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Discovery and evaluation of selective N-type calcium channel blockers: 6-Unsubstituted-1,4-dihydropyridine-5-carboxylic acid derivatives

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ABSTRACT

A structure–activity relationship study of 6-unsubstituted-1,4-dihydropyridine and 2,6-unsubstituted-1,4-dihydropyridine derivatives was conducted in an attempt to discover N-type calcium channel blockers that were highly selective over L-type calcium channel blockers. Among the tested compounds, (+)-4-(3,5-dichloro-4-methoxy-phenyl)-1,4-dihydro-pyridine-3,5-dicarboxylic acid 3-cinnamyl ester was found to be an effective and selective N-type calcium channel blocker with oral analgesic potential.

The N-type calcium channel ($Ca_v2.2$) is a well-established and characterized subtype of voltage-dependent calcium channels (VDCCs) that are distributed among central and peripheral nerve terminals and are known to control calcium influx into cells in response to changes in membrane potential. Activation of this channel triggers various physiological events, such as neuronal excitability and secretion of neurotransmitters (e.g., glutamate, substance P, or CGRP), in strong association with the pathological process of neuropathic pain and cerebral ischemia. Thus, blockade of the N-type VDCCs has been considered as a promising therapeutic target for these pathological conditions.[1]

The clinical treatment of pain, especially prolonged and neuropathic pain, is still a major challenge, and the efficacy of current analgesic drugs, including opioids, is often determined by undesired dose-limiting side effects, such as tolerance and physical dependence. Therefore, there is a strong need for a novel analgesic drug that does not cause these adverse effects.[2] The therapeutic potential of N-type VDCC blockers for neuropathic pain states has been proven in clinical and preclinical studies of N-type selective peptidic blockers, ω-conotoxins MVIIA, GVIA, and CVID.[3] These ω-conotoxins clearly showed therapeutic benefits, with minimal development of tolerance and addiction. However, their usage had several drawbacks related to peptide blockers, such as limited route of administration, challenging synthesis, and high costs.

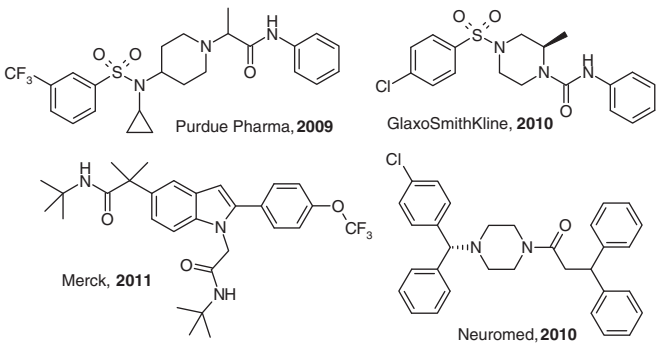

Figure 1. Recently reported N-type VDCC blockers.

Ziconotide, which is a synthetic version of ω-conotoxin MVIIA and is approved as an analgesic drug to treat intractable pain conditions, can be administered only through the intrathecal route, which has very low patient compliance.[3] Therefore, many efforts have been made to discover systemically efficacious small-molecule N-type VDCC blockers to overcome the limitations of conopeptides (recently reported small-molecule N-type VDCC blockers are shown in Fig. 1).[4]

As previously reported,[5] we performed a structure–activity relationship study of unique 1,4-dihydropyridine-5-carboxylate compounds. Our goal was to discover orally available potent N-type VDCC blockers with high selectivity over L-type channels to ensure minimal effects on the cardiovascular system. As a consequence of

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the structural optimization, 2-dimethoxymethyl-4-(3-chlorophenyl)-6-methyl-1,4-dihydropyridine-5-carboxylic acid (**1**) was identified as a promising N-type VDCC blocker that showed an efficacious analgesic potency with no detectable influence on systemic blood pressure at the effective dose.[5d] Therefore, further modification of **1** to achieve improvement in the N-type VDCC inhibitory activity with reduced blocking activity on the L-type channel is a potential strategy to obtain an analgesic drug candidate with minimal adverse effects.

