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Synthesis and evaluation of 6-pyrazoylamido-3*N*-substituted azabicyclo[3,1,0]hexane derivatives as T-type calcium channel inhibitors for treatment of neuropathic pain

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ABSTRACT

A new series of aryls, including benzo[*d*]imidazole/isoxazole/pyrazole, conjugated to 3*N*-substituted-azabicyclo[3.1.0]hexane derivatives were designed and synthesized as inhibitors of T-type calcium channels. Among the synthesized compounds, 3*N*-*R*-substituted azabicyclo[3.1.0]hexane carboxamide derivatives containing 5-isobutyl-1-phenyl-pyrazole ring exhibited potent and selective T-channel inhibition and good metabolic stability without CYP450 inhibition. Compounds **10d** and **10e** contained hydrophobic substituents at the 3*N*-position and exhibited potent in vitro efficacy, as well as neuropathic pain alleviation in rats.

1. Introduction

Chronic pain is common among people suffering from AIDS, cancer neuropathies, diabetic neuropathy, and complex regional pain syndrome. Neuropathic pain is sensed from the spontaneous and hypersensitive responses that persist after an original nerve injury has healed. The treatment failure for neuropathic pain is high, with only ~30–50% treated adequately.[1] Thus, the development of efficient and safe neuropathic pain drugs is necessary.

T-type calcium channels (T-channels) have been identified as novel targets for the development of medications that treat chronic and neuropathic pain, epilepsy, and Parkinson's disease (PD).[2] T-channels are low-voltage, gated ion channels that belong to one of three subtypes, including $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}), and $Ca_v3.3$ (α_{1I}), depending on their amino acid sequence.[3] Although the mechanisms leading to nociception are not well understood, in vivo and in vitro studies have identified a key role for T-channels in sensory transmission and pain perception.[4] Moreover, murine studies involving $Ca_v3.1$ and $Ca_v3.2$ KO mice[5,6] have indicated that T-channel inhibitors are efficacious in controlling absence epilepsy and neuropathic pain. The T-channel blocker, Mibefradil, was developed by Roche but withdrawn due to complications from drug-drug interactions. Recently, a centrally active, potent, and specific T-channel blocker, **ML218** (α_{1H}, IC_{50} = 310 nM in patch clamp assays) was discovered through a scaffold hopping approach.[7] Researchers at Merck also reported the lead compound **TTA-P2** to have potent anti-hyperalgesic activity in an animal model of neuropathic pain (Fig. 1).[8]

Previously, we reported on a potent T-channel inhibitor with anti-nociceptive effects in a neuropathic pain model.[9] To develop T-channel inhibitors with low hERG and CYP450 inhibition, we designed and synthesized 6-aryl-connected-3-*N*-substituted azabicyclo[3.1.0]hexane derivatives. The resulting compounds were evaluated for their in vitro T-channel inhibitory activities, as well as their ability to alleviate neuropathic pain in an in vivo spinal nerve ligation (SNL) rat model.

The structure of **I** was designed in this study based on the chemical structure of mibefradil and **ML218**. Compound **I** is a 3*N*-substituted azabicyclo[3,1,0]hexane conjugated to an amide linker to aromatic heterocycles, including benzimidazole, isoxazole, or pyrazole (Fig. 2).

2. Results and discussions

2.1. Chemistry

Synthesis of the azabicyclo[3.1.0]hexane 6-carbamoyl derivatives (**7a** and **7b**) containing 3-(1*H*-benzo[*d*]imidazol-2-yl)-*N*-methylpropan-1-amine is outlined in Scheme 1. A protection

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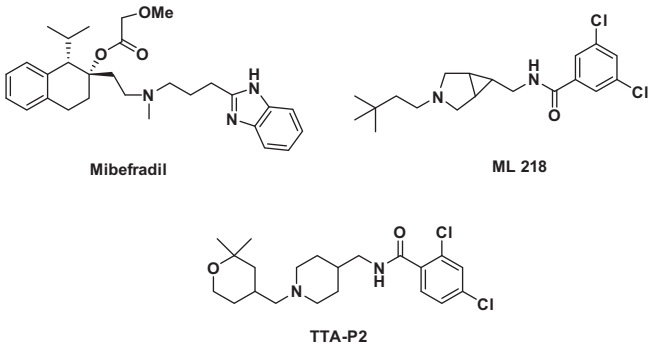


Figure 1. Structure of T-type calcium channel inhibitors.

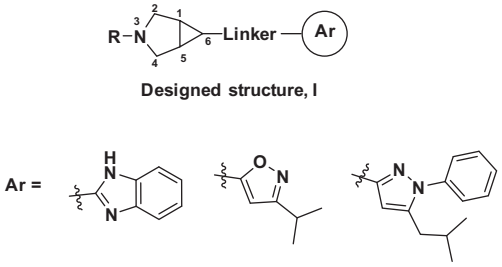


Figure 2. Structure of 3*N*-*R*-substitute azabicyclo[3.1.0]hexanes containing 6-aromatic heterocycles.

