channels, and their down-regulation has been implicated in several hyperexcitability-related disorders, including epilepsy, neuropathic pain, and tinnitus. Activators of these channels reduce the excitability of central and peripheral neurons, and, as such, have therapeutic utility. Here, we synthetically modified several moieties of the KCNQ2–5 channel activator retigabine, an anticonvulsant approved by the U.S. Food and Drug Administration. By introducing a CF3-group at the 4-position of the benzylamine moiety, combined with a fluorine atom at the 3-position of the aniline ring, we generated Ethyl (2-amino-3-fluoro-4-((4-(tri-fluoromethyl)benzyl)amino)phenyl)carbamate (RL648_81), a new KCNQ2/3-specific activator that is >15 times more potent and also more selective than retigabine. We suggest that RL648_81 is a promising clinical candidate for treating or preventing neurologic disorders associated with neuronal hyperexcitability.

Introduction

Epilepsy is the most common neuronal hyperexcitability disorder, affecting 1% of the world population. This neurologic condition is generally managed with sodium channel blockers or GABA receptor agonists (Bialer et al., 2010; Bialer and White, 2010). Although there are several drugs in clinical use with distinct mechanism of action, unfortunately approximately 30% of patients do not respond to these agents (Brodie et al., 2010; Sharma et al., 2015). Thus, there is an urgent need for drug development to broaden the treatment options.

KCNQ (or Kv7) channels play a critical role in maintaining neuronal excitability and have recently emerged as a potential target for the prevention and treatment of epilepsy and other hyperexcitability-related disorders, including neuropathic pain and tinnitus (Gribkoff, 2008; Miceli et al., 2008; Brown and Passmore, 2009; Wickenden and McNaughton-Smith, 2009; Wulff et al., 2009; Li et al., 2013; Grunnet et al., 2014). These noninactivating potassium channels are open at resting membrane potentials and function as a brake on the excitability of central and peripheral neurons (Brown and Adams, 1980; Robbins, 2001). The KCNQ family comprises five subunits (KCNQ1–5): KCNQ2–5 are confined to the nervous system, including the brainstem and inner ear, whereas KCNQ1 is limited to the heart and peripheral epithelial and smooth muscle cells (Howard et al., 2007). Genetic mutations in either KCNQ2 or KCNQ3 subunits are linked to benign familial neonatal convulsions, whereas noise-induced reduction in KCNQ2/3 channel activity leads to development of tinnitus in mice (Biervert et al., 1998; Jentsch, 2000; Li et al., 2013; Li et al., 2015). Moreover, pathologic reduction in KCNQ2/3 channel activity is involved in different classes of seizures, neuropathic pain, migraine, anxiety, attention deficient-hyperactivity disorder, schizophrenia, mania, and bipolar disease (Murro and Dalby-Brown, 2007; Hansen et al., 2008; Grunnet et al., 2014). As a result, KCNQ channel activators, which lead to the opening of these channels at more hyperpolarized potentials, have recently been employed to treat or prevent epilepsy (Gribkoff, 2008; Miceli et al., 2008; Xiong et al., 2008).

Several KCNQ channel openers are under active development for the management of hyperexcitability disorders (Dalby-Brown et al., 2013; Grunnet et al., 2014; Stott et al., 2014; Davoren et al., 2015). Retigabine, which activates all KCNQ2–5 channels, is the only anticonvulsant KCNQ activator that has been approved by the U.S. Food and Drug Administration (FDA) (Tatulian et al., 2001; Gunthorpe et al., 2012). However, recent data have shown severe side effects associated with retigabine, including urinary retention, blue skin discoloration, and retinal abnormalities.
(Jankovic and Ilickovic, 2013). As a result, the FDA has limited its use to patients who have not responded to alternative treatments.

The undesirable side effects are likely due to the poor selectivity of retigabine among KCNQ2–5 channels as well as metabolic degradation products of its aniline ring. For example, retigabine activates KCNQ4 and KCNQ5, which are not involved in the pathology of hyperexcitability-related disorders. KCNQ4 is the primary potassium channel in the smooth muscle of the bladder, where it regulates contractility (Jentsch, 2000; Greenwood and Ohya, 2009). Activation of KCNQ4 leads to membrane hyperpolarization and results in significantly reduced contractility, which may be the cause for urinary retention associated with the use of retigabine. Moreover, a form of dominant deafness arises from loss of function of KCNQ4 (Kharkovets et al., 2000), so the opening of these channels may affect hearing.

