Supporting Information

Design and development of selective competitive fluorescent ligands for the detection and visualization of Kv7.2/7.3 *in vitro*

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Experimental section

EXPERIMENTAL SECTION

Material and Apparatus

All raw materials were purchased from Aladdin, Macklin, Energy Chemical, Sinopharm Chemical Reagent Company and reagents of electrophysiology were purchased from Sigma-Aldrich (Mainland China). All compounds were purified by column chromatography. $CDCl_3$ and *d*6-DMSO were used as solvents to capture the NMR spectra. ^1H NMR (600 MHz) and ^{13}C NMR (125 MHz) spectra was acquired on a Bruker ASCEND 600 MHz NMR system (Germany). UV spectra were performed on UV-2600 spectrophotometer. The images of HEK293 cells and brain slices were captured by a fluorescence confocal microscopy (Nikon A1R MP). HRMS was obtained through AB SCIEX TripleTOF 5600+ mass spectrometer. The pH value was detected with a pH meter (PHS-3C). Electrophysiological data was obtained by patch clamp with a HEKA EPC10 amplifier (Harvard Bioscience, Holliston, MA, USA) with PatchMaster software. The cell viability after incubated with probes was measured by a Tecan Auatria GmbH A-5082 microplate reader. Fluorescent measurements were operated on F-4600 FL Spectrophotometer. Fluorescence polarization and FlexStation 3 multi-mode microplate reader assay was performed on FlexStation 3 (Molecular Device). The slices from hippocampus were cut on a vibratome (VT-1200, Leica, Germany). Rb^+ efflux assay was performed by an Ion Channel Reader 8000 (ICR8000; Aurora Biomed, Vancouver, Canada).

Chemistry

Synthesis of 20c

(*S*)-1-(4-bromophenyl)ethan-1-amine (4.27g, 21.35 mmol) and tiethylamine (4.3g, 42.7 mmol) were dissolved in 50 mL CH_2Cl_2, then di-*tert*-butyl pyrocarbonate (9.3 g, 42.7 mmol) was added into solution at 0℃ and stirred at 25℃ for 1 h. Saturated sodium bicarbonate solution and dichloromethane were added to extract the organic phase. The organic phase was combined and dried with anhydrous $MgSO_4$. Then, the solvent was removed and purified by column chromatography to obtain purified white product *tert*-butyl (*S*)-(1-(4-bromophenyl)ethyl)carbamate (6.34 g, 99%).

tert-Butyl (*S*)-(1-(4-bromophenyl)ethyl)carbamate (740 mg, 2.46 mmol), morpholine (2.1 g, 24.6 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (143 mg, 0.48 mmol), $Pd_2(dba)_3$ (113 mg, 0.12 mmol), K_3PO_4 (0.73 g, 3.44 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH_2Cl_2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl (*S*)-(1-(4-morpholinophenyl)ethyl)carbamate was obtained by column chromatography (285 mg, 38%).

tert-Butyl (*S*)-(1-(4-morpholinophenyl)ethyl)carbamate (285 mg, 0.93 mmol) was dissolved in 2 ml CH_2Cl_2. Trifluoroacetic acid (530 mg, 4.65 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(4-morpholinophenyl)ethan-1-amine (120 mg, 63%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, and 4-dimethylaminopyridine (2.3 mg, 0.02

mmol), (*S*)-1-(4-morpholinophenyl)ethan-1-amine (20 mg, 0.10 mmol) was successively added into the solution. After 20 min, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product (*S*)-7-(diethylamino)-*N*-(1-(4-morpholinophenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (23 mg, 51%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.10 (d, *J* = 7.4 Hz, 1H), 8.68 (s, 1H), 7.40 (d, *J* = 8.8 Hz, 1H), 7.31 (d, *J* = 8.1 Hz, 2H), 6.89 (d, *J* = 7.5 Hz, 2H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.49 (s, 1H), 5.24 (dd, *J* = 13.8, 6.8 Hz, 1H), 3.85 (s, 4H), 3.44 (d, *J* = 7.0 Hz, 4H), 3.14 (s, 4H), 1.56 (d, *J* = 6.7 Hz, 3H), 1.23 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.7 (s), 162.1 (s), 157.6 (s), 152.5 (s), 148.1 (s), 131.1 (s), 127.1 (s), 115.9 (s), 110.5 (s), 109.9 (s), 108.4 (s), 96.6 (s), 66.9 (s), 49.5 (s), 48.5 (s), 45.1 (s), 22.4 (s), 12.4 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{26}H_{31}N_3O_4$: 449.2315, found: 449.2315. Purity: 97.7%. HPLC: t_R = 3.52 min.

Synthesis of 21a

(*S*)-1-(3-Bromophenyl)ethan-1-amine (4.27g, 21.35 mmol) and triethylamine (4.3 g, 42.7 mmol) were dissolved in 50 mL CH_2Cl_2, then di-*tert*-butyl pyrocarbonate (9.3 g, 42.7 mmol) was added into solution at 0℃ and stirred at 25℃ for 1 h. Saturated $NaHCO_3$ solution and CH_2Cl_2 were added to extract the organic phase. The organic phase was combined and dried with anhydrous $MgSO_4$. Then, the solvent was removed and purified by column chromatography to obtain purified white product *tert*-butyl (*S*)-(1-(3-bromophenyl)ethyl)carbamate (6.2g, 97%).

tert-Butyl (*S*)-(1-(3-bromophenyl)ethyl)carbamate (800 mg, 2.67 mmol), *cis*-2,6-dimethylmorpholine (3.07 g, 26.7 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (155 mg, 0.52 mmol), $Pd_2(dba)_3$ (119 mg, 0.13 mmol), K_3PO_4 (0.79 g, 3.74 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH_2Cl_2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl ((*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)carbamate was obtained by column chromatography (810 mg, 91%).

tert-Butyl ((*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)carbamate (810 mg, 2.43 mmol) was dissolved in 2 ml CH_2Cl_2. Trifluoroacetic acid (1.38 g, 12.12 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethan-1-amine (333 mg, 61%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, and 4-dimethylaminopyridine (2.3 mg, 0.02 mmol), (*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethan-1-amine (23 mg, 0.10 mmol) was successively added into the solution. After 20 min, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product 7-(diethylamino)-*N*-((*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (30 mg, 63%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.16 (d, *J* = 7.5 Hz, 1H), 8.68 (s, 1H), 7.41 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 7.2 Hz, 1H), 6.93 (d, *J* = 8.3 Hz, 2H), 6.80 (s, 1H), 6.63

(d, *J* = 8.9 Hz, 1H), 6.50 (s, 1H), 5.25 (p, *J* = 6.7 Hz, 1H), 3.81 (s, 2H), 3.45 (q, *J* = 7.0 Hz, 6H), 2.44 (s, 2H), 1.57 (d, *J* = 6.7 Hz, 3H), 1.24 (dd, *J* = 15.6, 6.9 Hz, 12H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.78 (s), 162.19 (s), 157.64 (s), 152.51 (s), 148.14 (s), 131.10 (s), 129.48 (s), 117.50 (s), 114.48 (s), 114.02 (s), 110.43 (s), 109.91 (s), 108.42 (s), 96.59 (s), 71.64 (s), 53.43 (s), 49.46 (s), 45.07 (s), 22.70 (s), 19.06 (s), 12.44 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{28}H_{35}N_3O_4$: 477.2628, found: 477.2628. Purity: 95.5%. HPLC: t_R = 3.172 min.

Synthesis of 21b

tert-Butyl (*S*)-(1-(3-bromophenyl)ethyl)carbamate (800 mg, 2.67 mmol), *N*-ethylpiperazine (2.67 g, 26.7 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (155 mg, 0.52 mmol), $Pd_2(dba)_3$ (119 mg, 0.13 mmol), K_3PO_4 (0.79 g, 3.74 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH_2Cl_2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl (*S*)-(1-(3-(4-methylpiperazin-1-yl)phenyl)ethyl)carbamate was obtained by column chromatography (440 mg, 49%).

tert-Butyl (*S*)-(1-(3-(4-methylpiperazin-1-yl)phenyl)ethyl)carbamate (440 mg, 1.38 mmol) was dissolved in 2 ml CH_2Cl_2. Trifluoroacetic acid (786 mg, 6.90 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(3-(4-methylpiperazin-1-yl)phenyl)ethan-1-amine (335 mg, 99%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, then 4-dimethylaminopyridine (2.3 mg, 0.02 mmol), (*S*)-1-(3-(4-methylpiperazin-1-yl)phenyl)ethan-1-amine (22 mg, 0.10 mmol) was successively added into the solution. After 20 min, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product 7-(diethylamino)-*N*-((*S*)-1-(3-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (28 mg, 61%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.17 (d, *J* = 7.7 Hz, 1H), 8.68 (s, 1H), 7.41 (d, *J* = 8.9 Hz, 1H), 7.23 (d, *J* = 7.9 Hz, 1H), 6.96 (s, 1H), 6.91 (d, *J* = 7.6 Hz, 1H), 6.80 (d, *J* = 8.0 Hz, 1H), 6.63 (d, *J* = 8.5 Hz, 1H), 6.49 (s, 1H), 5.24 (t, *J* = 7.2 Hz, 1H), 3.45 (q, *J* = 7.0 Hz, 5H), 3.29 (s, 5H), 2.42 (s, 2H), 1.56 (d, *J* = 6.9 Hz, 3H), 1.24 (d, *J* = 6.6 Hz, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.2 (s), 161.5 (s), 157.0 (s), 151.9 (s), 147.5 (s), 144.3 (s), 130.5 (s), 128.8 (s), 117.1 (s), 114.2 (s), 113.8 (s), 109.8 (s), 109.3 (s), 107.8 (s), 96.0 (s), 54.2 (s), 48.8 (s), 48.0 (s), 44.4 (s), 29.1 (s), 22.1 (s), 11.8 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{27}H_{34}N_4O_3$: 462.2631, found: 462.2631. Purity: 96.2%. HPLC: t_R = 0.89 min.

Synthesis of 21c

tert-Butyl (*S*)-(1-(3-bromophenyl)ethyl)carbamate (2.1 g, 7 mmol), morpholine (6.09 g, 70 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (210 mg, 1.4 mmol), $Pd_2(dba)_3$ (320 mg, 0.35 mmol), K_3PO_4 (2.08 g, 9.8 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH2Cl2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl (*S*)-(1-(3-morpholinophenyl)ethyl)carbamate was obtained by column chromatography (1.38 g, 64%).

tert-Butyl (*S*)-(1-(3-morpholinophenyl)ethyl)carbamate (1.38 g, 4.5 mmol) was dissolved in 2 ml CH_2Cl_2. Trifluoroacetic acid (2.56 g, 22.5 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(3-morpholinophenyl)ethan-1-amine (800 mg, 86%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, then 4-dimethylaminopyridine (2.3 mg, 0.02 mmol), (*S*)-1-(3-morpholinophenyl)ethan-1-amine (20 mg, 0.10 mmol) was successively added into the solution. Then, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product (*S*)-7-(diethylamino)-*N*-(1-(3-morpholinophenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (26 mg, 58%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.11 (d, *J* = 5.2 Hz, 1H), 8.68 (d, *J* = 2.5 Hz, 1H), 7.41 (dd, *J* = 8.9, 2.5 Hz, 1H), 7.32 (d, *J* = 6.2 Hz, 2H), 6.92 (s, 2H), 6.63 (d, *J* = 8.9 Hz, 1H), 6.49 (s, 1H), 5.23 (d, *J* = 6.3 Hz, 1H), 3.87 (s, 4H), 3.48 – 3.40 (m, 4H), 3.15 (s, 4H), 1.59 – 1.53 (m, 3H), 1.24 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.2 (s), 161.6 (s), 157.0 (s), 151.9 (s), 147.5 (s), 130.5 (s), 128.8 (s), 109.8 (s), 109.3 (s), 107.8 (s), 96.0 (s), 66.3 (s), 48.7 (s), 44.4 (s), 29.1 (s), 22.1 (s), 11.8 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{26}H_{11}N_3O_4$: 449.2315, found: 449.2315. Purity: 98.3%. HPLC: t_R = 3.57 min.

Synthesis of 24a

tert-Butyl (*S*)-(1-(4-bromophenyl)ethyl)carbamate (2.1 g, 7 mmol), *N*-ethylpiperazine (7.0 g, 70 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (210 mg, 1.4 mmol), $Pd_2(dba)_3$ (320 mg, 0.35 mmol), K_3PO_4 (2.08 g, 9.8 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH_2Cl_2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl (*S*)-(1-(4-(4-methylpiperazin-1-yl)phenyl)ethyl)carbamate was obtained by column chromatography (600 mg, 27%).

Tert-Butyl (*S*)-(1-(4-(4-methylpiperazin-1-yl)phenyl)ethyl)carbamate (600 mg, 1.88 mmol) was dissolved in 2 ml CH_2Cl_2. Then, trifluoroacetic acid (1.07 g, 9.40 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(4-(4-methylpiperazin-1-yl)phenyl)ethan-1-amine (300 mg, 73%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, then 4-dimethylaminopyridine (2.3 mg, 0.02 mmol), (*S*)-1-(4-(4-methylpiperazin-1-yl)phenyl)ethan-1-amine (20 mg, 0.10 mmol) was successively added into the solution. After 20 min, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product (*S*)-7-(diethylamino)-*N*-(1-(4-(4-methylpiperazin-1-yl)phenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (28 mg, 61%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.09 (d, *J* = 7.1 Hz, 1H), 8.68 (s, 1H), 7.40 (d, *J* = 8.6 Hz, 1H), 7.30 (d, *J* = 7.5 Hz, 2H), 6.89 (d, *J* = 7.5 Hz, 2H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.49 (s, 1H), 5.27

– 5.19 (m, 1H), 3.45 (d, *J* = 6.7 Hz, 4H), 3.35 (s, 4H), 2.83 (s, 4H), 2.52 (s, 3H), 1.55 (d, *J* = 6.2 Hz, 3H), 1.23 (t, *J* = 6.5 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 148.1 (s), 131.1 (s), 127.2 (s), 116.7 (s), 110.5 (s), 109.9 (s), 108.4 (s), 96.6 (s), 54.5 (s), 48.5 (s), 48.3 (s), 45.1 (d, *J* = 17.4 Hz), 22.4 (s), 12.4 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{27}H_{34}N_4O_3$: 462.2631, found: 462.2631. Purity: 95.1%. HPLC: t_R = 0.92 min.

Synthesis of 24b

tert-Butyl (*S*)-(1-(4-bromophenyl)ethyl)carbamate (2.1 g, 7 mmol), *cis*-2,6-dimethylmorpholine (8.05 g, 70 mmol), 2-(di-*tert*-butylphosphino)-biphenyl (210 mg, 1.4 mmol), $Pd_2(dba)_3$ (320 mg, 0.35 mmol), K_3PO_4 (2.08 g, 9.8 mmol), was stirred without extra solvent at 80℃ overnight under argon. Then, the mixture was filtered and the filtrate was extracted with CH2Cl2 for three times. The solution was dried with anhydrous $MgSO_4$ and then removed by rotary evaporation to obtain the crude product. Product *tert*-butyl ((*S*)-1-(4-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)carbamate was obtained by column chromatography (960 mg, 43%).

tert-Butyl (*S*)-(1-(4-morpholinophenyl)ethyl)carbamate (960 mg, 2.87 mmol) was dissolved in 2 ml CH_2Cl_2. Trifluoroacetic acid (1.64 g, 14.37 mmol) was added into solution at 0℃ and stirred at room temperature. The reaction was monitored by TLC. When the reaction finished, saturated $NaHCO_3$ was added to quench the reaction and then CH_2Cl_2 was added to extracted the organic phase for three times. After dried with anhydrous $MgSO_4$, the solvent was removed by rotary evaporation and the crude product was purified by column chromatography to obtain purified product (*S*)-1-(4-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethan-1-amine (510 mg, 79%).