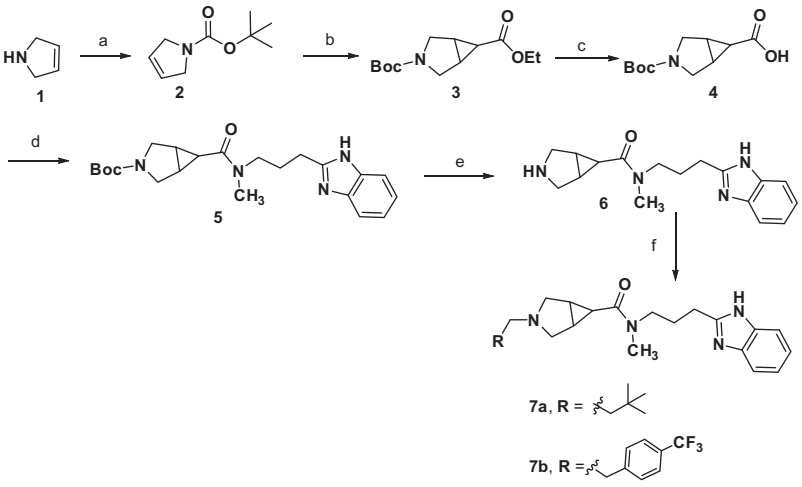
reaction of 3-pyrollidine **1** was performed using t-butoxycarbonyl (Boc) anhydride, followed by traction with ethyl diazoacetaten in the presence of $[Ru(OAc)_2]_2$ to generate a 43:22 mixture of exo-**3** and endo-**3** azabicyclo[3,1,0]hexane carboxylate. The exo-**3** and endo-**3** compounds were separated using column chromatography.[10] The stereochemistry of the exo-isomer was determined by NMR spectroscopy and compared with the results of previous studies.[11] Hydrolysis of exo-**3** with 2 N NaOH in ethanol generated the exo-6-carboxylic acid derivative **4**. Following this, compound **4** was coupled with (1*H*-benzo[*d*]imidazol-2-yl)-*N*-methylpropan-1-amine in the presence of the hydroxybenzotriazole (HOBT) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) under nitrogen to give compound **5**. Compound **6** was then generated by deprotection of the Boc group of compound **4** with trifluoro acetic acid. The reductive alkylation of **6** with corresponding aldehydes and sodium triacetoxyborohydride generated the 3*N*-substitited azabicyclo[3,1,0]hexane carbamoyl alkyl, which was linked to 1*H*-benzo[*d*]imidazole analogues to generate compounds **7a** and **7b**.

The 3-substituted azabicyclo[3,1,0]hexane carbamoyl derivatives (**10a**–**f**) containing the 5-isobutyl-1-phenyl-pyrazole moiety were synthesized according to Scheme 2. An amide coupling reaction of azabicyclo[3,1,0]hexane 6-carboxlyic acid **4** was performed with 5-isobutyl-1-phenyl-pyrazole-3-methylamine to yield compound **8**. The Boc group of compound **8** was subsequently deprotected with 1.25 M hydrochloric acid in methanol to yield compound **9**. Multiple aldehydes, including 3,3-dimethylpropyl aldehyde, benzaldehyde, and p-trifluoromethylphenylethyl aldehyde, were incubated with derivative **9** to generate analogues with (3,3-dimethyl)pentyl **10a**, benzyl **10b**, and *p*-trifluoromethyl phenylethyl **10c** at the 3*N* position of the azabicyclo[3,1,0]hexane-6-cabamoyl linked 5-isobutyl-1-phenyl-pyrazole motif. Acylation of **9** with benzoyl chloride and 2-phenyacetyl chloride yielded **10c** and **10d**. The phenylsulfone derivative **10f** was synthesized by reacting **9** with benzenesulfonyl chloride.

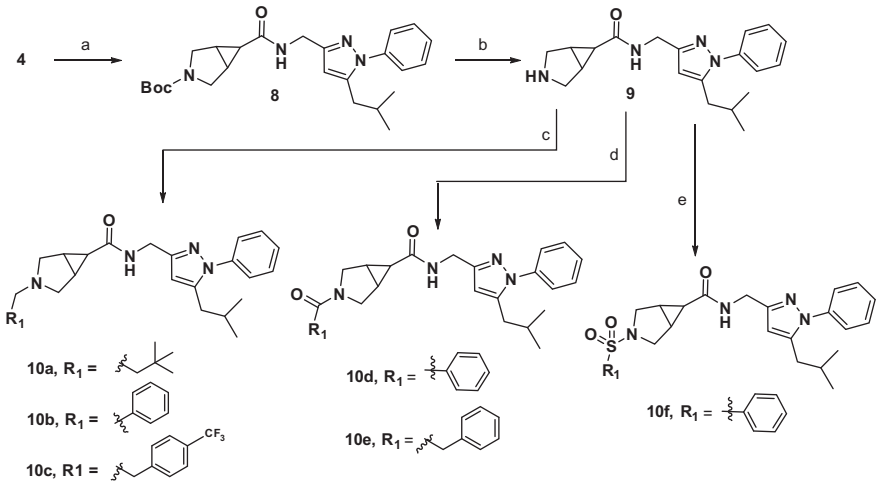
The arylcarbamoyl derivatives were synthesized according to Scheme 3. The azabicyclo[3,1,0]hexane-6-methyl amine **14** intermediate that contained a 3,3-dimethyl butyl at the 3*N*-position of the bicyclic ring was prepared from the reduction of **13**. Compound **4** was converted to compound **12** via amination with 2.0 M ammonia in methanol, followed by Boc deprotection. Compound **12** underwent subsequent reductive alkylation to generate compound **13**. Amide coupling of the bicyclic methylamine derivative **14** with 5-isobutyl-1-phenyl-pyrazol-3-carboxylic acid and 5-iso-propyl-isooxazol-3-carboxlyic acid yielded **15a** and **15b**.

2.2. Biological evaluation

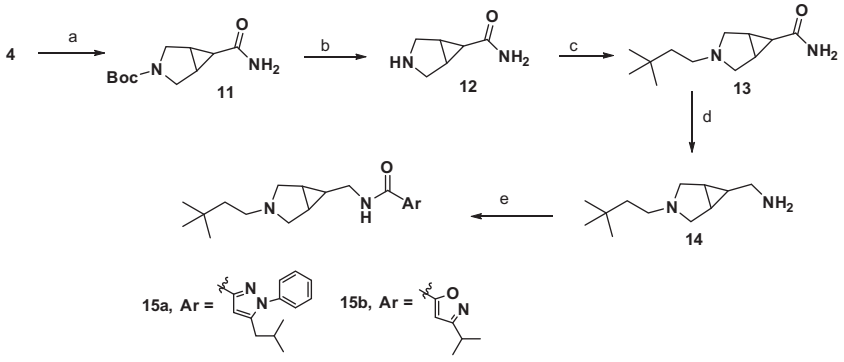
All of the 3*N*-substituted-azabicyclo[3.1.0]hexane derivatives containing 1*H*-benzo[*d*]imidazole (**7a**–**b**), 5-isobutyl-1-phenyl-pyrazole (**10a**–**f**, **15a**), or 5-iso-propyl-isooxazol (**15b**) at the 6-position of the core skeleton were evaluated for their T-channel (α_{1G}, α_{1H}) inhibitory activity using an FDSS6000 fluorescence-based HTS assay on HEK293 cells.[12] The results are summarized in Table 1.