In addition to expression in the central nervous system, KCNQ4 and KCNQ5 are also found in skeletal muscle (Jentsch, 2000; Iannotti et al., 2010; Iannotti et al., 2013). Accordingly, there is an urgent need for the development of potent and selective KCNQ2/3 channel activators, which, unlike retigabine, do not activate KCNQ4 and KCNQ5 channels.

To achieve this aim, we synthesized and evaluated several novel KCNQ2/3-specific channel activators. To maximize potency, we manipulated the different chemical components of “zones” of retigabine (Fig. 1). Particularly, by introducing a CF₃-group in zone 1 at the 4-position of the benzylamine moiety, combined with a fluorine atom in zone 2 at the 3-position of the aniline ring, we generated Ethyl (2-amino-3-fluoro-4-((4-(trifluoromethyl)benzyl)amino)phenyl)carbamate (RL648_81), a new KCNQ2/3-specific activator that is 3 times more potent and also more selective than Ethyl (2-amino-3-fluoro-4-((4-fluorobenzyl)amino)phenyl)carbamate (SF0034), a recently described retigabine analog with increased potency and selectivity for KCNQ2/3 channels (Kalappa et al., 2015). We propose that RL648_81 is a promising clinical candidate for the treatment or prevention of hyperexcitability-related neurologic disorders.

**Materials and Methods**

**Constructs and Chemicals.** The KCNQ2, KCNQ3, KCNQ4, KCNQ5, and KCNQ2 (W236L) constructs as well as the PIPKI-g90 plasmid used in this study have been described previously elsewhere (Soh and Tzingounis, 2010; Kalappa et al., 2015) and were generously provided by Dr. Anastassios Tzingounis (University of Connecticut, Storrs, CT). Buffers and salts were purchased from Sigma-Aldrich (St. Louis, Missouri, MO). Compounds stock solutions (20 mM) were made in dimethylsulfoxide. All stock solutions were stored at −20°C. On the day of experiment, fresh working drug concentrations were prepared from stock solutions by dissolving them in physiologic buffer solution.

**Cell Culture and Transfection.** Chinese hamster ovary (CHO) cells were plated on glass coverslips in 35-mm culture dishes and were incubated and maintained at 37°C in a humidified incubator.
with 5% CO₂. To get a heterologous KCNQ2/3 configuration, CHO cells were transiently transfected with human KCNQ2 and KCNQ3 subunits cDNA in a 1:1 (0.5 μg:0.5 μg) ratio with 0.5 μg of green fluorescent protein plasmid using lipofectamine transfection reagent (Thermo Fisher Scientific, Waltham, MA) and used for recording within 24 to 72 hours after transfection. For homomeric KCNQ4 and KCNQ5 channels, 1 μg of plasmid cDNA was used. To record robust KCNQ5 currents, we cotransfected KCNQ5 with pIREs-deRed-PIPKI isoforms consisting of (in mM): 132 K-gluconate, 10 KCl, 4 Mg₂⁺ATP, 20 HEPES, and 1 EGTA-KOH, pH 7.2–7.3. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of (in mM): 144 NaCl, 2.5 KCl, 2.25 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 22 d-glucose, pH 7.2–7.3. Osmolarity was adjusted to 300–305 mOsm and pH to 7.2–7.3 with NaOH.

We conducted electrophysiology experiments at room temperature (22–25°C). Patch pipettes of borosilicate glass (BF150-110-10; Sutter Instrument Company, Novato, CA) were pulled to a tip resistance of 4–6 MΩ. Patch pipettes were filled with a solution consisting of (in mM): 132 K-gluconate, 10 KCl, 4 Mg₂⁺ATP, 20 HEPES, and 1 EGTA-KOH, pH 7.2–7.3. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of (in mM): 144 NaCl, 2.5 KCl, 2.25 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 22 d-glucose, pH 7.2–7.3. Osmolarity was adjusted to 300–305 mOsm and pH to 7.2–7.3 with NaOH.

The cells were clamped at −85 mV, and currents were elicited by 1-second depolarization potentials in 10 mV increments, from −105 to +15 mV, followed by a return step to −70 mV. Currents elicited by each voltage step were measured and used to generate the conductance-voltage (G-V) curves, as described in the figure legends. These values are adjusted for the calculated junction potential, which was −15 mV. Series resistance was compensated by 75%.

To quantify the potency of the tested compounds, we measured the shift in V₁/₂ at increasing concentrations and used a Hill equation fit to calculate their EC₅₀, which is the concentration of the compound that produces a half-maximal shift in V₁/₂. Data were acquired through an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 2 kHz, sampled at 10 kHz using the pClamp 10 data acquisition system (Molecular Devices).

