Support Information

Synthesis and Biological Evaluation of a Novel Sigma-1 Receptor ligands for Treating Neuropathic Pain: 6-hydroxy-2-phenylpyridazinones

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Table 1. Ratio of K_i values without or with phenytoin

	Ratio K_i without / with phenytoin*
(+) SKF 10047	2.82
S1RA	0.65
Compound **54**	0.73

*: The concentration of phenytoin was 1mM

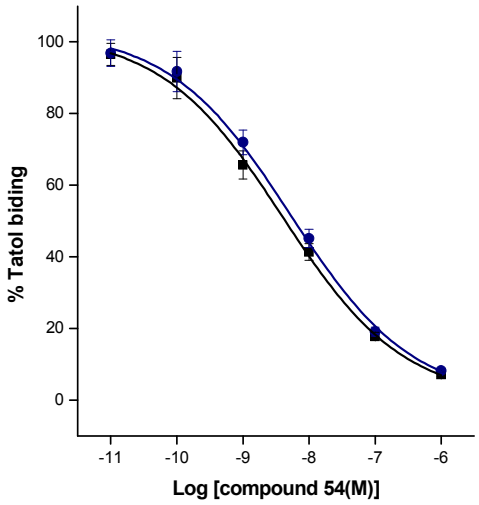


Figure 1. Inhibition of compound **54** with increasing concentrations of ligands (■) and the inhibition in the presence of 1mM phenytoin (●)

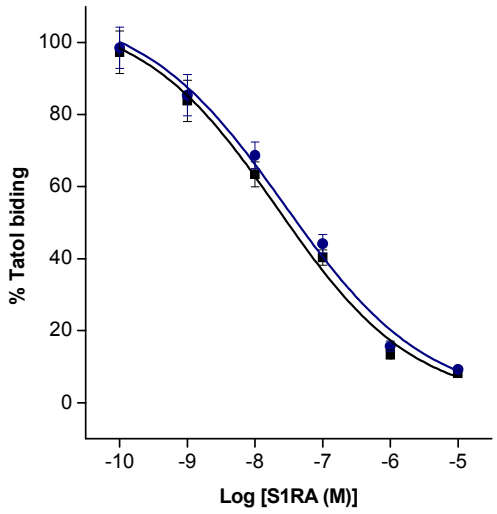


Figure 2. Inhibition of compound **S1RA** with increasing concentrations of ligands (■) and the inhibition in the presence of 1mM phenytoin (●)

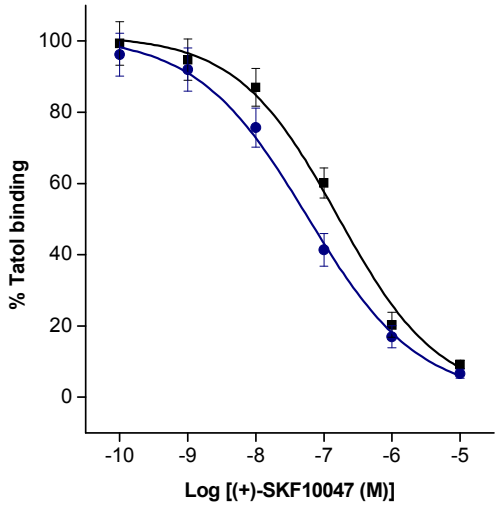


Figure 3. Inhibition of compound (+)-SKF-10047 with increasing concentrations of ligands (■) and the inhibition in the presence of 1mM phenytoin (●)

Selectivity of Compound 54 for Additional Receptors and Ion Channels

Selectivity of compound 54 for additional receptors

In vitro binding assay

General procedures: All of the new compounds were dissolved in 5% DMSO. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Compounds were tested at least three times over 1 μM. The following specific radioligands and tissue sources were used: (1) Serotonin 5-HT_{1A} receptor, [^{3}H]-8-OH-DPAT (187.4 Ci/mmol, Perkin-Elmer), and rat cerebral cortex; (2) Serotonin 5-HT_{2A} receptor, [^{3}H]-ketanserin (60.0 Ci/mmol, Perkin-Elmer), and rat cerebral cortex; (3) Serotonin 5-HT transporter, [^{3}H]-paroxetine (22.9 Ci/mmol; Perkin-Elmer), and rat cerebral cortex; (4) Noradrenaline transporter, [*N*-methyl-^{3}H]-nisoxetine (25.0 Ci/mmol; Perkin-Elmer), and rat hippocampal membrane; (5) Histamine H_1 receptor, [^{3}H]-mepyramine (20.0 Ci/mmol; Perkin-Elmer), and guinea pig cerebellum; (6) Histamine H_3 receptor, [^{3}H]-N-α-methylhistamine (50.0 Ci/mmol; Perkin-Elmer), and rat cerebral cortex; (7) Adrenergic α_1 receptor, [^{3}H]-prazosin (85.4 Ci/mmol; Perkin-Elmer), and rat cerebral cortex; (8) Adrenergic α_2 receptor, [^{3}H]-rauwolscine (73.0 Ci/mmol; Perkin-Elmer), and rat cerebral cortex; (9) NMDA receptor, [^{3}H]-glycine (60.0 Ci/mmol; Perkin-Elmer), and rat hippocampal membrane; (10) μ-opioid receptor, [^{3}H]-diprenorphine (45.0 Ci/mmol; Perkin-Elmer), and human μ-opioid receptor-expressing CHO cells. (11) Cannabinoid CB_1 receptor, [^{3}H]-CP-55940 (148 Ci/mmol; Perkin-Elmer), and human CB_1 receptor-expressing CHO cells. (12) Cannabinoid CB_2 receptor, [^{3}H]-CP-55940 (148 Ci/mmol; Perkin-Elmer), and human CB_2 receptor-expressing HEK cells.