Scheme 1. Regents and reaction conditions. (a) $(Boc)_2O$, CH_2Cl_2; (b) ethyl diazoate, $[Rh(OAc)_2]_2$, CH_2Cl_2; (c) 2*N* NaOH, EtOH; (d) 3-(1*H*-benzo[*d*]imidazol-2-yl)-*N*-methylpropan-1-amine, HOBt, EDCI; (e) TFA, CH_2Cl_2; (f) RCHO, $NaBH(OAc)_3$, CH_2Cl_2.



Scheme 2. Reagents and reaction conditions. (a) (5-Isobutyl-1-phenyl-1*H*-pyrazol-3-yl)methanamine, HOBt, EDCI; (b) 1.25 M HCl (MeOH); (c) R_1CHO, $NaBH(OAc)_3$, CH_2Cl_2; (d) RCOCl, TEA, CH_2Cl_2; and (e) R_1SO_2Cl, TEA, CH_2Cl_2.



Scheme 3. Reagents and reaction conditions. (a) 2.0 M NH_3 in MeOH, HOBt, EDCI, DIPEA, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) K_2CO_3, 1-chloro-3,3-dimethylbutane, DMF, CH_3CN, reflux, 12 h; (d) LAH (ether), CH_2Cl_2, reflux; and (e) $ArCO_2H$, HOBt, EDCI.

All compounds exhibited similar levels of α_{1G} and α_{1H} T-channel inhibition, in vitro. Based on those data, we investigated the structure–activity relationship (SAR) of the 6-aryl-connected-3-*R*-substituted-azabicyclo[3.1.0]hexane derivatives (**7a** and **7b**) containing 1*H*-benzo[*d*]imidazole. The 1*H*-benzo[*d*]imidazole motif is also present in mibefradil, which had low inhibitory activity against the two T-channel subtypes. The inhibitory activity of the 5-isobutyl-1-phenyl-pyrazole-conjugated analogues (**10a–f**) exhibited increased channel inhibition compared with the 1*H*-benzo[*d*]imidazole analogues (**7a–b**). With the exception of **10f** (inhibition of α_{1G} = 34.1% and α_{1H} = 25.5%), compounds containing a 6-phenylsulfonyl group had moderate to high levels of inhibition. The inhibitory activities of compounds **10b–c** that contain aromatic benzyl or phenethyl groups were superior to **10a**, which contained a bulky alkyl-substituted compound. Benzoyl- (**10d**, α_{1G} = 80.4%, α_{1H} = 90.3%) and phenacyl- (**10e**, α_{1G} = 81.0%, α_{1H} = 89.9%) substituted analogues had the highest inhibitory activities that were also superior to that of mibefradil.

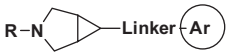
When the linker of compound **10a** was altered from –CONH-CH_2– to –CH_2-NHCO–, the inhibitory activity increased to that of **15a**. However, the 5-iso-propyl-isooxazol derivative of **15b** had reduced inhibitory activities.

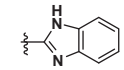
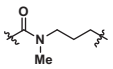
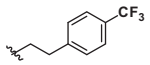
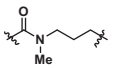
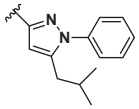
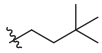
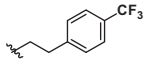
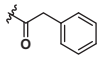
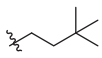
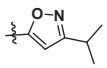
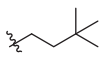
To investigate the selectivity of compounds to other channels, the IC_{50} values of the 5-isobutyl-1-phenyl-pyrazole containing analogues **10a**, **10c–e**, and **15a** against T-type and N-type calcium channels, as well as the hERG channel were determined using the patch clamp method[13] (Table 2). With the exception of compound **10e**, all compounds selectively inhibited the T-type channel. Compounds **10d** and **10e** exhibited weak hERG inhibition, as indicated by a half-inhibitory concentration of 15–20 μM. Compound **10d** was the most potent T-channel inhibitor.

With the exception of compound **10b**, all compounds in this series exhibited good stability in human microsomes. The inhibitory effect of these compounds on CYP450 enzymes was also low, suggesting that undesirable drug-drug interactions did not occur. It has been reported that the 2D6-subtype enzyme metabolizes drugs targeting the central nervous system, and the 3A4 enzyme metabolizes over 50% of clinical drugs. Thus, compounds **10a**, **10d**, and **10e** are potentially good drug candidates and were investigated further (Table 3).

Based on their biological properties, compounds **10a** and **10d** were selected for in vivo evaluation. Although compound **10e** exhibited highest inhibitory potency of T-type calcium channel, compound **10e** was excluded for the in vivo test due to the high inhibitory potency of N-type calcium channel (60.6%, 10 μM), and high inhibition of CYP-450 2C9 (remaining % = 24.8). Behavioral testing for neuropathic pain was performed in rats (n = 25) using the spinal nerve ligation (SNL) model.[14] After 14 days of surgical manipulation, compounds **10a** and **10d** were administrated orally, and rats were assessed for reductions in neuropathic pain, as

Table 1
Percent T-type (α_{1G} and α_{1H}) calcium channel inhibition by 6-aryl-connected-3-*R*-substituted-azabicyclo[3.1.0]hexanes



Compd	Ar	R	Linker	FDSS % inhibition (10 μM)[a]	
				α_{1G}	α_{1H}
7a				28.4	23.4
7b				49.2	58.7
10a				56.9	48.0
10b				76.5	79.5
10c				60.5	64.6
10d				80.0	90.2
10e				81.0	89.9
10f				34.1	25.5
15a				64.3	61.9
15b				38.2	23.7
Mibefradil				79.2	76.0

[a] % inhibition was obtained using an FDSS6000 fluorescence-based HTS assay on HEK293 cells.