**Whole-Cell Patch Clamp Electrophysiology.** We used whole-cell patch clamp electrophysiology to assess the effects of retigabine and of the synthesized compounds on KCNQ channel currents. We conducted electrophysiology experiments at room temperature (22–25°C). Patch pipettes of borosilicate glass (BF150-110-10; Sutter Instrument Company, Novato, CA) were pulled to a tip resistance of 4–6 MΩ. Patch pipettes were filled with a solution consisting of (in mM): 132 K-gluconate, 10 KCl, 4 Mg₂⁺ATP, 20 HEPES, and 1 EGTA-KOH, pH 7.2–7.3.

**Data Analysis and Statistics.** We used the Boltzmann function to fit the G-V curves and determine the maximal conductance (Gₘₐₓ) and half-maximal activation voltage (V₁/₂) of KCNQ currents, where:

\[ G = G_{\text{max}}/[1 + e^{-(V - V_{1/2})/k}] \]  

and k is the slope factor. To calculate the dependence of the shift in V₁/₂ with the concentration of different KCNQ channel activators, we measured the V₁/₂ of KCNQ2/3 currents in presence of various concentrations of compounds (100 nM to 30 μM). We then fitted the agonist dependence of the shift of the V₁/₂ obtained by different concentrations with a Hill equation, where \[ \Delta V_{1/2} = V_{1/2 \text{max}} + x \text{EC}_{50} \text{max} \times \text{EC}_{50} \text{max} \times [\text{Activator}] / ([\text{Activator}] + \text{EC}_{50} \text{max}). \]

**Fig. 3.** SF0034 is 5 times more potent than retigabine in activating KCNQ2/3 channel currents. CHO cells transiently expressing heterologous KCNQ2/3 channels were clamped at −85 mV, and KCNQ2/3 currents were elicited by a 1-second depolarization step, in 10 mV increments from −105 to +15 mV, followed by a return step to −70 mV; the voltage protocol is shown below A1. (A1,B1) Representative current traces of KCNQ2/3 channels recorded at −85 mV, −105 mV, and −70 mV, and half-maximal activation voltage (V₁/₂) of KCNQ2/3 currents calculated from normalized G-V Boltzmann curves at control and at increasing concentration of retigabine (Li et al., 2005).

**A1**

- **Control**
- **KCNQ2/3**
- **Retigabine (100 nM)**

**B1**

- **Control**
- **SF0034 (100 nM)**

**A2**

- **Control**
- **1 μM**
- **10 μM**

**B2**

- **Control**
- **100 nM**
- **1 μM**
- **10 μM**

**C**

- **SF0034**
- **Retigabine**

**D**

- **SF0034**
- **Retigabine**

All data are presented as mean values ± S.E.M. Statistical significance between control and test conditions was determined using two-way ANOVA followed by Tukey’s post-hoc test. Detailed values are found in the Supplemental Data (values for main figures).
using Student’s *t* test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer post hoc test for multiple comparisons was performed as needed. Data analysis and statistical tests were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) and Origin2015 (OriginLab, Northampton, MA).

**Results**

Retigabine and its derivative SF0034 shift the voltage-dependent opening of KCNQ channels toward more hyperpolarized potentials, leading to increased K⁺ currents at resting membrane potential (Tatulian et al., 2001; Kalappa et al., 2001). Addition of a trifluoromethyl group at the 3- or 4-position of the phenyl ring in zone 1 significantly increased the potency in activating KCNQ2/3 currents. CHO cells transiently expressing heterologous KCNQ2/3 channels were clamped at −85 mV, and KCNQ2/3 currents were elicited by a 1-second depolarization step, in 10 mV increments from −105 to +15 mV followed by a return step to −70 mV; the voltage protocol is shown below. Representative current traces of KCNQ2/3 channels recorded at increasing membrane potentials in the absence or presence of 100 nM NR561_40 (A1), 100 nM NR561_50 (B1), 100 nM NR579_04 (C1), and 100 nM NR561_62 (D1). Representative curves of normalized G-V relationship of KCNQ2/3 currents at control and at increasing concentration of NR561_40 (A2), NR561_50 (B2), NR579_04 (C2), and NR561_62 (D2). Summary bar graphs representing half-activation (V½) of KCNQ2/3 currents calculated from normalized G-V Boltzmann curves at control and at increasing concentration of NR561_40 (A3), NR561_50 (B3), NR579_04 (C3), and NR561_62 (D3). Representative curves showing half-activation shift (ΔV½) by NR561_40 (EC50 0.91 ± 0.08 μM, n = 4–9; red) (A4), by NR561_50 (EC50 0.74 ± 0.07 μM, n = 4–9; red) (B4), by NR579_04 (EC50 NA, n = 4–9; red) (C4), and by NR561_62 (EC50 1.48 ± 0.18 μM, n = 4–9; red) (D4). NR561_40 and NR561_50 showed similar potency as SF0034 in activating KCNQ2/3 channel currents. NR561_62 showed 2 to 3 times lower potency than SF0034 whereas NR579_04 did not activate KCNQ2/3 currents at all. Curves were fitted with the Hill equation, and EC50 values were calculated. Error bars represent the mean ± S.E.M. *P < 0.05, **P < 0.01. Detailed values are found in the Supplemental Data (values for main figures).
To generate KCNQ2/3 channel activators with increased potency and selectivity, we partitioned retigabine’s chemical structure into three distinct zones (Fig. 1). During the first round of chemical synthesis we mainly modified zones 1 and 3, whereas in the second iteration of our structure-activity relationship (SAR) analysis, we combined the beneficial modifications found for zones 1 and 3 with previously known beneficial modifications on zone 2, which led to the development of SF0034 (Fig. 1; Kalappa et al., 2015).