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (26 mg, 0.10 mmol) were dissolved in 2 mL dichloromethane and 0.5 mL tetrahydrofuran, then 4-dimethylaminopyridine (2.3 mg, 0.02 mmol), (*S*)-1-(4-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethan-1-amine (20 mg, 0.10 mmol) was successively added into the solution. After 20 min, EDC (26 mg, 0.14 mmol) were added into the reaction and stirred overnight at 25°C. The solvent was removed by rotary evaporation to obtain the crude product. The purified product 7-(diethylamino)-*N*-((*S*)-1-(4-((*2S,6R*)-2,6-dimethylmorpholino)phenyl)ethyl)-2-oxo-2*H*-chromene-3-carboxamide was obtained by column chromatography (30 mg, 63%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.55 (s, 1H), 7.26 (s, 1H), 7.10 (s, 2H), 6.77 (s, 2H), 6.65 (s, 1H), 6.49 (s, 1H), 6.36 (s, 1H), 5.10 (s, 1H), 3.64 (s, 2H), 3.30 (s, 8H), 1.43 (s, 3H), 1.09 (d, *J* = 5.5 Hz, 12H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.1 (s), 161.5 (s), 157.0 (s), 151.8 (s), 147.5 (s), 130.4 (s), 126.5 (s), 115.3 (s), 109.9 (s), 109.2 (s), 107.8 (s), 95.9 (s), 71.0 (s), 54.3 (s), 47.8 (s), 44.4 (s), 21.8 (s), 18.4 (s), 11.8 (s). HRMS: m/z $[M+H]^+$ calcd for $C_{28}H_{35}N_3O_4$: 477.2628, found: 477.2628. Purity: 99.5%. HPLC: t_R = 2.20 min.

Fluorescence and UV spectroscopy

20 μL of probes (1 mM in DMSO) were added into the prepared 2 mL DMSO/H_2O or glycerol/H_2O solutions with different volume ratio. The fluorescence and UV spectra were captured by F-4600 fluorospectro photometer and UV-2600 spectrophotometer, respectively.

Cell culture and transient transfection

The KCNQ2/3 stably transfected HEK293 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 900 μg/ml G418 and 100 μg/ml Hygromycin B and HEK293 cells were cultivated in DMEM with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C.

KCNQ1-5 transiently transfected HEK293 cells for Rb^+ efflux assay was cultured in medium without G418 and Hygromycin B. Cells were passaged at 2-day to 3-day intervals and before recording and imaging, the cells were plated and adhered on 1 cm^3 glass coverslips.

Cell membrane preparation

The first sixth generations of KCNQ2/3 stably transfected HEK293 cells line were used to obtain the cell membrane. KCNQ2/3 stably transfected HEK293 cells were cultured in 10-cm dished. Then the proteins from HEK293 cells were extracted using RIPA (Thermo,U.S.). Then cell lysate was centrifuged at 40 000g for 20 min at 4 °C for two times. The supernatant was discarded and the pellet containing cell membrane was resuspended in assay buffer (50mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) and aliquoted in 1.5 ml tube and stored in -80°C before use. The concentration of cell membrane was quantified using BCA Protein Assay Reagent Kit (Thermo, U.S.).

Fluorescent polarization assay

50 μL of KCNQ2/3 stably transfected HEK293 cell membrane (0.1 mg/mL) in assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM KCl) was incubated with 50 μL different concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 μM) of probes for 5 min at 37°C. Then the fluorescent polarization of the solutions was detected at 405 nm with Flexstation 3 (Molecular Device). The equilibrium binding constant (Kd) was calculated by nonlinear curve fit to one site binding saturation equation.

Cytotoxicity test

Eight thousand HEK293 cells were seeded in 96-well plate in 100 μL fresh medium at 37℃ in a 5% CO_2 condition. Each well was cultivated with different concentrations of tested compounds for 24 h. Then treated with 10 μL of MTT solution for 4 h. After discarding the medium contained MTT of each well, 150 μL DMSO was added. OD values (Optical density) of each well was measured with a Tecan Auatria GmbH A-5082 Microplate Reader at 490 nm. Each well was duplicated for five times.

Electrophysiological experiment

The currents of Kv7.2/7.3 channel, Nav1.5 channel and Nav1.7 channel were recorded by a whole-cell patch clamp. A micropipette puller (Sutter Instrument, Novato, CA, USA) was used to pull the pipettes with resistance about 4 MΩ.

For whole-cell recording of Kv7.2/7.3 channel in Kv7.2/7.3 stably transfected HEK293 cells, the bath solution contained 140 mM NaCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM glucose, 4.7 mM KCl, 3 mM Mg-ATP and 5 mM HEPES (pH 7.4). The pipette was filled with 130 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 3 mM NaCl, and 10 mM HEPES, 3 mM Mg-ATP and 5 mM glucose (pH 7.4). Membrane potential was held at -80 mV for 200 ms, then cell was hold at -80 to 40 mV for 1s with 13 sweeps and following return to -80 mV for 500 ms.

Nav1.5 channel: the external solution contained 137 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM KCl and 10 mM glucose (pH 7.4). The recording pipette solution contained 100 mM KF, 40 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES and 5 mM EGTA (pH 7.2). Membrane potential was held at -130 mV then -10 mV for 15 ms and hyperpolarized to -130 mV.

Nav1.7 channel: the external solution contained 4 mM KCl, 70 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4). The recording pipette solution contained

100 mM KF, 40 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES and 5 mM EGTA (pH 7.2). Membrane potential was held at -130 mV then -10 mV for 15 ms and hyperpolarized to -130 mV.

The whole-cell patches assays were carried out at a room temperature and the data was analyzed by Origin 8.6 and Igor Pro software.

Rb^+ efflux assay

The Rb^+ efflux assay was performed based on the published protocols.[1, 2] 20,000 cells/well stably transfected Kv7.2/7.3 HEK293 cells or transiently transfected Kv7.1-7.5 HEK293 cells were seeded in poly-D-lysine-coated 96-well microplates and incubated at 37°C with 5% CO_2 overnight. Each well was loaded with Rb^+ loading buffer (5.4 mM RbCl, 150 mM NaCl, 25 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.8mM NaH_2PO_4, pH 7.4) for 3 h at 37°C and then gently washed with washing buffer (5.4 mM KCl, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, 0.8 mM NaH_2PO_4, pH 7.4) for three times. In experiments screening for KCNQ blockers, the cells were incubated with depolarization buffer (50 mM KCl, 130 mM NaCl, 25 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.8 mM NaH_2PO_4, pH 7.4) contained 10 μM compounds for 10 min. For Rb^+ efflux measurements, 200 μl supernatant of each well was collected and transferred to a new 96-well plate. 200 μl of lysis buffer (25 mM HEPES, 1 mM $MgCl_2$, 0.8mM NaH_2PO_4, 2 mM $CaCl_2$, 1-1.5% Triton X-100) was added into each well to lyse cell completely at 37°C for 20 min. The Rb^+ level of cell lysates and cell supernatants were quantified by an Ion Channel Reader 8000 (ICR8000; Aurora Biomed, Vancouver, Canada). Reading of a whole 96-well plate takes about 20 min.

FlexStation 3 multi-mode microplate reader assay for TPRV1 and TPRV3 channels

The changes of intracellular calcium level ($[Ca^{2+}]_i$) in TRPV1 or TRPV3 transfected HEK293 cells were measured by fluorescent calcium-sensitive dyes (Cal-520 PBX Calcium Assay Kit, AAT Bioquest, CA, USA). The fluorescent signals were read by FlexStation3 Multi-Mode Microplate Reader (Molecular Devices, San Francisco, USA). For recording intracellular calcium level, 30000 cells/well TRPV1 or TRPV3 transfected HEK-293 cells were seeded in 96-well black-walled plates and grown for overnight at 37°C with 5% of CO_2. Then, cells were loaded with the dye for 1.5 hours at 37°C with 5% of CO_2. Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.1 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 4 mM $NaHCO_3$ and 20 mM HEPES, pH 7.4) was used during the experiment. All drugs including test compounds, agonist and antagonist were applied at six-fold working concentration. The compounds were added onto cells at 17 seconds and then, corresponding TRP agonists was added at the time point of 100 seconds and the relative fluorescence unit values were continuously measured for 80 seconds. The wavelength of excitation light was set as 485 nm and the wavelength of emission was set at 525 nm, the 515 nm wavelength was cut off and fluorescence intensity was measured at an interval of 1.6 seconds.[3]

Confocal imaging

KCNQ2/3 transfected HEK293 cells or non-transfected HEK293 cells were loaded with 10 μM probes for 2 minutes. After washed three times with PBS, the images of cells were captured by a Nikon A1R MP confocal fluorescence microscope with 40X objective (λex: 405 nm, λem: 470-520 nm).

Animals

C57BL/6N mice (6 weeks) were purchased from Beijing Vital River Laboratory Technology Co. Ltd (Beijing China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University Health Science Center and were carried out in compliance with national and institutional guidelines for the care and use of laboratory animals.

Preparation of hippocampal brain slices

C57BL/6 mice were sacrificed by decapitation after a short period of inhalation of anesthetic diethyl ether. The dissociated brains were fixed in 4% paraformaldehyde/PBS at 4℃ for 12 h. After cryo-protection in 10% sucrose/PBS (wt/vol) at 4℃ for 12 h, 20% sucrose/PBS (wt/vol) at 4℃ for 12 h and 30% sucrose/PBS (wt/vol) at 4℃ for 12 h, 70 μm thickness of coronal slices from hippocampus were cut on a vibratome (VT-1200, Leica, Germany) and the brain slices were transferred into PBS for staining.

Immunofluorescence

Hippocampal brain slices were incubated with probe for 1 min and washed with PBS for three times and then incubated with blocking solution (10% goat serum (abs933, Lot#201018, absin) in PBS contained 0.5% TritonX-100) for 60 min. Then the slices were incubated with (0.2 mg/ml) anti-KCNQ2 antibody (Santa Cruz Biotechnology sc-271852) with a dilution rate about 1:50 after overnight culture at 4°C and washed with PBS for three times. Then the slices were covered by PBS with Alexa Fluor 488 Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody (Thermo Fisher A-11001) with a dilution rate about 1:200 for 1h at 37℃. Finally, the slices were washed with PBS three times and placed on glass slides for confocal imaging.

Molecular docking

To reveal the binding model of probes and Kv7.2 channels, we built a complex of probes and Kv7.2 channels. The crystal structures of Kv7.2 channel with or without the ligand retigabine (PDB code: 7CR2 and 7CR0) were used in molecular docking experiments. Retigabine was removed from the crystal structure and all hydrogen atoms was added.

The chemical structures of probes **20c**, **21a**, **21b**, **21c**, **24a** and **24b** were optimized by SYBYL (Tripos, St Louis, MO, USA) based on previous protocol.[4, 5]

The molecular docking was performed on the program GOLD Suite 5.1). The retigabine was extracted from the complex and the new ligands (**20c**, **21a**, **21b**, **21c**, **24a** and **24b**) were fit into the binding pocket of retigabine in Kv7.2 channel. The ChemScore and GoldScore was selected to fitness scoring function. The exported complexes were edited by UCSF Chimera 1.15 software.

Supplementary Figures

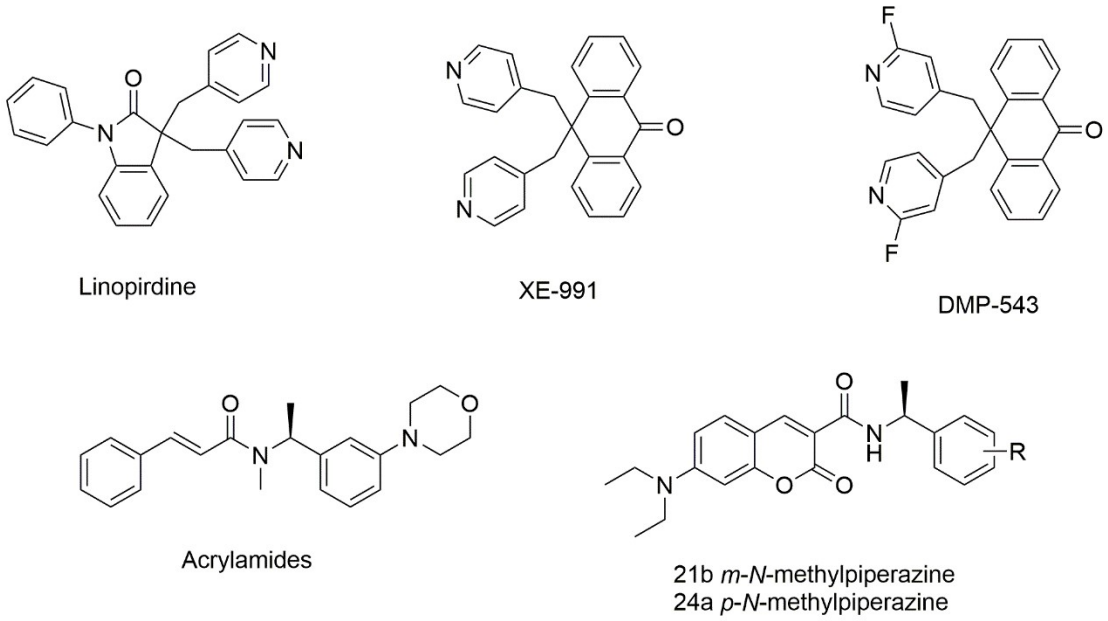


Figure S1 Structure of published Kv7.2/7.3 inhibitors and probe **21b** and **24a**.

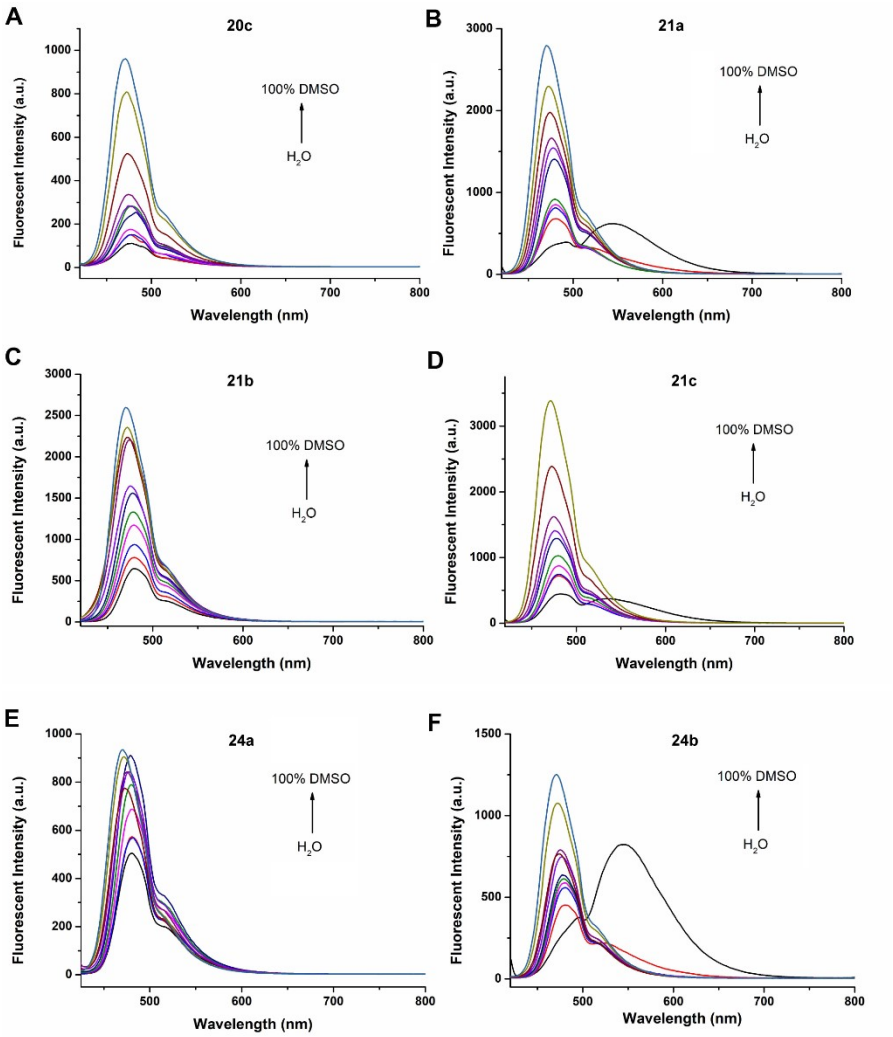


Figure S2. Fluorescent spectra of 10 μM **20c** (A), **21a** (B), **21b** (C), **21c** (D), **24a** (E), **24b** (F) in different DMSO/water mixtures.

