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Discovery of Novel Retigabine Derivatives as Potent KCNQ4 and KCNQ5 Channel Agonists with Improved Specificity

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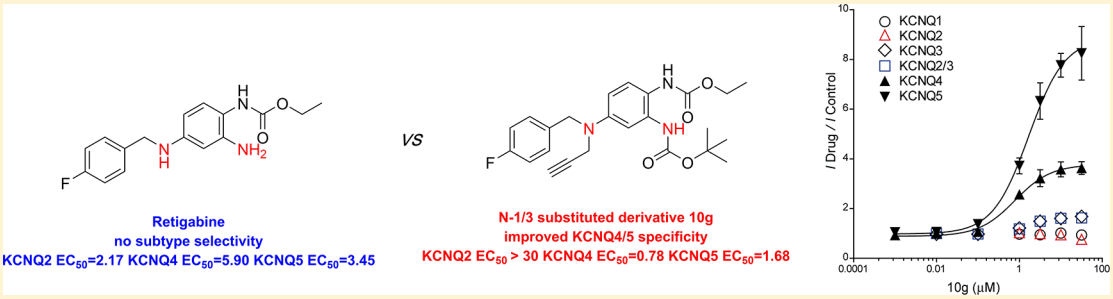
Supporting Information



ABSTRACT: Recent research suggests that KCNQ isoforms, particularly the KCNQ4 and KCNQ5 subtypes expressed in smooth muscle cells, are involved in both establishing and maintaining resting membrane potentials and regulating smooth muscle contractility. Retigabine (RTG) is a first-in-class antiepileptic drug that potentiates neuronal KCNQ potassium channels, but poor subtype selectivity limits its further application as a pharmacological tool. In this study, we improved the subtype specificity of retigabine by altering the N-1/3 substituents and discovered several compounds that show better selectivity for KCNQ4 and KCNQ5 channels. Among these compounds, **10g** is highly selective for KCNQ4 and KCNQ5 channels without potentiating KCNQ1 and KCNQ2 channels. These results are an advance in the exploration of small molecule modifiers that selectively activate different KCNQ isoforms. The developed compounds could also serve as new pharmacological tools for elucidating the function of KCNQ channels natively expressed in various tissues.

KEYWORDS: *KCNQ channel, retigabine, subtype selectivity, agonist*

The Kv7 (KCNQ) subfamily of voltage-gated potassium channels consists of five members (KCNQ1−5) and plays important roles in many excitable cells, such as neurons, cardiac myocytes, and vascular smooth muscle cells.[1−4] KCNQ1 is the most divergent and is primarily expressed in cardiac tissues. The KCNQ2−5 subtypes are predominantly found in various central and peripheral neurons.[5,6] Specifically, the KCNQ2/3 heterotetramers are considered to be the molecular basis for generating M currents that exert inhibitory control over neuronal firing.[7] Therefore, the neuronal KCNQ2/3 channels represent interesting targets for the treatment of diseases that involve altered neuronal excitability, such as epilepsy and chronic pain.[8−11] The small molecule retigabine (RTG, Figure 1) is an anticonvulsant drug, and it activates KCNQ2−KCNQ5 channels.[12,13] However, the poor subtype selectivity of retigabine leads to undesirable side effects, such as urinary retention, which limit its clinical use.[14,15]

It has also been suggested that KCNQ isoforms expressed in smooth muscle cells are involved in establishing and maintaining the resting membrane potential as well as in regulating smooth muscle contractility. KCNQ channels have been identified in multiple smooth muscle cells, including vascular smooth muscle such as thoracic artery, carotid artery, femoral artery, and portal vein; and visceral smooth muscle such as gastrointestinal smooth muscle, bladder detrusor, respiratory smooth muscle, and uterine smooth muscle.[16−20] Experiments using an array of pharmacological KCNQ channel modulators have supported the crucial role of these channels in regulating smooth muscle contractility.[21−23] Therefore, recent reports have refocused attention on the smooth muscle

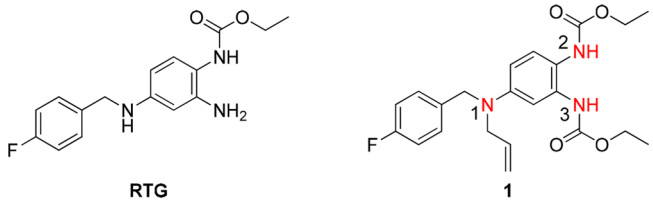


Figure 1. Structures of **RTG** and **1**.