Based on the site of modification, we also categorized first-generation compounds into three classes. Class I compounds had substitutions at the phenyl ring in zone 1, class II compounds had substitutions at the methylene group in zone 1, and class III compounds featured substitutions in zone 3 (Fig. 2). Our guiding principle for this round of SAR studies was to modulate both steric and, in particular, electronic features in zone 1 by introducing fluoride, trifluromethyl, and, importantly, a novel pentafluorosulfanyl group, which is considered a “super-trifluoromethyl” substituent (Wipf et al., 2009; Mo et al., 2010; Alvarez et al., 2015). These modifications led to our class I analogs NR561_29, NR561_40, NR561_45, and NR561_50 (see Supplemental Data for full chemical names).

As part of our class II analog design, we introduced substituents at the benzylic methylene group, designed to slow down metabolic degradation (trifluromethyl: NR579_04; deuterium: NR561_87). Moreover, we introduced a preliminary series of heterocycle analogs of the phenyl group in zone 1, that is, a more electron-rich thiophene (NR579_46) and a more electron-deficient thiazole (NR579_38). Finally, our class III design modified the steric size of the ethylcarbamate in zone 3 by introducing an iso-propyl group (NR561_62) and the solubilizing oxetane derivatives NR579_45 and NR579_36 (Sprachman and Wipf, 2012; Skoda et al., 2014). For synthesis details, full chemical names, and spectroscopic information on these compounds, see the Supplemental Data.

**Incorporation of Highly Fluorinated Substituents at the Phenyl Ring of Retigabine Increases the Potency of KCNQ2/3 Channel Activation.** To set the control conditions for assessing the potency and selectivity of the newly synthesized compounds, we first evaluated the ability of two standard activators, retigabine and SF0034, in potentiating KCNQ2/3 channel-mediated K+ currents under our assay conditions (Fig. 3). We transiently expressed heterologous KCNQ2/3 channels in CHO cells and tested the effect of increasing concentrations (100 nM to 30 μM) of retigabine and SF0034 on KCNQ2/3 currents. We employed whole-cell patch clamp electrophysiologic techniques (Kalappa et al., 2015). We found that 100 nM SF0034 increased KCNQ2/3 currents at hyperpolarized potentials, whereas 100 nM retigabine failed to show any effect (Fig. 3A1 and Fig. 3B1). Consistent with our previous studies (Kalappa et al., 2015), SF0034 was approximately 5 times more potent than retigabine in shifting the V1/2 of KCNQ2/3 currents (retigabine: EC50 3.3 ± 0.8 μM, n = 4–11; SF0034: EC50 0.60 ± 0.06 μM; n = 5–21, P < 0.01) (Fig. 3, A–D).

Next, we tested the ability of the newly synthesized compounds to shift the V1/2 of KCNQ2/3 currents. We used different concentrations of these analogs to evaluate whether the new substituents resulted in gain or loss of potency in activating KCNQ2/3 currents compared with retigabine and SF0034. A concentration of 100 nM NR561_40 (CF3-group at the 4-position of the phenyl ring) increased the KCNQ2/3 currents at hyperpolarized potentials (Fig. 4A). Consistent with this finding, a Boltzmann fit of the G-V relationship revealed a significant shift in the V1/2 toward hyperpolarized potentials in the presence of 100 nM or 10 μM NR561_40 without altering Gmax (Gmax ns: control: 0.95 ± 0.015, n = 9; 100 nM: 0.99 ± 0.043, n = 5; 10 μM: 0.97 ± 0.108, n = 4) (Fig. 4A2–4).

