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Original article

Design, synthesis and biological activity of pyrazolo[1,5-a]pyrimidin-7(4H)-ones as novel Kv7/KCNQ potassium channel activators

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ABSTRACT

Voltage-gated Kv7/KCNQ/M-potassium channels play a pivotal role in controlling neuronal excitability. Genetic reduction of KCNQ channel activity as a result of mutations causes various human diseases such as epilepsy and arrhythmia. Therefore, discovery of small molecules that activate KCNQ channels is an important strategy for clinical intervention of membrane excitability related disorders. In this study, a series of pyrazolo[1,5-a]pyrimidin-7(4H)-ones (PPOs) have been found to be novel activators (openers) of KCNQ2/3 potassium channels through high-throughput screening by using atomic absorption rubidium efflux assay. Based on structure–activity relationship (SAR), the substituted PPOs have been optimized. The 5-(2,6-dichloro-5-fluoropyridin-3-yl)-3-phenyl-2-(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-7(4H)-one (**17**) was identified as a novel, potent, and selective KCNQ2/3 potassium channel opener by patch-clamp recording assay.

1. Introduction

Voltage-gated KCNQ2 (Kv7.2) and KCNQ3 (Kv7.3) are two members of potassium channels that belong to the 6-transmemberance gene family of ion channels [1,2]. KCNQ2/3 heterotetramers are believed to underlie the molecular basis of M-type K^+ currents found in neuronal system [3]. Mutations of KCNQ2 and KCNQ3 genes were identified to cause a form of neonatal human epilepsy [4,5]. Genetic, physiological and pharmacological evidence now exist to support a role for KCNQ channels in control of neuronal excitability, suggesting that activation of KCNQ2/3 channels might be useful in potential treatment of hyperexcitability-related neuronal disorders [1]. Importantly, KCNQ/Kv7/M-channels are found to be expressed not only in the central nervous system [6–9] but also in sensory neurons involved in pain transduction [10], suggesting that KCNQ2/3 channels as potentially attractive targets for potential treatment of neuropsychiatric disorders such as migraine, epilepsy, and neuropathic pain [1,11–15].

Pharmacological targeting of M-channels is of great clinical interest. Anticonvulsant compound retigabine [ethyl 2-amino-4-(fluoro(phenyl)methylamino)phenylcarbamate; D-23129] as the first reported KCNQ2/3 opener has shown to be efficacious and safe in two Phase III trials in patients with refractory epilepsy [16]. Retigabine (also known as Potiga or Ezogabine) recently filed by Valeant Pharmaceuticals is currently being reviewed by FDA for approval for treatment of epilepsy. A number of recent studies have been reported that retigabine can relieve pain behaviors in animal models of neuropathic and inflammatory pain [17–20]. This compound is also under investigation in patients with post-herpetic neuralgia (PHN), a painful and common complication of shingles [21].

Over the past years, several series of novel molecules known as M-channel openers have been reported as shown in Fig. 1. These activators include: (i) BMS-204352, originally developed as a KCa activator which interacts with retigabine binding site but with a noticeably strong action on KCNQ4 (Kv7.4) channels [22,23]; (ii) acrylamides with an enantiomer-specific effect, which also interacts at retigabine binding site with a strong effect on KCNQ4 and KCNQ5 (Kv7.5) subunits [24]; (iii) the non-steroidal analgesics diclofenac and meclofenamate [25] and other compounds derived from these [26],which may act at a different site from retigabine;

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[1] Equally contributed to this work.

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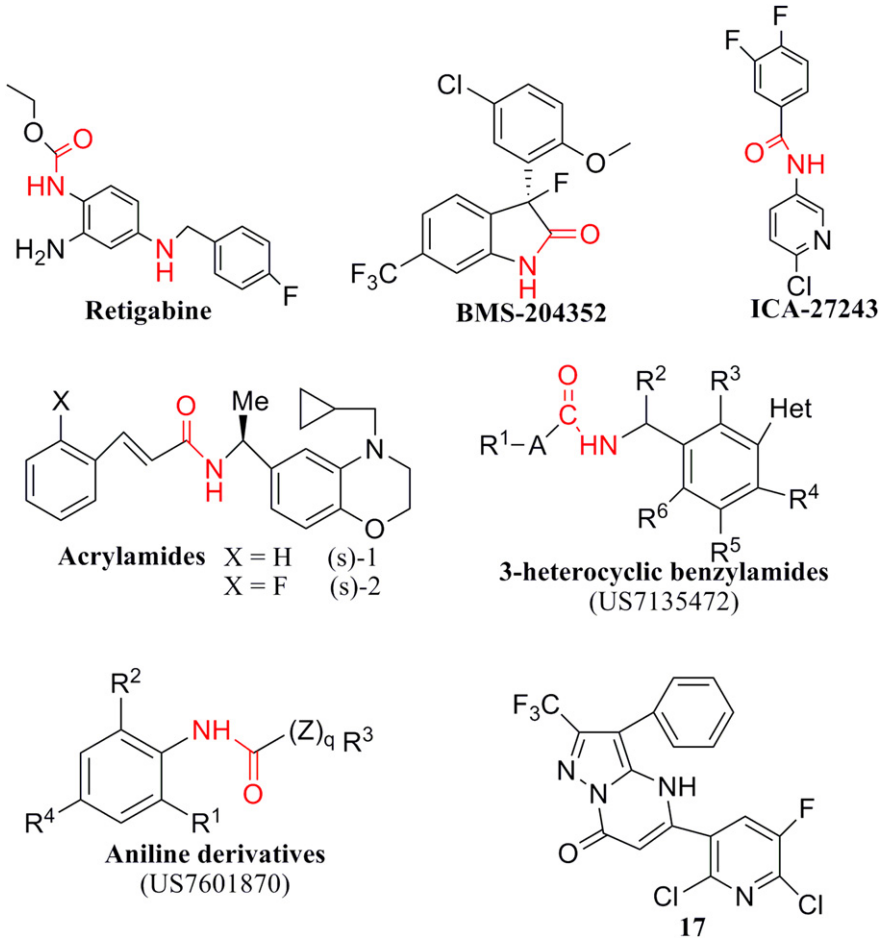


Fig. 1. Novel compound **17** and other known KCNQ/M channel openers.

(iv) a benzamide derivative ICA-27242, selective for KCNQ2/3 over KCNQ4 or KCNQ5 channels [27], which is effective against a broad range of epileptic protocols in animal experiments, with no apparent effect on the water-maze cognition test [28]; and (v) zinc pyrithione which interacts with the S5–S6 domains but at a different (extracellular) site from retigabine, does not require the S5-tryptophan and enhances cardiac KCNQ1 currents as well as KCNQ2/Q3 currents [29,30].

Previous chemical compounds share some structural similarity with amide group, and most of them are analogues of retigabine. In an effort to find new chemical structures of KCNQ/M-channel openers, we designed and synthesized a novel serial of pyrazolo[1,5-a]pyrimidin-7(4H)-ones (PPOs) and identified compounds **6** and **7** (Fig. 2) as initial leads that were evaluated in atomic absorption Rb^+ efflux assay. Further optimization of the series led to identification of more potent KCNQ/M-channel openers. Among them, compound **17** (Fig. 1) showed a potent activity by activating KCNQ2/3 channels at concentrations comparable to retigabine, and was much less effective at activating KCNQ1 which may cause cardiac side effects,

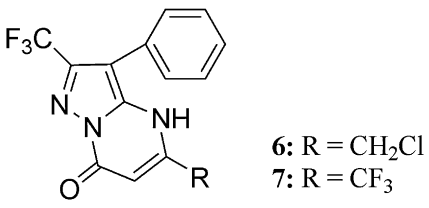


Fig. 2. Structure of lead compounds **6** and **7**.