Table 2
In vitro activities of compounds **10a**, **10c**, **10d**, **10e**, and **15a** against T-type (α_{1G} and α_{1H}) and N-type calcium channels, as well as the hERG channel

Compd	T-type Ca^{2+} channel (IC_{50})[a]		N-type Ca^{2+} channel	hERG channel
	α_{1G} (μM)	α_{1H} (μM)	% inhibition (10 μM)[b]	IC_{50} (μM)
10a	11.03 ± 2.21	8.20 ± 0.84	38.3	2.60 ± 0.53
10b	4.76 ± 0.77	10.79 ± 2.49	42.2	1.43 ± 0.40
10d	5.91 ± 0.37	2.89 ± 0.27	48.4	15.00 ± 3.61
10e	2.73 ± 0.12	2.50 ± 0.37	60.6	20.80 ± 8.96
15a	10.08 ± 0.96	8.87 ± 1.25	37.4	3.13 ± 0.93

[a] IC_{50} values (±SD) were determined from dose–response curves using the patch clamp method.
[b] % inhibition was obtained using the patch clamp method.

indicated by cold and mechanical allodynia. The neuropathic pain drug, gabapentin, was used as the reference for such assays.

Compound **10a** exhibited the most significant reduction in mechanical allodynia, which began 3 h after administration and progressed for 5 h (Fig. 3A and B). Moreover, compound **10a** reduced cold allodynia 3-fold more than did gabapentin after 1 h and continued for 5 h (Fig. 3C and D). Compound **10d** also caused a 2-fold reduction on cold allodynia, compared with gabapentin (Fig. 4A and B); however, **10d** increased mechanical allodynia. However, the increase in mechanical allodynia remained lower than that of gabapentin (Fig. 4C and D). The caveats of compound **10d** were that it had poor bioavailability ($F = 7.6\%$) and brain-to-plasma ratios (B/P = 0.12) when administered orally (Table 4).

3. Conclusions

In this study, the synthesis and SAR analysis of aryls (6-benzo[*d*] imidazole/isoxazole/pyrazole) conjugated to 3*N*-substituted-

Table 3
Inhibitory effects of compounds on CYP-expressing microsomes

Compd	% control of CYP-450 (10 μM)[a]				HLM[f] Stability
	2D6[b]	2C9[c]	2C19[d]	3A4[e]	Remaining %
7b	27.7	51.4	42.3	61.4	76.9
10a	126.0	72.8	100.7	82.3	76.3
10b	56.2	118.0	30.3	102.3	5.8
10d	94.0	64.6	60.8	74.6	78.5
10e	83.0	24.8	65.5	80.4	57.8
15a	55.9	87.1	84.2	129.7	93.8

[a] Values represent the percentage of remaining activity from triplicate experiments.
[b] Quinidine.
[c] Sulfaphenazol.
[d] Miconazole.
[e] Ketoconazole.
[f] HLM, human liver microsome.

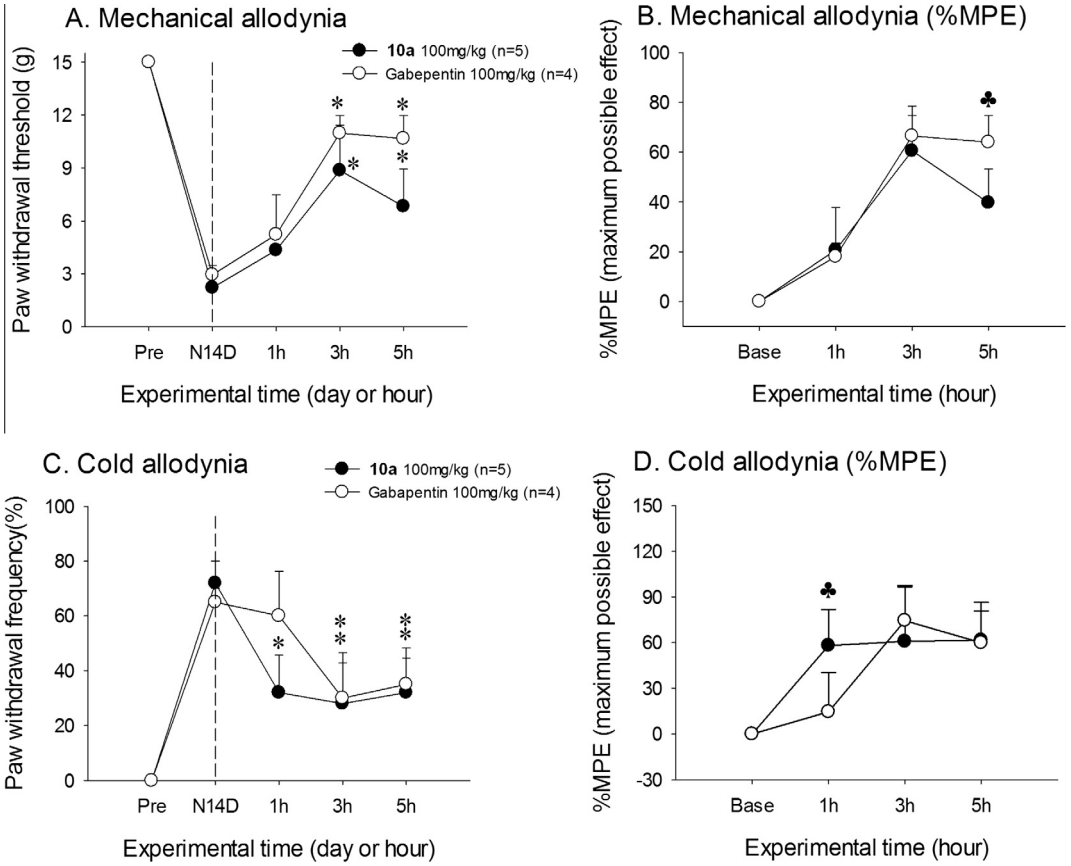


Figure 3. Assessments of mechanical (A and B) and cold allodynia (C and D) after oral administration of gabapentin (○, 100 mg/kg, $n = 4$) or **10a** (●, 100 mg/kg, $n = 5$) to neuropathic pain-induced rats. D, days after neuropathic injury (N); and h, hours after gabapentin or **10a** administration. $^{*}P < 0.05$ (gabapentin) and $^{*}P < 0.05$ (**10a**) versus pre-administration value (paired t-test). $^{\clubsuit}P < 0.05$ gabapentin versus **10a** (unpaired t-test).