Similarly, we measured the shift in V1/2 in the presence of increasing concentrations of NR561_40 (100 nM to 30 μM) and calculated the EC50 values (Fig. 4A2). NR561_40 showed a similar potency as SF0034 (Fig. 5A).

Because retigabine has only one fluorine atom at the 4-position of the phenyl ring, these results suggested that increasing the steric bulk and electron-withdrawing properties at this position improves the potency at KCNQ2/3 channels. Like SF0034, first-generation class I compounds NR561_40, NR561_50, and NR561_29 showed 4 to 5 times higher potency than retigabine but showed a significantly lower maximal ΔV1/2 compared with NR561_40 and SF0034. Maximal ΔV1/2 values were calculated from the observed shift in V1/2 at 10 μM concentrations.
Fig. 6. NR561_50 does not activate KCNQ4 and KCNQ5 channel currents. CHO cells transiently expressing heterologous homomeric KCNQ4 or KCNQ5 channels were clamped at −85 mV, and currents were elicited by a 1-second depolarization step, in 10 mV increments from −105 to +15 mV, followed by a return step to −70 mV; the voltage protocol is shown below A1. (A1, B1) Representative current traces of KCNQ4 (A1) and KCNQ5 (B1) channels recorded at increasing membrane potentials in absence and in presence of 100 nM NR561_50. (A2, B2) Representative curves of normalized G-V relationship of KCNQ4 (B1) and KCNQ5 (B2) currents at control, 100 nM, and 1 μM NR561_50. (A3, B3) Summary bar graph representing half-activation voltage ($V_{1/2}$) of KCNQ4 (A3) and KCNQ5 (B3) channel currents calculated from normalized G-V relationship of KCNQ4 (B1) and KCNQ5 (B2) currents at control, 100 nM, and 1 μM NR561_50. (C1, D1) Representative current traces of KCNQ4 (C1) and KCNQ5 (D1) channels recorded at increasing membrane potentials in absence and in presence of 100 nM NR561_40. (C2, D2) Representative curves of normalized G-V relationship of KCNQ4 (C2) and KCNQ5 (D2) currents at control, 100 nM, and 1 μM NR561_40. (C3, D3) Representative current traces of KCNQ4 (C3) and KCNQ5 (D3) channels recorded at increasing membrane potentials in absence and in presence of 100 nM NR561_29. (C4, D4) Representative curves of normalized G-V relationship of KCNQ4 (C4) and KCNQ5 (D4) currents at control, 100 nM, and 1 μM NR561_29.
of the compound (Fig. 5B). This result suggested that a large steric size in zone 1 might limit the potency/efficacy at KCNQ2/3 channels (Fig. 5).

Furthermore, when either the smaller CF3-group or the larger SF5-group were introduced at the 3-position of the phenyl ring, the resulting NR561_50 (Fig. 4B) and NR561_45 both showed similar potency but a lower maximal $\Delta V_{1/2}$ compared with NR561_40 and SF0034. From this result we concluded that the exact position of the fluorinated groups at the phenyl ring plays a critical role in determining the potency/efficacy of KCNQ2/3 activators at KCNQ2/3 channels.

Likewise, we tested class II and class III compounds for their potency and efficacy at shifting the $V_{1/2}$ of KCNQ2/3 currents. The class II compounds NR579_38, NR579_46, and NR561_87 shifted the $V_{1/2}$ of KCNQ2/3 channels but did not show any improvement in EC50 values relative to retigabine. Interestingly, NR579_04 failed to show any effect on KCNQ2/3 channel currents, even at concentrations as high as 10 $\mu$M, which demonstrates the need for a small linker between the phenyl ring and the benzylic amine (Fig. 4C and Fig. 5). Among class III compounds, only NR561_62 demonstrated potency that was 2- to 3-fold better than retigabine; the more hydrophilic substituents were not tolerated (Fig. 4D).

Taken together, our SAR results demonstrate that the position and the steric size of fluorinated groups in zone 1 of retigabine are critical determinants of compound potency and efficacy. Addition of CF3- and SF5-groups in positions 3 and 4 of the phenyl ring of retigabine generates KCNQ2/3 activators with increased potency over retigabine. Incorporation of a trifluoromethyl substituent specifically at the para-position of the benzylamine moiety resulted in the maximal improvement in potency and efficacy at KCNQ2/3 channels, eclipsing that of SF0034. Manipulation of the retigabine structure as probed in class II and III compounds did not provide further improvements in KCNQ2/3 channel potency, with the exception of NR561_62, where the ethyl group was replaced with a more lipophilic iso-propyl chain in the carbamate moiety.