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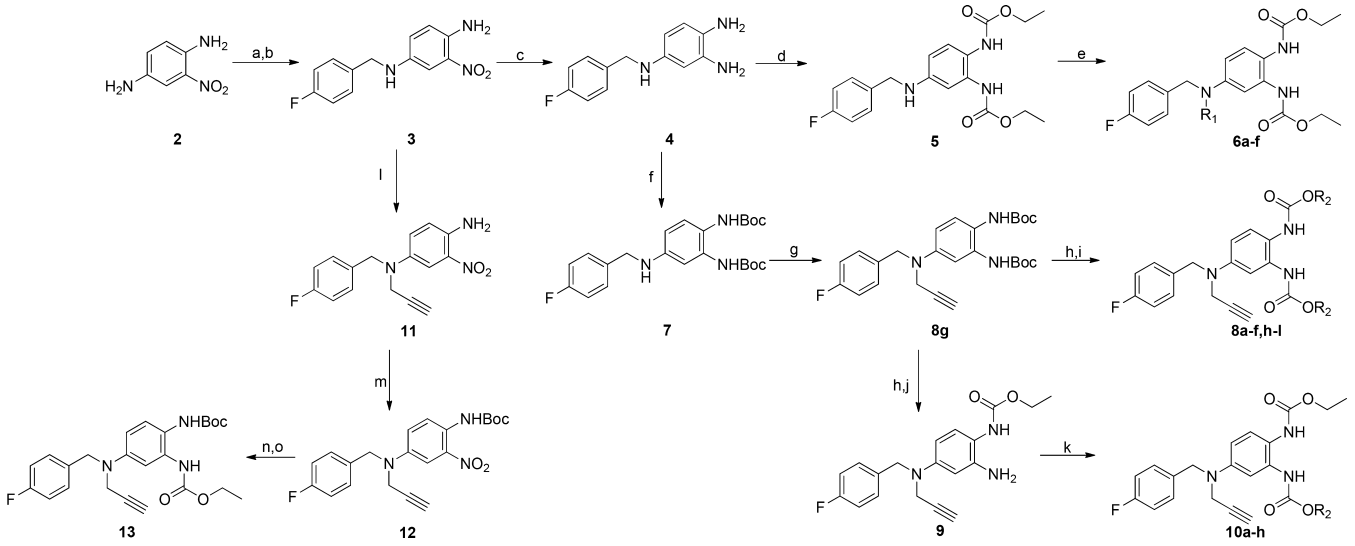
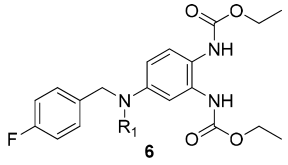
Scheme 1. Synthesis of the N-1/3 Retigabine Derivatives 6a–f, 8a–l, 10a–h, and 13[a]



[a]Reagents and conditions: (a) 4-fluorobenzaldehyde, cat. p-TsOH, toluene, 120°C, 77.2%; (b) $NaBH_4$, 1,4-dioxane:MeOH (4:1), rt, 94%; (c) H_2, Pd/C, MeOH, rt, 100%; (d) ethyl chloroformate, DIPEA, 1,4-dioxane, rt, 90%; (e) R_1Br, DIPEA, 60°C, DMF; (f) Boc_2O, $NaHCO_3$, H_2O/THF (1:2), rt, 85%; (g) 3-bromo-1-propyne, DIPEA, 60°C, DMF, 90%; (h) TFA, DCM, 0°C; (i) $ClCOOR_2$, DIPEA, 1,4-dioxane, rt; (j) ethyl chloroformate, DIPEA, 1,4-dioxane, rt, 50%; (k) $ClCOOR_3$, DIPEA, 1,4-dioxane, rt; (l) 3-bromo-1-propyne, DIPEA, 60°C, DMF, 85%; (m) Boc_2O, DMAP, THF, 80 °C, 35%; (n) Zn, NH_4Cl, MeOH, H_2O, reflux; (o) ethyl chloroformate, DIPEA, rt, 60% over two steps.