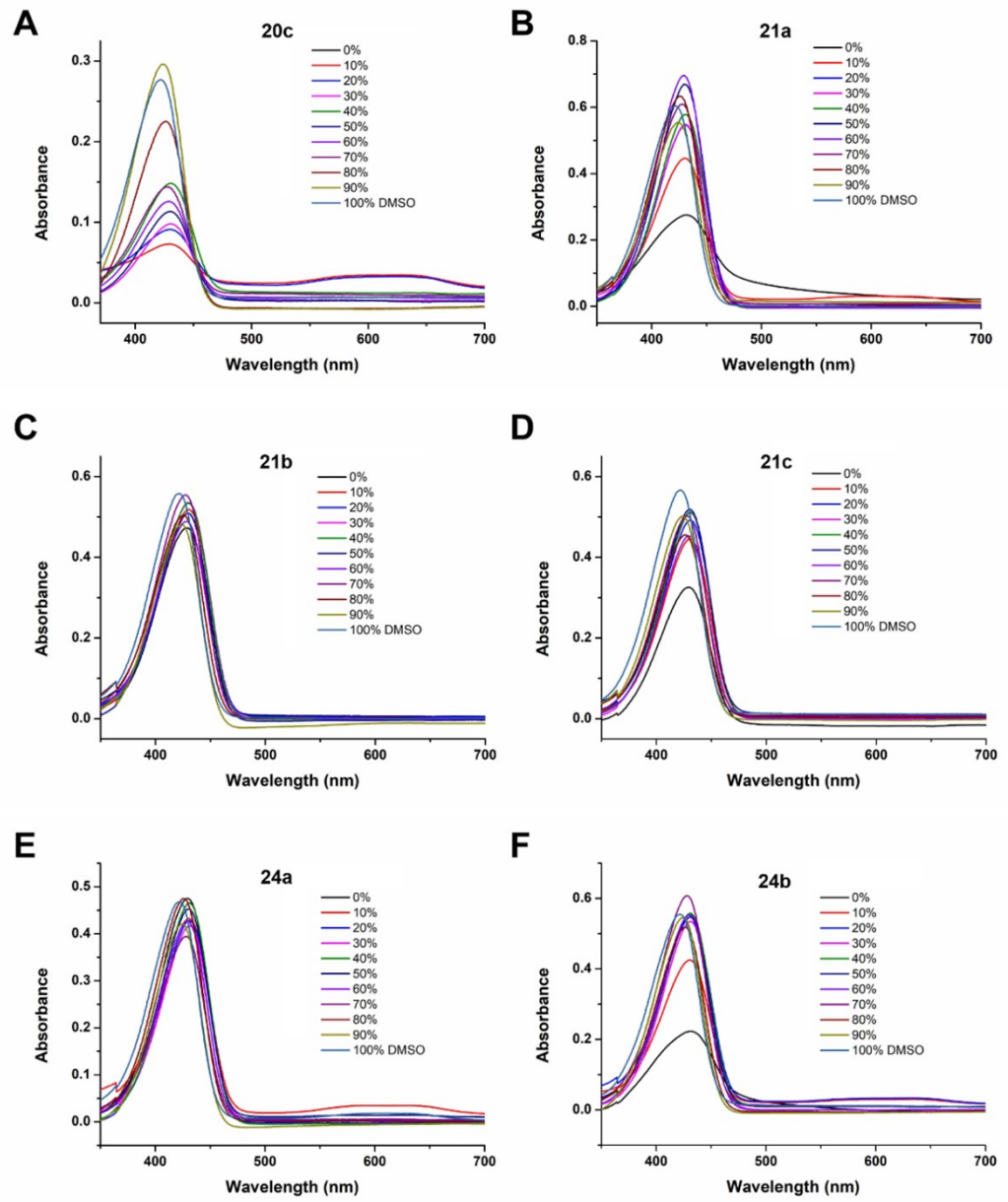


Figure S3. Absorption spectra of 10 μM **20c** (A), **21a** (B), **21b** (C), **21c** (D), **24a** (E), **24b** (F) in different DMSO/water mixtures.

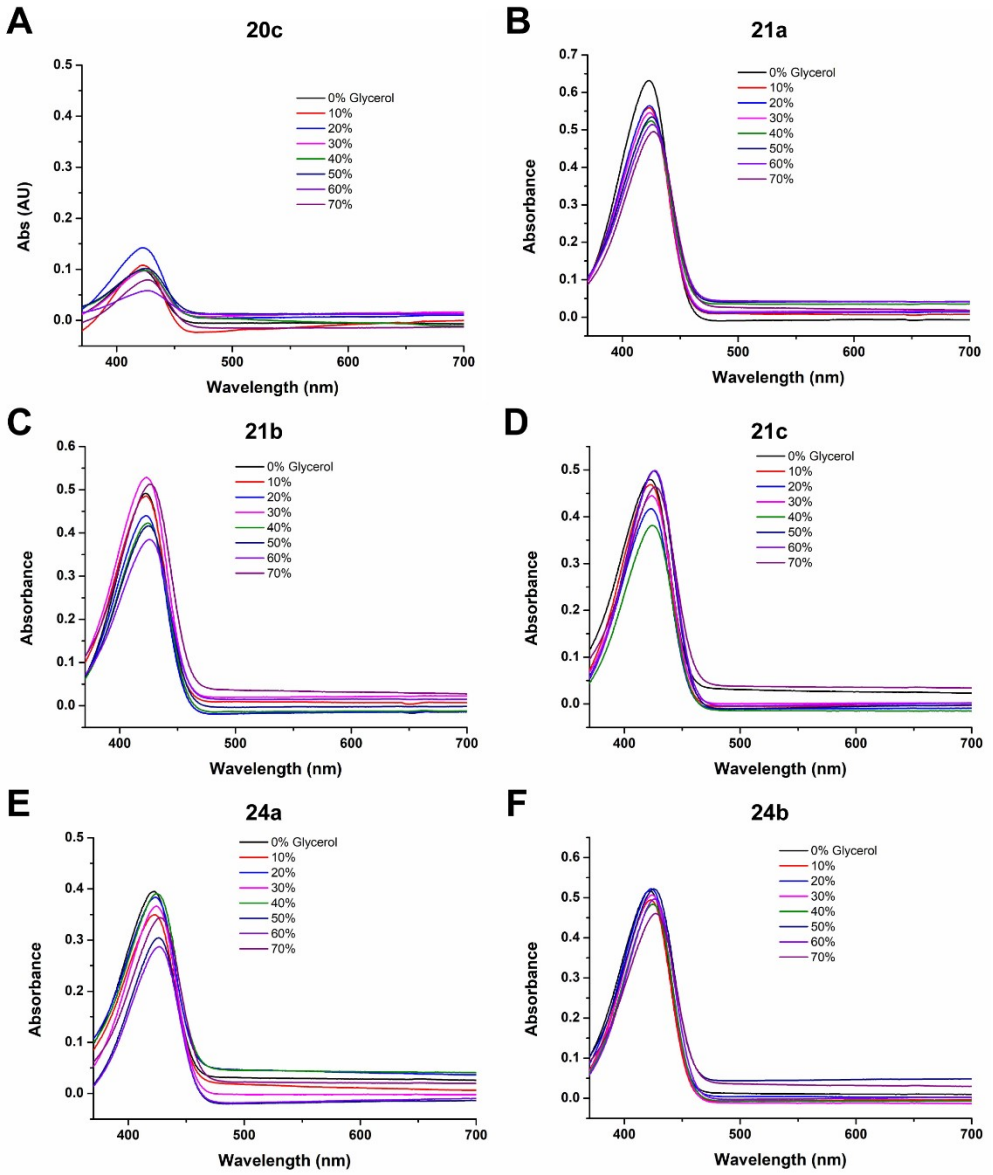


Figure S4. Absorption spectra of 10 μM **20c** (A), **21a** (B), **21b** (C), **21c** (D), **24a** (E), **24b** (F) in different H_2O/glycerol mixtures.

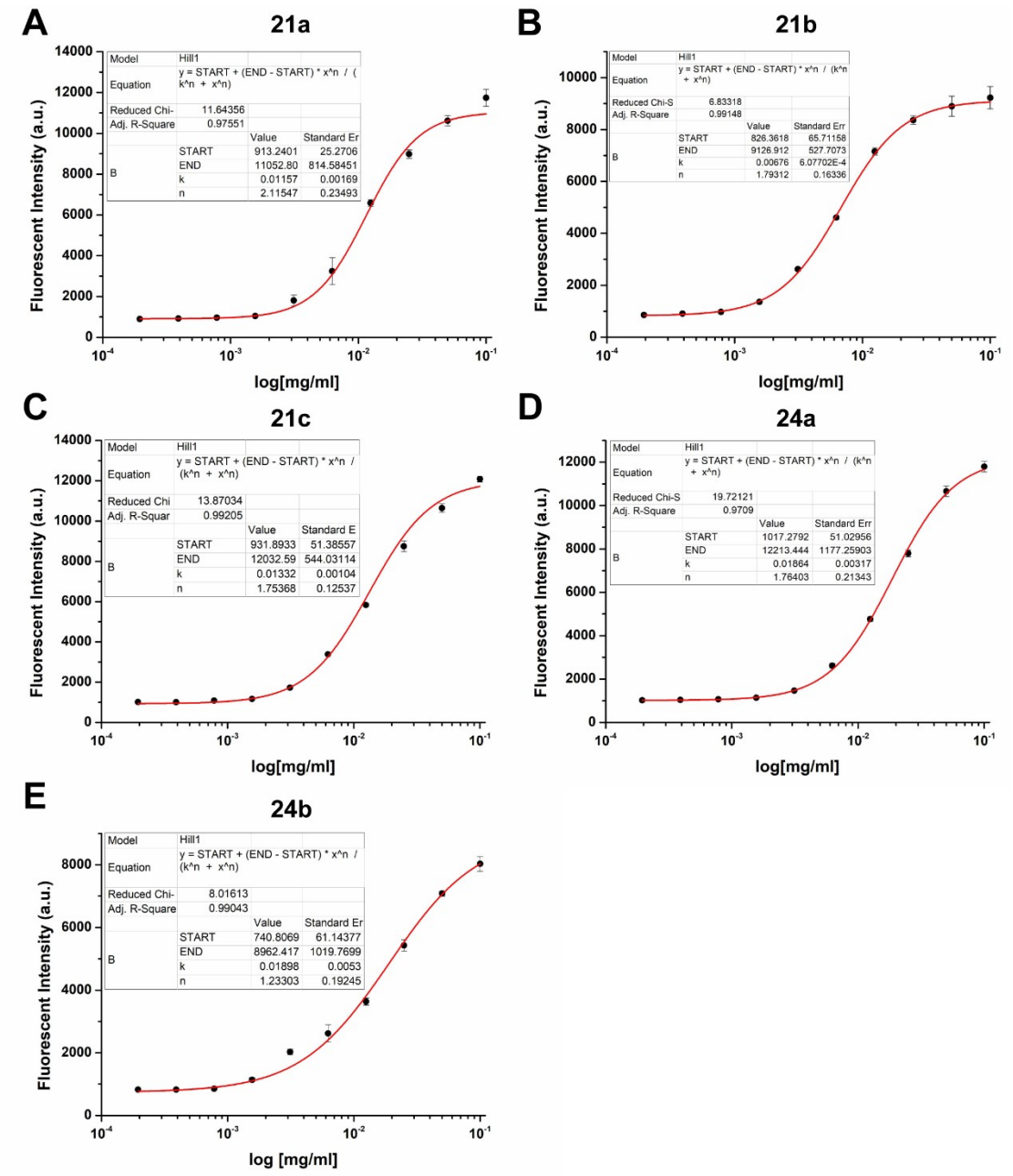


Figure S5. Nonlinear curve fit between the fluorescence intensity of 10 μM **21a**, **21b**, **21c**, **24a**, **24b** and different concentrations (from 0 to 1.0 mg/ml) of Kv7.2/7.3 stably transfected cell membrane. Em: 460 nm, Ex: 405 nm. Nonlinear curves are fitted by the Origin 86 software with Hill1 equation. The results are presented as means ± SEM with three replicates (n = 3).

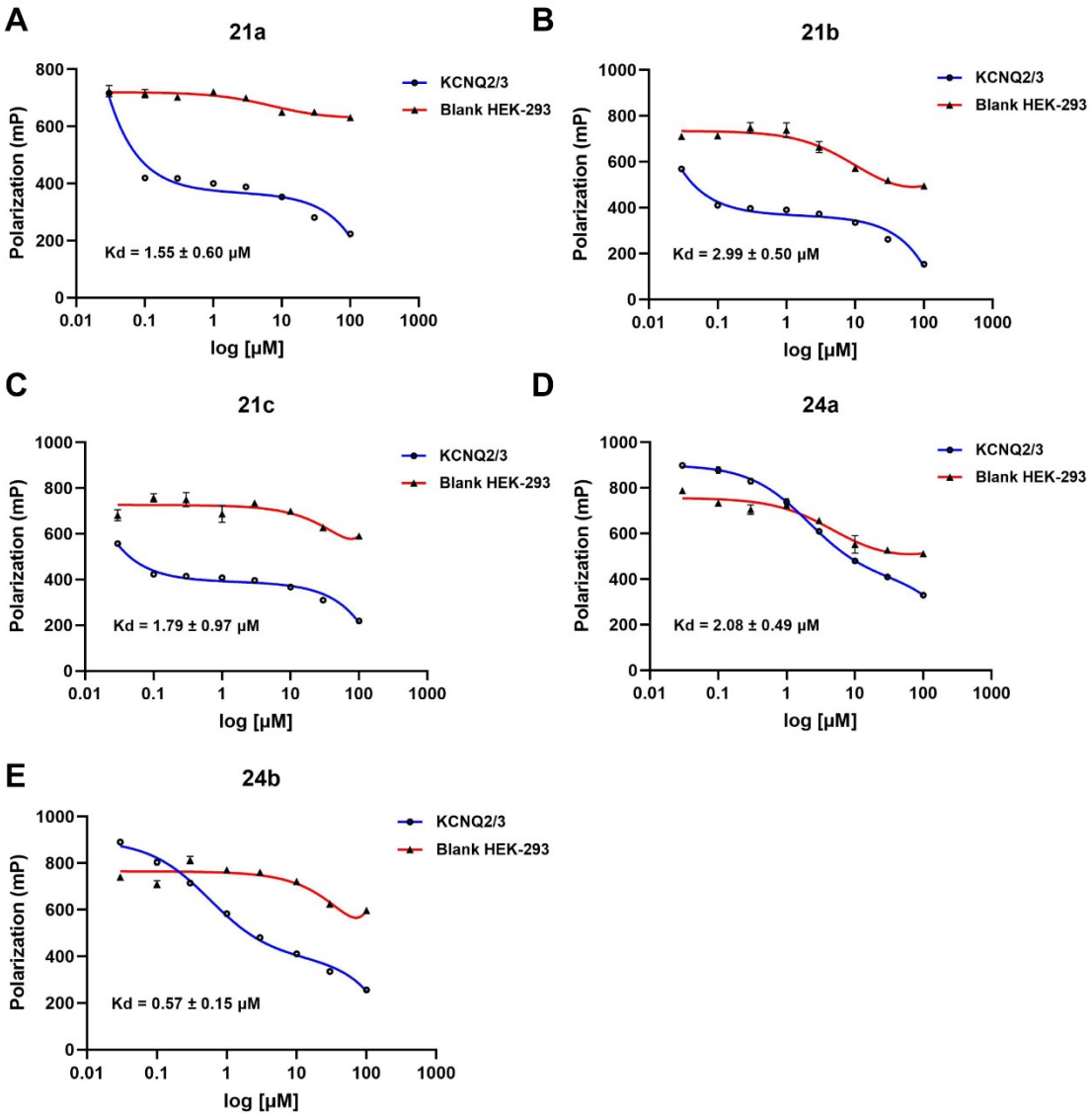


Figure S6. Fluorescence polarization analysis of KCNQ2/3 stably transfected HEK293 cell membrane (blue curves) or blank HEK293 cell membrane (red curves) with different concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 μM) of **21a** (A), **21b** (B), **21c** (C), **24a** (D), **24b** (E). The equilibrium binding constant (K_d) was calculated by nonlinear curve fit to one site binding saturation equation. K_d value of **21a**, **21b**, **21c**, **24a**, **24b** with KCNQ2/3 channels were calculated as 1.55, 2.99, 1.79, 2.08 and 0.57 μM, respectively. Nonlinear curves are fitted by the GraphPad Prism 5 software with one site binding saturation equation. The results are presented as means ± SEM with three replicates (n = 3), Em: 460 nm, Ex: 405 nm.