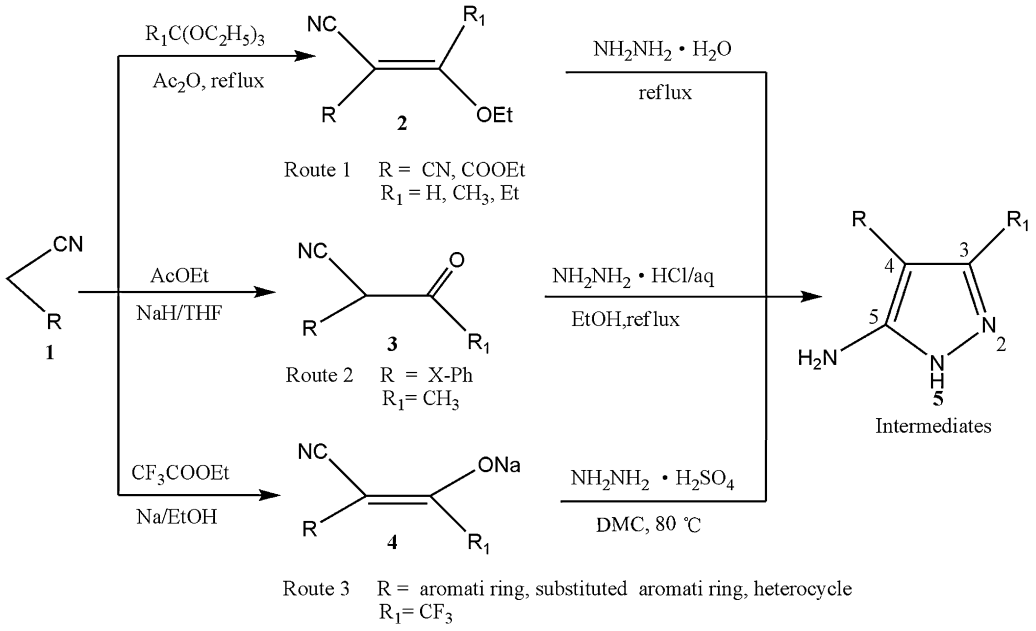
assessed by using both high-throughput screening and whole-cell patch-clamp recordings techniques. Herein, we report on the synthesis and biological evaluation of the PPOs.

2. Results and discussion

2.1. Chemistry

The synthesis of PPOs was accomplished in a two-step process (Scheme 1 and Scheme 2). 5-Amino pyrazoles (**5**) are important intermediates, which can be prepared through three synthetic routes as shown in Scheme 1. The commercially available substituted acetonitrile was reacted with ethyl orthoformate or it's analogs in reflux acetic anhydride, then hydrazine hydrate was added dropwise to obtain target intermediate (R_1 = H, R_1 = CH_3 or R_1 = Et; R = CN or COOEt) in good yield as depicted in Route 1 [31–33]. In the Route 2 and the Route 3, the similar reactions were carried out by using different starting materials and reaction conditions [34,35]. When R_1 is methyl, and R is substituted aromatic ring, good yields were obtained by using Route 2. Most of 5-amino pyrazole intermediates **5** were prepared through optimized Route 3, especially when R is an aromatic ring, substituted aromatic ring or heterocycle, and R_1 is trifluoromethyl. Thus, substituted acetonitrile was reacted with ethyl trifluoroacetate in ethanol in the presence of fresh sodium ethoxide, and then was reacted with hydrazine sulfate in dimethyl carbonate (DMC) in the presence of 3 Å molecular sieve at 80 °C, as described in Route 3.

In the second step, the synthesis of a library of PPOs was accomplished via parallel synthesis by reacting various



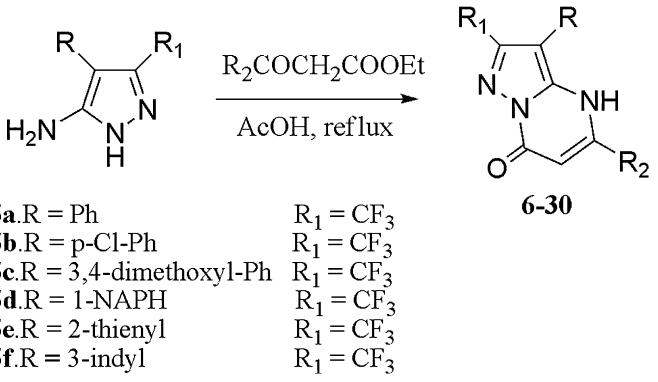
Scheme 1. Synthesis of substituted 5-amino pyrazoles.

intermediates **5** with respective β-ketoester in acetic acid at reflux, as described in Scheme 2 [36]. We usually obtained target compounds in satisfactory purity and good yields by scrubbing final productions with acetic acid and then water. Some of the compounds were further purified by prepared HPLC. The compounds were characterized by LC-MS, HPLC, IR, NMR (^{1}H and ^{13}C) and HRMS.

2.2. Biology

2.2.1. Atomic absorption Rb^+ efflux assay

We established and used an automatic Rb^+ efflux system for high-throughput screening of the synthesized compounds [37]. Chinese hamster ovary (CHO) cells stably expressing KCNQ2 and KCNQ3 were used for functional screening. Retigabine (RTG), the known KCNQ2/3 channel opener, was used as a control to validate the assay. As expected, retigabine increased Rb^+ efflux by activating KCNQ2/3 channels stably expressed in CHO cells, with an EC_{50} in the range of 0.30–0.50 μM. All synthesized compounds were evaluated for modulation of KCNQ2/3 channels using the same Rb^+ efflux assay. The results are summarized in Table 1. Among them, compounds **6**, **7**, **17** and **18** all showed the enhanced Rb^+ efflux of KCNQ2/3 channels in response to membrane depolarization in the presence of increased extracellular potassium, with EC_{50} values of 2.27 ± 1.02 μM, 1.46 ± 0.36 μM, 0.06 ± 0.01 μM and 0.15 ± 0.02 μM, respectively. The compound **7** and **17** showed similar efficacy when compared with retigabine, and the compound **6** and **18** showed less efficacy than retigabine (Fig. 3).



Scheme 2. Synthesis of pyrazolo[1,5-a]pyrimidin-7(4H)-ones (PPOs).

KCNQ1 is a member of the KCNQ potassium channel family, which, when coexpressed with the auxiliary subunit KCNE1 (minK), forms the important cardiac repolarizing potassium current, slowly activated delayed rectifier potassium current (I_{Ks}) [38]. Mutations in KCNQ1 that decrease the conductance of the complex prolong the cardiac action potential, leaving individuals susceptible to long QT syndrome [39–41].