Table 1. Structures of the N-1 Derivatives 6a–f and Their Effects on KCNQ2, 4, and 5 Channels



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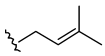
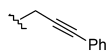
Cpds	R_1	I/I_0[a]		
		KCNQ2	KCNQ4	KCNQ5
1		0.30±0.00	2.21±0.15	1.49±0.24
6a		0.87±0.06	1.85±0.25	1.36±0.13
6b		0.66±0.16	3.63±0.24	1.37±0.10
6c		0.85±0.04	2.36±0.60	1.28±0.16
6d		1.19±0.06	1.49±0.08	1.05±0.05
6e		0.94±0.05	5.68±1.11	3.20±0.27
6f		1.26±0.03	1.54±0.21	1.13±0.03

[a]The testing concentration was 10 μM. Each compound was tested in more than four cells.

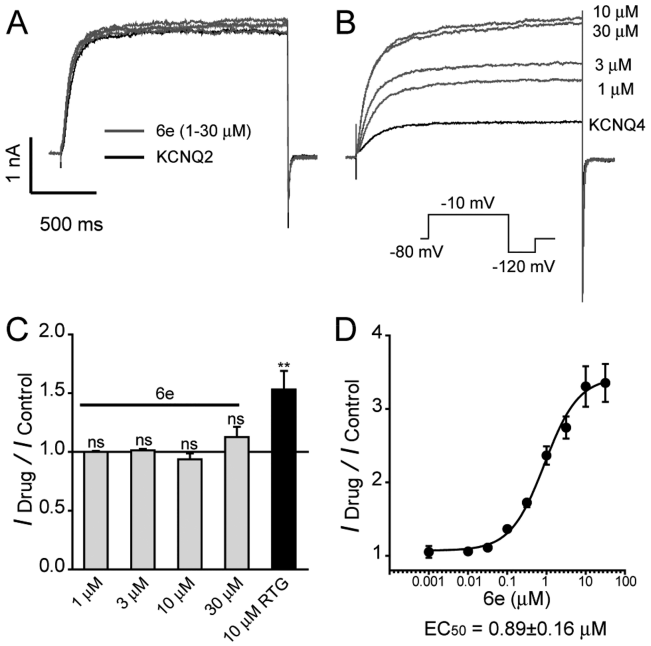


Figure 2. Effect of **6e** on KCNQ2 and KCNQ4 channels. (A,B) Representative current traces of KCNQ2 (A) and KCNQ4 (B) channels activated by **6e** using different drug concentrations as indicated are shown. (C) Histogram plotting **6e** and RTG effect on KCNQ2 currents measured at −10 mV ($n \geq 4$). (D) Concentration–response relationships of **6e** on homomeric KCNQ4 currents. The Hill coefficient was 0.33.

isoforms of KCNQ channels. It is worth noting that most of the visceral tissues that have been tested show high expression of the KCNQ4 and KCNQ5 subtypes, which suggests that KCNQ4 and KCNQ5 channels could be potential targets for visceral smooth muscle-related diseases such as irritable bowel syndrome and overactive bladder syndrome.[24] To date, no compounds that selectively activate KCNQ4 and KCNQ5 channels without activating neuronal KCNQ2 and KCNQ3 channels have been developed for clinical use.