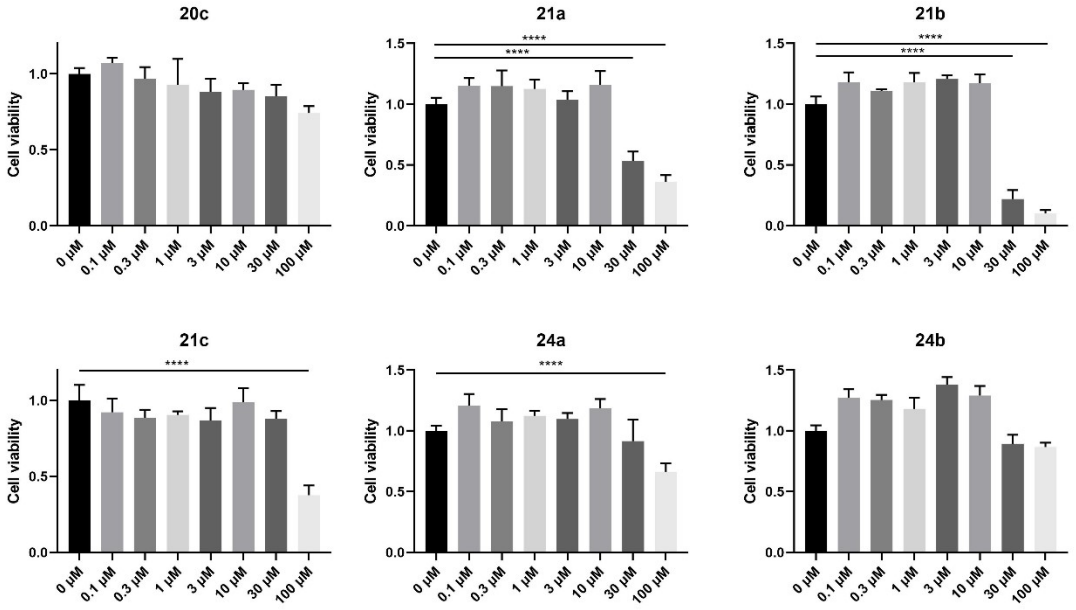


Figure S7. Cell viability of HEK293 cells incubated with different concentrations (0, 0.1, 0.3, 1, 3, 10, 30, 100 µM) of **20c**, **21a**, **21b**, **21c**, **24a**, **24b**. The data are presented as the means ± SEM from five replicates (n = 5). ****p<0.0001.

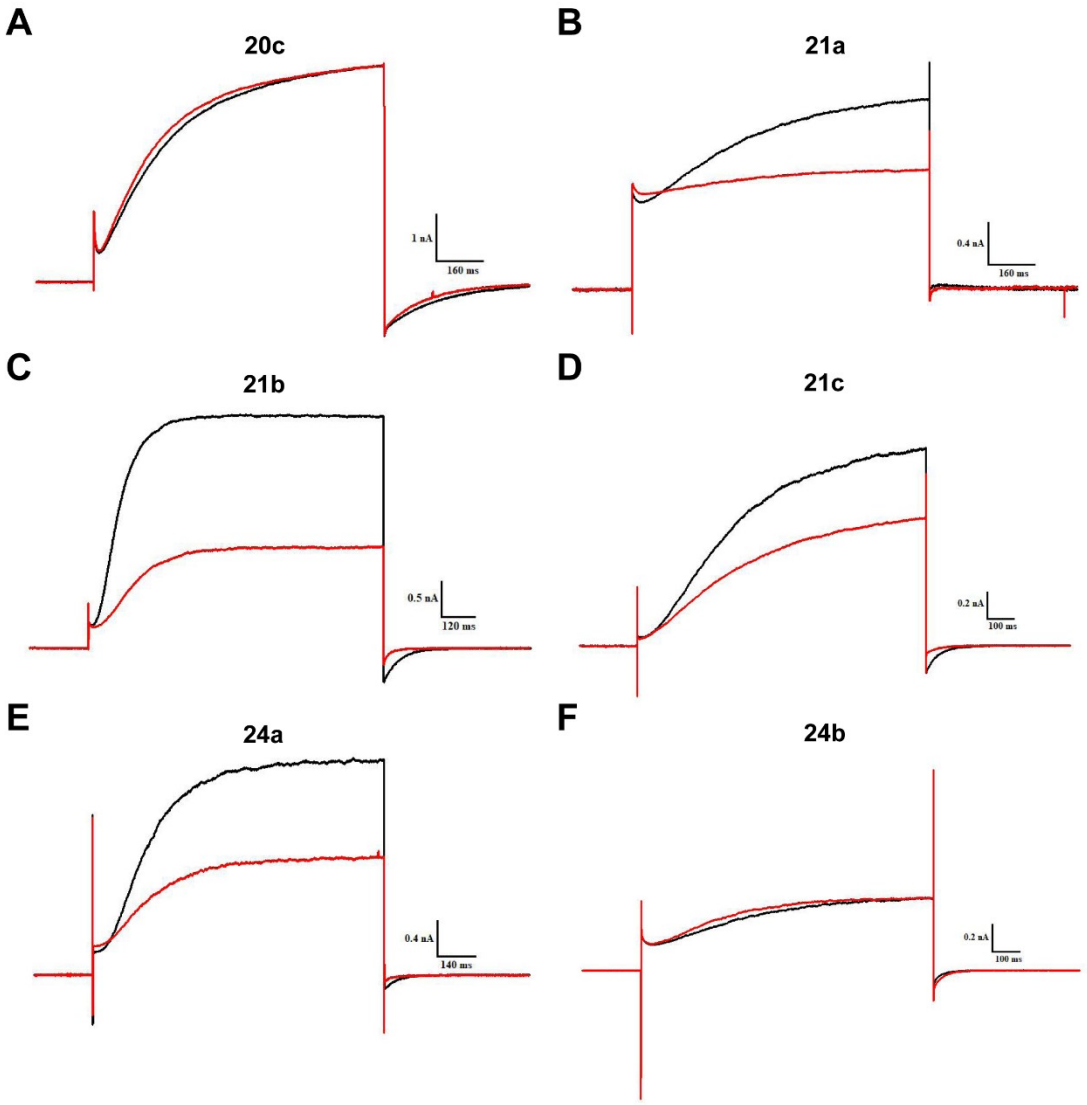


Figure S8. The inhibitory effect of **20c** (A, 100 μM), **21a** (B, 10 μM), **21b** (C, 10 μM), **21c** (D, 10 μM), **24a** (E, 10 μM), **24b** (F, 10 μM) on Kv7.2/7.3 channels in HEK-293 cells. Black trace: control; red trace: 10 μm probes.

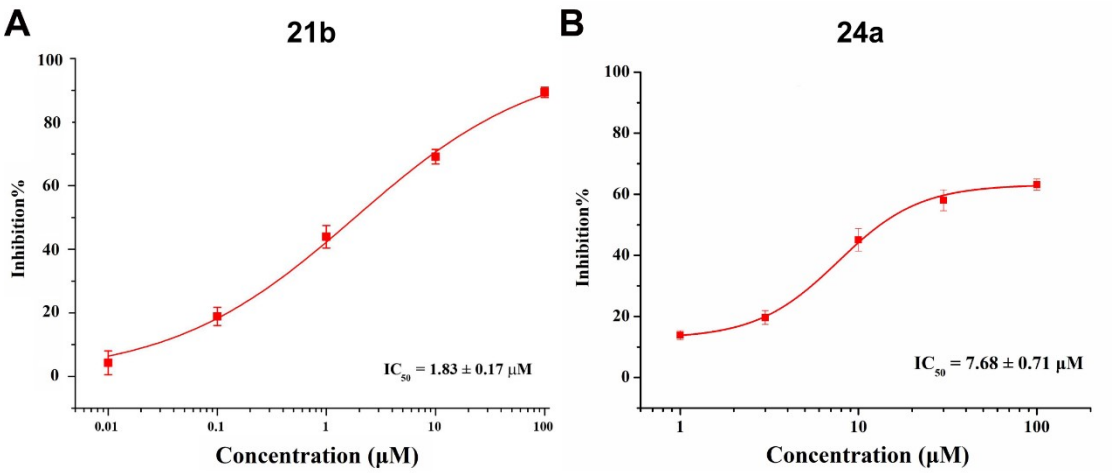


Figure S9. Dose-dependent inhibition of Kv7.2/7.3 channels by 21b and 24a in HEK293 cells. The IC_{50} values of **21b** (A) and **24a** (B) were fitted by a Logistic fitting and calculated as 1.83 ± 0.17 μM and 7.68 ± 0.71 μM respectively. These values are the mean ± SEM (n=4).

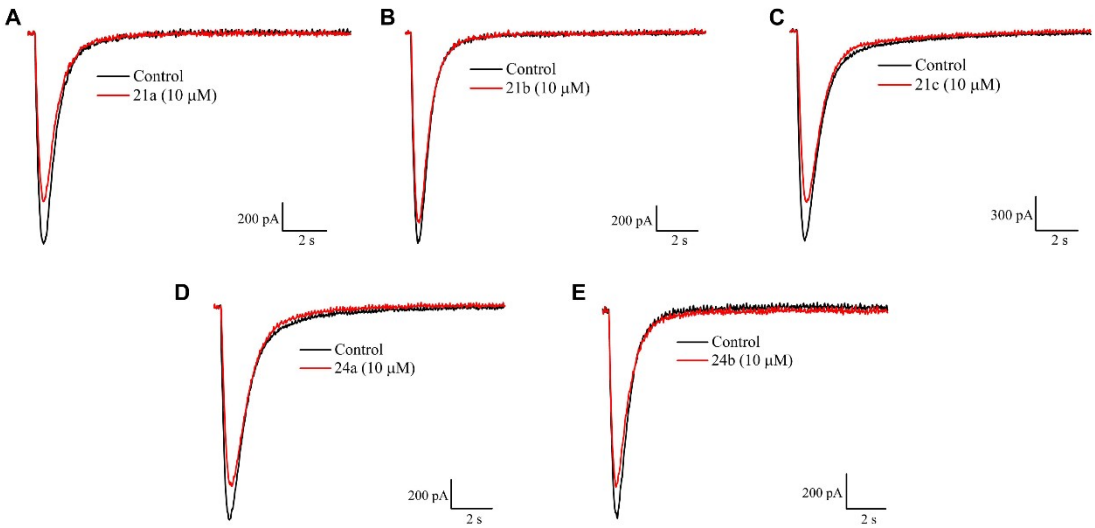


Figure S10. Selectivity of 10 μM **21a** (A), **21b** (B), **21c** (C), **24a** (D) and **24b** (E) on Nav1.5 channels expressed in HEK293 cells by whole-cell patch assay.

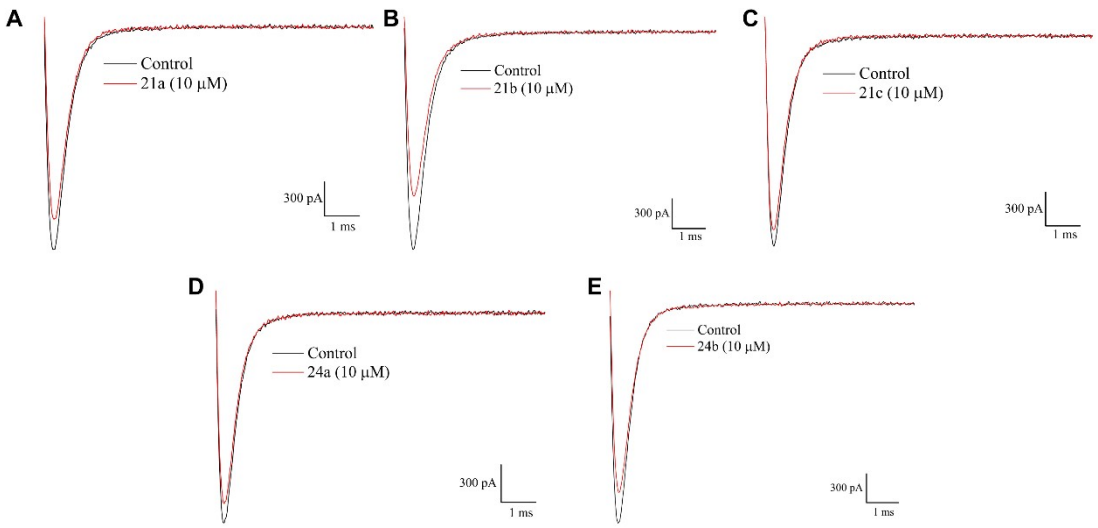


Figure S11. Selectivity of 10 μM **21a** (A), **21b** (B), **21c** (C), **24a** (D) and **24b** (E) on Nav1.7 channels expressed in HEK293 cells by whole-cell patch assay.

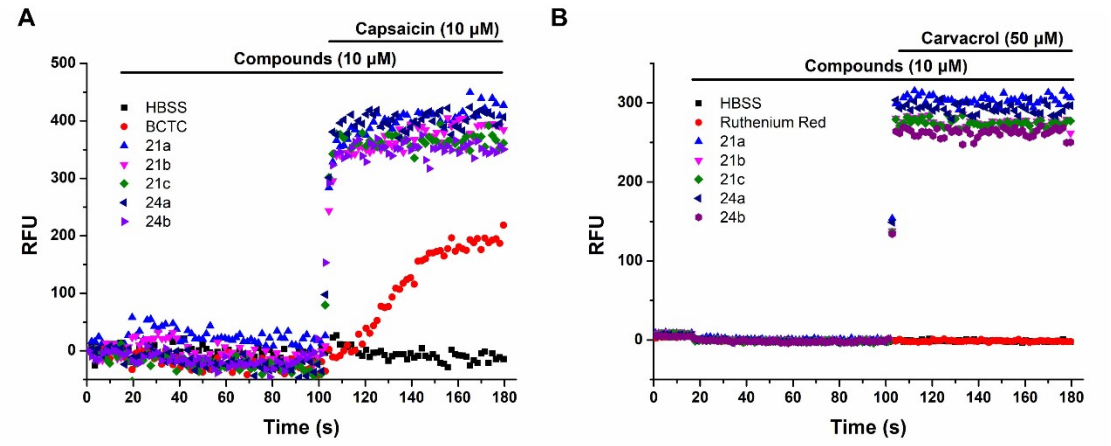


Figure S12. Selectivity of 10 μM **21a**, **21b**, **21c**, **24a** and **24b** on TRPV1 and TRPV3 channels. Inhibition and activation of intracellular calcium rise in TRPV1-expressing (A) and TRPV3-expressing (B) HEK-293 cells by 10 μM **21a**, **21b**, **21c**, **24a** and **24b** in FlexStation3 calcium fluorescence assay, in comparison with TRPV1 antagonist BCTC (1 μM), TRPV1 agonist capsaicin (10 μM), TRPV3 antagonist Ruthenium red (20 μM) and TRPV3 agonist carvacrol (50 μM).

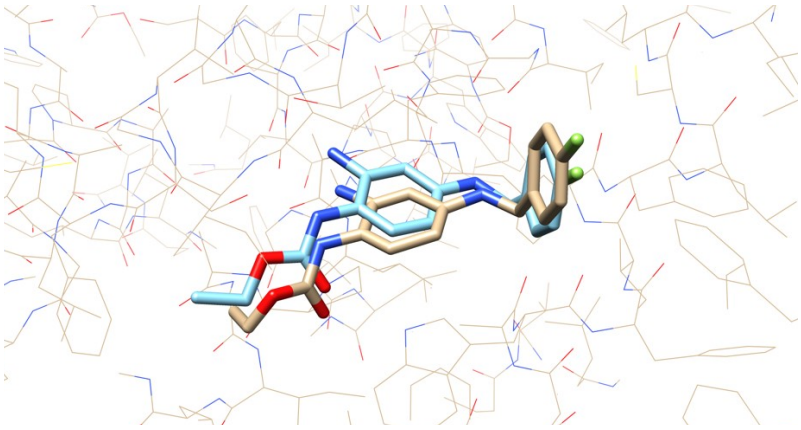


Figure S13. Crystal structure of retigabine bound to Kv7.2 channel was superimposed with docking result of retigabine bound to Kv7.2. Retigabine were shown in stick (cream in PDB file and cyan in docking result respectively), rest of residues were shown in wire.