Serotonin 5-HT_{1A} receptor.[1] Rat cerebral cortex was homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.7) using an ULTRA TURAX homogenizer, and was then centrifuged at 32000 g for 10 min. The resulting pellet was then resuspended in the same buffer, incubated for 10 min at 37 °C, and centrifuged at 32000 g for 10 min. The final pellet was resuspended in Tris-HCl buffer containing 10 μM Pargyline, 4 mM $CaCl_2$, and 0.1% ascorbic acid. For total binding, to each

assay tube was added 900 μL of the tissue suspension, 50 μL of 0.5 nM [^{3}H]-8-OH-DPAT, 50 μL of Tris-HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbic acid. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.5 nM [^{3}H]-8-OH-DPAT, and 50 μL of 10 μM serotonin. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.5 nM [^{3}H]-8-OH-DPAT, 50 μL of 1 μM compound. The tubes were incubated at 37 °C for 30 min. The incubation was followed by a rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Serotonin 5-HT$_{2A}$ receptor.[1] Rat cerebral cortex was homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.7) using an ULTRA TURAX homogenizer, and centrifuged at 32000g for 20 min. The resulting pellet was resuspended in the same quantity of the buffer centrifuged for 20 min. The final pellet was resuspended in 50 volumes of the Tris-HCl buffer. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.6 nM [3H]-ketanserin, and 50 μL Tris-HCl buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.6 nM [^{3}H]-ketanserin, and 50 μL of 10 μM methisergide. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.6 nM [^{3}H]-ketanserin, 50 μL of 1 μM compound. The tubes were incubated at 37 °C for 15 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Serotonin 5-HT transporter.[2-3] Rat cerebral cortex was homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.7) containing 150 mM NaCl and 5mM KCl, using an ULTRA TURAX homogenizer, and centrifuged at 20000 g for 20 min. The resulting pellet was resuspended in the same quantity of the buffer centrifuged for 20

min. The final pellet was resuspended in 50 volumes of the Tris-HCl buffer. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-paroxetine, and 50 μL Tris-HCl buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-paroxetine, and 50 μL of 10 μM paroxetine. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.6 nM [^{3}H]-paroxetine, 50 μL of 1 μM compound. The tubes were incubated at 37 °C for 10 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Noradrenaline transporter.[3-4] Rat hippocampal membrane was homogenized in 20 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) using an ULTRA TURAX homogenizer, and centrifuged twice for 10 min at 35,000g with resuspension of the pellet in fresh buffer. The final pellet was resuspended in membrane buffer (20 mM HEPES, 145 mM NaCl, 5 mM KCl, pH 7.4). For total binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM *N*-methyl-[^{3}H]-nisoxetine, and 50 μL of membrane buffer. For nonspecific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM *N*-methyl-[^{3}H]-nisoxetine, and 50 μL of 1 μM desipramine. For specific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM *N*-methyl-[^{3}H]-nisoxetine, 50 μL of 1 μM compound. The tubes were incubated at 30°C for 8h. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Histamine H_1 receptor.[1] Guinea pig cerebellum was homogenized in 20 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) using an ULTRA TURAX homogenizer, and centrifuged twice for 10 min at 50,000g with resuspension of the pellet in fresh buffer. The final pellet was resuspended in phosphate buffer. For total binding, to