By introducing a CF_3 group at the 4-position of the benzylamine moiety and a fluorine atom at the 3-position of the aniline ring of retigabine, Tzounopoulos et al. generated SF0034 and RL648_81 as new KCNQ2/3-specific activators, which are more potent and more selective than retigabine.[25,26]

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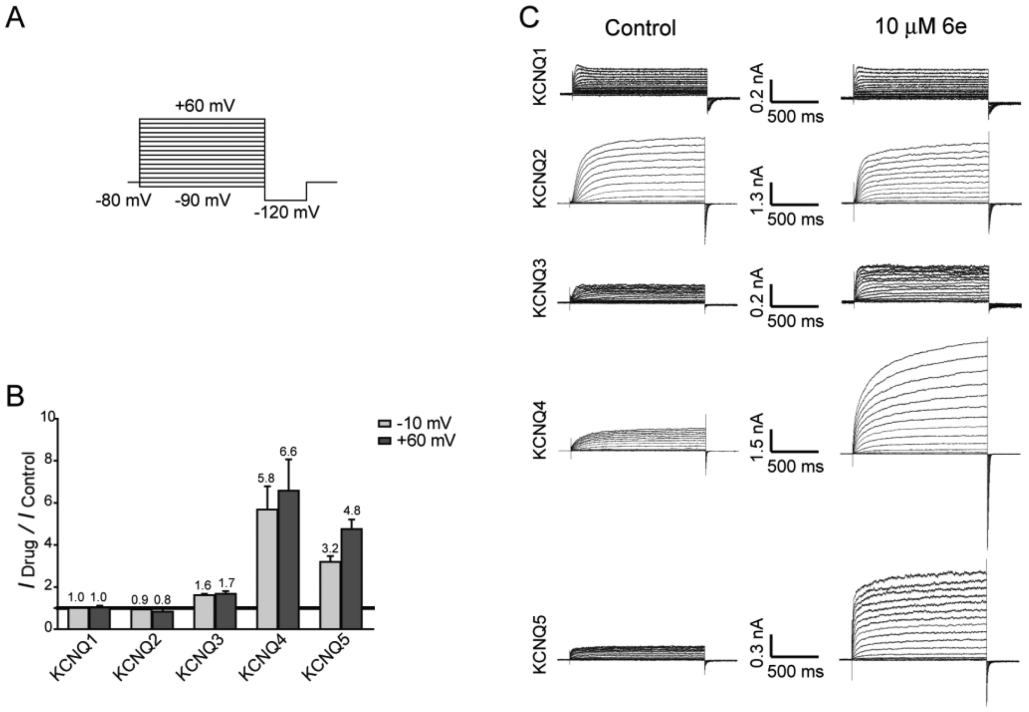

Figure 3. Subtype selectivity of **6e** for KCNQ channels. (A) In the voltage protocol, cells were held at −80 mV and stepped to a series of voltages ranging from −90 to +60 mV in 10 mV increments for a 1500 ms pulse followed by stepping to −120 mV for 500 ms. (B) Histogram showing the effects of **6e** on different subtypes of KCNQ with currents measured at −10 and +60 mV. (C) Whole-cell currents of CHO cells transfected individually with KCNQ1 to KCNQ5 were recorded in the absence (left panels) and presence (right panels) of 10 μM **6e**.

During our previous studies on KCNQ modulators, P-RTG, an RTG derivative that incorporates a propargyl group at the N-1 position, did not show any changes in potentiation potency, subtype selectivity, or molecular determinants on KCNQ channels compared to RTG.[27] In addition, we found that certain chemical modifications at the N-1 and N-3 positions of retigabine gave rise to a KCNQ4 and KCNQ5 channel agonist with improved subtype specificity (compound **1**, Figure 1).[28] This compound activated the current of the KCNQ4 and KCNQ5 channels at a concentration of 10 μM by 2.12- and 1.49-fold, respectively. Meanwhile, compound **1** inhibited the current of the KCNQ2 channel by 70%. This encouraged us to further explore this class of retigabine derivatives to improve KCNQ4 and KCNQ5 selectivity. Here, we report the synthesis of a series of N-1- and N-3-substituted retigabine derivatives via structural modification of **1** and the evaluation of their activity toward different KCNQ channel subtypes. We found several compounds that selectively activate KCNQ4 and KCNQ5 channels without activating KCNQ2 channels. These results therefore provide hope for the further development of subtype-specific KCNQ channel agonists.