The effect of compound **17** on KCNQ1 channels stably expressed in human embryonic kidney 293 (HEK293) cells was also assessed using an Rb^+ efflux assay. Retigabine exhibited no activation of KCNQ1 concentrations up to 100 μM. Znpy pyrithione [29], the known KCNQ2/3 as well as KCNQ1 channel opener, increased Rb^+ efflux of KCNQ1 channels with EC_{50} values of 2.27 ± 1.02 μM. The compound **17** showed the enhanced Rb^+ efflux of KCNQ1 channels with EC_{50} value of 49.29 ± 7.04 μM, which is about 800 times higher compared to the EC_{50} values for KCNQ2/3 channels (Fig. 4). Thus the compound **17** is a KCNQ2/3 subtype-selective opener over KCNQ1 channels.

To further confirm the effect of compounds **6**, **7**, **17** and **18**, we then utilized the gold standard assay of patch-clamp recordings.

2.2.2. Whole-cell patch-clamp recordings

In the CHO cells stably expressing KCNQ2 and KCNQ3, depolarizing voltage steps elicited typical outward whole-cell currents of KCNQ/M-channels that displayed the characteristic of slow activation, slow deactivation and no inactivation. The compound **7** (100 μM), compound **6** (100 μM), compound **17** (10 μM) and compound **18** (10 μM) significantly enhanced KCNQ2/3 currents at −40 mV with similar potency compared with 10 μM retigabine (Fig. 5). The left panel in Fig. 5 shows the typical current traces elicited from cells recorded by the perforated patch-clamp technique. The cells were held at −80 mV for 500 ms, and were then depolarized to −40 mV for 800 ms, followed by an 800 ms-pulse

Table 1
The half effective concentration (EC_{50}) of the tested PPOs compounds on KCNQ2/3 channel activation using atomic absorption Rb^+ efflux assay.

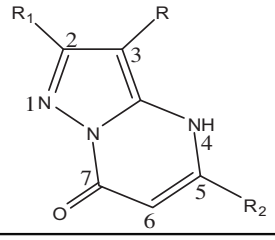
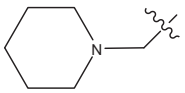
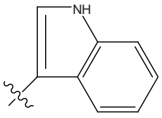
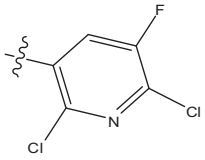
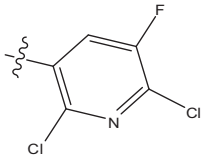
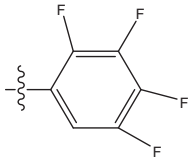
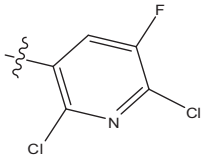
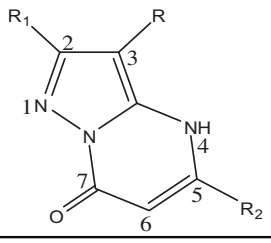
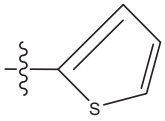
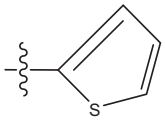
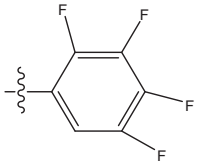
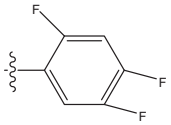
Cpd[a] no.				EC_{50}[b](μM)
	R	R_1	R_2	
6	Ph	CF_3	CH_2Cl	2.27 ± 1.02
7	Ph	CF_3	CF_3	1.46 ± 0.36
8	$COOC_2H_5$	CF_3	CH_2Cl	–[c]
9	Ph	CF_3		–
10	Ph	CF_3	Ph	–
11	Ph	Me	CH_2Cl	–
12	Ph	H	CF_3	–
13	Ph	CF_3	Me	–
14		CF_3	CF_3	–
15	1-NAPH	CF_3	CH_2Cl	1.25 ± 1.02
16	1-NAPH	CF_3	CF_3	4.98 ± 1.86
17	Ph	CF_3		0.06 ± 0.01
18	1-NAPH	CF_3		0.15 ± 0.02
19	p-ClPh	CF_3	CH_2Cl	2.33 ± 1.36
20	Ph	CF_3	p-CH_3OPh	1.93 ± 0.9
21	Ph	CF_3	$(CH_3)_3C$	6.19 ± 0.59
22	Ph	CF_3	CHF_2	4.29 ± 1.28
23	Ph	CF_3		0.60 ± 0.19
24	Ph	CF_3	3-NO_2Ph	0.57 ± 0.24
25	3,4-dimethoxylPh	CF_3		0.23 ± 0.03

Table 1 (*continued*)

Cpd[a] no.				EC_{50}[b](μM)
	R	R_1	R_2	
26		CF_3	3-NO_2Ph	0.58 ± 0.67
27		CF_3		0.16 ± 0.17
28	1-NAPH	CF_3	$(CH_3)_3C$	19.3 ± 1.48
29	p-ClPh	CF_3	CF_3	2.06 ± 1.68
30	p-ClPh	CF_3		0.21 ± 0.1

[a] Cpd = compound.
[b] These values are the mean ± S.E.M ($n = 3$).
[c] "–": No activity. (Screened at a concentration of 100 μM, and the compounds induced the rubidium responses by less than 15% when compared to the control).

back to −80 mV. Compounds **6**, **7**, **17** and **18** significantly enhanced both the activation current and the deactivation tail current of KCNQ2/3 channels. The right panel in Fig. 5 shows the time course of KCNQ2/3 currents recorded at −40 mV. Retigabine was used as control. The enhancement developed rapidly and reached a steady state soon after drug application. Removal of the drug from external solution resulted in a large extent recovery.

2.3. In vitro structure–activity relationship of pyrazolo[1,5-a] pyrimidin-7(4H)-ones (PPOs) compounds as Kv7/KCNQ modulators

Based on two initial lead compounds, we designed and synthesized about 120 analogues to develop SAR. In order to explore both steric and electronic effects at the active site, various modifications and substitutions were introduced, particularly to the 2-position, 3-position and 5-position of the PPOs. The representative compounds and their EC_{50} values on KCNQ2/3 channel activity are shown in the Table 1. Fig. 6 summarizes the SAR analysis in terms of the functional groups conferring activity of KCNQ/M activation. When the C-2 was substituted by a hydrogen or methyl group (compounds **11** and **12**), the activity was abolished completely, indicating that a trifluoromethyl group at the 2-position is required for the activity. As for the 3-position, substitution with a phenyl or naphthyl group afforded similar activity (compounds **6**, **7** and **17** vs **15**, **16** and **18**, respectively), and a thienyl group was also well tolerated (compounds **26** and **27**). However, replacement of the phenyl with an ester (compound **8**) or a big heterocycle, such as indyl in compound **14**, led to loss of activity.