each assay tube was added 900 μL of membranes, 50 μL of 1nM [^{3}H]-mepyramine, and 50 μL of phosphate buffer. For nonspecific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM [^{3}H]-mepyramine, and 50 μL of 10 μM promethazine. For specific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM [^{3}H]-mepyramine, 50 μL of 1 μM compound. The tubes were incubated at 30 °C for 60 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Histamine H_3 receptor.[5] Rat cerebral cortex was homogenized in 20 volumes of ice-cold membrane buffer (5 mM Tris-HCl, pH 7.5; 5 mM EDTA and protease inhibitor) using an ULTRA TURAX homogenizer, and centrifuged at 40000g for 30 min at 4 °C. The resulting membrane pellets were washed by resuspension in membrane buffer (50 mM Tris-HCl, pH 7.5; 0.6 mM EDTA, 5 mM $MgCl_2$ and protease inhibitors) and centrifuged for 20 min. The final pellet was resuspended in 50 volumes of the Tris-HCl buffer. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-N-α-methylhistamine, and 50 μL of membrane buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-N-α-methylhistamine, and 50 μL of 10 μM thioperamide. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-N-α-methylhistamine, 50 μL of 1 μM compound. The tubes were incubated at 25 °C for 30 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Adrenergic α_1 receptor.[1] Rat cerebral cortex was homogenized in 20 volumes of ice-cold Tris-HCl buffer containing 5 mM EDTA (50mM, pH 7.7) using an ULTRA TURAX homogenizer and centrifuged at 44000g for 20 min at 4 °C. The resulting

pellet was resuspended in the same quantity of the buffer centrifuged for 20 min. The final pellet was resuspended in 50 volumes of the Tris-HCl buffer. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-prazosin, and 50 μL of Tris-HCl buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-prazosin, and 50 μL of 10 μM prazosin. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-prazosin, 50 μL of 1 μM compound. The tubes were incubated at 25 °C for 60 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Adrenergic α_2 receptor.[1] Rat cerebral cortex was homogenized in 20 volumes of ice-cold Tris-HCl buffer containing 5 mM EDTA (50 mM, pH 7.7) using an ULTRA TURAX homogenizer and centrifuged at 44000g for 20 min at 4 °C. The resulting pellet was resuspended in the same quantity of the buffer centrifuged for 20 min. The final pellet was resuspended in 50 volumes of the Tris-HCl buffer. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-rauwolscine, and 50 μL of Tris-HCl buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-rauwolscine, and 50 μL of 10 μM rauwolscine. For specific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-rauwolscine, 50 μL of 1 μM compound. The tubes were incubated at 25 °C for 60 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

NMDA receptor.[6] Rat hippocampal membrane was homogenized in 20 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) using an ULTRA TURAX homogenizer,

and centrifuged twice for 10 min at 50,000g with resuspension of the pellet in fresh buffer. The final pellet was resuspended in phosphate buffer. For total binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM [^{3}H]-glycine with 10 μM glutamic acid, and 50 μL of phosphate buffer. For nonspecific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM [^{3}H]-glycine with 10 μM glutamic acid, and 50 μL of 1 μM glycine. For specific binding, to each assay tube was added 900 μL of membranes, 50 μL of 1 nM [^{3}H]-glycine with 10 μM glutamic acid, 50 μL of 1 μM compound. The tubes were incubated at 30°C for 30 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

μ-opioid receptor.[7] A suspension of membranes from human *μ*-opioid receptor-expressing CHO cells in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and 10% sucrose was used. For total binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 0.33 nM [^{3}H]-diprenorphine, and 50 μL of Tris-HCl buffer. For nonspecific binding, to each assay tube was added 900 μL of the tissue suspension, 50 μL of 1 nM [^{3}H]-diprenorphine, and 50 μL of 10 μM naloxone. For specific binding, to each assay tube was added 900 μL of the suspension, 50 μL of 0.33 nM [^{3}H]-diprenorphine, 50 μL of 1 μM compound. The tubes were incubated at room temperature for 150 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Cannabinoid CB_1 receptor.[8-9] Human CB1 receptor-expressing CHO cells were scraped in 10ml cold buffer (20mM HEPES, 10mM EDTA, pH 7.5), homogenized in an ULTRA TURAX homogenizer, and centrifuged twice for 10 min at 32,000g with resuspension of the pellet in fresh buffer. For total binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, and 50 μL of buffer

(50mM Tris-HCl, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol 2.5mM EDTA, 5mM $MgCl_2$, 0.05% fatty acid free BSA, pH 7.4). For nonspecific binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, and 50 μL of 10 μM CP-55940. For specific binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, 1 μM compound. The tubes were incubated at 30°C for 90 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Cannabinoid CB_2 receptor.[8-9] Human CB2 receptor-expressing HEK cells were scraped in 10ml cold buffer (20mM HEPES, 10mM EDTA, pH 7.5), homogenized in an ULTRA TURAX homogenizer, and centrifuged twice for 15 min at 30,000g with resuspension of the pellet in fresh buffer. For total binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, and 50 μL of buffer (50mM Tris-HCl, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol 2.5mM EDTA, 5mM $MgCl_2$, 0.05% fatty acid free BSA, pH 7.4). For nonspecific binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, and 50 μL of 10 μM CP-55940. For specific binding, to each assay tube was added 900 μL of membranes, 50 μL of 0.5 nM [^{3}H]-CP-55940, 50 μL of 1 μM compound. The tubes were incubated at 30°C for 90 min. The incubation was followed by rapid vacuum filtration through Whatman GF/B glass filters, and the filtrates were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Scintillation fluid (3.0 mL) was added, and the radioactivity bound was measured using a Beckman LS 6500 liquid scintillation counter.