In this Letter, we focused on the introduction of a hydrogen atom at the 2 and 6 positions of the 1,4-dihydropyridine structure of **1** to improve the activity of the N-type VDCCs and to reduce inhibitory activity at the L-type channels.

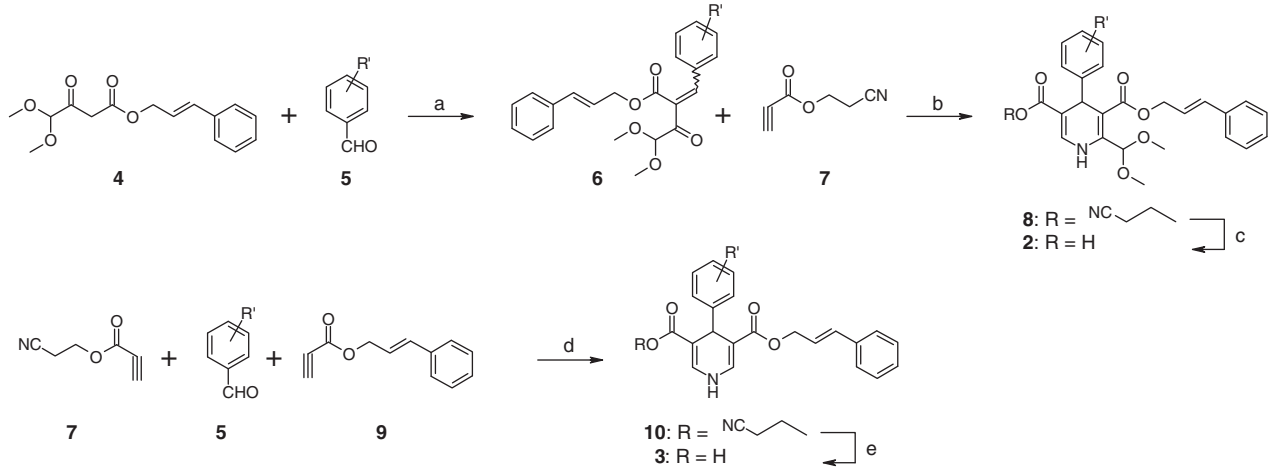
The synthetic methods for the synthesis of 6-unsubstituted-1, 4-dihydropyridine derivatives are shown in Scheme 1. 4,4-Dimethoxy-2-benzylidene-3-oxo-butyricacid cinnamyl ester (**6**) was obtained using Knoevenagel condensation of 4,4-dimethoxy-3-oxobutyric acid cinnamyl ester (**4**) with corresponding benzaldehyde (**5**). The 2-propanol solution of **6** was heated with propynoic acid 2-cyanoethyl ester (**7**) in the presence of ammonium acetate to obtain 1,4-dihydropyridine (**8**), which was treated with 1 M NaOH to yield a 2-dimethoxymethyl-6-unsubstituted-1,4-dihydropyridine-5-carboxylicacid derivative (**2**). A four-component cyclizing reaction that used the corresponding benzaldehyde (**5**), **7**, propynoic acid cinnamyl ester (**9**), and ammonium acetate resulted in the formation of 2, 6-unsubstituted-1,4-dihydropyridine-3,5-dicarboxylicacid3-cinnamylester5-(2-cyanoethylester) (**10**). Subsequent hydrolysis provided 2,6-unsubstituted-1,4-dihydropyridine-5-carboxylicacid (**3**). Preparative HPLC equipped with a chiral column (Daicel Chiralcel OD) followed by hydrolysis was used to separate and obtain the optical isomer of the 3′,5′-dichloro-4′-methoxy-phenyl derivative **3f** from 4-(3,5-dichloro-4-methoxy-phenyl)-1,4-dihydro-pyridine-3,5-dicarboxylic acid 3-cinnamyl ester 5-(2-cyanoethyl) ester (**10f**).[6]

