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The structure–activity relationship study on 2-, 5-, and 6-position of the water soluble 1,4-dihydropyridine derivatives blocking N-type calcium channels

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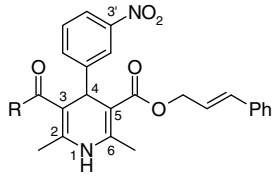
ABSTRACT

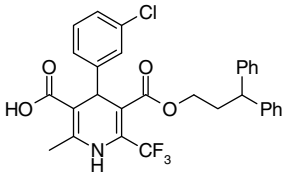
In order to find an injectable and selective N-type calcium channel blocker, we have performed the structure–activity relationship (SAR) study on the 2-, 5-, and 6-position of 1,4-dihydropyridine-3-carboxylate derivative APJ2708 (**2**), which is a derivative of Cilnidipine and has L/N-type calcium channel dual inhibitory activities. As a consequence of the optimization, 6-dimethylacetal derivative **7** was found to have an effective inhibitory activity against N-type calcium channels with more than 170-fold lower activity for L-type channel compared to that of APJ2708.

Calcium ions act as an intracellular signal and play a critical role in variety of biological functions including muscle contraction, release of neurotransmitters, calcium-dependent gene transcription, and the regulation of neuronal excitability. Several types of voltage-dependent calcium channels have been identified in native tissues and classified into at least five subtypes based on their pharmacological and functional properties: N-($Ca_V2.2$), L-($Ca_V1.1$-$Ca_V1.4$), P/Q-($Ca_V2.1$), R-($Ca_V2.3$) and T-type ($Ca_V3.1$-$Ca_V3.3$).[1]

The N-type calcium channel is a well established and characterized subtype which is expressed on nerve endings to regulate neuronal overexcitement and pain signals. Therefore, agents which inhibit the N-type calcium channel have been shown to be effective therapies for several pathological states including ischemic brain injury and neuropathic pain.[2] In fact, Ziconotide, which is the chemically synthesized version of 25-residue-peptide marine toxin ω-conotoxin MVIIA, has approved as an analgesic drug for severe chronic pain treatment. However, although this drug has been shown a non-addictive analgesic effect without opiates-like tolerance, its clinical use is limited because Ziconotide could be administered only via intrathecal route with least compliance for a patient.[3] Thus, systemically available N-type calcium channel blocker is still strongly needed and many small molecules were reported mostly for obtaining oral drug candidates.[4] An injectable N-type calcium channel blocker with high aqueous solubility is also in demand, especially for a patient who cannot take medicine orally (see Table 1).



Cilnidipine (**1**); R = OCH_2CH_2OMe
APJ2708 (**2**) ; R = OH



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In order to find a selective N-type calcium channel blockers with the least effects on cardiovascular system, we have performed a structure–activity relationship study on APJ2708 (**2**), which is a derivative of Cilnidipine (**1**) and has a unique 1,4-dihydropyridine-3-carboxylate structure with dual blockage activities at the L/N-type calcium channels. As a consequence of the optimization on 4-, 5-, and 6-position of 1,4-dihydropyridine structure in **2**, **3** was found as a promising N-type calcium channel blocker possessing potent analgesic effect in vivo with a 40-fold lower activity against L-type calcium channels than that of **2**.[4g] In this study, we made further optimization of 2-, 5-, and 6-position of 1,4-dihydropyridine ring in **2** to find a selective N-type calcium channel blocker with high aqueous solubility.