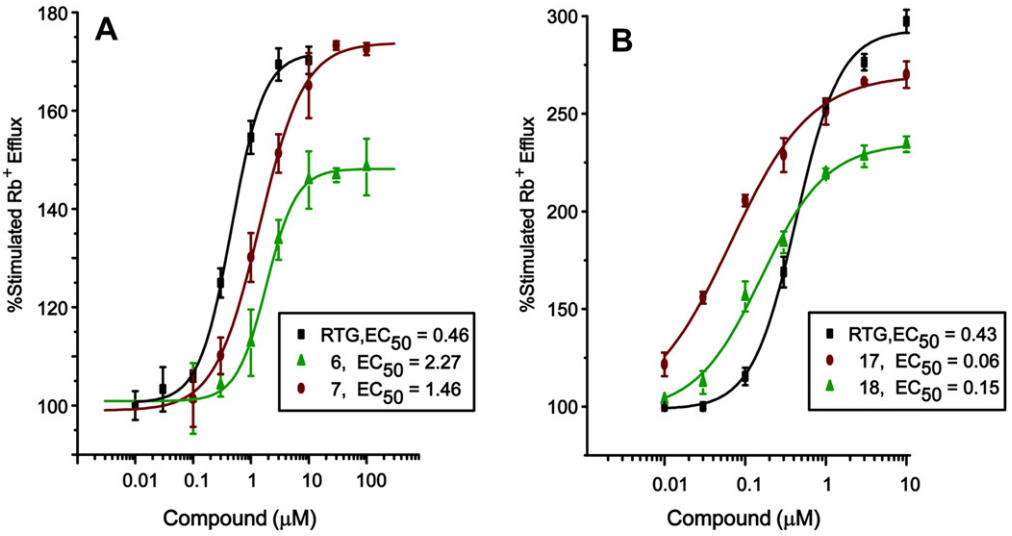


Fig. 3. Rb^+ efflux in KCNQ2/3 cells induced by retigabine and our compounds. A: Positive control retigabine, $EC_{50} = 0.46 \pm 0.01$ μM ($n = 3$); EC_{50} values of compounds **6** and **7** are 2.27 ± 1.02 μM and 1.46 ± 0.36 μM, respectively ($n = 3$); B: Positive control retigabine, $EC_{50} = 0.43 \pm 0.03$ μM ($n = 3$); EC_{50} values of compounds **17** and **18** are 0.06 ± 0.01 μM and 0.15 ± 0.02 μM, respectively ($n = 3$).

Introduction of a 4-Cl group to the phenyl ring did not affect the activity (compounds **19** and **29** vs. **6** and **7**), but a 3,4-dimethoxyl group caused 4-fold decrease of the activity (compounds **25** vs. **17**). The 5-position is the most important site for activity. Substitution with a methyl or phenyl group abolished the activity (compounds **13** and **10**). When the hydrogen of the methyl of compound **13** was replaced by different kinds and different amounts of halogen atoms as shown in compounds **6**, **7**, **15**, **16**, and **22**, or replaced by three methyls as shown in compounds **21** and **28**, all of them regained activity. However, halogen atoms substitutions showed more activation than the corresponding three methyls substitutions, indicating that electron-withdrawing halogens are the preferred substituents. Modifications of aromatic ring at C-5 of compound **10** are required for activity. As judged from EC_{50} values of compounds **23**, **24** in comparison with compound **20**, it appears that electron-withdrawing substituents on the aromatic ring have better channel–enhancement activity than electron-donating group. Compound **17** and **18** have the best EC_{50} (0.06 ± 0.01 μM and 0.15 ± 0.02 μM), which indicates that pyridyl with halogen electron-withdrawing substituents at C-5 is beneficiary for activity.

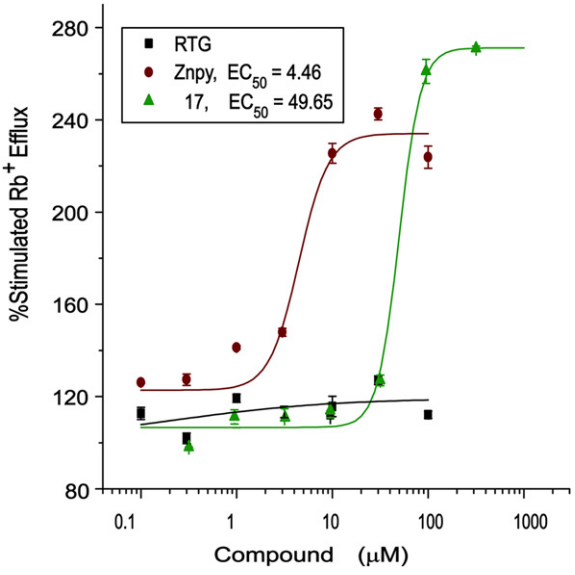


Fig. 4. Rb^+ efflux in KCNQ1 expressing cells induced by control drugs and the compound **17**. Positive control retigabine, no activity ($n = 3$); Znpy pyrithione, $EC_{50} = 2.27 \pm 1.02$ μM ($n = 3$); The compound **17**, $EC_{50} = 49.29 \pm 7.04$ μM ($n = 3$).

3. Conclusion

A novel series of substituted pyrazolo[1,5-a]pyrimidin-7(4H)-ones (PPOs) were synthesized and investigated for their activities in modulation of KCNQ2/3 channels. Through a preliminary SAR campaign, we have identified **17** and **18** with similar potency to retigabine. The results reveal that the PPOs as a new class of KCNQ2/3 potassium channel openers lead to warrant for further studies. Further design and synthesis of the related compounds, and the *in vivo* activity together with detailed mechanistic and pharmacokinetics studies on compound **17** are in progress in our laboratory.

4. Experimental methods

4.1. Synthesis

4.1.1. General procedures

All commercial reagents and solvents from Sigma–Aldrich and Fisher were used without further purification. Melting points were determined on a Digital Melting Point apparatus and were uncorrected. ^1H NMR spectra were recorded on Varian Inova-600 or Bruker Avance II 500 MHz NMR spectrometers using tetramethylsilane (TMS) as an internal standard. Chemical shift data for the proton resonances were reported in parts per million (ppm) relative to internal standard TMS (0.0), and coupling constants (J) were reported in hertz (Hz). ^{13}C NMR spectra were recorded on Bruker Avance II 500 MHz NMR spectrometers. Low resolution mass spectra (LRMS) were obtained using an ABI 3200 Qtrap mass spectrometer, and high resolution mass spectra (HRMS) were obtained using a Waters Micro Q-Tof mass spectrometer. Thin-layer chromatography (TLC) was run using Silica Gel GF254 plates, and plates were visualized under UV light. Analytical HPLC was performed using an Agilent 1200 equipped with a Diode Array Detector (DAD). Prepared HPLC was performed using an Agilent 1100 on a semi-prepared C18 chromatographic column (9.6 × 250 mm). The Parallel Synthesis was performed on Büchi Syncore® Reactor with Rack R-24 and