IMR-32 human neuroblastoma cells for N-type VDCCs[7] and a rat thoracic aorta ring for L-type VDCCs were used to perform in vitro characterizations of the synthesized compounds.[8] Our study was initiated by the replacement of the 6-methyl group in compound **1** with a hydrogen atom to afford **2a** (Table 1). Interestingly, **2a** showed improved inhibitory activity for N-type VDCC (IC_{50} = 1.1 μM) and reduced activity at the L-type channels (44% inhibition at 10 μM) compared with those of **1**, indicating that a sterically least hindered hydrogen atom at the 6-position is important for the inhibitory activity at N-type VDCCs as well as for selectivity over L-type channels. Thus, further modification was performed on the substituent groups in the 4-phenyl moiety in **2a** to optimize activities at both the N- and L-type VDCCs. The derivatives possessing chlorine atoms at the 4′-position (**2b**) and 3′,5′-positions (**2c**) showed further improvement in N/L selectivity (7.9% and 4.0% inhibition for L-type VDCCs at 10 μM, respectively), with effective inhibitory activity at the N-type (IC_{50} = 1.1 and 1.0 μM, respectively). The 3′,4′-dichloro derivative (**2d**) had a submicromolar IC_{50} value of 0.52 μM for N-type VDCCs. The compound with a methoxy group at the 4′-position (**2e** and **2f**) and the 4′-trifluoromethyl derivative (**2g**) resulted in improved inhibitory activity at N-type VDCCs (IC_{50} = 1.5, 1.2, and 1.1 μM, respectively), with decreased activity at L-type channels (26%, 20%, and 17% inhibition at 10 μM, respectively) compared with those of **1**, but their N/L-selectivity profiles were not comparable with those of **2b** and **2c**.

Further introduction of a hydrogen atom was made at the 2-position of the 1,4-dihydropyridine ring in **2a** to obtain the 2,6-unsubstituted-1,4-dihydropyridine derivative **3a** (Table 2), which showed effective N-type activity with equivalent inhibitory activity at the L-type VDCCs compared with that of **1** (IC_{50} = 1.6 and 9.1 μM, respectively). Its 4′-chloro derivative **3b** had improved N-type blocking activity and selectivity over those of L-type channels (16% inhibition at 10 μM). Another derivative with two chlorine atoms at the 3′- and 4′-positions (**3c**) showed submicromolar activity for N-type VDCCs (IC_{50} = 0.72 μM). The introduction of a trifluoromethyl group at the 3′-position (**3d**) resulted in a 1.2 μM IC_{50} value for the N-type. The 3′-chloro derivative with another electron-donating group at the 4′-position (**3e**) had an IC_{50} value of 1.3 μM for N-type VDCCs, with reduced inhibitory activities for the L-type compared with that of **3a** (15% inhibition at 10 μM). Finally, the derivative possessing two chlorine atoms at the 3′- and 5′-positions with a 4′-methoxy group (**3f**) had improved activity for the N-type VDCCs (IC_{50} = 0.58 μM) and reduced activity for the L-types (19% inhibition at 10 μM) compared with those of **3a**.

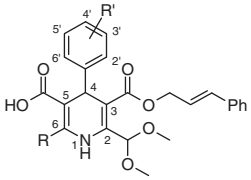
As a consequence of the structural optimization, all of the tested 6-unsubstituted-1,4-dihydropyridine and 2,6-unsubstituted-1,4-dihydropyridine derivatives showed improved N-type inhibitory activity and selectivity over those of the L-types compared with **1**, which indicated the crucial role of the hydrogen atom at the 6-position for these bioactivities. Among the derivatives, **2b**, **2c,** and **3f** were found to be particularly selective and effective N-type calcium channel blockers.