Selectivity of compound 54 for ion channels

Voltage-gated sodium channel Nav 1.7

The inhibitory activity of compound **54** to voltage-gated sodium channel Nav 1.7 was performed according to Macsari, I and *et al.*[10] by using human voltage-gated sodium channel Nav 1.7 expressing HEK293 cells, with some minor modifications.

Solutions. For Nav 1.7 recording, the extracellular Ringer`s solution is composed of 140mM NaCl, 4mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM D-Glucose monohydrate, 10mM HEPES, pH=7.4 with NaOH. For the DRG neuron measurement, 100mM $CdCl_2$ and 0.5μM TTX were involved to block the Ca^{2+} currents and TTXs-Nav currents. The internal pipette solution contains 145mM CsCl, 0.1mM $CaCl_2$, 2mM $MgCl_2$, 10mM NaCl, 0.5mM Na_2-GTP, 2mM Mg-ATP, 1.1mM EGTA, 10mM HEPES, pH 7.2 with CsOH.

Cell culture. Human voltage-gated sodium channel Nav 1.7 expressing HEK293 cells were cultured in DMEM medium supplemented with 10% FBS and 1.2mg/ml G418 in culture flasks. Cells grew in a humidified incubator at 37°C under 5% carbon dioxide. To maintain electrophysiological performances, cell density must not exceed 80%.

Electrophysiological recordings. To measure voltage-gated sodium channel Nav 1.7, the membrane potential was hold at -90mV, then depolarized to 0mV for 50ms to activate Nav1.7 currents. This voltage-clamp pulse protocol was performed during the experiment. An interpulse interval of 10 seconds allows recovery from inactivation.

The electrophysiological recordings were obtained under visual control under microscope (Olympus IX71, Japan). The amplifier (HEKA EPC10, Germany) was used for the recording of the electrophysiological signal. Offset potentials were nulled directly before formation of a seal. No leak subtraction was made. Cell (in pF) was made from whole-cell capacitance compensation. The data were stored and analyzed with Patchmaster software. All of the experiments were performed at room temperature. Fast perfusion system should be used to apply drugs directly to the cell in seconds. Each concentration was perfused over 5 minutes, or until the current reached a steady- state level. After the final concentration of reference agent tested, the reference compound should be washout out with extracellular solution for 5 minutes. The following criteria were used to determine data acceptability: (1) Access resistance ≤ 10 M\. (2) Membrane resistance ≥ 500 M\. (3) No apparent rundown for peak current.

Within each cellular recording, the current responses to test compound addition

were normalized to the vehicle control (Icompound / Ivehicle control) and the ratios of inhibition were calculated as following equation:

% inhibition = 1 - (current response / maximal control tail current) × 100 %

TRPV-1 protein

The inhibitory activity of compound **54** to TRPV-1 protein was performed according to M. Quintanar-Audelo and *et al*.[11-12] by using Human TRPV-1 protein expressing HEK293 cells, with some minor modifications.

Solutions. For whole cell patch clamp recordings, the external solution is composed of 140mM NaCl, 5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, 5mM glucose, pH 7.4 with NaOH. The internal solution contains 140mM CsCl, 5mM $CaCl_2$, 1mM Mg-ATP, 10mM HEPES, 10mM EGTA, 2mM $MgCl_2$, pH 7.2 with CsOH. The $[Ca^{2+}]_I$ was clamped to 85 nM (calculated with WebMaxC v2.1).

Cell culture. Human TRPV-1 protein expressing HEK293 cells were cultured in DMEM medium supplemented with 10% FBS and 1.2mg/ml G418 in culture flasks. Cells grew in a humidified incubator at 37°C under 5% carbon dioxide. To maintain electrophysiological performances, cell density must not exceed 80%.

Electrophysiological recordings. To measure TRPV-1 current, cells were held at 0 mV and repeated voltage commands stepping to −100 mV for 20 ms followed by a voltage ramp to 100 mV in 100 ms were given at a 1 sec interval.