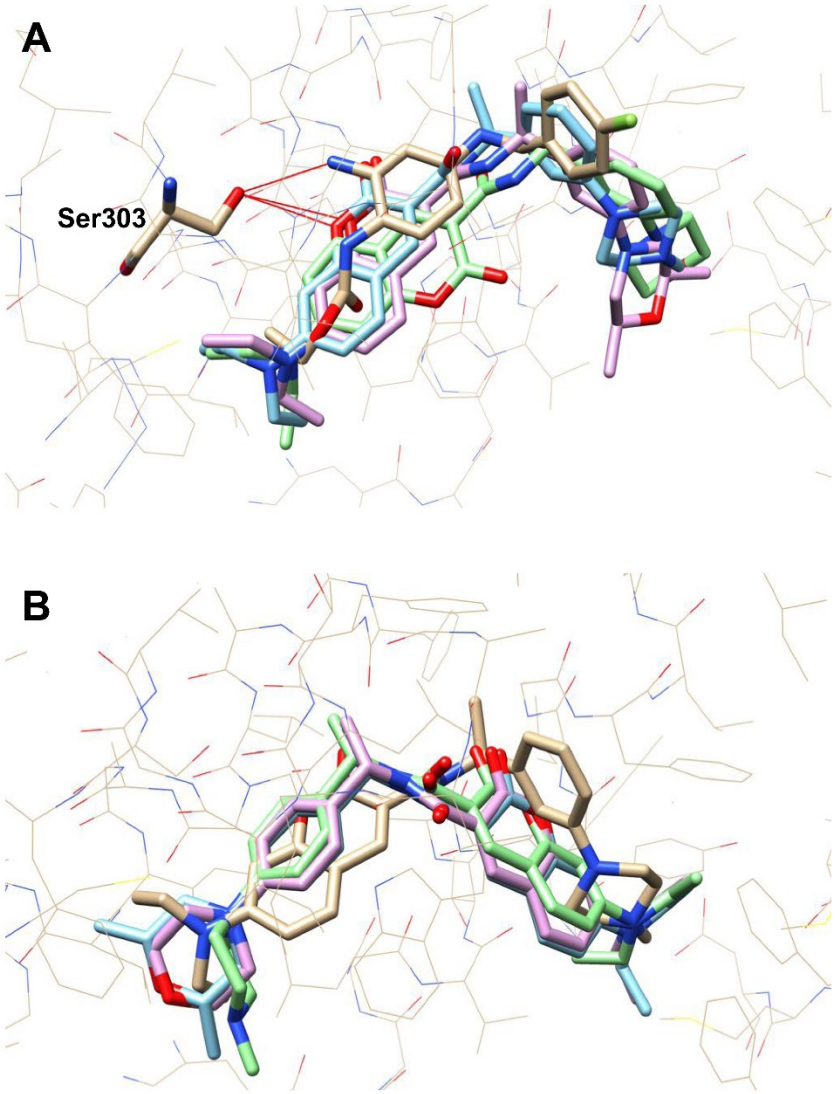


Figure S14. (A) Crystal structure of retigabine bound to Kv7.2 was superimposed with docking result of probe **21a**, **21b** and **21c**. Retigabine, **21a**, **21b** and **21c** was shown in stick (cream, purple, cyan and green, respectively). Ser303 was shown in stick and rest of residues were shown in wire. Potential intermolecular hydrogen bond (red line) was predicted by Chimera 1.13.1 (B) Docking result of probe **21b** bound to Kv7.2 was superimposed with docking result of probe **20c**, **24a** and **24b**. Probes **21b**, **20c**, **24a**, and **24b** were shown in stick (cream, purple, green and cyan respectively). Rest of residues were shown in wire.

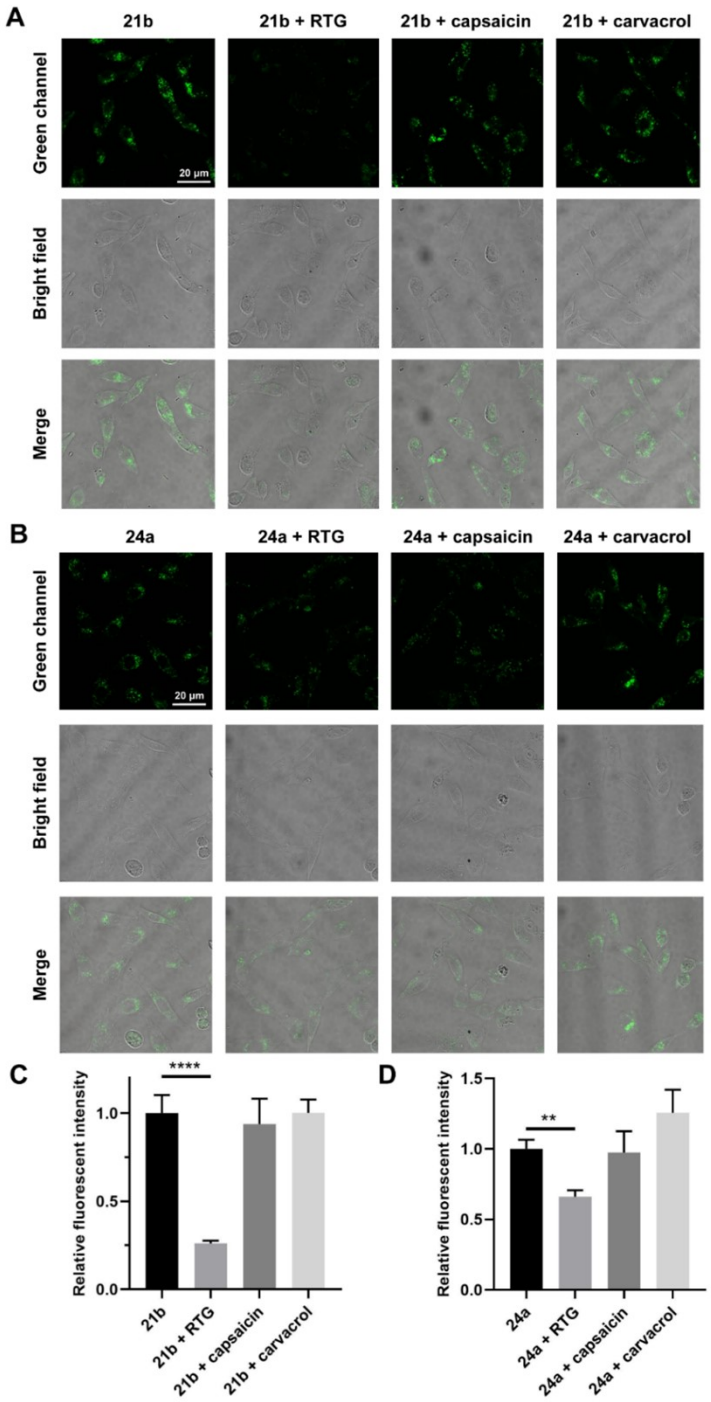


Figure S15. Competitive binding assay of **21b** and **24a** by confocal imaging. (A) Confocal imaging of **21b** (10 μM) and co-incubation with 10 μM retigabine (RTG, KCNQ activator), capsaicin (TRPV1 agonist) or carvacrol (TRPV3 agonist) in Kv7.2/7.3 stably transfected HEK293 cells. (B) Confocal imaging of **24a** (10 μM) and co-incubation with 10 μM retigabine (RTG, KCNQ activator), capsaicin (TRPV1 agonist) or carvacrol (TRPV3 agonist) in Kv7.2/7.3 stably transfected HEK293 cells. (C) The change of fluorescent intensity in groups of **Figure S15A**. (D) The change of fluorescent intensity in groups of **Figure S15B**. Scale bars (white): 20 μm. The results are presented as means ± SEM (n = 3-6). ****$p<0.0001$, **$p<0.01$.

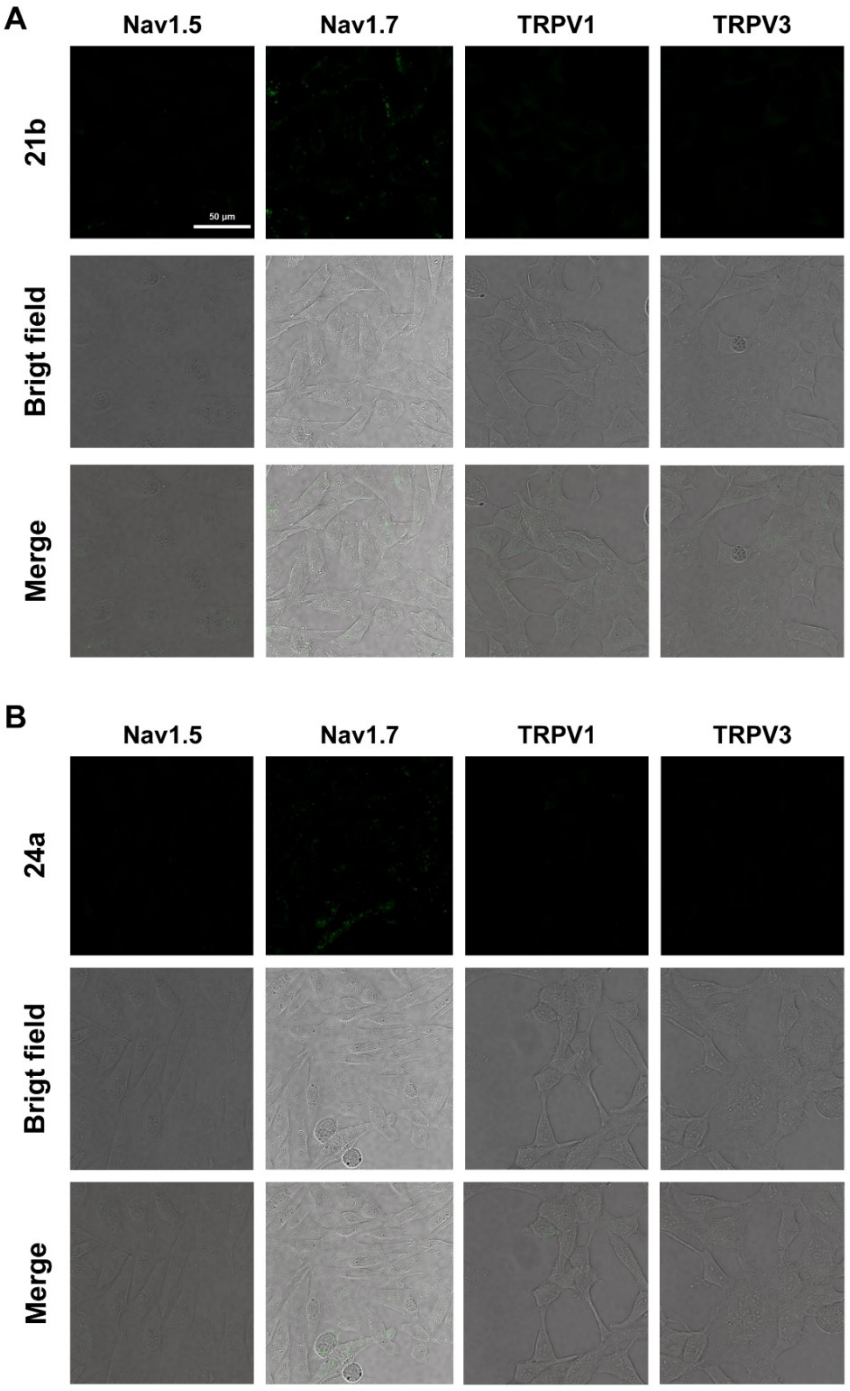


Figure S16. Confocal imaging of 10 μM probe **21b** (A) and **24a** (B) in Nav 1.5 and Nav1.7 stably transfected HEK 293 cells, TRPV1 and TRPV3 transfected HEK 293 cells. Scale bars (white): 50 μm.

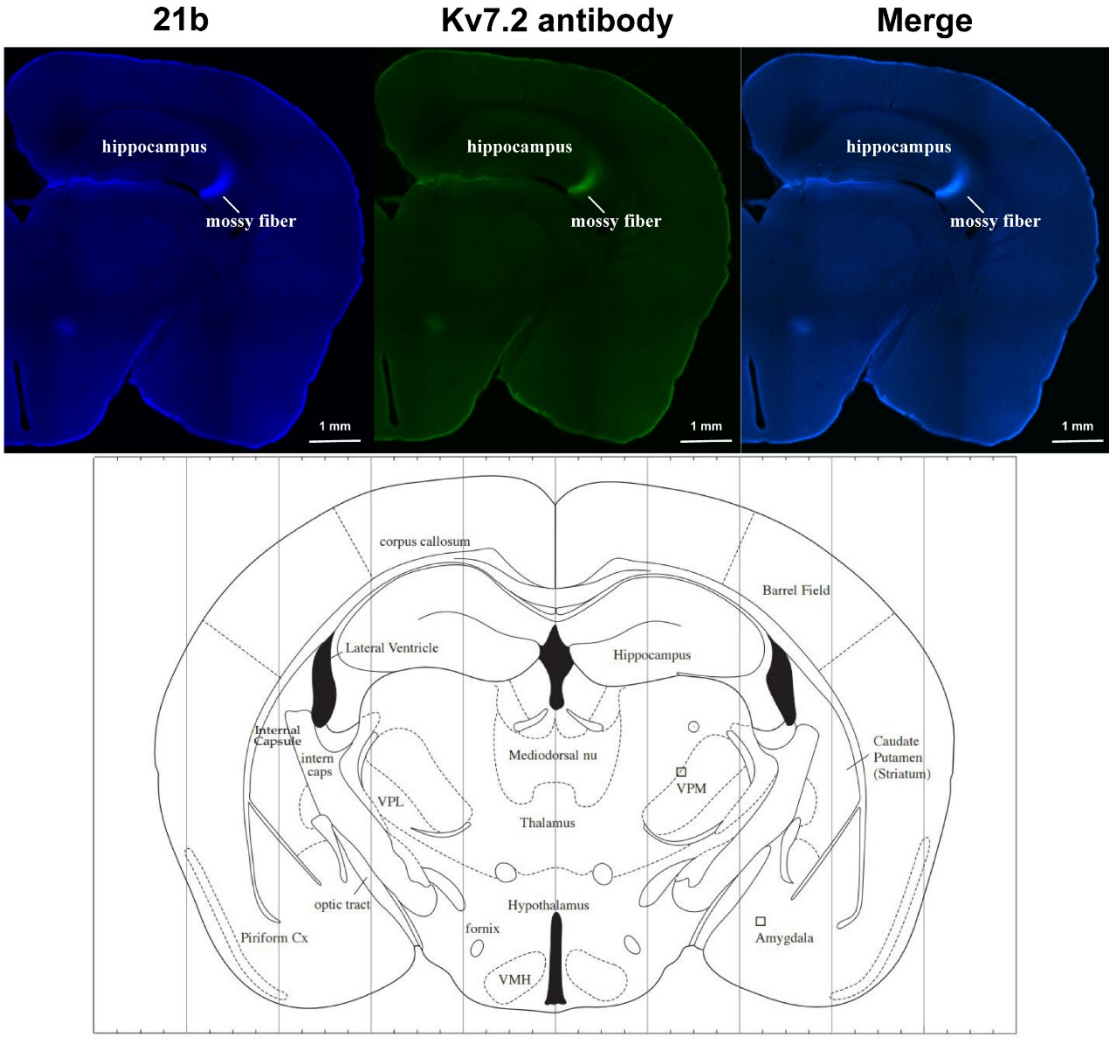
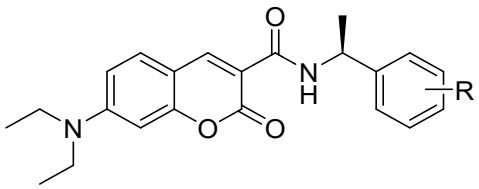


Figure S17. Confocal imaging of 10 μM **21b** with anti-KCNQ2 antibody in hippocampal brain slices. (A) Large image of right hippocampal brain slice of mouse after incubated with **21b** (blue) and anti-Kv7.2 antibody (green). Scale bars (white): 1 mm. (B) The mouse brain slice containing hippocampus in stereotaxic coordinates.[7]

Table S1. The effect of 21a, 21b, 21c and 24a on Kv7.2/7.3 current.



compounds	R	%control[a]
21a	*m*-2,6-cis-dimethylmorpholine	27.2 ± 10.1
21b	*m*-*N*-methylpiperazine	69.6 ± 9.5
21c	*m*-morpholine	34.5 ± 12.1
24a	*p*-*N*-methylpiperazine	45.1 ± 7.4

[a] Kv7.2/7.3 current in the presence of 10 μM test compound/compound free control current at -40 mV. These values are the mean ±SEM (n=3-6).