First, compounds **6a**−**f** were synthesized to evaluate the effect of various substituents at the N-1 position of **1**. All compounds were synthesized according to Scheme 1. Compound **3** was synthesized from the commercially available compound **2** and 4-fluorobenzaldehyde via a reductive amination reaction. Compound **3** was then catalytically hydrogenated over Pd/C. Intermediately, compound **4** was treated with ethyl chloroformate to give **5**. The reaction of compound **5** with the appropriate alkyl bromide reagent produced **6a**−**f**.

With these N-1-substituted analogues in hand, their effects on the KCNQ2, 4, and 5 channels were first assessed. Electrophysiology experiments were conducted using the whole-cell patch clamp technique, and compound effects on the amplitude of the outward current (I/I_0) were analyzed. I_0 is the amplitude of the outward current in the absence of a compound. I is the amplitude of the outward current in the presence of a compound. Compounds resulting in $I/I_0 > 1$ were defined as activators, while compounds that gave $I/I_0 < 1$ were defined as inhibitors. All compounds were tested at a concentration of 10 μM, and the testing voltage was −10 mV unless otherwise stated. As shown in Table 1, the results indicate that N-1 substitution is important for activation and subtype selectivity. Branching on the allylic group (providing increased length and steric hindrance) had a detrimental effect on KCNQ2 inhibition, but the ability to activate KCNQ4 and KCNQ5 channels was retained. Among the newly synthesized derivatives, the N-1 propargyl-substituted compound **6e** displayed the best agonist potency. At a concentration of 10 μM, **6e** caused 5.68-fold and 3.20-fold increases in the KCNQ4 and KCNQ5 channels, respectively. However, there was no significant change in the outward current when we applied **6e** to KCNQ2 channels; for comparison, RTG increased KCNQ2 currents by 1.6-fold (Figure 2C). To determine the specific potency of **6e** on the KCNQ2 and KCNQ4 channels, the concentration−response relationship of **6e** was established for the KCNQ2 and KCNQ4 currents. As demonstrated in Figure 2B,D, **6e** enhanced KCNQ4 currents in a concentration-dependent manner. The EC_{50} of **6e** for KCNQ4 was determined to be 0.89 ± 0.16 μM. In contrast, **6e** (1−30 μM) did not affect the current amplitude of homomeric KCNQ2 currents (Figure 2A) at similar concentrations. In fact, **6e** at a concentration of 30 μM slightly potentiated KCNQ2 currents by 2 ± 0.9%.

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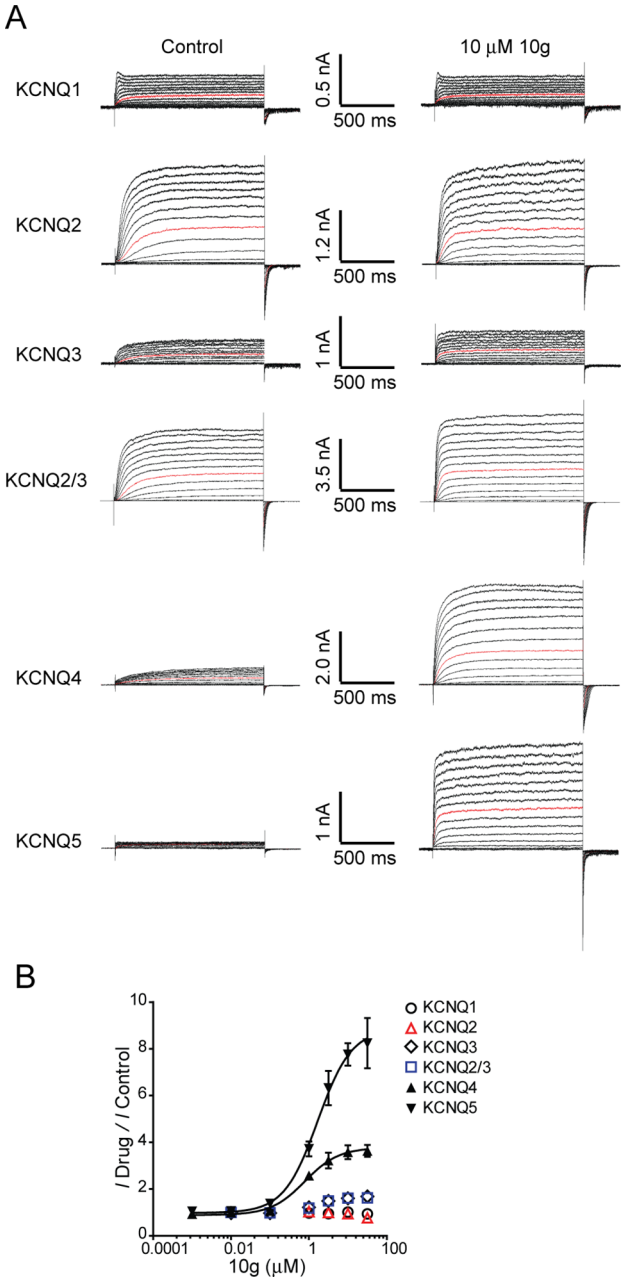