**KCNQ2/3 Selectivity of CF3- and SF5-Containing Analogs.** Next we determined the selectivity profile of the class I compounds that showed increased potency for KCNQ2/3 channels, namely, NR561_40, NR561_50 and NR561_29. To assess the selectivity of these analogs, we quantified their effect on homomeric KCNQ4 and KCNQ5 channels. First, we tested NR561_50 at homomeric KCNQ4 channels. A concentration of 100 nM NR561_50 failed to alter the $V_{1/2}$ of KCNQ4 channels (Fig. 6A). A Boltzmann fit of the G-V relationship in presence of 100 nM or 1 $\mu$M compound did not shift the $V_{1/2}$ of KCNQ4 channels (Fig. 6A). Similarly, NR561_50 did not shift the $V_{1/2}$ of KCNQ5 channels (Fig. 6B). Although 100 nM NR561_40 and NR561_29 did not change the $V_{1/2}$, at 1 $\mu$M concentration both NR561_40 and NR561_29 shifted the $V_{1/2}$ of KCNQ4 currents toward more hyperpolarized potentials (Fig. 6, C and E). Furthermore, NR561_40 and NR561_29 increased KCNQ5 currents and shifted the $V_{1/2}$ to more hyperpolarized potentials at 100 nM and 1 $\mu$M (Fig. 6, D and F).

Taken together, NR561_50 proved to be selective for KCNQ2/3 channels whereas NR561_40 and NR561_29 were found to be less selective. This profile suggests that incorporation of a trifluoromethyl group at the meta-position of the benzylamine generates activators with enhanced KCNQ2/3 selectivity.

**Incorporation of a Fluorine Substituent in Zone 2 in Analogs with CF3- and SF5-Functions in Zone 1 Generates the Most Potent KCNQ2/3 Activator Yet Described.** Because incorporation of a fluorine atom at the 3-position of the aniline ring of retigabine improved the potency and selectivity at KCNQ2/3 channels (Kalappa et al., 2015), we hypothesized that addition of fluorinated groups in zone 2 might also further improve the potency and selectivity of class I compounds. To test our hypothesis, we selected the most potent and efficacious compounds based on the first-generation SAR (Fig. 5) and introduced a fluorine atom at the 3-position of the aniline. In this fashion, RL648_81 was obtained as the fluorinated analog of NR561_40, RL648_73 as the fluorinated analog of NR648_50, RL648_86 as the fluorinated analog of NR561_62, and RL673_02 as the fluorinated analog of NR561_45 (Fig. 7). For synthetic, full chemical names, and spectroscopic details, see the Supplemental Data.

We first evaluated the effect of different concentrations of RL648_81 at KCNQ2/3-mediated currents (Fig. 8A). Application of 100 nM RL648_81 robustly shifted the $V_{1/2}$ of KCNQ2/3 channels toward hyperpolarized potentials. Also, increased concentrations of 1 $\mu$M or 10 $\mu$M RL648_81 caused a more robust shift in $V_{1/2}$ compared with the shift caused by 1 $\mu$M or 10 $\mu$M SF0034 (Fig. 8A–C). Evaluation of the EC50 revealed that RL648_81 is 3 times more potent than SF0034 at shifting the voltage dependence of KCNQ2/3 channels to more hyperpolarized potentials (Fig. 8D; EC50 0.19 ± 0.02 $\mu$M, n = 5). This shift in $V_{1/2}$ was not associated by changes in $G_{\text{max}}$ (Fig. 8A1; $G_{\text{max}}$ control: 0.96 ± 0.012; $G_{\text{max}}$ nor : control: 0.96 ± 0.012; $G_{\text{max}}$ nor : nor: 0.89 ± 0.02; $G_{\text{max}}$ nor : nor: 0.95 ± 0.02).