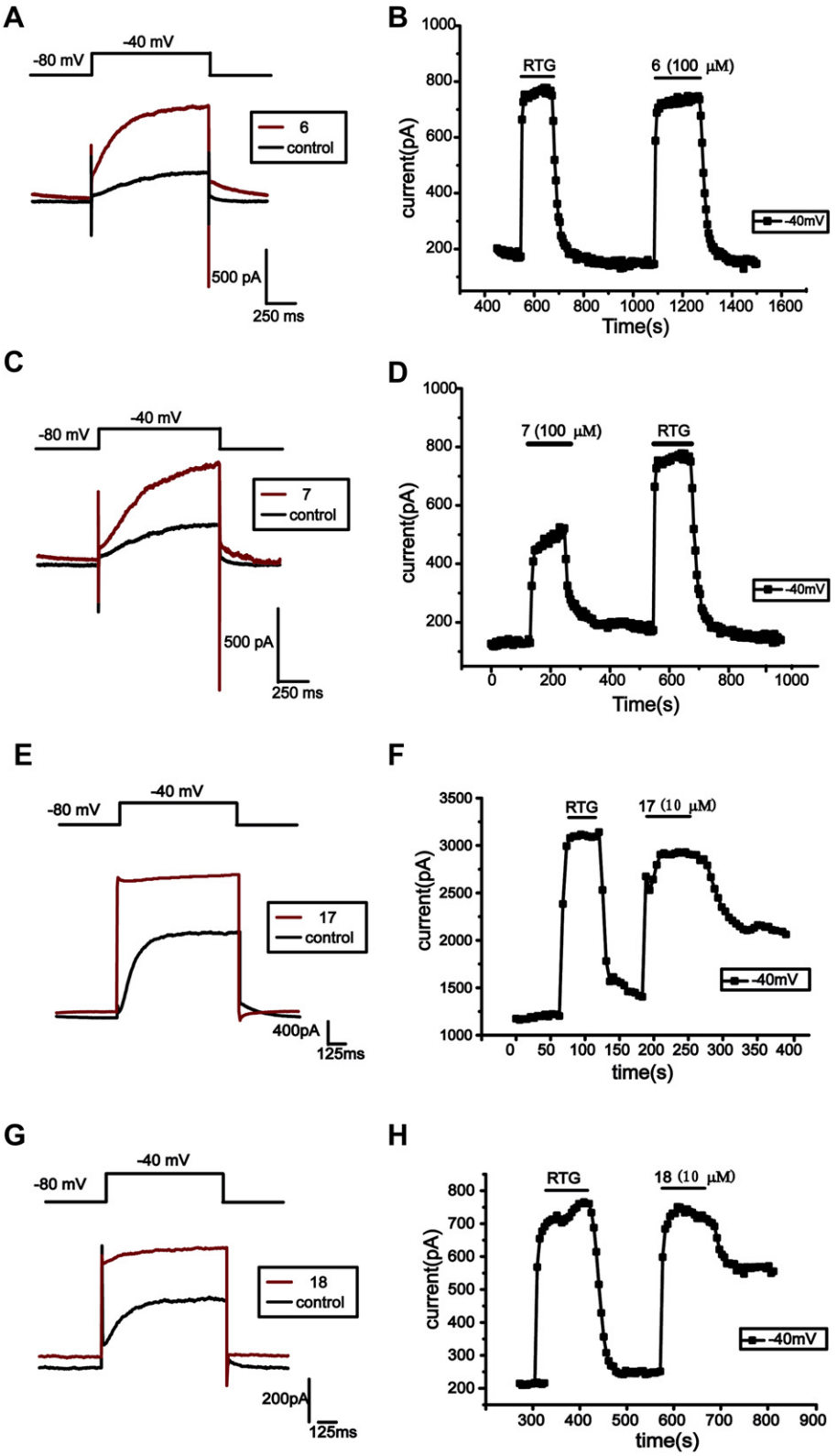


Fig. 5. Activation of KCNQ2/3 channel currents by lead compounds. The KCNQ2/3 current traces in the left panel (A, C, E, G) were elicited by the voltage protocols. The cells were held at −80 mV for 500 ms, and were then depolarized to −40 mV for 800 ms, followed by an 800 ms-pulse back to −80 mV. The time courses of KCNQ2/3 currents recorded at −40 mV were shown in the right panel (B, D, F, H). The concentrations of compounds were tested at used 100 μM for compound **6** and compound **7**, and at 10 μM for compound **17**, compound **18** and retigabine, respectively.

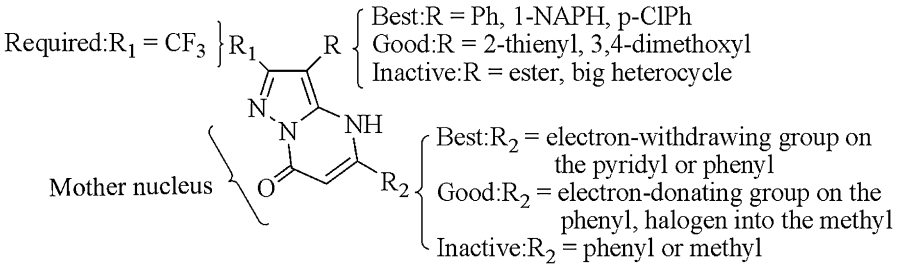


Fig. 6. Structure–activity analysis of PPOs as KCNQ/M-channel openers.

Syncore® Polyvap, Vacuum Pump V-700 professional with Vacuum controller V-855 (Switzerland). IR spectra were recorded on KBr disks with a Shimadzu FTIR 8400S spectrometer.

4.1.2. Examples of the general procedure for the preparation of inter mediates of activity compounds

*4.1.2.1. 4-Phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine (**5a**).* 2-Phenylacetonitrile (11.7 g, 100 mmol) was added to a fresh solution of Na (2.3 g, 100 mmol) in absolute alcohol (25 ml), then ethyl trifluoroacetate (17.0 g, 120 mmol) was added dropwise at about 50 °C. The mixture of reaction was refluxed for 2 h. After this period, the solvent was removed under vacuum. The solid was dissolved in water (20 ml), washed by CH_2Cl_2 (4 × 15 ml) and the solution was concentrated to dryness under reduced pressure to obtain white solid (21 g, 90%), which was used in the next step without further purification. The white solid (11.8 g, 50 mmol), hydrazine sulfate (12.9 g, 100 mmol), and 3 Å molecular sieve (10 g) in 120 ml DMC were stirred at 80 °C for 4 h, and monitored by TLC (2:1 petroleum ether/EtOAc, $R_f = 0.3$). After this period, the solid was filtered off and washed with some DMC. The solution was reclaimed, and the residue was recrystallized from isopropyl ether to obtain the target compound as a white solid (7.5 g, 63%); ESI-Ms (*m*/*z*): 228.2 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.39 (m, 2H, Ph-H), 7.28 (m, 3H, Ph-H), 5.20 (brs, 2H, NH_2), 12.33 (brs, 1H, NH); HRMS (ESI) calcd for $C_{10}H_9F_3N_3$ ($[M + H]^+$), 228.0749; found 228.0740.

*4.1.2.2. 4-(4-Chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-5-amine (**5b**).* Starting from 2-(4-chlorophenyl) acetonitrile; Yield: 45%; white needle crystal; ESI-Ms (*m*/*z*): 262.1 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.44 (m, 2H, Ph-H), 7.29 (d, 2H, $J = 8.5$, Ph-H), 12.37 (brs, 1H, NH), 5.31 (brs, 2H, NH_2); HRMS (ESI) calcd for $C_{10}H_8ClF_3N_3$ ($[M + H]^+$), 262.0359; found 262.0345.

*4.1.2.3. 4-(3,4-Dimethoxyphenyl)-3-(trifluoromethyl)-1H-pyrazol-5-amine (**5c**).* Starting from 2-(3,4-dimethoxyphenyl)acetonitrile; Yield: 52%; white power; ESI-Ms (*m*/*z*): 288.1 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 6.95 (d, 1H, $J = 8$, Ph-H), 6.79 (m, 2H, Ph-H), 3.74 (d, 6H, $J = 5.5$, OCH_3), 12.26 (brs, 1H, NH), 5.14 (brs, 2H, NH_2); HRMS (ESI) calcd for $C_{12}H_{13}F_3N_3O_2$ ($[M + H]^+$), 288.0960; found 288.0966.