Figure 4. Dose−response curves of **10g** on different KCNQ isoforms. (A) Representative traces of homomeric KCNQ1−KCNQ5 channels and heteromeric KCNQ2/3 channels, before and after application of 10 μM **10g**. (B) Dose−response relationship of **10g** on homomeric KCNQ1−KCNQ5 channels and heteromeric KCNQ2/3 channels.

To determine the specificity of **6e** on other KCNQ subtypes, we individually expressed and then tested KCNQ1 to KCNQ5 in CHO-K1 cells using the whole cell voltage-clamp technique. The depolarized voltage ranged from −90 to +60 mV in 10 mV increments as described in Figure 3A. Except for KCNQ1 and KCNQ2, the outward currents of all KCNQ subtypes were potentiated by extracellular treatment with 10 μM **6e** (Figures 3B and 4C). The provided histogram illustrates that the current amplitude produced by **6e** decreased in the following order: KCNQ4 > KCNQ5 > KCNQ3.

Next, the N-1 propargyl substituent was maintained, and the N-2 and N-3 substituents were varied. Two new series of N-2- and N-3-substitued analogues (**8** and **10**) were prepared via a similar synthetic strategy (Scheme 1). Compound **4** was treated with Boc_2O to give **7**. The reaction of compound **7** with 3-bromo-1-propyne produced **8g**. Deprotection of **8g** followed by treatment with the appropriate chloroformate gave the final compounds **8a−f** and **8h−l**. Deprotection of **8g** followed by treatment with ethyl chloroformate formed intermediate **9**. Compounds **10** were synthesized in the same way from **9**.

Accordingly, the two new series of synthesized compounds (**8** and **10**) were assessed for their effects on the KCNQ2, 4, and 5 channels, and the results are shown in Table 2. When R_2 and R_3 remained the same, the effects of the substituents were determined from compounds **8a−l**. The identities of the functional groups at N-2 and N-3 were very important. For the alkyl substituted compounds **8a−j**, a critical difference was observed between the activity of the methyl and tertiary butyl analogues (**8a** and **8g**) and the rest of the analogues; **8a** and **8g** behaved as weak activators, while the others inhibited the KCNQ2 channel. Changing the length or steric hindrance of the chain caused an ambiguous effect on the activation of KCNQ4 and KCNQ5. In general, alkane-substituted compounds were better agonists than cycloalkane- and arene-substituted compounds. Among these compounds, the bis-tertiary butyl-substituted compound **8g** displayed the best agonist potency and selectivity.