azabicyclo[3.1.0]hexane derivatives were reported. Among such compounds, a new series of 6-pyrazoyl-connected-3*N*-*R*-substituted-azabicyclo[3.1.0]hexanes were identified as potent T-channel inhibitors. The 5-isobutyl-1-phenyl-pyrazole-containing analogues (**10a**–**f**) had strong T-channel inhibitory activity compared with benzo[*d*]imidazole or isoxazole rings. SAR analysis suggested that the benzyl, benzoyl, phenethyl, and 1-pheylacetyl groups attached to the 3-*N* substituent of azabicyclo[3.1.0]hexanes improved in vitro activity. This series of compounds selectively inhibited T-channels, exhibited good metabolic stability, and had low CYP450 liability. Among the compounds evaluated, **10a** and **10d** significantly reduced cold allodynia in an SNL rat model; however, **10d** exhibited the lowest bioavailability ($F = 7.6\%$) and brain-to-plasma ratio (B/P = 0.12) during oral administration. Therefore 5-isobutyl-1-phenyl-pyrazole conjugated 3*N*-benzoyl substituted azabicyclo[3.1.0]hexanes (**10d**) is identified to be a potent and selective T-type calcium channel blocker for pain treatments. Based on these data, additional assessments of this class of compounds will be performed for the development of neuropathic pain treatments.

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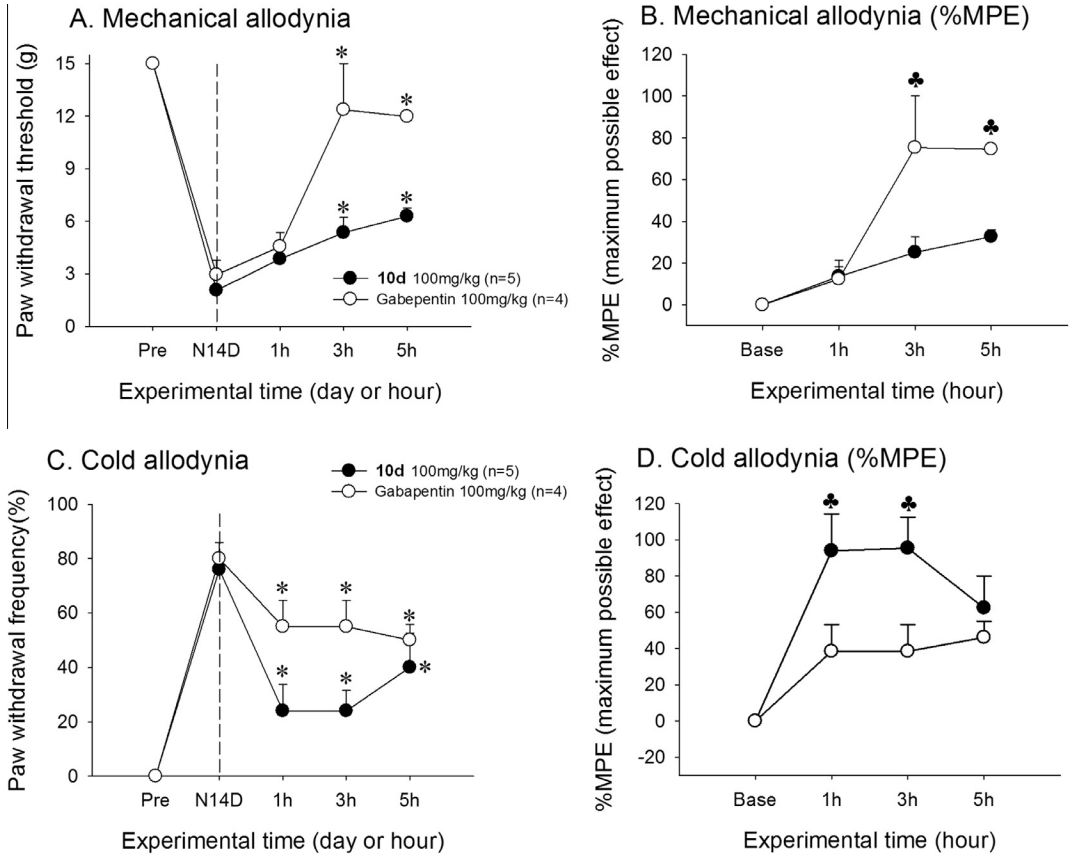

Figure 4. Assessments of mechanical (A and B) and cold allodynia (C and D) after oral administration of gabapentin (○, 100 mg/kg, $n = 4$) or **10d** (●, 100 mg/kg, $n = 5$) to neuropathic pain-induced rats. D, days after neuropathic injury (N); and h, hours after gabapentin or **10d** administration. $^{*}P < 0.05$ (gabapentin) and $^{\#}P < 0.05$ (**10d**) versus pre-administration value (paired t-test). $^{\clubsuit}P < 0.05$ gabapentin versus **10d** (unpaired t-test).

Table 4
Pharmacokinetic parameters following intravenous ($n = 4$) and oral ($n = 4$) administration (10 mg/kg) of **10d** to male rats

	10d (mean ± SD[a])	
	iv	Oral
$AUC_{0-\infty}$(μg min/ml)	231.30 ± 29.75	17.62 ± 6.95
AUC_{last}(μg min/ml)	231.23 ± 29.70	16.38 ± 8.03
Terminal half-life (min)	19.90 ± 0.67	65.14 ± 24.6
C_{max} (μg/ml)	—	0.28 ± 0.20
T_{max} (min)		26 (15 ~ 30)[b]
CL (ml/min/kg)	43.77 ± 5.52	—
MRT (min)	18.20 ± 3.04	—
Brain-to-plasma ratio (B/P) at 2 h	0.29	0.12
F (%)	7.6	

Abbreviations: $AUC_{0-\infty}$, total area under the plasma concentration–time curve from time zero to infinity; AUC_{last}, total area under the plasma concentration–time curve from time zero to the previous time; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}; CL, time-averaged total body clearance; MRT, mean residence time; F, bioavailability.

[a] SD: Standard deviation.

[b] Median (range) for T_{max}.