Table S2. Rb^+ efflux assay of probes on Kv7.1-7.5. (n=3)

Compounds	Efficacy (%) @ 10 μM[a]					
	Kv7.1[b]	Kv7.2[b]	Kv7.3[b]	Kv7.2/7.3[c]	Kv7.4[b]	Kv7.5[b]
20c	142 ± 14	124 ± 3	120 ± 14	117 ± 5	238 ± 24	208 ± 13
21a	26 ± 1	97 ± 5	172 ± 7	103 ± 7	263 ± 12	250 ± 8
21b	82 ± 3	28 ± 9	67 ± 5	46 ± 2	210 ± 7	139 ± 5
21c	78 ± 6	84 ± 10	176 ± 13	98 ± 7	164 ± 1	114 ± 11
24a	93 ±10	72 ± 8	28 ± 8	53 ± 12	247 ± 1	185 ± 10
24b	59 ± 2	57 ± 0	115 ± 3	95 ± 4	128 ± 6	99 ± 9
Retigabine[d]	136 ± 5	190 ± 15	121 ± 5	123 ± 8	193 ± 12	145 ± 13

[a] The efficacy of probes was calculated by $E = [(F_{cpd} - F_{basal})/(F_d - F_{basal})] * 100$

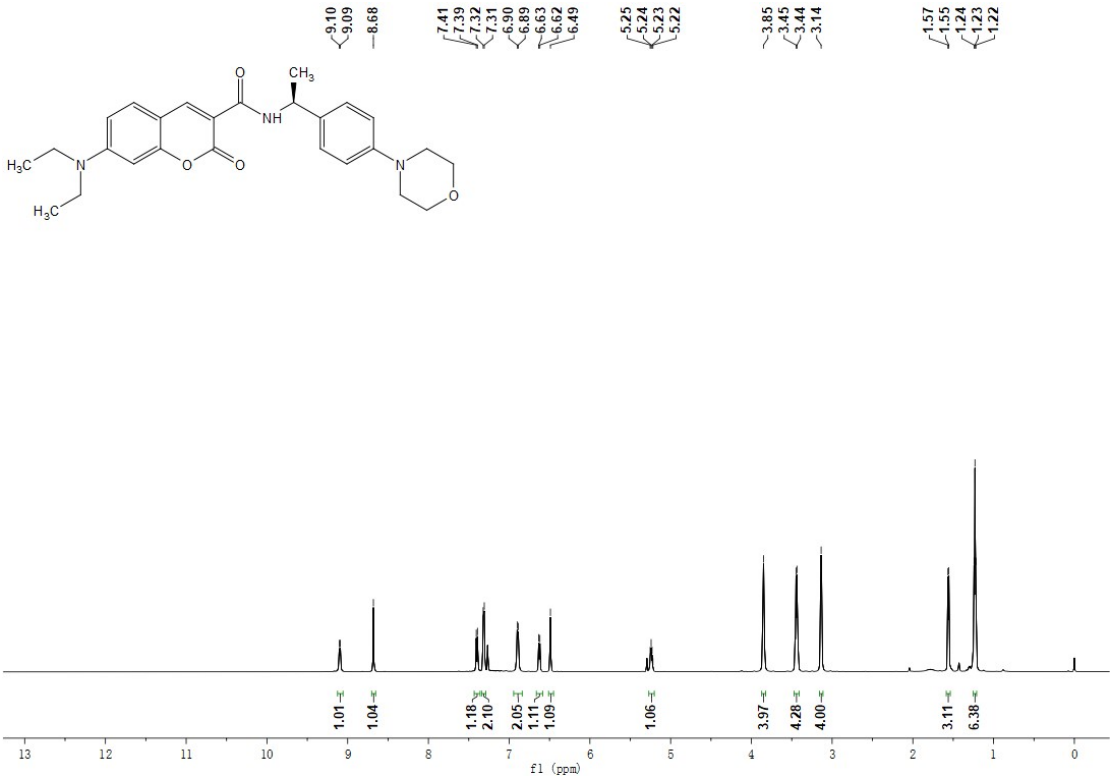
[b] Transiently transfected HEK293 cells.

[c] Stably transfected Kv7.2/7.3 HEK293 cells.

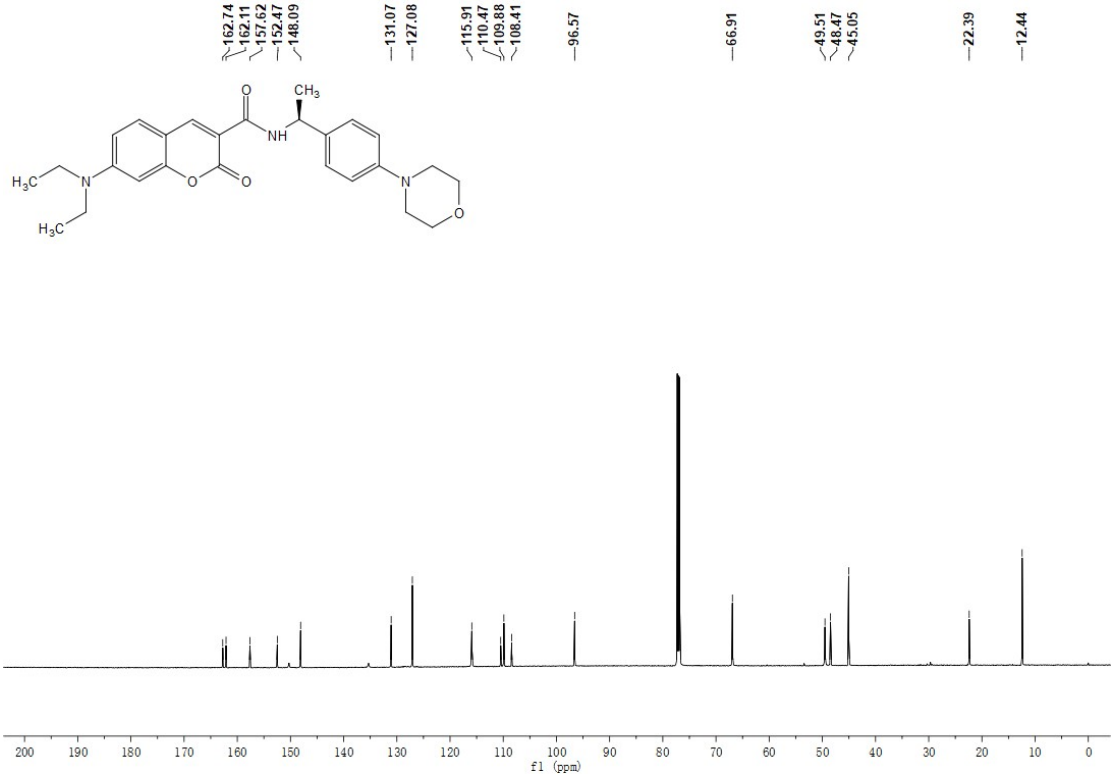
[d] Positive control

NMR and HRMS Spectra

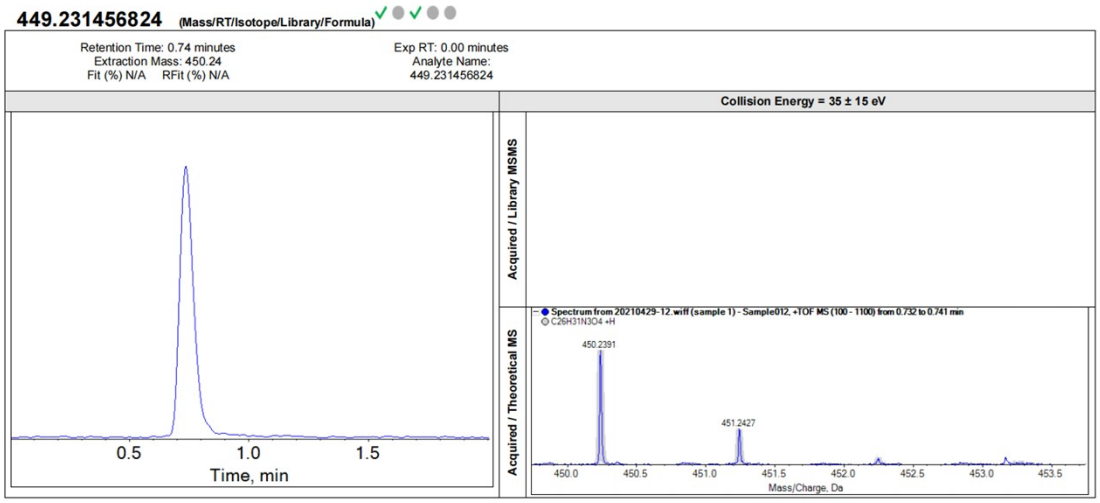
^{1}H NMR spectrum of 20c



^{13}C NMR spectrum of 20c

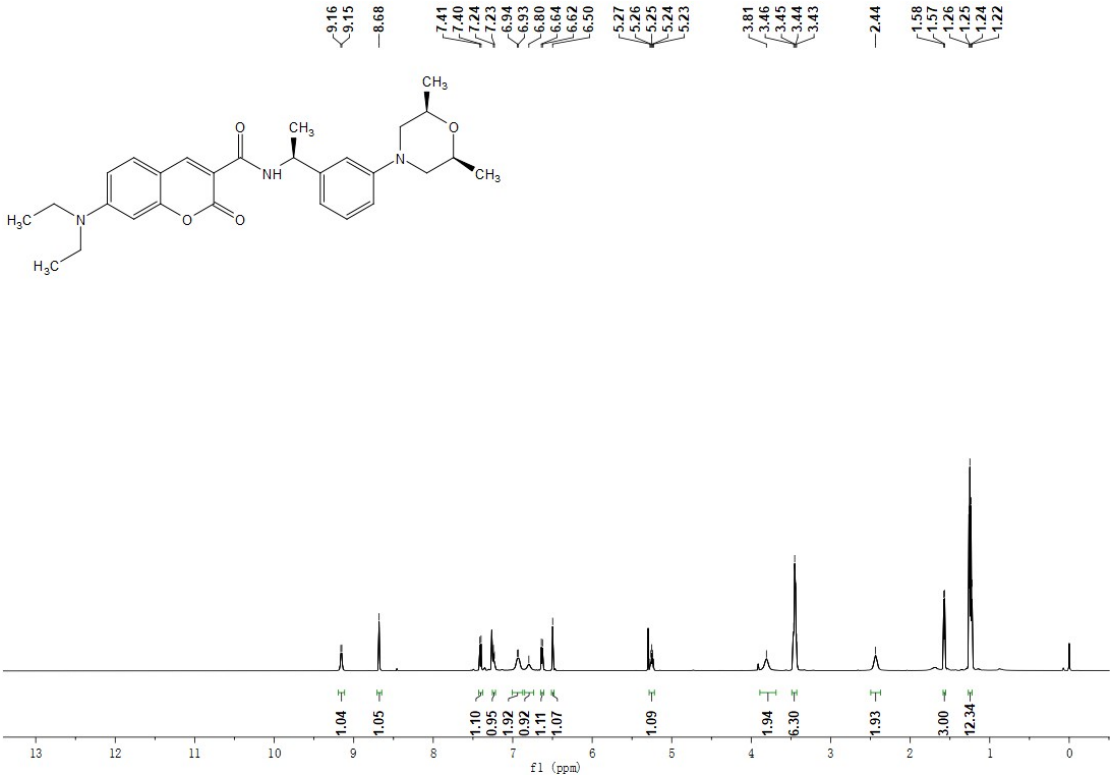


HRMS of 20c

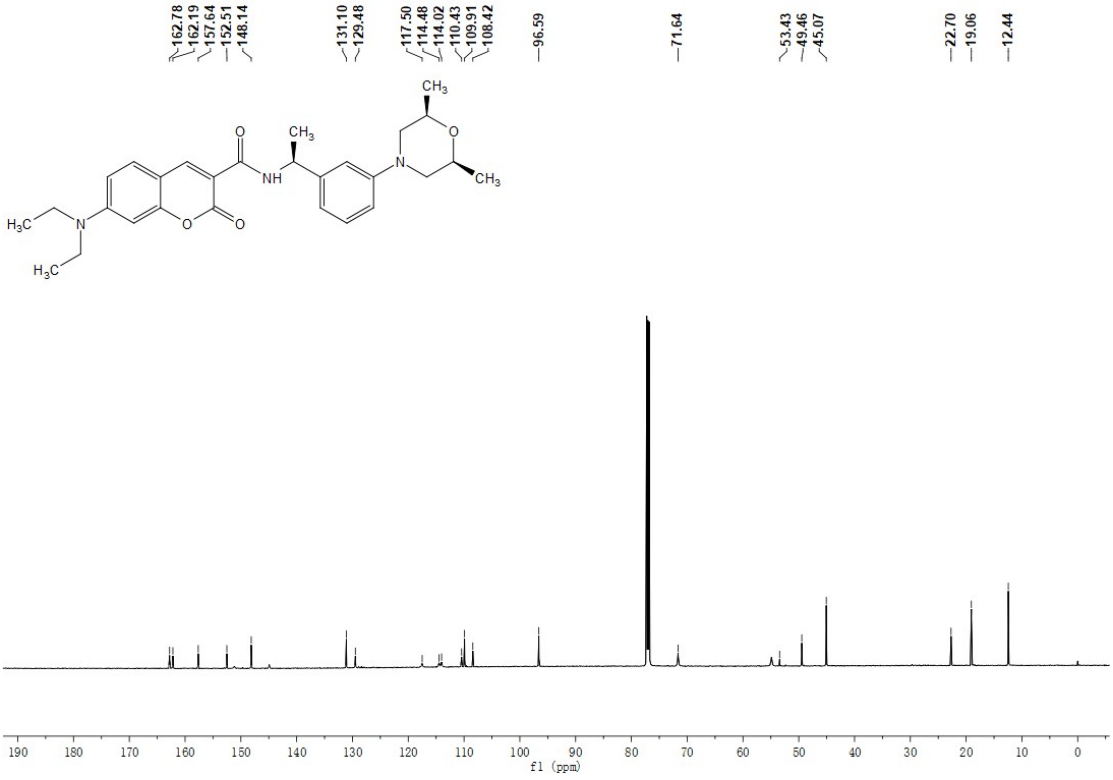


Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
449.231456824	97%	C26H31N3O4	21564	5	450.2387	450.2391	0.8	0.00	0.74	0.74	0.6%	N/A