Further biological evaluations were performed on the two enantiomers of **3f**. (−)**-3f** had an IC_{50} of 0.84 μM for the N-type channel, with 28% inhibition of the L-type channel at 10 μM. In addition, (**+**)**-3f** had equipotent inhibitory activity at the N-type VDCCs (IC_{50} = 0.72 μM) compared with that of **3f** and showed excellent



Scheme 1. Reagents and conditions: (a) Cat. piperidine, benzene, reflux; (b) $AcONH_4$, *i*-PrOH, 80 °C; (c) 1 M NaOH, MeOH; (d) $AcONH_4$, *i*-PrOH, 80 °C; (e) 1 M NaOH, MeOH.

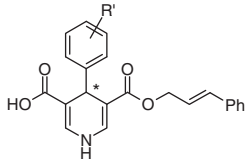
Table 1
Activity table of dihydropyridine derivatives



Compound	R	R′	N-type IMR-32 IC_{50}, μM	L-type Magnus IC_{50}, μM or inh. % at 10 μM
1	Me	3′-Cl	3.0	8.1
2a	H	3′-Cl	1.1	44%
2b	H	4′-Cl	1.1	7.9%
2c	H	3′,5′-Cl	1.0	4.0%
2d	H	3′,4′-Cl	0.52	31%
2e	H	3′-Cl, 4′-OMe	1.5	26%
2f	H	3′,5′-Cl, 4′-OMe	1.2	20%
2g	H	3′-CF_3	1.1	17%

In vitro inhibition against N-type (calcium influx using IMR-32 cells) and L-type (Magnus method) calcium channels.

Table 2
Activity table of optically pure dihydropyridine derivatives



Compound	R′	N-type IMR-32 IC_{50}, μM	L-type Magnus IC_{50}, μM or inh. % at 10 μM
3a	3′-Cl	1.6	9.1
3b	4′-Cl	1.2	16%
3c	3′,4′-Cl	0.75	41%
3d	3′-CF_3	1.2	40%
3e	3′-Cl, 4′-OMe	1.3	15%
3f	3′,5′-Cl, 4′-OMe	0.58	19%
(−)-**3f**	3′,5′-Cl, 4′-OMe	0.84	28%
(+)-**3f**	3′,5′-Cl, 4′-OMe	0.72	6.5% (IC_{50} = 44 μM)

In vitro inhibition against N-type (calcium influx using IMR-32 cells) and L-type (Magnus method) calcium channels.

Table 3
Inhibitory activity of the test compound in phase II pain responses following footpad injection of formalin in rats

Compound	Rat formalin test	
	Dose	Inhibition%
(+)-**3f**	3 mg/kg, po	35 ± 11% (n = 5)
Gabapentin	100 mg/kg, po	33 ± 12% (n = 3)

Inhibition% versus that of the control.

selectivity over the L-types (IC_{50} = 44 μM). As a result, (**+**)**-3f** was found to be a promising N-type VDCC blocker with submicromolar activity and the best selectivity over the L-type VDCCs among the tested compounds.

This result motivated us to use a rat formalin-induced pain model to evaluate (**+**)**-3f** in vivo using rat formalin-induced pain model (Table 3). Our previously reported compounds were used to confirm the applicability of this model for N-type calcium channel blockers.[5a,b] (**+**)**-3f** (3 mg/kg po) displayed significant analgesic efficacy that was comparable to that of Gabapentin (100 mg/kg, po), which was indicative of its effective pain-relieving potential.[9]

In summary, in a structure–activity relationship study at the 2- and 6-positions of the 1,4-dihydropyridine ring of **1**, the derivatives **2b**, **2c**, and (**+**)**-3f** were found to be effective and selective N-type VDCC blockers with potent analgesic efficacy. These compounds could be interesting research tools and possibly promising drug candidates for the control of severe to moderate pain states with negligible effects on blood pressure.