When R_2 was maintained as ethyl and the R_3 group was varied, the effects of substituents can be seen by comparing compounds **10a−h**. Unlike compounds **8** and with the exception of **10a**, which was a weak inhibitor of KCNQ2, compounds **10** had no agonist activity on KCNQ2 and displayed significant activator potency on the KCNQ4 and KCNQ5 channels. Increasing the length or steric hindrance of the alkyl chain had beneficial effects on compound activity (**10a** vs **10c** vs **10e** and **10e** vs **10f** vs **10g**). Among the compounds in this series, the N-2 ethyl, N-3 tertiary butyl-substituted compound **10g** exhibited the best agonist potency. In addition, an N-2 and N-3 substituent exchanged compound (**13**) of **10g** was designed and synthesized (Scheme 1). Compound **13** displayed agonist activities comparable to that of **10g**, and the activity of **13** toward KCNQ4 was even greater than that of **10g**.

Compounds **8g**, **10g**, and **13** were selected for further testing based on their potent activation of the KCNQ4 and KCNQ5 channels. The concentration−response relationships of **8g**, **10g**, and **13** were then established for the KCNQ4 and KCNQ5 currents. Analysis of the dose−response curves revealed that the EC_{50} values of the selected compounds ranged from 0.78 to 2.43 μM (Table 3). Compound **10g** displayed the best agonist potency against KCNQ4 and KCNQ5 with EC_{50} values of 0.78 and 1.68 μM, respectively. Then, the evaluation of **10g** against all other subtypes was completed (Figure 4). To characterize the activation of KCNQ2/3 heteromeric channels by **10g**, we generated a r-KCNQ2/3 tandem construct (see Experimental Section in the Supporting Information). Unfortunately, we observed a maximum increase of approximately 1.50-fold in the outward currents of the KCNQ3 and KCNQ2/3 channels. Notably, at a similar concentration range (1−30 μM), **10g** did not affect the current amplitude of homomeric KCNQ2 currents. Compound **10g** actually slightly inhibited KCNQ2 currents at higher concentrations (30 μM) (Figure 4), making its effect on the KCNQ channels more specific than that of RTG.

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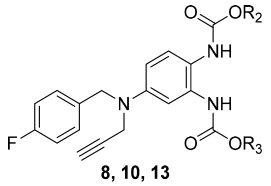
Table 2. Structures of N-2/3 Derivatives 8, 10, and 13 and Their Effects on the KCNQ2, 4, and 5 Channels



8, 10, 13

			I/I_0[a]		
Cpds	R_2	R_3	KCNQ2	KCNQ4	KCNQ5
8a	Me	Me	1.37 ± 0.11	4.77 ± 0.44	2.89 ± 0.07
8b	allyl	allyl	0.67 ± 0.02	2.21 ± 0.11	5.26 ± 0.53
8c	*n*Pr	*n*Pr	0.78 ± 0.04	2.58 ± 0.56	2.75 ± 0.84
8d	*i*Pr	*i*Pr	0.35 ± 0.04	1.33 ± 0.06	3.11 ± 0.93
8e	*n*Bu	*n*Bu	0.77 ± 0.02	2.75 ± 0.40	4.13 ± 1.09
8f	*i*Bu	*i*Bu	0.83 ± 0.04	1.52 ± 0.17	1.73 ± 0.10
8g	*t*Bu	*t*Bu	1.00 ± 0.09	3.05 ± 0.74	5.32 ± 0.39
8h	cyclopropyl	cyclopropyl	0.64 ± 0.03	1.51 ± 0.04	2.55 ± 0.38
8i	cyclobutyl	cyclobutyl	0.26 ± 0.06	1.21 ± 0.09	1.33 ± 0.23
8j	cyclopentyl	cyclopentyl	0.21 ± 0.05	0.88 ± 0.07	1.25 ± 0.14
8k	Ph	Ph	0.91 ± 0.02	1.22 ± 0.05	1.16 ± 0.04
8l	Bn	Bn	1.25 ± 0.01	3.02 ± 0.28	3.57 ± 0.31
10a	Et	Me	0.61 ± 0.05	2.56 ± 0.47	3.34 ± 0.54
10b	Et	Allyl	0.96 ± 0.04	3.68 ± 0.36	4.67 ± 0.69
10c	Et	*n*Pr	1.07 ± 0.03	3.95 ± 0.89	5.81 ± 0.62
10d	Et	*i*Pr	0.94 ± 0.06	5.01 ± 0.83	5.71 ± 0.34
10e	Et	*n*Bu	1.06 ± 0.03	4.16 ± 0.60	3.62 ± 0.31
10f	Et	*i*Bu	1.04 ± 0.04	5.19 ± 0.64	4.84 ± 0.32
10g	Et	*t*Bu	1.16 ± 0.04	6.37 ± 0.84	4.58 ± 0.32
10h	Et	Bn	0.97 ± 0.15	1.27 ± 0.03	2.57 ± 0.67
13	*t*Bu	Et	1.07 ± 0.06	7.71 ± 0.50	4.59 ± 0.56