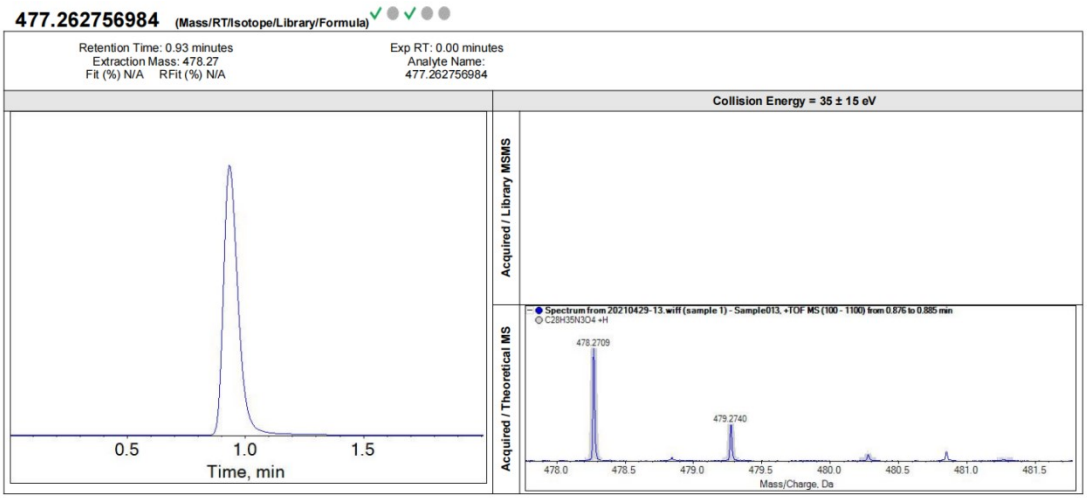
^{1}H NMR spectrum of 21a



^{13}C NMR spectrum of 21a

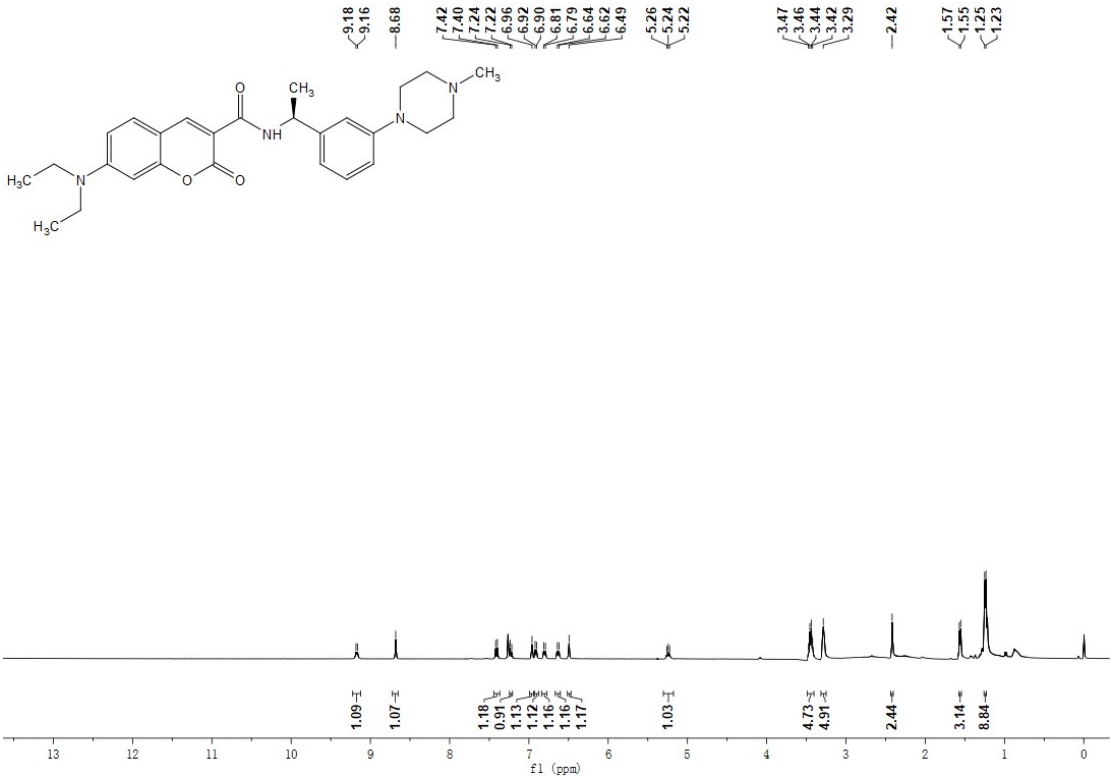


HRMS of 21a

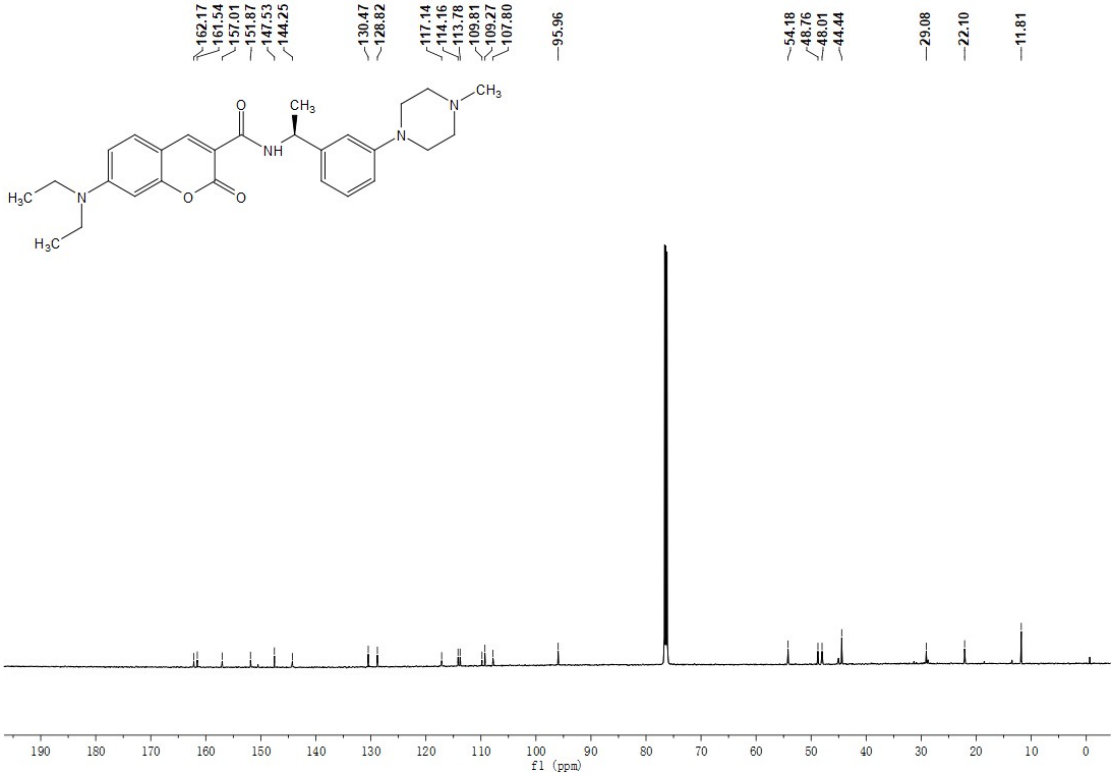


	Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
✓●✓●●	477.262756984	93%	C28H35N3O4	1173433	5	478.2700	478.2709	1.7	0.00	0.93	0.93	0.7%	N/A

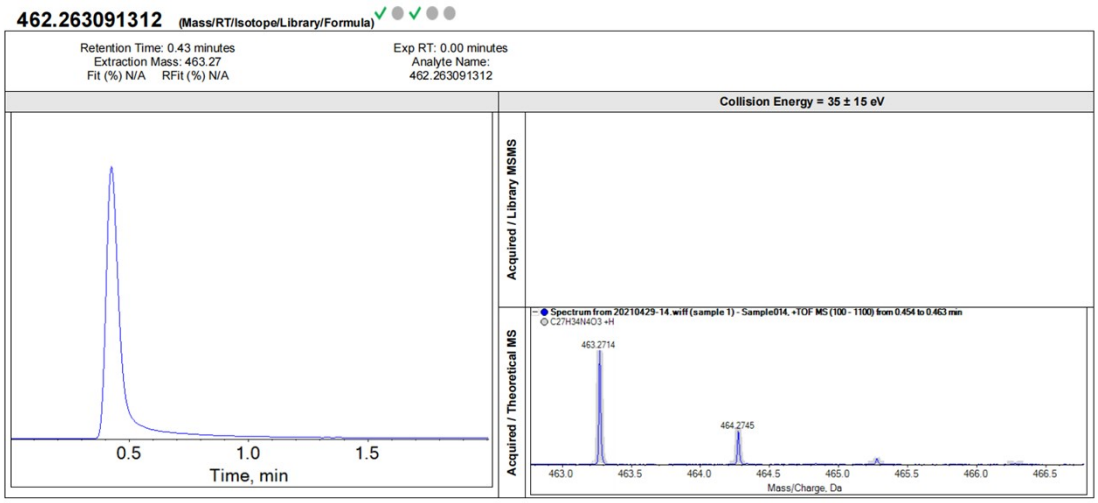
^{1}H NMR spectrum of 21b



^{13}C NMR spectrum of 21b

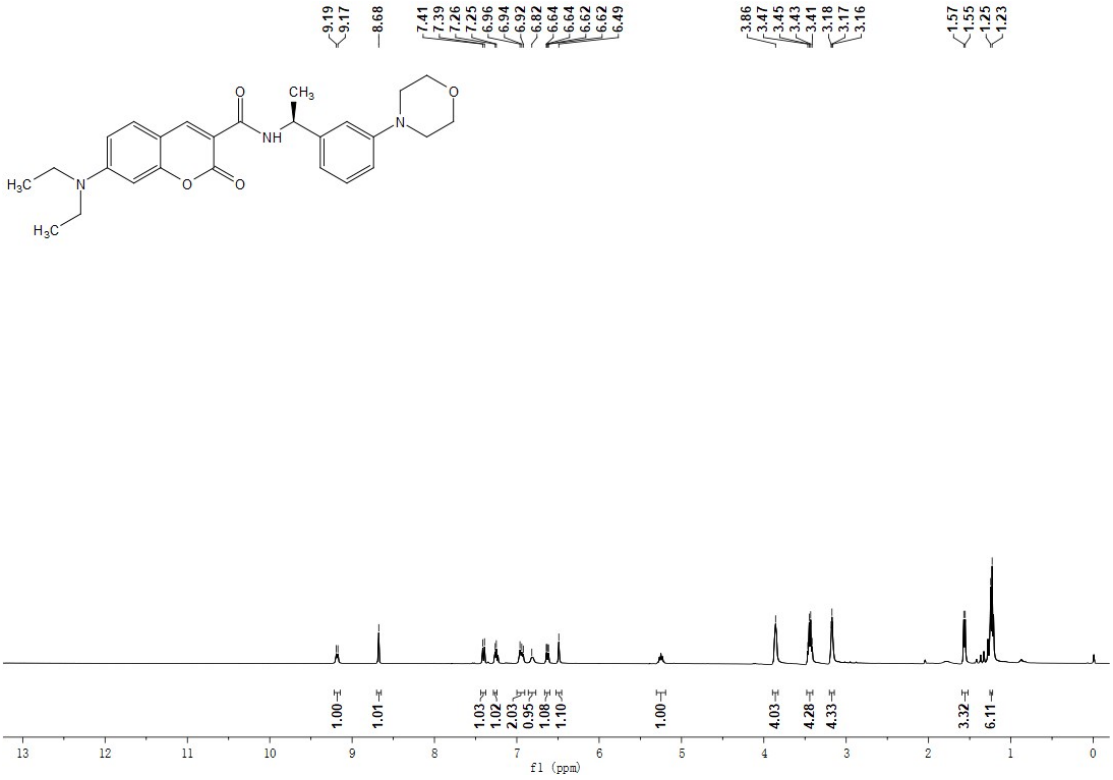


HRMS of 21b

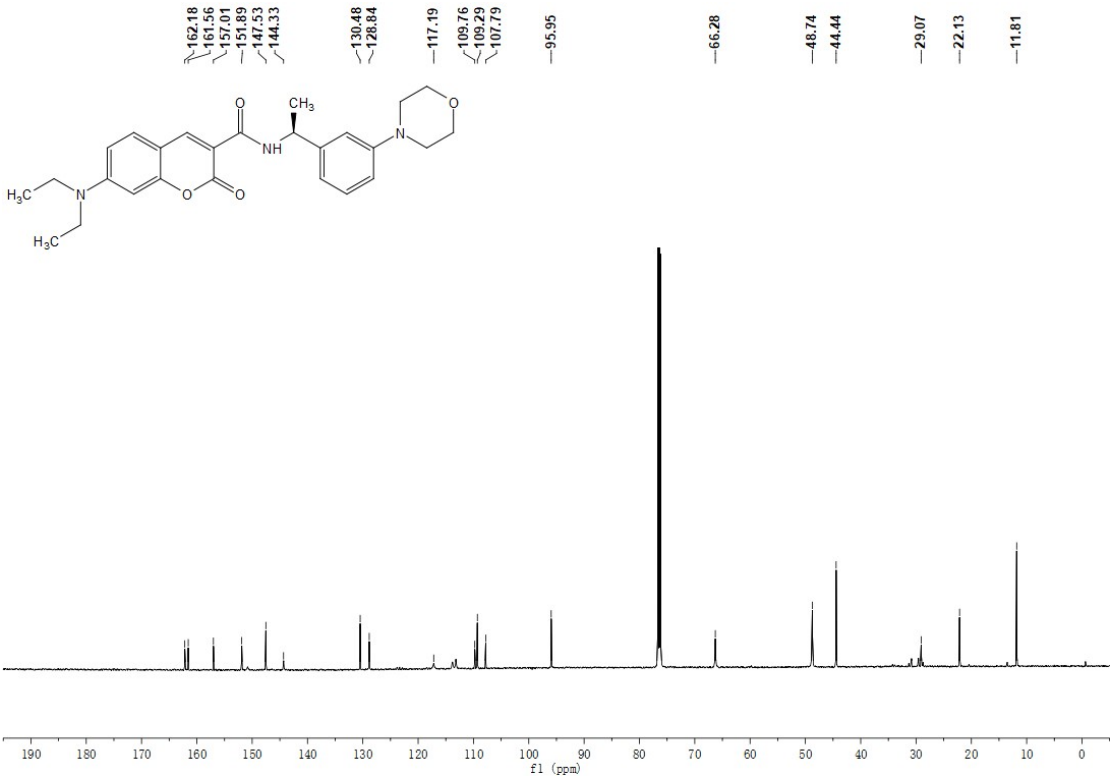


Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
462.263091312	89%	C27H34N4O3	742810	5	463.2704	463.2714	2.3	0.00	0.43	0.43	3.1%	N/A