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6. *The synthesis of (+)-**3f** was performed as follows:* Step 1. Synthesis of racemic 4-(3,5-dichloro-4-methoxy-phenyl)-1,4-dihydro-pyridine-3,5-dicarboxylic acid 3-cinnamyl ester 5-(2-cyanoethyl) ester (**10f**): 3,5-dichloro-4-methoxy-benzaldehyde (**5**; 318 mg, 1.86 mmol), propynoic acid 2-cyanoethyl ester (**7**; 229 mg, 1.86 mmol), propynoic acid cinnamyl ester (**9**; 351 mg, 1.88 mmol), and ammonium acetate (270 mg, 3.50 mmol) were dissolved in 2-propanol and stirred overnight at 80 °C. The solvent was evaporated under reduced pressure, and the residue was extracted three times with EtOAc from water. The title compound (116 mg, 0.23 mmol, 12.2%) was obtained after purification by silica gel chromatography (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): 2.56 (2H, t), 3.83 (3H, s), 3.83 (3H, s), 4.18–4.40 (2H, m), 4.63–4.83 (2H, m), 4.87 (1H, s), 6.23 (1H, dt), 6.34 (1H, br d), 6.58 (1H, d), and 7.23-7.47 (9H, m). MS (ESI, *m*/*z*) 511 $(M-H)^-$.
Step 2. Optical separation of **10f**: the racemic mixture of **10f** (3.50 g, 6.89 mmol; ca 100 mg for each injection) was separated by HPLC using a chiral column (semi-preparative HPLC system: HITACHI L-6200 system; Daicel Chiralcel OD, Daicel Chemical Industries Inc.; 250 × 0.46 cm ID; *n*-hexane/ethanol = 85/15; flow rate: 6 mL/min; detection: 254 nm). The fraction between 88 and 102 min was collected to obtain (**+**)-**10f** (1.39 g, 2.70 mmol, 39.2%). Enantiomeric excess: 99.7%, (analytical HPLC system: HITACHI L-6200 system; column: Daicel Chiralcel OD-H, 25 × 0.46 cm ID; Solvent: *n*-hexane/EtOH = 80:20; Flow rate: 1.0 mL/min; Detection: 254 nm), $[\alpha]D^{25}$ = +12.05 (*c* = 0.95, MeOH).
Step 3. Synthesis of (+)-4-(3,5-dichloro-4-methoxy-phenyl)-1,4-dihydro-pyridine-3,5-dicarboxylic acid 3-cinnamyl ester (+)-**3f**): 1.37 g (2.66 mmol) of (**+**)-**10f** was dissolved in MeOH (50 mL), and 1 M NaOH (8 mL) was added. After stirring for 2 h, 1 M HCl (8 mL) was added, and the solvent was removed under reduced pressure. After extracting three times with EtOAc, the organic layer was dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 500/1) to obtain the title compound (1.08 g, 2.34 mmol, 88.0%). purity: 97.1% (analytical HPLC system: HITACHI L-6200 system; C-18 reversed-phase column: YMC-Pack ODS AM, 15 × 0.46 cm ID, YMC Co., Ltd; solvent: water/acetonitrile = 50:50–100:0 in 30 min; flow rate: 1.0 mL/min; detection: 254 nm, $[\alpha]D^{25}$ = +51.12 (*c* 1.00, MeOH). ^{1}H NMR (DMSO-d_6) δ: 3.76(3H, s), 4.61–4.78(2H, m), 4.73(1H, s), 6.33(1H, dt), 6.56(1H, d), 7.20–7.48(9H, m), and 9.27(1H, t). HR–MS (FAB, *m*/*z*) calcd 458.0562 $(M-H)^-$ observed 458.0565.
7. Measurement of N-type calcium channel ($Ca_V2.2$) inhibitory activity was performed as follows (previously reported in the Ref. 5): Human neuroblastoma cells IMR-32 were obtained from the American Type Culture Collection and cultured with a medium, which comprised the following: a phenol red-free Eagle's minimum essential medium containing Earle's salts (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate (pH 6.5) (GIBCO), antibiotic/antimycotic mixture (GIBCO), and 10% fetal calf serum (Cell Culture Technologies). For measurement of intracellular calcium concentrations, 3 mL of 1×10^5 cells/mL IMR-32 cells were spread on the glass bottom of a dish (Iwaki Glass Co., Ltd) having a diameter of 35 mm that had been treated with poly-L-lysine (SIGMA) and collagen (COLLAGEN VITROGEN 100; Collagen Co.). One day after the culture, 1 mM dibutyl-cAMP and 2.5 μM 5-bromo-2-deoxyuridine (SIGMA) were added to express N-type calcium channels. After culturing for an additional 10–14 days, the cells were subjected to the assay.