The electrophysiological recordings were obtained under visual control under microscope (Olympus IX71, Japan). The amplifier (HEKA EPC10, Germany) was used for the recording of the electrophysiological signal. Offset potentials were nulled directly before formation of a seal. No leak subtraction was made. Cell (in pF) was made from whole-cell capacitance compensation. The data were stored and analyzed with Patchmaster software. All of the experiments were performed at room temperature. Fast perfusion system should be used to apply drugs directly to the cell in seconds. Each concentration was perfused over 5 minutes, or until the current reached a steady- state level. After the final concentration of reference agent tested, the reference compound should be washout out with extracellular solution for 5 minutes. The following criteria were used to determine data acceptability: (1) Access

resistance ≤ 10 M\. (2) Membrane resistance ≥ 500 M\. (3) No apparent rundown for peak current.

Within each cellular recording, the current responses to test compound addition were normalized to the vehicle control (Icompound / Ivehicle control) and the ratios of inhibition were calculated as following equation:

% inhibition = 1 - (current response / maximal control tail current) × 100 %

S 20

Table 2. Binding affinities for the additional receptors and ion channels of compound **54**

Receptor / Ion Channel	% Inhibition*
5-HT_{1A}	30.81± 4.1
5-HT_{2A}	51.47± 3.8
5-HT transporter	45.34± 0.4
Noradrenaline transporter	31.89± 0.4
H_1	11.91± 1.4
H_3	35.65± 3.2
α_1	15.28± 1.2
α_2	10.56± 0.9
NMDA	27.68± 2.1
μ-opioid	25.29± 2.4
CB_1	19.28± 2.4
CB_2	38.56± 1.9
$Nav_{1.7}$	24.56± 1.2
TRPV-1	23.34 ± 1.9

Note: *, % inhibition was determined at the concentration of 1 μM.

The data of compound 54 in Phase I (0-15 min) of the mice formalin test

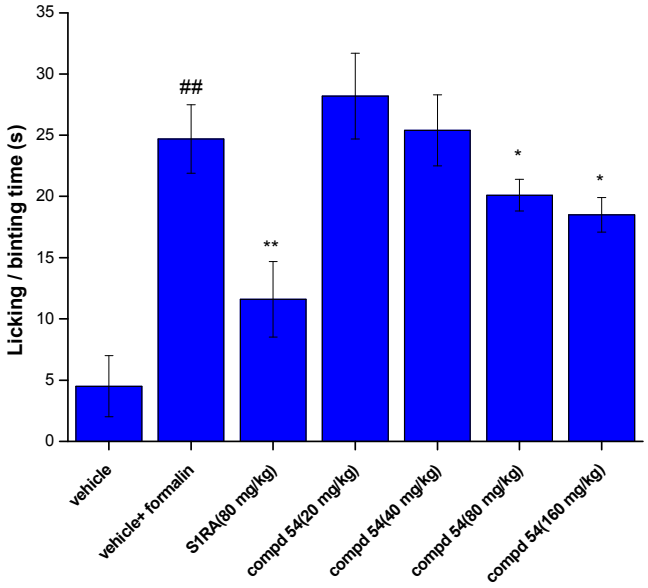
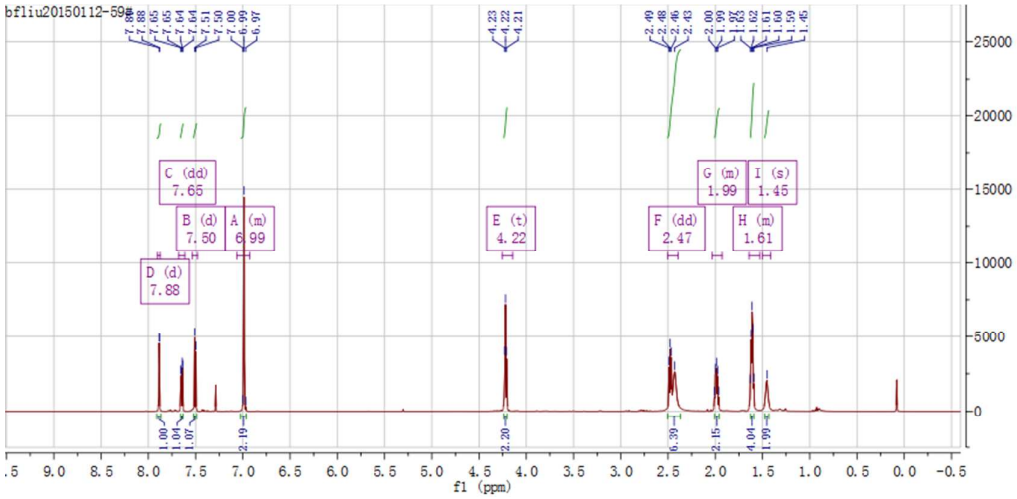
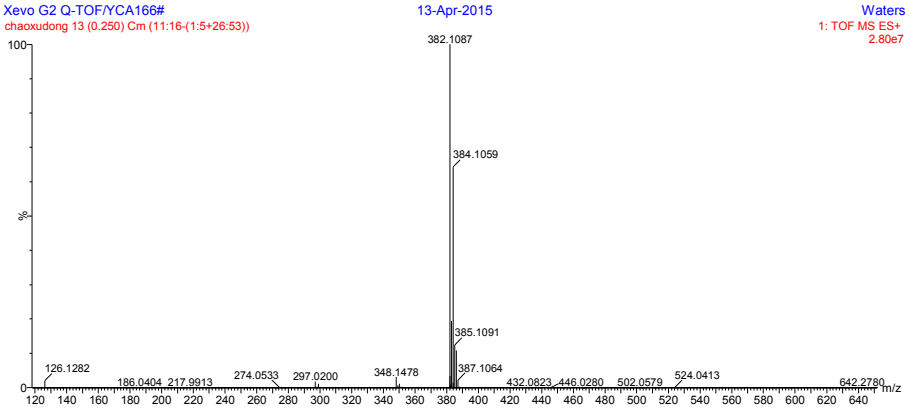