4. Experimental section

4.1. Chemistry

4.1.1. General

All solvents and commercially available reagents were used without additional purification. Reactions were monitored by analytical thin layer chromatography (TLC, Merck, silica gel 60 F_{254}) with UV light (254 nm). Visualization was performed by incubation with p-methoxy anisaldehyde (PMA) solution and heating with a heat gun. Flash column chromatography was performed using a silica gel (Merck, 230–400 mesh), and NMR spectra (^{1}H and ^{13}C) were recorded on Bruker Avance 300 or 400 MHz spectrometers. Mass spectra (MS) were measured with a Q-TOF SYNAPT G2 (Waters MS Technologies, Manchester, UK).

4.1.2. *Tert*-Butyl-6-(3-(1*H*-benzo[*d*]imidazol-2-yl)-*N*-methylpropanecarbamoyl)-3-azabicyclo [3.1.0]hexane (5)

Compound **4** (162.3 mg, 0.714 mmol), hydroxybenzotriazole (116.5 mg, 0.857 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (165 mg, 0.857 mmol) were dissolved in 3.0 mL of methylene chloride, and 3-(1*H*-benzo[*d*]imidazol-2-yl)-*N*-methylpropan-1-amine (148.7 mg, 0.785 mmol) was added dropwise. After stirred for 3.5 h, the mixture was extracted with methylene chloride after adding water and saturated sodium bicarbonate. The organic layer was dried with anhydrous sodium sulfate, filtered, concentrated under reduced pressure and separated by column chromatography (DCM–MeOH = 20:1) to obtain 212 mg (74.5%) of the compound **5**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.65 (s, 9H), 1.69 (s, 1H), 1.70 (t, $J = 3.06$ Hz, 1H), 2.01 (t, $J = 2.55$ Hz, 2H), 2.20 (m, 2H), 2.87 (dd, $J = 6.12$ HZ, 2.85 Hz, 2H), 3.23 (s, 3H), 3.51 ~ 3.76 (m, 6H), 7.23 (dd, $J = 7.53$ Hz, 2.79 Hz, 2H), 7.51 (br s, 1H), 7.72 (br s, 1H), 11.62 (bs, 1H).

4.1.3. 6-(3-(1*H*-Benzo[*d*]imidazol-2-yl)-*N*-methylpropane)-3-carbamoyl-azabicyclo[3.1.0] hexane hydrochloride (6)

1.25 *M* hydrochloric acid solution (9.76 mL, 12.2 mmol) dissolved in methanol was added dropwise to the compound **5** (217 mg, 0.544 mmol) and stirred for 3 h. The completion of the

reaction was confirmed by TLC (hexane–ethyl acetate = 1:2). After the reaction was completed, the reaction mixture was concentrated under reduced pressure to obtain 210 mg (99%) of the target compound **6**. ^{1}H NMR (300 MHz, $CDCl_3$) 1.17 (s, 4H), 2.21(m, 4H), 3.00 (s, 3H), 7.51 (m, 2H), 7.79 (m, 2H), 9.59 (br s, 1H), 10.29 (br s, 1H), 14.8 (s, 2H).

4.1.4. 3-[3-(3,3-Dimethylbutyl)-3-azabicyclo[3.1.0]hexane-6-carboxamido]-2(*N*-methyl)propyl-1*H*-benzo[*d*]imidazol (7a)

Compound **6** (100.6 mg, 0.270 mmol) and a molecular sieve were dried in vacuum and dissolved in 2 mL of methylene chloride. Then, triethylamine (38 μL, 0.270 mmol) was added dropwise at 0 °C. After adding 3,3-dimethylbutyraldehyde (33 μL, 0.270 mmol) dropwise, the mixture was stirred at room temperature for 1 h. Sodium triacetoxyborohydride (171 mg, 0.810 mmol) was added and stirred for 2 h. The reaction mixture was diluted with methylene chloride and extracted several times with saturated sodium bicarbonate. The organic layer was dried with anhydrous magnesium sulfate, filtered, concentrated under reduced pressure and separated by column chromatography (CH_2Cl_2–MeOH = 15:1) to obtain 63.1 mg (61%) of the target compound **7a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.9 (br, 1H), 7.60 (br, 2H), 7.25–7.19 (m, 2H), 3.55 (t, J = 6.09 Hz, 2H), 3.23–3.17 (m, 5H), 2.86 (t, J = 6.06 Hz, 2H), 2.52–2.43 (m, 4H), 2.29 (br, 1H), 2.08 (s, 2H), 2.00 (br, 2H), 1.40 (t, J = 8.22 Hz, 2H), 0.94 (s, 9H) ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 174.4, 154.6, 139.4, 125.6, 121.7, 54.8, 51.5, 46.1, 42.3, 35.5, 29.8, 29.6, 29.3, 26.5, 25.7, 25.0, 20.4.

4.1.5. 3-[4-(Trifluoromethyl)phenylethyl]-3-azabicyclo[3.1.0]hexane-6-carboxamido-2-(*N*-methyl)propyl-1*H*-benzo[*d*]imidazol (7b)

Compound **7b** was synthesized in a manner similar to that of **7a** with a yield of 53.5 mg (40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.64–7.56 (m, 4H), 7.35–7.30 (m, 2H), 7.25–7.22 (m, 2H), 3.55 (t, J = 5.91 Hz, 2H), 3.23–3.20 (m, 4H), 2.99–2.97 (m, 1H), 2.90–2.84 (m, 4H), 2.80–2.76 (m, 2H), 2.55 (d, J = 8.82 Hz, 2H), 2.22 (s, 1H), 2.13–2.10 (m, 3H), 2.01 (br, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.3, 154.6, 144.6, 129.1, 128.9, 125.2, 125.1, 121.9, 55.9, 54.4, 46.9, 46.2, 35.5, 35.0, 26.3, 25.6, 24.9, 20.2.