4.1.3. General procedures for preparation of PPOs compounds

*4.1.3.1. 5-(Chloromethyl)-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**6**).* A solution of intermediate **5a** (0.227 g, 1 mmol) and ethyl 4-chloro-3-oxobutanoate (0.165 g, 1 mmol) was made with 5 ml acetic acid. The mixture was refluxed for 4 h, cooled to room temperature. The solid was filtered off by filtration and the cake was washed with acetic acid and water, to obtain the target compound (0.25 g, Yield: 76%, Purity > 95% by HPLC–DAD) as a pale yellow solid. mp 256.7–257.5 °C; ESI-Ms (*m*/*z*): 328.0 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.54 (m, 3H, Ph-H), 7.44 (m, 2H, Ph-H), 6.17 (s, 1H, 6-CH=), 4.69 (s, 2H, CH_2Cl), 12.73 (brs, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d6) δ: 41.18 (CH_2Cl), 97.88 (6-C=H), 104.10 (3-C), 156.00 (7-C=O), 150.99, 142.03 (5-C), 140.69 (2-C), 130.79 (Ph-C), 129.16 (Ph-C), 128.88 (Ph-C),128.09 (Ph-C), 122.87 (CF_3); HRMS (ESI) calcd for $C_{14}H_{10}ClF_3N_3O$ ($[M + H]^+$), 328.0464; found 328.0455.

*4.1.3.2. 3-Phenyl-2,5-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**7**).* The compound **7** (0.18 g, Yield: 49%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 4,4,4-trifluoro-3-oxobutanoate (0.18 g, 1 mmol) as a white solid. mp 318–320 °C; ESI-Ms (*m*/*z*): 348.2 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.55 (m, 2H, Ph-H), 7.43 (m, 3H, Ph-H), 6.04 (s, 1H, 6-CH=); HRMS (ESI) calcd for $C_{14}H_8F_6N_3O$ ($[M + H]^+$), 348.0572; found 348.0560.

*4.1.3.3. 3-Phenyl-5-(piperidin-1-ylmethyl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**9**).* A solution of compound **6** (0.325 g, 1 mmol) and piperidine (0.1 g, 1.2 mmol) in 8 ml DMF in the presence of K_2CO_3 (0.28 g, 2 mmol) was stirred at 50 °C for 1 h, and cooled to room temperature. The mixture was added 30 ml water and extracted with EtOAc (2 × 20 ml). The combined organic layers were washed with water, dried over Na_2SO_4, and concentrated under reduced pressure to obtain pure compound **30** (0.30 g, 90%) as a white solid. mp >300 °C; ESI-Ms (*m*/*z*): 377.2 $[M + H]^+$; ^1H NMR (500 MHz, CD_3OD) δ: 7.55 (d, 2H, Ph-H), 7.42 (m, 2H, Ph-H), 7.33 (m, 1H, Ph-H), 5.83 (s, 1H, 6-CH=), 4.10 (s, 2H, CH_2), 3.30 (m, 4H, piperidine 2-CH_2 and 6-CH_2), 1.82 (m, 4H, piperidine 3-CH_2 and 5-CH_2), 1.66 (t, 2H, piperidine 4-CH_2). HRMS (ESI) calcd for $C_{19}H_{20}F_3N_4O$ ($[M + H]^+$), 337.1589; found 337.1585.

4.1.4. General procedures of parallel synthesis of PPOs compounds

*4.1.4.1. 5-(2,6-Dichloro-5-fluoropyridin-3-yl)-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**17**).* A solution of intermediate **5a** (0.227 g, 1 mmol) and ethyl 3-(2,6-dichloro-5-fluoropyridin-3-yl)-3-oxopropanoate (0.28 g, 1 mmol) in acetic acid (8 ml) was added to a teat glass (40 ml) of Syncore® Reactor, keeping the temperature at 125 °C and the rotational speed of the device at 300 rpm for 4 h. After this period, the solvent of reaction was concentrated to half under reduced pressure by Syncore® Polyvap, and cooled to about 15 °C gradually. The mixture was filtered and the solid was washed with acetic acid and water to pH 7, to obtain the compound **17** (0.35 g, Yield: 80%, Purity: 98% by HPLC–DAD) as a white floccular solid. mp >300 °C; ESI-Ms (*m*/*z*): 443.2 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.52 (m, 3H, Ph-H), 7.47 (m, 2H, Ph-H), 6.17 (s, 1H, 6-CH=), 8.41 (d, 1H, $J = 8$, pyridine 4-CH=), 12.90 (brs, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d6) δ: 99.33 (6-C=H), 104.56 (3-C), 155.63 (7-C=O), 152.57, 154.63 (5-C), 140.75 (2-C), 130.65 (Ph-C), 129.22 (Ph-C), 128.89 (Ph-C), 127.94 (Ph-C), 122.86 (CF_3), 146.91 (pyridine-C), 142.59 (pyridine-C), 137.94 (pyridine-C), 130.44 (pyridine-C), 130.26 (pyridine-C);

HRMS (ESI) calcd for $C_{18}H_9Cl_2F_4N_4O$ ([M + H]$^+$), 443.0090; found 443.0085; IRν_{max} (KBr):1701 (C=O), 1629, 1558, cm^{-1}.

*4.1.4.2. 5-(Chloromethyl)-3-(naphthalen-1-yl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**15**).* The compound **15** (0.245 g, 65%) was obtained by the reaction of intermediate **5d** (0.277 g, 1 mmol) and 4-chloro-3-oxobutanoate (0.165 g, 1 mmol) as a white solid. mp >300 °C; ESI-Ms (*m*/*z*): 378.1 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 12.58 (s, 1H, NH), 8.10 (d, 1H, *J* = 8, NAPH-H), 8.05 (d, 1H, *J* = 8.5, NAPH-H), 7.63 (t, 1H, *J* = 7, NAPH-H), 7.58 (m, 3H, NAPH-H), 7.49 (m, 1H, NAPH-H), 6.17 (s, 1H, 6-CH=), 4.57 (s, 2H, CH_2Cl); ^{13}C NMR (125 MHz, DMSO-d6) *δ*: 41.13 (CH_2Cl), 97.98 (6-C=H), 101.49 (3-C), 156.22 (7-C=O), 150.71, 142.87 (5-C), 141.55 (2-C), 133.75, 133.17, 130.34, 129.76, 128.77, 127.28, 126.72, 126.00, 125.81, 125.62, 125.43, 122.83 (CF_3), 41.13 (CH_2Cl); HRMS (ESI) calcd for $C_{18}H_{12}ClF_3N_3O$ ([M + H]$^+$), 378.0621; found 378.0609.

*4.1.4.3. 3-(Naphthalen-1-yl)-2,5-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**16**).* The compound **16** (0.175 g, 44%) was obtained by the reaction of intermediate **5d** (0.277 g, 1 mmol) and ethyl 4,4,4-trifluoro-3-oxobutanoate (0.18 g, 1 mmol) as a white solid. mp >300 °C; ESI-Ms (*m*/*z*): 398.2 [M + H]$^+$; ^{1}H NMR (600 MHz, CD_3OD) *δ*: 8.03 (d, 1H, *J* = 8.4, NAPH-H), 7.98 (d, 1H, *J* = 7.8, NAPH-H), 7.59 (t, 1H, *J* = 6.6, NAPH-H), 7.51 (m, 3H, NAPH-H), 7.45 (m, 1H, NAPH-H), 6.44 (s, 1H, 6-CH=); HRMS (ESI) calcd for $C_{18}H_{10}F_6N_3O$ ([M + H]$^+$), 398.0728; found 398.0730.