The medium for the IMR-32 cells thus prepared was replaced with 1 mL of phenol red-free Eagle's minimum essential medium (GIBCO) containing 2.5 μM fura-2/AM (Dojin Kagaku, Co.) and Earle's salts supplement, and the incubation was performed at 37 °C for 30 min. Next, the medium was replaced with a recording medium (20 mM HEPES–KOH, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $mgCl_2$, 1.8 mM $CaCl_2$, and 13.8 mM D-glucose). A fluorescence microscope (Nikon Corporation) and an image analysis device ARGUS 50 (Hamamatsu Photonics) were used to analyze the intracellular calcium concentrations. To prevent the activation of the L-type calcium channels in the differentiated IMR-32 cells, a recording medium containing 1 μM of a selective L-type calcium channel blocker, nifedipine, was used throughout the experiment. Then, a stimulating medium containing 60 mM KCl (KCl was substituted for equimolar NaCl in the recording medium) was rapidly given by the Y-tube method for 6 s. The change in the intracellular calcium concentration was expressed as the N-type calcium channel activity (please see Takahara, A.; Fujita, S.; Moki, K.; Ono, Y.; Koganei, H.; Iwayama, S.; Yamamoto, H. Hypertens Res. 2003, 26, 743). Then, 60 mmol/L KCl solution was applied repeatedly at 5 min intervals, and 0.1, 1, or 10 μM of a test compound was applied 4–5 min before the application of 60 mmol/L KCl. The antagonistic activity of each test compound on the N-type calcium channel was expressed as a 50% inhibitory concentration (IC_{50}), which was calculated as previously reported (Dohmoto, H; Takahara, A; Uneyama, H; Yoshimoto, R. J Pharmacol Sci. 2003, 91(2), 163) using data from at least two independent experiments.
It should be noted that the tested compounds were evaluated at three concentrations to prevent "run-down" of the cells used in the experiments, on the basis of the reproducibility data of the KCl-induced responses in the absence of drugs in our preliminary study.
8. Measurement of L-type calcium channel ($Ca_V1.1$–$Ca_V1.4$) inhibitory activity was performed as follows (previously reported in the Ref. 5): Male Sprague–Dawley rats (7 weeks old) were used. The thoracic aorta was isolated, cleared of adhering periadventitial fat, and cut into rings of 3 mm width. The endothelium was removed by gently rubbing the luminal surface. The ring was mounted in an organ bath filled with warmed (37 °C), oxygenated (95% O_2/5% CO_2) Tyrode's solution (pH 7.4). The ring was equilibrated under a resting tension of 2 g for 1 h. Next, the ring was incubated in a high K^+ solution (containing 50 mM KCl; KCl was substituted for equimolar NaCl in the Tyrode's solution) and then in Tyrode's solution for 45 min each. The solution was replaced with high K^+ solution again. After attaining the maximum contraction reaction, the test compound was cumulatively added at intervals of 90 min to attain concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M for the normal screening protocol and of 10^{-7}, 10^{-6}, 10^{-5}, $10^{-4.5}$, and 10^{-4} M for compound (**+**)-**3f**. The antagonistic activity of each test compound on the L-type calcium channel was expressed as a 50% inhibitory concentration (IC_{50}), which was calculated as previously reported (Dohmoto, H; Takahara, A; Uneyama, H; Yoshimoto, R. J Pharmacol Sci. 2003, 91(2), 163) using data from at least two independent experiments.
9. The experiments were performed as reported in the reference Koganei, H.; Shoji, M.; Iwata, S. *Biol. Pharm. Bull.* **2009**, *32*, 1695.