Figure 4. Anti-allodynic effect of S1RA and compound **54** in phase I (0−15 min) of the mice formalin test. Each column and vertical line represents mean ±SEM of the values obtained in at least 10 animals. Statistically significant differences: $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs* vehicle; * $p < 0.05$, ** $p < 0.01$ *vs* vehicle +formalin (Two-Way ANOVA followed by Newman−Keuls test)

^{1}H NMR of compound 54



HRMS analysis of compounds 54



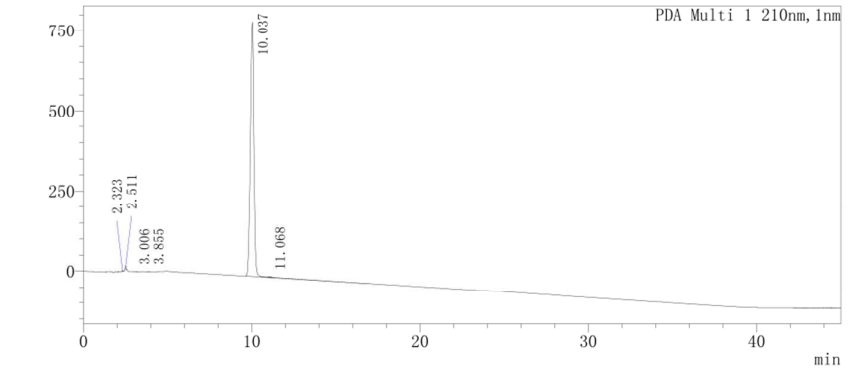
HPLC analysis of compounds 54

SHIMADZU LabSolutions 分析报告

〈样品信息〉

样品名	: 新药曹旭东s201401201-21_约1mg每ml		
样品ID	:		
数据文件名	: 新药曹旭东s201401201-1_约1mg每ml（中性稀释剂）		
方法文件名	: s201401201-1方法.lcm		
批处理文件名	: 20150212日曹旭东s20141201-1纯度检查.lcb		
样品瓶号	: 1-95	样品类型	: 未知
进样体积	: 5 uL		
分析日期	: 2015/2/12 14:24:43	分析者	: wgh
处理日期	: 2015/2/13 14:50:30	处理者	: wgh

〈色谱图〉



〈峰表〉

PDA Ch1 210nm

峰号	保留时间	面积	面积%	高度	浓度	化合物名
1	2.323	12033	0.102	2344	0.102	
2	2.511	40626	0.346	9430	0.346	
3	3.006	2883	0.025	266	0.025	
4	3.855	4583	0.039	378	0.039	
5	10.037	11669750	99.316	783696	99.316	
6	11.068	20193	0.172	1620	0.172	
总计		11750067	100.000	797734		

X-Ray Crystal Data of Compound 54

The single-crystal growth was carried out in co-solvent of methanol and acetone at room temperature.

Table 3 Crystal data and structure refinement for SF20150715_ZYC_0m.

Identification code	sf20150715_zyc_0m
Empirical formula	C18 H26 Cl3 N3 O4
Formula weight	454.77
Temperature	296 K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P -1
Unit cell dimensions	a = 8.7813(3) Å □= 97.6772(14)°.
	b = 10.4026(3) Å □= 91.8626(13)°.
	c = 12.2094(4) Å □ = 05.0210(12)°.
Volume	1064.92(6) $Å^3$
Z	2
Density (calculated)	1.418 Mg/m^3
Absorption coefficient	0.459 mm^{-1}
F(000)	476
Crystal size	0.39 x 0.32 x 0.3 mm^3
Theta range for data collection	1.687 to 27.519°.
Index ranges	-11<=h<=11, -13<=k<=13, -15<=l<=15
Reflections collected	17671
Independent reflections	4873 [R(int) = 0.0167]
Completeness to theta = 26.000°	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7456 and 0.1631
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4873 / 0 / 259
Goodness-of-fit on F^2	1.028
Final R indices [I>2sigma(I)]	R1 = 0.0364, wR2 = 0.1002