[a]The testing concentration was 10 μM. Each compound was tested in more than four cells.

Table 3. Potency (EC_{50}, μM) of RTG, 8g, 10g, and 13 against KCNQ4, KCNQ5, and KCNQ2

	RTG	8g	10g	13
KCNQ4	5.90 ± 0.18	2.03 ± 0.14	0.78 ± 0.14	1.42 ± 0.04
KCNQ5	3.45 ± 0.28	2.43 ± 0.17	1.68 ± 0.12	1.37 ± 0.11
KCNQ2	2.17 ± 0.07	>30	>30	>30

Investigation of the influence of an activator on $V_{1/2}$ is very important for fully understanding the effects of an activator on channels. The effects of **6e** and **10g** on voltage-dependent activation were then analyzed. As described previously,[13] the current−voltage *G-V* curves suggest that one effect of RTG is to produce a 20−30 mV negative shift in the KCNQ current activation curve. However, unlike RTG, **6e** and **10g** only slightly affected the voltage-dependent activation curves of the KCNQ2 and KCNQ4 channels (data shown in Supplementary Table 1). Particularly at −60 mV or at more negative membrane potentials, the drug **10g** does not induce channel opening (data shown in Supplementary Figure 5). We speculated that the RTG-induced *G-V* shift should be sensitive to structure alterations around N-1, N-2, and N-3 and that the major influence of this class of compounds is on the current amplitude.

The previously reported KCNQ activators have low or no selectivity for KCNQ2−5 channels. Their undesirable side effects are likely due to poor KCNQ2−5 channel selectivity, and nonselective KCNQ modulators may be likely to cause side effects when used clinically. Recently, certain compounds have been reported to be selective for KCNQ4, 5 or KCNQ2 channels. For example, fasudil did not affect KCNQ2 and KCNQ2/3 currents but enhanced KCNQ4 and KCNQ4/5 channels,[29] while it originally acted as a potent rho-kinase inhibitor to suppress proliferation/migration and induce apoptosis in urothelial cancer cells.[30] AaTXK$\beta_{(2-64)}$, a peptide activator isolated from scorpion toxin, increased the maximal currents in homomeric KCNQ4 and heteromeric KCNQ2/3 channels but showed no effect on homomeric KCNQ3 channels.[31] In this study, we synthesized a series of selective activators for KCNQ4 and KCNQ5 channels based on the structural core of RTG, an approved antiepileptic drug. Among these newly synthesized derivatives, compound **10g** was found to be the most potent activator of KCNQ4 and KCNQ5 and exhibited EC_{50} values of 0.78 and 1.68 μM, respectively, with no enhancement of the current amplitude for KCNQ2. This result provides a new platform for developing selective KCNQ modulators. However, as a KCNQ4 and KCNQ5-selective probe compound, **10g** will be a useful tool for elucidating the mechanism of the interaction between the compounds and channels and for determining the contributions of different KCNQ channel subtypes in various tissues. The slight increase in the outward currents of the KCNQ3 and KCNQ2/3 channels will be an issue in regard to possible side effects; thus, additional experiments are needed to clarify these results. Therefore, experiments are in progress in our lab to further improve the compound specificity.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00315.

Experimental procedures and characterization data for new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Cpds, compounds; p-TsOH, *p*-toluenesulfonic acid; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide.; Boc_2O, di-*tert*-butyl dicarbonate; DCM, dichloromethane; rt, room temperature; DMAP, 4-(dimethylamino)pyridine.

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