*4.1.4.4. 5-(2,6-Dichloro-5-fluoropyridin-3-yl)-3-(naphthalen-1-yl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**18**).* The compound **18** (0.271 g, 55%) was obtained by the reaction of intermediate **5d** (0.277 g, 1 mmol) and ethyl 3-(2,6-dichloro-5-fluoropyridin-3-yl)-3-oxopropanoate (0.28 g, 1 mmol) as a white solid. mp >300 °C; ESI-Ms (*m*/*z*): 493.3 [M + H]$^+$; ^{1}H NMR (600 MHz, CD_3OD) *δ*: 8.01 (d, 1H, *J* = 3.6, NAPH-H), 7.99 (d, 1H, *J* = 3.6, NAPH-H), 7.59 (t, 1H, *J* = 6.6, NAPH-H), 7.51 (m, 3H, NAPH-H), 7.45 (m, 1H, NAPH-H), 6.12 (s, 1H, 6-CH=), 7.96 (d, 1H, *J* = 8.4, pyridine 4-CH=); HRMS (ESI) calcd for $C_{22}H_{11}Cl_2F_4N_4O$ ([M + H]$^+$), 493.0246; found 493.0252.

*4.1.4.5. 5-(4-Methoxyphenyl)-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**20**).* The compound **20** (0.288 g, 75%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 3-(4-methoxyphenyl)-3-oxopropanoate (0.222 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 386.4 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 3.83 (s, 3H, CH_3O), 6.12 (s, 1H, 6-CH=), 12.49 (brs, 1H, NH), 7.08 (d, 2H, *J* = 9, Ph-H), 7.71 (m, 2H, Ph-H), 7.42–7.51 (m, 5H, Ph-H); ^{13}C NMR (125 MHz, DMSO-d6) *δ*: 95.25 (6-C=H), 104.44 (3-C), 156.07 (7-C=O), 152.93, 162.06, 141.00 (2-C), 130.84, 129.78, 128.93, 128.54, 123.02 (CF_3), 130.35, 114.48, 55.96 (CH_3O); HRMS (ESI) calcd for $C_{20}H_{15}F_3N_3O_2$ ([M + H]$^+$), 386.1116; found 386.1110.

*4.1.4.6. 5-tert-Butyl-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**21**).* The compound **21** (0.217 g, 65%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 4,4-dimethyl-3-oxopentanoate (0.172 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 336.2 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.50 (m, 2H, Ph-H), 7.45 (m, 1H, Ph-H), 7.40 (d, 2H, *J* = 7, Ph-H), 5.85 (d, 1H, *J* = 1.5 6-CH=), 11.48 (brs, 1H, NH), 1.33 (s, 9H, CH_3); HRMS (ESI) calcd for $C_{17}H_{17}F_3N_3O$ ([M + H]$^+$), 336.1324; found 336.1322.

*4.1.4.7. 5-(Difluoromethyl)-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**22**).* The compound **22** (0.223 g, 68%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 4,4-difluoro-3-oxobutanoate (0.166 g, 1 mmol) as a white needle crystal. ESI-Ms (*m*/*z*) 330.3 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.53 (m, 3H, Ph-H), 7.46 (m, 2H, Ph-H), 6.96 (t, 1H, *J* = 53, CHF_2), 6.22 (s, 1H, 6-CH=); ^{13}C NMR (125 MHz, DMSO-d6) *δ*: 95.12 (6-C=H), 104.92 (3-C), 155.98 (7-C=O), 146.07, 141.08 (2-C), 130.80 (Ph-C), 129.06 (Ph-C), 128.83 (Ph-C), 128.13 (Ph-C), 122.86 (CF_3), 110.57 (CHF_2); HRMS (ESI) calcd for $C_{14}H_9F_5N_3O$ ([M + H]$^+$), 330.0666; found 330.0660.

*4.1.4.8. 3-Phenyl-5-(2,3,4,5-tetrafluorophenyl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**23**).* The compound **23** (0.178 g, 40%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 3-oxo-3-(2,3,4,5-tetrafluorophenyl) propanoate (0.264 g, 1 mmol) as a white powder. ESI-Ms (*m*/*z*) 428.2 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.51 (m, 2H), 7.44 (m, 3H), 7.80 (m, 1H), 12.88 (brs, 1H, NH), 6.16 (s, 1H, 6-CH=); HRMS (ESI) calcd for $C_{19}H_9F_7N_3O$ ([M + H]$^+$), 428.0634; found 428.06324.

*4.1.4.9. 5-(3-Nitrophenyl)-3-phenyl-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**24**).* The compound **24** (0.280 g, 70%) was obtained by the reaction of intermediate **5a** (0.227 g, 1 mmol) and ethyl 3-(3-nitrophenyl)-3-oxopropanoate (0.237 g, 1 mmol) as a white needle crystal. ESI-Ms (*m*/*z*) 401.3 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.50 (m, 3H, Ph-H), 7.46 (m, 2H, Ph-H), 8.55 (t, 1H, *J* = 2, Ph-H), 8.40 (m, 1H, Ph-H), 8.18 (m, 1H, Ph-H), 7.83 (t, 1H, *J* = 8, Ph-H), 6.29 (s, 1H, 6-CH=), 12.81 (brs, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d6) *δ*: 96.86 (6-C=H), 104.19 (3-C), 155.43 (7-C=O), 147.49, 140.65 (2-C), 130.37, 128.51, 128.18, 127.93, 122.47, 123.29, 125.37, 130.16, 133.98, 134.99; HRMS (ESI) calcd for $C_{19}H_{12}F_3N_4O_3$ ([M + H]$^+$), 401.0861; found 401.0868.

*4.1.4.10. 5-(2,6-Dichloro-5-fluoropyridin-3-yl)-3-(3,4-dimethoxyphenyl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**25**).* The compound **25** (0.186 g, 37%) was obtained by the reaction of intermediate **5c** (0.287 g, 1 mmol) and ethyl 3-(2,6-dichloro-5-fluoropyridin-3-yl)-3-oxopropanoate (0.28 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 503.1[M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 3.80 (s, 3H, CH_3O), 3.76 (s, 3H, OCH_3), 7.09 (d, 1H, *J* = 8, Ph-H), 7.00 (m, 1H, Ph-H), 6.83 (m, 1H, Ph-H), 6.15 (s, 1H, 6-CH=), 8.40 (d, 1H, *J* = 8, pyridine 4-CH=), 12.85 (brs, 1H, NH); HRMS (ESI) calcd for $C_{20}H_{13}Cl_2F_4N_4O_3$ ([M + H]$^+$), 503.0301; found 503.0307.

*4.1.4.11. 5-(3-Nitrophenyl)-3-(thiophen-2-yl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**26**).* The compound **26** (0.172 g, 42%) was obtained by the reaction of intermediate **5e** (0.233 g, 1 mmol) and ethyl 3-(3-nitrophenyl)-3-oxopropanoate (0.237 g, 1 mmol) as a violet solid. ESI-Ms (*m*/*z*) 407.1 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.22 (m, 1H, thienyl-H), 7.30 (m, 1H, thienyl-H), 7.74 (m, 1H, thienyl-H), 6.33 (s, 1H, 6-CH=), 7.84 (t, 1H, *J* = 8, Ph-H), 8.20 (d, 1H, *J* = 8, Ph-H), 8.41 (m, 1H, Ph-H), 8.57 (s, 1H, Ph-H), 12.87 (brs, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d6) *δ*: 96.73, 97.08, 155.34, 147.52, 141.59, 123.22, 125.36, 127.18, 127.52, 127.96, 129.82, 130.19, 134.08, 134.88, 141.88, 122.27; HRMS (ESI) calcd for $C_{17}H_{10}F_3N_4O_3S$ ([M + H]$^+$), 407.0426; found 407.0420.