4.1.6. 3-[3-(3,3-Dimethylbutyl)-3-azabicyclo[3.1.0]hexane-6-carboxamido]methyl-5-isobutyl-1-phenyl-1*H*-pyrazole (10a)

Compound **10a** was synthesized in a manner similar to that of **7a** with a yield of 63.9%. ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.53–7.40 (m, 5H), 6.24 (br, 1H), 6.16 (s, 1H), 4.50 (d, J = 5.07 Hz, 2H), 3.11 (d, J = 9.03 Hz, 2H), 2.53 (d, J = 7.14 Hz, 2H), 2.42 (t, J = 8.40 Hz, 2H), 2.36 (d, J = 8.79 Hz, 2H), 1.99 (s, 2H), 1.89–1.80 (m, 2H), 1.35 (t, J = 8.40 Hz, 2H), 0.90 (s, 9H), 0.89 (d, J = 7.32 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$), δ 172.5, 162.3, 149.4, 144.3, 139.8, 129.1, 128.0, 125.7, 104.8, 54.9, 51.5, 42.4, 37.8, 35.2, 29.8, 29.6, 28.4, 25.1, 23.8, 22.4. HRMS [ESI$^+$] m/z calcd for $C_{26}H_{38}N_4O$ [M+H]$^+$: 423.3046, found: 423.3115.

4.1.7. 3-(3-Benzyl-3-azabicyclo[3.1.0]hexane-6-carboxamido) methyl-5-isobutyl-1-phenyl-1*H*-pyrazole (10b)

Compound **10b** was synthesized in a manner similar to that of **7a**, with a yield of 75.7%. ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.53–7.40 (m, 5H), 7.34–7.22 (m, 5H), 6.43 (br, 1H), 6.17 (s, 1H), 4.51 (d, J = 5.07 Hz, 2H), 3.61 (s, 2H), 3.02 (d, J = 8.97 Hz, 2H), 2.52 (d, J = 7.14 Hz, 2H), 2.45 (d, J = 8.49 Hz, 2H), 1.99 (s, 2H), 1.95 (s, 1H), 1.91–1.78 (m, 1H), 0.89 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$), δ 172.6, 149.5, 144.3, 139.9, 139.4, 129.1, 128.5, 128.2, 128.0, 126.9, 125.8, 104.8, 58.8, 54.3, 37.8, 35.2, 28.4, 25.2, 23.6, 22.4.

4.1.8. 3-(3-Benzoyl-3-azabicyclo[3.1.0]hexane-6-carboxamido) methyl-5-isobutyl-1-phenyl-1*H*-pyrazole (10d)

Compound **10d** was synthesized in a manner similar to that of **10b** using **9** and benzoyl chloride (125 μL, 1.08 mmol) to obtain 360 mg (83.2%). ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.48–7.30 (m, 10H), 6.94 (t, J = 4.98 Hz, 1H), 6.13 (s, 1H), 4.45 (d, J = 5.1 Hz, 2H), 4.19 (d, J = 12.4 Hz, 1H), 3.66 (dd, J = 3.06, 10.9 Hz, 1H), 3.55–3.47 (m, 2H), 2.48 (d, J = 7.14 Hz, 2H), 2.10 (d, J = 10.2 Hz, 2H), 1.85–1.76 (m, 1H), 1.31 (t, J = 3.0 Hz, 1H), 0.85 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$), δ 170.6, 170.3, 149.2, 144.3, 139.7, 136.6, 130.0, 129.1, 128.3, 128.1, 127.0, 125.8, 104.8, 51.1, 47.6, 37.7, 35.1, 28.4, 25.4, 25.1, 23.7, 22.4. HRMS [ESI$^+$] m/z calcd for $C_{27}H_{30}N_4O$ [M+Na]$^+$: 466.2261. found: 466.2269.

4.1.9. 5-Isobutyl-1-phenyl-3-[3-(2-phenylacetyl)-3-azabicyclo [3.1.0]hexane-6-carboxamido] methyl-1*H*-pyrazole (10e)

Compound **10e** was synthesized in a manner similar to that of **10d**, with a yield of 82.1%. ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.51–7.22 (m, 10H), 6.67 (t, J = 4.98 Hz, 1H), 6.15 (s, 1H), 4.47 (d, J = 5.16 Hz, 2H), 3.85 (d, J = 12.3 Hz, 1H), 3.65–3.56 (m, 4H), 3.49 (td, J = 3.9, 12.3 Hz, 1H), 2.50 (d, J = 7.17 Hz, 2H), 2.16–2.07 (m, 2H), 1.87–1.78 (m, 1H), 1.17 (t, J = 3.0 6 Hz, 1H), 0.88 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$), δ 170.6, 170.2, 149.1, 144.4, 139.7, 134.4, 129.2, 128.9, 128.7, 128.2, 126.9, 125.8, 104.9, 49.0, 48.1, 42.3, 37.7, 35.1, 28.4, 26.5, 25.3, 24.1, 22.4.

4.1.10. 5-Isobutyl-1-phenyl-3-(3-benzenesulfonyl-3-azabicyclo [3.1.0]hexane-6-carboxamido) methyl-1*H*-pyrazole (10f)

Compound **10f** was prepared in a similar manner to that for **10d** using benzenesulfonyl chloride to obtain 70.4 mg (79.4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, J = 8.01 Hz, 2H), 7.62–7.37 (m, 8H), 6.73 (br, 1H), 6.15 (s, 1H), 4.46 (d, J = 5.07 Hz, 2H), 3.59 (d, J = 9.51 Hz, 2H), 3.09 (d, J = 9.3 Hz, 2H), 2.51 (d, J = 7.17 Hz, 2H), 2.01 (s, 2H), 1.89–1.75 (m, 1H), 1.52 (t, J = 2.88 Hz, 1H), 0.87 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 149.0, 144.4, 139.8, 136.1, 132.9, 129.2, 129.1, 128.1, 127.5, 125.8, 104.8, 49.6, 37.8, 35.1, 28.4, 24.5, 24.3, 22.4.

4.1.11. (3-(3,3-Dimethylbutyl)-3-azabicyclo[3.1.0]hexan-6-yl) methanamine (14)

Compound **13** (170 mg, 0.808 mmol) was dissolved in DCM (7.42 mL) and lithium aluminum hydride (1*M* in ether, 3.23 mL, 3.23 mmol) was added 0 °C, and the reaction mixture was refluxed for 20 h. After the reaction mixture was cooled down to 0 °C and diluted with dichloromethane, and sodium sulfate hydrate wad added carefully. The reaction mixture was filtered by celite, and residual solution was concentrated in vacuo to give compound **14** (130 mg, 82.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.09 (d, J = 8.79 Hz, 2H), 2.53 (d, J = 6.96 Hz, 2H), 2.44 (t, J = 8.04 Hz, 2H), 2.32 (d, J = 8.16 Hz, 2H), 1.50 (br, 2H), 1.39 (t, J = 8.31 Hz, 2H), 1.34–1.29 (m, 1H), 1.24 (br, 2H), (s, 9H).