R indices (all data)	R1 = 0.0427, wR2 = 0.1063
Extinction coefficient	n/a
Largest diff. peak and hole	0.337 and -0.289 e.Å^{-3}

Table 4 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for SF20150715_ZYC_0m. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
Cl(1)	10352(1)	13549(1)	3906(1)	66(1)
Cl(2)	10824(1)	12834(1)	6299(1)	67(1)
O(1)	7142(2)	8759(1)	7020(1)	58(1)
O(2)	3147(1)	6056(1)	3607(1)	51(1)
N(1)	6205(2)	8556(1)	5206(1)	38(1)
N(2)	5174(2)	7851(1)	4320(1)	42(1)
N(3)	2260(1)	3538(1)	374(1)	35(1)
C(1)	6196(2)	8137(2)	6238(1)	42(1)
C(2)	4977(2)	6925(2)	6341(1)	48(1)
C(3)	3982(2)	6242(2)	5490(1)	48(1)
C(4)	4148(2)	6763(2)	4466(1)	42(1)
C(5)	3232(2)	6637(2)	2592(1)	50(1)
C(6)	2209(2)	5588(2)	1702(1)	45(1)
C(7)	2869(2)	4377(2)	1471(1)	44(1)
C(8)	552(2)	2803(2)	340(1)	47(1)
C(9)	2(2)	2027(2)	-810(2)	56(1)
C(10)	986(2)	1062(2)	-1175(2)	57(1)
C(11)	2713(2)	1821(2)	-1099(2)	54(1)
C(12)	3246(2)	2585(2)	47(2)	52(1)
C(13)	7243(2)	9772(1)	4925(1)	37(1)
C(14)	7018(2)	10111(2)	3874(1)	46(1)
C(15)	7990(2)	11259(2)	3570(1)	51(1)
C(16)	9173(2)	12104(2)	4301(1)	44(1)
C(17)	9383(2)	11780(2)	5345(1)	43(1)
C(18)	8441(2)	10613(2)	5666(1)	44(1)
O(3)	3248(2)	-1468(2)	-781(2)	73(1)
O(4)	4179(2)	-316(2)	1428(1)	77(1)
Cl(3)	-2721(1)	4590(1)	1410(1)	51(1)

Table 5 Bond lengths [Å] and angles [°] for SF20150715_ZYC_0m.

Cl(1)-C(16)	1.7269(16)
Cl(2)-C(17)	1.7330(15)
O(1)-C(1)	1.2346(19)
O(2)-C(4)	1.3462(18)
O(2)-C(5)	1.4454(19)
N(1)-N(2)	1.3805(16)
N(1)-C(1)	1.3879(19)
N(1)-C(13)	1.4442(18)
N(2)-C(4)	1.2883(19)
N(3)-H(3)	0.9800
N(3)-C(7)	1.4972(19)
N(3)-C(8)	1.4930(18)
N(3)-C(12)	1.5006(19)
C(1)-C(2)	1.451(2)
C(2)-H(2)	0.9300
C(2)-C(3)	1.330(2)
C(3)-H(3A)	0.9300
C(3)-C(4)	1.425(2)
C(5)-H(5A)	0.9700
C(5)-H(5B)	0.9700
C(5)-C(6)	1.513(2)
C(6)-H(6A)	0.9700
C(6)-H(6B)	0.9700
C(6)-C(7)	1.515(2)
C(7)-H(7A)	0.9700
C(7)-H(7B)	0.9700
C(8)-H(8A)	0.9700
C(8)-H(8B)	0.9700
C(8)-C(9)	1.519(2)
C(9)-H(9A)	0.9700
C(9)-H(9B)	0.9700
C(9)-C(10)	1.519(3)
C(10)-H(10A)	0.9700
C(10)-H(10B)	0.9700
C(10)-C(11)	1.511(3)
C(11)-H(11A)	0.9700

C(11)-H(11B)	0.9700
C(11)-C(12)	1.509(2)
C(12)-H(12A)	0.9700
C(12)-H(12B)	0.9700
C(13)-C(14)	1.397(2)
C(13)-C(18)	1.393(2)
C(14)-H(14)	0.9300
C(14)-C(15)	1.379(2)
C(15)-H(15)	0.9300
C(15)-C(16)	1.380(2)
C(16)-C(17)	1.380(2)
C(17)-C(18)	1.393(2)
C(18)-H(18)	0.9300
O(3)-H(3B)	0.8500
O(3)-H(3C)	0.8500
O(4)-H(4A)	0.8500
O(4)-H(4B)	0.8501