*4.1.4.12. 5-(2,3,4,5-Tetrafluorophenyl)-3-(thiophen-2-yl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**27**).* The compound **27** (0.217 g, 50%) was obtained by the reaction of intermediate **5e** (0.233 g, 1 mmol) and ethyl 3-oxo-3-(2,3,4,5-tetrafluorophenyl) propanoate (0.264 g, 1 mmol) as a violet solid. ESI-Ms (*m*/*z*) 434.3 [M + H]$^+$; ^{1}H NMR (500 MHz, DMSO-d6) *δ*: 7.18 (d, 1H, *J* = 2.5, thienyl-H), 7.25 (d, 1H, *J* = 3.0, thienyl-H), 7.68 (m, 1H, thienyl-H), 7.86 (s, 1H, Ph-H), 6.17 (s, 1H, 6-CH=); HRMS (ESI) calcd for $C_{17}H_7F_7N_3OS$ ([M + H]$^+$), 434.0198; found 434.0204.

*4.1.4.13. 5-tert-Butyl-3-(naphthalen-1-yl)-2-(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)–one (**28**).* The compound **28** (0.212 g, 55%) was obtained by the reaction of intermediate **5d** (0.277 g, 1 mmol) and ethyl 4,4-dimethyl-3-oxopentanoate (0.172 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 386.2 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 8.05 (m 2H, NAPH-H), 7.63 (m 1H, NAPH-H), 7.55 (m, 3H, NAPH-H), 7.45 (m, 1H, NAPH-H), 5.86 (d, 1H, $J = 2.0$, 6-CH═), 11.35 (brs, 1H. NH), 1.23 (s, 9H, CH_3); HRMS (ESI) calcd for $C_{21}H_{19}F_3N_3O$ ($[M + H]^+$), 386.1480; found 386.1488.

*4.1.4.14. 3-(4-Chlorophenyl)-2,5-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**29**).* The compound **29** (0.266 g, 70%) was obtained by the reaction of intermediate **5b** (0.261 g, 1 mmol) and ethyl 4,4,4-trifluoro-3-oxobutanoate (0.18 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 382.1 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.65 (m, 2H, Ph-H), 7.50 (m, 2H, Ph-H), 6.17 (s, 1H, 6-CH═); HRMS (ESI) calcd $C_{14}H_7ClF_6N_3O$ ($[M + H]^+$), 382.0182; found 382.0190.

*4.1.4.15. 3-(4-Chlorophenyl)-2-(trifluoromethyl)-5-(2,4,5-trifluorophenyl)pyrazolo[1,5-a]pyrimidin-7(4H)-one (**30**).* The compound **30** (0.155 g, 35%) was obtained by the reaction of intermediate **5b** (0.261 g, 1 mmol) and ethyl 3-oxo-3-(2,4,5-trifluorophenyl)propanoate (0.246 g, 1 mmol) as a white solid. ESI-Ms (*m*/*z*) 444.2 $[M + H]^+$; ^1H NMR (500 MHz, DMSO-d6) δ: 7.73 (d, 2H, $J = 7$, Ph-H), 7.54 (m, 4H, Ph-H), 6.16 (s, 1H, 6-CH═), 12.61 (brs, 1H. NH); HRMS (ESI) calcd $C_{19}H_9ClF_6N_3O$ ($[M + H]^+$), 444.0338; found 444.0333.

4.2. Biological assay

4.2.1. Materials

Retigabine (Purity > 98% by HPLC–DAD) was synthesized according to the method outlined in US patent 5,384,330 in the Department for Development of New Drugs, School of Pharmacy, Hebei Medical University, and was verified by MS and NMR analysis. All other materials were of the highest quality available. Synthesized compounds were resolved in 100% DMSO Dulbecco's modified Eagle medium (DMEM) for cell culture and all antibiotics were purchased from Invitrogen.

4.2.2. Cell culture

Stable CHO cells expressing KCNQ2/3 channels were grown in DMEM supplemented with 10% fetal calf serum, 1× NEAA, 600 mg/ml G418 and 600 mg/ml hygromycin B. Stable HEK293 cells expressing KCNQ1 channels were grown in DMEM supplemented with 10% fetal calf serum and 600 mg/ml G418. Cells were plated at a density of 20,000 cells/well in 96-well microplates and incubated overnight at 37 °C with 5% CO_2 [37].

4.2.3. Atomic absorption Rb^+ efflux assay

For Rb^+ loading, the cell culture medium was discarded gently. The monolayer cell was washed once with 200 μl of Rb^+ Load Buffer and then cells were loaded by application of 200 μl of Rb^+ loading buffer per well and incubated for 3 h at 37 °C, 5% CO_2. The Rb^+ loading buffer contained: 5.4 mM RbCl, 5 mM glucose, 25 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 2 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH). Following 3 h incubation, the Rb^+ loading buffer was removed and cells were washed gently three times with wash buffer. Wash buffer contained: 5.4 mM KCl, 25 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, and 2 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH) [42].

In experiments screening for KCNQ openers, tool drug or compound was added to depolarization buffer. The depolarization buffer (20 mM K^+) contained: 20 mM KCl, 25 mM HEPES, 130 mM NaCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 2 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH). The wash buffer was then replaced with 200 μl of depolarization buffer. Channel activation was maintained for 10 min. Supernatant (200 μl from each well) was collected and transferred to a new 96-well plate before measurement [43].

The concentration of Rb^+ in the cell supernatants was determined using an automated Ion Channel Reader (ICR) 8000 flame atomic absorption spectrometer (Aurora Biomed, Vancouver). One hundred microliter cell supernatants were processed automatically from the 96-well plates and injected into an air-acetylene flame followed by 150 μl of Rb^+ sample analysis buffer (Aurora Biomed). The amount of Rb^+ in the sample was measured by absorption at 780 nm using a hollow cathode lamp as a light source and a PMT detector. A calibration curve covering the range of 0–5 mg/l (or ppm) Rb^+ in the sample analysis buffer was generated with each set of two 96-well plates. Reading of a whole 96-well plate took about 30 min [42].

4.2.4. Whole-cell patch-clamp recordings

Perforated-patch recordings were made at room temperature (20–22 °C). Pipettes were pulled from borosilicate glass capillaries and had resistance of 2–3 MΩ when filled with internal solution. Currents were recorded using an EPC-10 amplifier (HEKA, Germany). The external solution used to record KCNQ2/3 channel currents contained (in mM): NaCl 160, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 20, and pH 7.4 adjusted with NaOH. The standard internal solution contained (in mM): KCl 175, $MgCl_2$ 5, HEPES 5, BAPTA 0.1, K_2ATP 3, NaGTP 0.1, pH 7.4 adjusted with KOH. Recording pipette was first front-filled with the standard internal solution, then backfilled with the same internal solution containing amphotericin B (120 ng/ml) [20,43].

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