**Summary bar graph representing half-activation voltage ($V_{1/2}$) of KCNQ4 (C3) and KCNQ5 (D3) channel currents calculated from normalized G-V Boltzmann curves at control, 100 nM, and 1 $\mu$M NR561_40.** Error bars represent the mean ± S.E.M. *$P <$ 0.05; **$P <$ 0.01. Detailed values are found in the Supplemental Data (values for main figures).
Fig. 8. RL648_81 is 3 times more potent than SF0034 in activating KCNQ2/3 channel currents and does not potentiate KCNQ4 and KCNQ5 channel currents. CHO cells transiently expressing heterologous KCNQ2/3 channels or homomeric KCNQ4 and KCNQ5 channels were clamped at −85 mV, and currents were elicited by a 1-second depolarization step, in 10 mV increments from −105 to +15 mV, followed by a return step to −70 mV; the voltage protocol is shown in A1 (left). (A1) Representative current traces of KCNQ2/3 channels recorded at increasing membrane potentials in absence and presence of 100 nM or 1 μM RL648_81. (A2) Representative current traces of KCNQ4 and KCNQ5 channels recorded in absence and presence of 100 nM RL648_81. (A3) Representative curves of normalized G-V relationship of KCNQ2/3 currents at control and at increasing concentration of RL648_81. (A4) Summary bar graph representing half-activation (V1/2) of KCNQ2/3 currents calculated from normalized G-V Boltzmann curves at control and at increasing concentration of RL648_81. (A5) Summary bar graph representing half-activation voltage shift (∆V1/2) by RL648_81 (EC50 0.19 ± 0.02 μM, n = 4–11; red) and SF0034 (EC50 0.60 ± 0.06 μM, n = 5–21; black) in a concentration-dependent manner (100 nM to 30 μM). Curves
Fig. 9. Conserved residue tryptophan at S5 of KCNQ2-5 subunit is critical for potentiation effect of RL648_81 at KCNQ2 currents. CHO cells transiently expressing homomeric KCNQ2WT and KCNQ2(W236L) channels were clamped at −85 mV, and currents were elicited by a 1-second depolarization step, in 10 mV increments from −105 to +15 mV, followed by a return step to −70 mV; the voltage protocol is shown below A1. (A1, A2) Representative current traces of KCNQ2WT (A1) and KCNQ2(W236L) (B1) channels recorded at increasing membrane potentials in absence and presence of 10 μM RL648_81. (A2, B2) Representative curves of normalized G-V (conductance-voltage) relationship of KCNQ2WT (A2) and KCNQ2(W236L) (B2) calculated from normalized G-V Boltzmann curves at control and at increasing concentrations of RL648_81. Mutation of W236 at KCNQ2 subunit abolished the potentiation effect of RL648_81 at KCNQ2 currents. Error bars represent the mean ± S.E.M. *P < 0.05; **P < 0.01. Detailed values are found in the Supplemental Data (values for main figures).

The conserved residue, tryptophan (W) in the intracellular end of the S5 helix, W236, is critical for the enhancing effect of retigabine (Schenzer et al., 2005). To determine whether the gating effect of RL648_81 also requires the same W residue, we examined the influence of RL648_81 in KCNQ2 channels that lack W236. Indeed, although RL648_81 had a strong gating effect on KCNQ2 channels, this efficacy was abolished upon substitution of W236 to L (Fig. 9, A and B). These results suggest that RL648_81, like retigabine and SF0034, requires W236 to exert its enhanced gating properties.

Taken together, our SAR investigations led to the discovery of several highly selective and potent channel agonists, including RL648_81, the most potent KCNQ2/3-specific channel activator reported to date, which has demonstrated 3 times higher potency than SF0034. Moreover, RL648_73 and RL648_86, which are two times more potent than SF0034, represent useful secondary lead compounds. Absorption, distribution, metabolism, excretion/ toxicity data for SF0034 were fitted with a Hill equation, and EC_{50} values were calculated. (B2) Representative current traces of KCNQ4 currents at increasing membrane potentials in absence and in presence of 100 nM RL648_81. (B2) Representative curves of normalized G-V relationship of KCNQ4 currents at control, 100 nM, 1 μM, and 10 μM NL648_81. (B2) Summary bar graph representing half-activation (V_{1/2}) of KCNQ4 currents calculated from normalized G-V relationship curves at control and at increasing concentration of RL648_81. (B3) Summary bar graph representing half-activation voltage (V_{1/2}) of KCNQ4 currents calculated from normalized G-V Boltzmann curves at control and at increasing concentrations of RL648_81. Mutation of W236 at KCNQ2 subunit abolished the potentiation effect of RL648_81 at KCNQ2 currents. Error bars represent the mean ± S.E.M. *P < 0.05; **P < 0.01. Detailed values are found in the Supplemental Data (values for main figures).
are not available yet, but in view of the undesirable side effects associated with retigabine, we suggest that these more potent, selective, and electronically deactivated KCNQ2/3 activators may be attractive candidates for treating or preventing hyperexcitability disorders at lower dosage and with reduced side effects.