C(4)-O(2)-C(5)	116.61(12)
N(2)-N(1)-C(1)	122.96(12)
N(2)-N(1)-C(13)	111.85(11)
C(1)-N(1)-C(13)	125.18(12)
C(4)-N(2)-N(1)	118.36(12)
C(7)-N(3)-H(3)	107.2
C(7)-N(3)-C(12)	110.91(12)
C(8)-N(3)-H(3)	107.2
C(8)-N(3)-C(7)	112.95(12)
C(8)-N(3)-C(12)	110.97(12)
C(12)-N(3)-H(3)	107.2
O(1)-C(1)-N(1)	122.59(15)
O(1)-C(1)-C(2)	122.23(14)
N(1)-C(1)-C(2)	115.17(13)
C(1)-C(2)-H(2)	119.0
C(3)-C(2)-C(1)	122.02(15)
C(3)-C(2)-H(2)	119.0
C(2)-C(3)-H(3A)	121.5
C(2)-C(3)-C(4)	117.05(15)
C(4)-C(3)-H(3A)	121.5

O(2)-C(4)-C(3)	116.56(13)
N(2)-C(4)-O(2)	119.05(13)
N(2)-C(4)-C(3)	124.39(14)
O(2)-C(5)-H(5A)	110.2
O(2)-C(5)-H(5B)	110.2
O(2)-C(5)-C(6)	107.44(13)
H(5A)-C(5)-H(5B)	108.5
C(6)-C(5)-H(5A)	110.2
C(6)-C(5)-H(5B)	110.2
C(5)-C(6)-H(6A)	109.5
C(5)-C(6)-H(6B)	109.5
C(5)-C(6)-C(7)	110.94(14)
H(6A)-C(6)-H(6B)	108.0
C(7)-C(6)-H(6A)	109.5
C(7)-C(6)-H(6B)	109.5
N(3)-C(7)-C(6)	112.98(12)
N(3)-C(7)-H(7A)	109.0
N(3)-C(7)-H(7B)	109.0
C(6)-C(7)-H(7A)	109.0
C(6)-C(7)-H(7B)	109.0
H(7A)-C(7)-H(7B)	107.8
N(3)-C(8)-H(8A)	109.7
N(3)-C(8)-H(8B)	109.7
N(3)-C(8)-C(9)	109.93(13)
H(8A)-C(8)-H(8B)	108.2
C(9)-C(8)-H(8A)	109.7
C(9)-C(8)-H(8B)	109.7
C(8)-C(9)-H(9A)	109.2
C(8)-C(9)-H(9B)	109.2
H(9A)-C(9)-H(9B)	107.9
C(10)-C(9)-C(8)	112.12(16)
C(10)-C(9)-H(9A)	109.2
C(10)-C(9)-H(9B)	109.2
C(9)-C(10)-H(10A)	109.7
C(9)-C(10)-H(10B)	109.7
H(10A)-C(10)-H(10B)	108.2
C(11)-C(10)-C(9)	109.69(14)
C(11)-C(10)-H(10A)	109.7

C(11)-C(10)-H(10B)	109.7
C(10)-C(11)-H(11A)	109.4
C(10)-C(11)-H(11B)	109.4
H(11A)-C(11)-H(11B)	108.0
C(12)-C(11)-C(10)	111.35(15)
C(12)-C(11)-H(11A)	109.4
C(12)-C(11)-H(11B)	109.4
N(3)-C(12)-C(11)	110.82(13)
N(3)-C(12)-H(12A)	109.5
N(3)-C(12)-H(12B)	109.5
C(11)-C(12)-H(12A)	109.5
C(11)-C(12)-H(12B)	109.5
H(12A)-C(12)-H(12B)	108.1
C(14)-C(13)-N(1)	118.28(13)
C(18)-C(13)-N(1)	122.34(13)
C(18)-C(13)-C(14)	119.37(14)
C(13)-C(14)-H(14)	119.9
C(15)-C(14)-C(13)	120.15(14)
C(15)-C(14)-H(14)	119.9
C(14)-C(15)-H(15)	119.5
C(14)-C(15)-C(16)	120.92(15)
C(16)-C(15)-H(15)	119.5
C(15)-C(16)-Cl(1)	119.92(13)
C(17)-C(16)-Cl(1)	121.07(12)
C(17)-C(16)-C(15)	119.00(15)
C(16)-C(17)-Cl(2)	120.45(12)
C(16)-C(17)-C(18)	121.30(14)
C(18)-C(17)-Cl(2)	118.24(12)
C(13)-C(18)-H(18)	120.4
C(17)-C(18)-C(13)	119.23(14)
C(17)-C(18)-H(18)	120.4
H(3B)-O(3)-H(3C)	109.5
H(4A)-O(4)-H(4B)	109.5

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