4.1.12. 3-{2-[3-(3,3-Dimethylbutyl)-3-azabicyclo[3.1.0]hexane-6-yl]methyl}carbamoyl-5-isobutyl-1-phenyl-1*H*-pyrazole (15a)

Compound **15a** was synthesized in a manner similar to that of **5** using 5-Isobutyl-1-phenylpyrazole-3-carboxylic acid[15] to obtain 109 mg (84.2%). ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.48–7.34 (m, 5H), 7.01 (br, 1H), 6.71 (s, 1H), 3.23 (t, J = 6.09 Hz, 2H), 3.00 (d, J = 8.73 Hz, 2H), 2.47 (d, J = 7.02 Hz, 2H), 2.34 (t, J = 8.13 Hz, 2H), 2.22 (d, J = 8.4 9 Hz, 2H), 1.84–1.75 (m, 1H), 1.37 (br, 1H), 1.30 (br, 4H), 0.83 (br, 15H). ^{13}C NMR (75 MHz, $CDCl_3$), δ 162.0, 146.8, 144.9, 139.4, 129.2, 128.6, 125.9, 106.5, 55.1, 51.6, 42.3, 41.4, 35.1, 29.7, 29.6, 28.2, 22.3, 21.6, 19.7.

4.1.13. 3-{2-[3-(3,3-Dimethylbutyl)-3-azabicyclo[3.1.0]hexane-6-yl]methyl}carbamoyl-5-isopropyl-isoxazole (15b)

The compound **15b** was synthesized in a manner similar to that of **15a** with a yield of 74%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (s, 1H), 6.70 (br, 1H), 3.30 (t, J = 6.87 Hz, 2H), 3.17–3.08 (m, 3H), 2.42 (t, J = 8.37 Hz, 2H), 2.31 (d, J = 8.25 Hz, 2H), 1.51 (br, 1H), 1.38 (s, 2H), 1.35 (br, 2H), 1.31 (d, J = 6.96 Hz, 6H), 0.89 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 163.1, 156.1, 105.2, 55.0, 51.7, 42.2, 41.9, 29.8, 29.6, 26.5, 21.7, 19.3.

4.2. Biological evaluation

4.2.1. Evaluation of T-channel inhibition

T-channel inhibition was assessed using the FDSS6000 assay and whole cell patch clamp method.[15]

4.2.2. Evaluation of hERG channel inhibition

Inhibition of the hERG channel was performed using an auto-patch clamp assay method with CHO cells. CHO-K1 cells expressing hERG channels from an inducible Tet-On gene expression system (CHO-K1 Tet-On hERG cells) were purchased from IonGate Biosciences GmbH (Frankfurt, Germany). The NPC-16 Patch liner (Nanion Technologies, München, Germany) automated patch-clamp device was used for whole-cell recordings, as described previously.[13]

4.2.3. CYP inhibition assay

Inhibition by CYP (2D6, 2C9, 2C19 and 3A4) was performed using the Vivid CYP450 kit (Invitrogen, Madison, WI, USA) in a 96-well format, according to the manufacturer's instructions. Briefly, test compounds and positive controls (quinidine, sulfaphenazole, miconazole and ketoconazole, diluted in water) were mixed with the Master Pre-Mix (CYP450 BACULOSOMES regeneration system) and incubated for 15 min at 37 °C. Following incubation, the inhibition reaction was initiated by adding the Vivid CYP450 substrates and NADPH buffer. A fluorescence plate reader was used to measure enzymatic activity.

4.2.4. Evaluation of compound stability using human liver microsomes

To determine the stability of compounds, pooled human liver microsomes (HLMs) containing 20 mg/mL protein (BD Biosciences, Bedford, MA, USA) were incubated at 37 °C with a 1 M solution containing test compounds, potassium phosphate buffer (PPB), and the cofactor NADPH (#44332000, Oriental Yeast Co., Tokyo, Japan, 1.2 mM). The reaction was terminated after a 30 min incubation by the addition of cold acetonitrile containing 0.1 μg internal standard. Samples were centrifuged and supernatants analyzed by LC MS/MS.

4.2.5. Evaluation of in vivo efficacy of neuropathic pain in a rat model

In vivo assessments of neuropathic pain reduction were performed using two behavioral tests (mechanical and cold allodynia) in a rat model of spinal nerve injury.[16] After the postoperative behavioral tests, animals were treated orally with 100 mg/kg **10a**, **10d** or gabapentin. Behavioral tests were performed 1, 3, and 5 h after administration of the compound.

Mechanical allodynia was assessed using a 50% paw withdrawal threshold after application of von Frey filaments five times to the hind paws. The 50% withdrawal threshold was determined using the up-down method of Dixon.[17] and was calculated as follows: 10 ($X + kd$)/104, where X was the value of the final von Frey hair used (in log units), k was the tabular value for the pattern of positive/negative responses, and d was the mean difference between stimuli in log units (0.17).

Cold allodynia was tested by quantifying brisk paw withdrawal in response to acetone treatment. An acetone bubble was formed at the end of a polyethylene tube connected to a syringe. The syringe was applied to the plantar surface of the hind paw, and the bubble was touched to the heel. Acetone was applied five times to the hind paw at 2 min intervals, and the frequency of paw withdrawal was expressed as a percentage [(no. of trials accompanied by brisk foot withdrawal/total no. of trials) × 100].

The results of behavioral tests were expressed as the %MPE. For example, paw withdrawal thresholds were converted to %MPE using a cutoff of 15 g (the threshold for normal rats) to define the maximal effect: (post drug threshold_baseline threshold)/(cut-off_baseline threshold) × 100. %MPE values approaching 100 indicated normal mechanical thresholds (i.e., at or near 15 g), while values approaching 0 indicated allodynia. The results of the cold allodynia tests were also expressed as %MPE.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.06.006.

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