**Discussion**

Epilepsy is a hyperexcitability disorder that affects approximately 65 million people worldwide (Bialer and White, 2010). Despite the availability of more than 20 antiepileptic drugs, 25%–40% of epileptics are refractory to the treatment (Brodie, 2010). Moreover, even when currently available drugs are helpful, they are not without adverse effects (Bialer et al., 2010; Thurman et al., 2011). Therefore, there is an urgent need for novel drugs with improved therapeutic index characterized by increased potency and reduced toxicity.

Retigabine, which stabilizes the open state of KCNQ2-5 channels and shifts the voltage-dependence toward more hyperpolarized potentials (Wuttke et al., 2005), is an FDA-approved KCNQ channel activator that serves as an add-on for the treatment of resistant partial-onset seizures (Gunthorpe et al., 2012). However, recently identified side effects of retigabine, including retinal abnormalities, skin discoloration, and urinary retention, are significantly limiting its clinical use.

Here, we disclose the preparation and biological analysis of RL648_81 and related analogs, and report that this compound is the most potent KCNQ2/3-specific channel activator known to date. Due to its higher yet selective agonism on KCNQ2/3 channels, RL648_81 is expected to be more effective at a lower dose than retigabine and its analog SF0034 in preventing seizures. In addition, RL648_81 does not activate either KCNQ4 or KCNQ5. Because of its enhanced specificity over retigabine, RL648_81 is expected to have fewer side effects.

Although the mechanism by which retigabine-mediated toxicity influences skin and retina remains poorly understood, one hypothesis is that UV radiation may cause photodegradation and oxidation of retigabine’s aniline ring, which may lead to the formation of colored deposits in skin and eyes. The incorporation of electron-withdrawing highly fluorinated substituents significantly reduces the highest occupied molecular orbital energy of RL648_81 (−8.33 eV) versus retigabine (−8.06 eV), and thus should render RL648_81 less prone to formation of reactive metabolites (Kawai et al., 2007).

The trifluoromethylated RL648_81 is also expected to be more resistant to photodegradation and therefore less toxic to the eye and to the skin (Dow et al.; 2006). In particular, the electronic properties of RL648_81 (Supplemental Fig. 2C) compared with retigabine (Supplemental Fig. 2A) suggest that its aniline π-system as well as all three attached nitrogen atoms are considerably more electron-deficient. Furthermore, the CF3-substituent is a stronger deactivator of the benzylamine π-system, and also presents a greater steric barrier to potential cytochrome P450-induced arene hydroxylation. The electrostatic potential map for RL673_02 (Supplemental Fig. 2B) and RL648_73 (Supplemental Fig. 2D) is closely related to that of RL648_81, with the SF5-substituent in RL673_02 providing even greater steric and electronic deactivation of the compound.

The conserved residue W236 is necessary for the enhancing effect of retigabine, SF0034, and RL648_81 on the gating properties of KCNQ2 channels (Schenzer et al., 2005). Whereas it was thought that the critical property of W was its hydrophobicity (Wuttke et al., 2005), recent studies have revealed that the ability of W to form H-bonds with the carbonyl/carbamate oxygen atom present in retigabine makes this contact critical (Kim et al., 2015). These studies suggest that the strength of the H-bond between retigabine and W236 determines the potency of retigabine. In part, the improved potency of SF0034 and RL648_81 is probably due to their ability to form stronger H-bonds with W236 than retigabine. However, besides W265, other residues are important for the gating effects of retigabine, such as L272, L314, and L338 (KCNQ3 numbering) and G301/G340 (KCNQ2/KCNQ3 numbering) (Schenzer et al., 2005; Wuttke et al., 2005; Lange et al., 2009). Thus, based on our SAR studies, we also suggest that the activity of analogs can be further modulated by hydrophobic interactions at the benzylamine moiety, which the carbamate-W236 interaction positions into the vicinity of L272, L314, and L338 (Lange et al., 2009), and that the more lipophilic RL648_81 (logP = 3.4) is a better fit than retigabine (logP = 2.5) for this hydrophobic pocket at the pore-forming S5 inner loop and S6 helix domains, located near the intracellular voltage-operated gate of KCNQ2–5 channels.

In conclusion, our investigation of fluorinated substituents on retigabine, in particular the effect of highly fluorinated polar and lipophilic CF3- and SF5-groups in the benzylamine portion of the KCNQ2-5 channel activator, yielded a series of submicromolar affinity activators with exquisite selectivity for KCNQ2/3. We propose that the combination of increased potency and selectivity of RL648_81, as well as its structural features and modified electrostatic properties, may provide a solution to the problems associated with the undesirable side effects associated with retigabine.

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**Authorship Contributions**

**Participated in research design:** Kumar, Aizenman, Wipf, Tzounopoulos.

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**References**


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