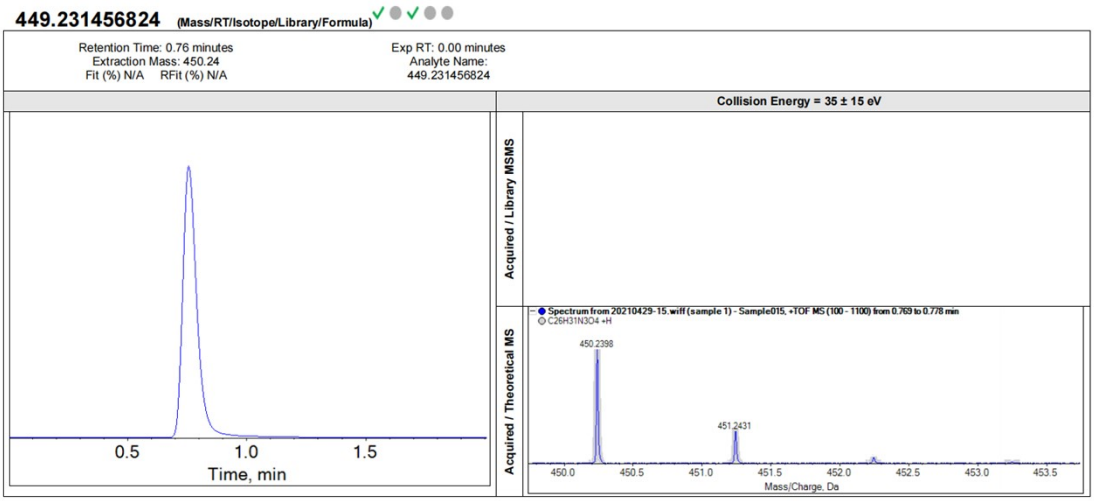
^{1}H NMR spectrum of 21c



^{13}C NMR spectrum of 21c

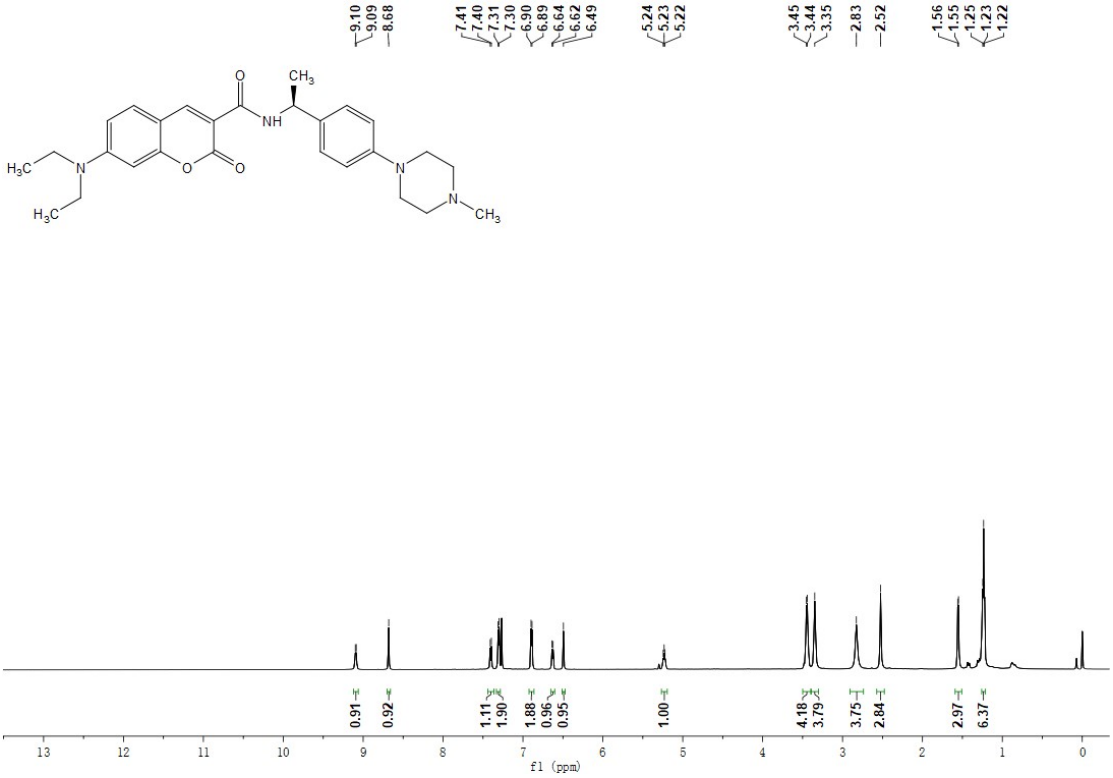


HRMS of 21c

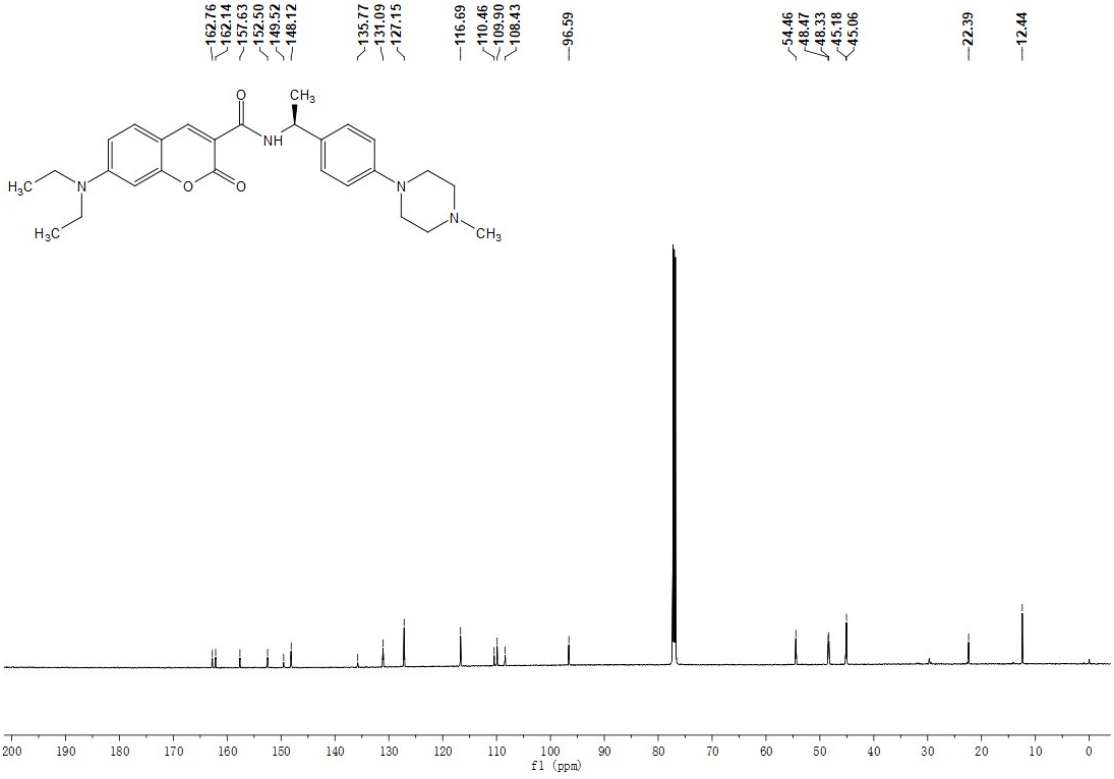


Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
449.231456824	89%	C26H31N3O4	2214948	5	450.2387	450.2398	2.4	0.00	0.76	0.76	2.7%	N/A

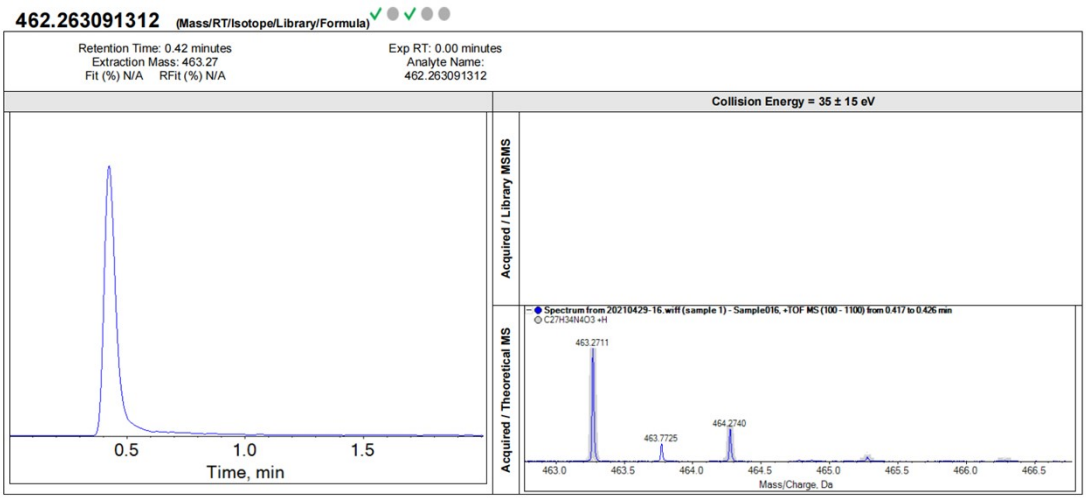
^{1}H NMR spectrum of 24a



^{13}C NMR spectrum of 24a

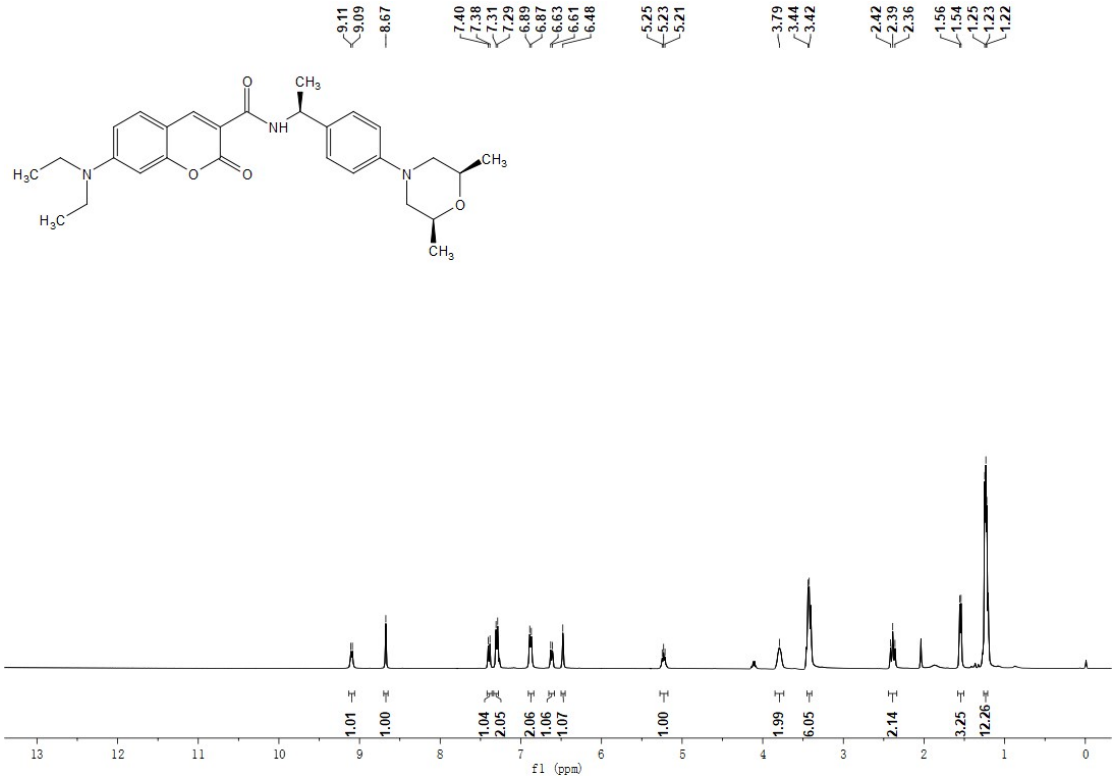


HRMS of 24a

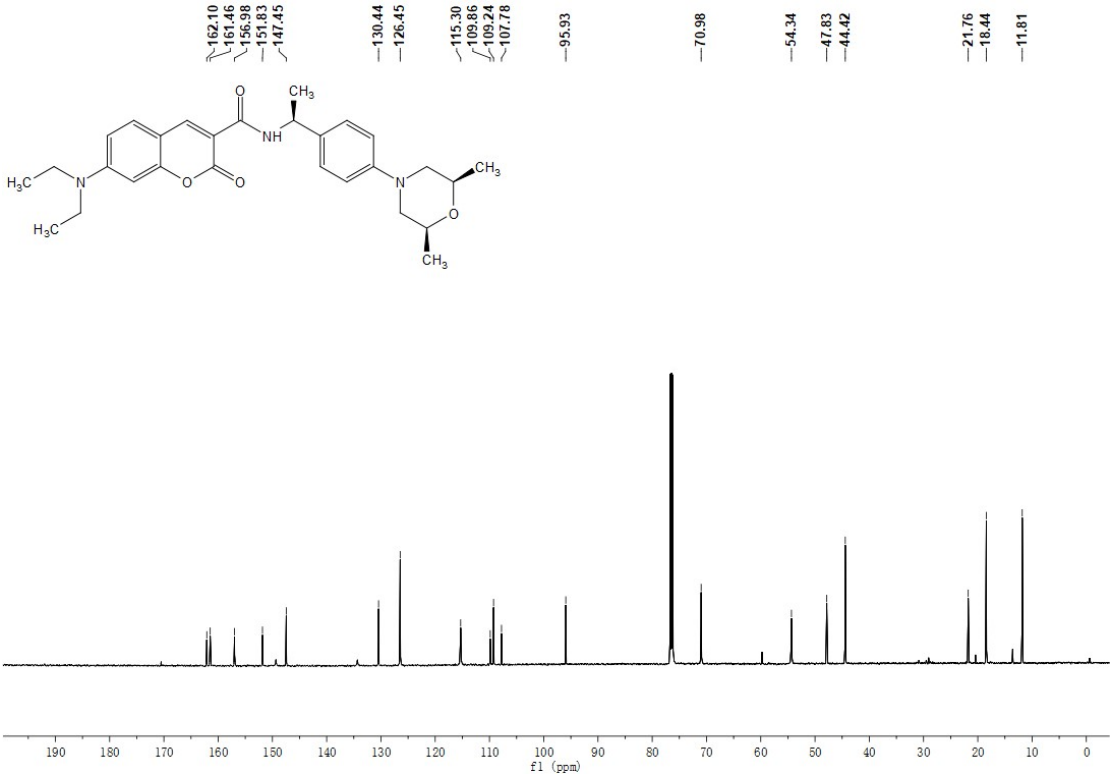


Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
462.263091312	91%	C27H34N4O3	120594	5	463.2704	463.2711	1.5	0.00	0.42	0.42	3.8%	N/A

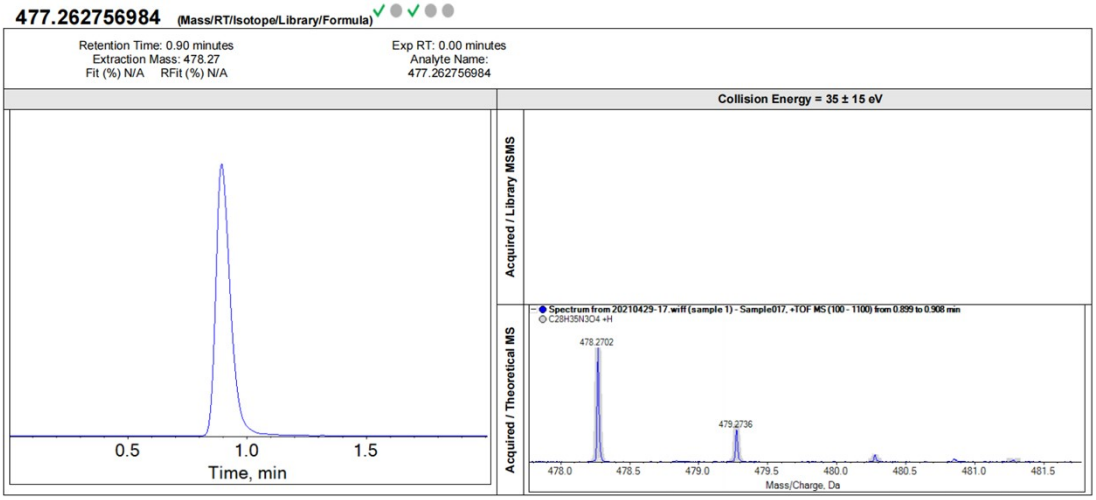
^{1}H NMR spectrum of 24b



^{13}C NMR spectrum of 24b



HRMS of 24b



	Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score (%)
✓●✓●●	477.262756984	94%	C28H35N3O4	142889	5	478.2700	478.2702	0.4	0.00	0.90	0.90	5.0%	N/A

Reference:

1. K. Wang, B. McIlvain, E. Tseng, D. Kowal, F. Jow, R. Shen, H. Zhang, Q. J. Shan, L. He, D. Chen, Q. Lu and J. Dunlop, *Assay Drug Dev Technol*, 2004, **2**, 525-534.
2. F. Jow, E. Tseng, T. Maddox, R. Shen, D. Kowal, J. Dunlop, B. Mekonnen and K. Wang, *Assay Drug Dev Technol*, 2006, **4**, 443-450.
3. Q. Zhou, Y. Shi, H. Qi, H. Liu, N. Wei, Y. Jiang and K. Wang, *FASEB J*, 2020, **34**, 12338-12353.
4. X. Liu, S. Jiang, L. H. Kong, R. R. Ye, L. Xiao, X. J. Xu, Q. He, Y. Y. Wei, Z. X. Li, H. J. Sun, Q. Xie, X. Xu, Y. Lu, Y. J. Wang, W. Li, W. Fu, Z. B. Qiu, J. G. Liu and L. M. Shao, *Acs Chem Neurosci*, 2021, **12**, 1018-1030.
5. L. Xiao, Y. Wang, M. Zhang, W. Wu, L. Kong, Y. Ma, X. Xu, X. Liu, Q. He, Y. Qian, H. Sun, H. Wu, C. Lin, H. Huang, R. Ye, S. Jiang, R. F. Ye, C. Yuan, S. Fang, D. Xue, X. Yang, H. Chen, Y. Zheng, L. Yu, Q. Xie, L. Zheng, W. Fu, W. Li, Z. Qiu, J. Liu and L. Shao, *J Med Chem*, 2019, **62**, 11054-11070.