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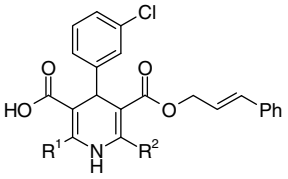
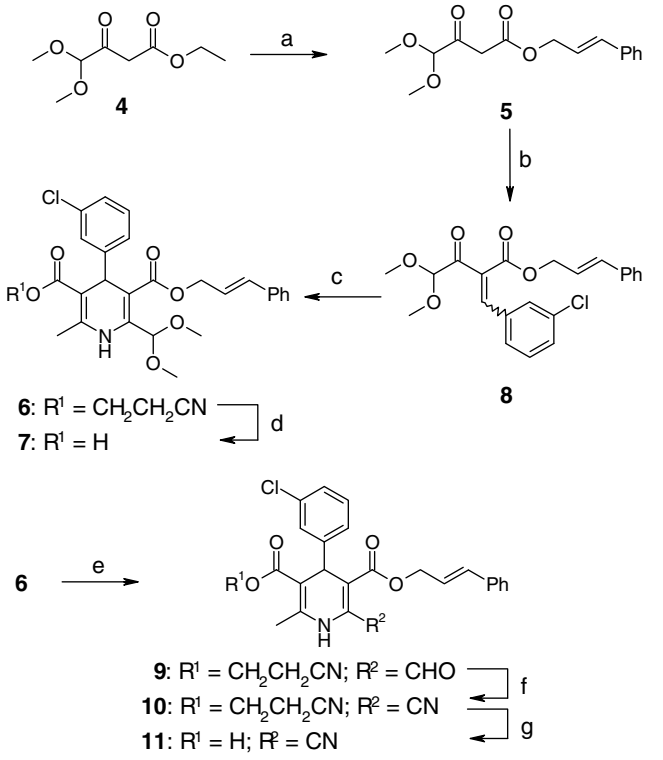
Table 1
Activity table of dihydropyridine derivatives



Compound	R^1	R^2	N-type IMR-32 IC_{50}, μM	L-type Magnus IC_{50}, μM
Cilnidipine (**1**)			1.6	0.0011
AJP2708 (**2**)			3.5	0.046
3			1.7	1.8
12a	Me	Me	1.0	0.020
12b	Me	Et	0.81	0.007
11	Me	CN	5.8	2.0
7	Me	$CH(OMe)_2$	3.0	8.1
12c	$CH(OMe)_2$	Me	3.9	1.3

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

The 1,4-dihydropyridine-3-carboxylate derivatives were synthesized using Hantzsch 1,4-dihydropyridine synthesis reaction as previously reported (Scheme 1).[4g,5] The 4,4-dimethoxy-3-oxo-butyric acid cinnamyl ester (**5**) was obtained by heating 4,4-dimethoxy-3-oxo-butyric acid ethyl ester (**4**)[6] and cinnamyl alcohol together to conduct the transesterification. The 1,4-dihydropyridine dicarboxylic acid 2-cyanoethyl ester 5-cinnamyl ester (**6**) was obtained with two step reactions, Knoevenagel condensation with 3-chlorobenzaldehyde followed by 1,4-dihydropyridine ring construction using 3-amino-2-butenoic acid 2-cyanoethyl ester. The



Scheme 1. Reagents: (a) Cinnamyl alcohol, toluene; (b) 3-Chloro-benzaldehyde, i-PrOH; (c) 3-Amino-2-butenoic acid 2-cyanoethyl ester, i-PrOH; (d) 1 M NaOH, MeOH; (e) 6 M HCl, acetone; (f) NH_2OH, AcONa, AcOH. Then Ac_2O; (g) 1 M NaOH, MeOH.

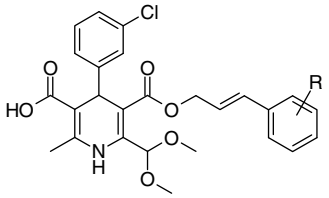
selective deprotection of 2-cyanoethyl ester group of **6** was performed by the treatment with 1 M sodium hydroxide solution to give **7**.[4g] The treatment of **6** under acidic condition yielded 6-aldehyde derivative **9** which was converted into cyanide **10** in the presence of acetic anhydride. Final selective hydrolysis with 1 M sodium hydroxide gave **11**. The rest of reported derivatives were synthesized with same method as **7** from the corresponding substituted cinnamyl alcohol, β-ketoester, enamine and benzaldehyde as starting materials.

The biological evaluation of the synthesized 1,4-dihydropyridine-3-carboxylate derivatives was performed using the procedure as previously described.[4g,h] Their inhibitory activities at the N-type calcium channel were determined using fluorescence based Ca^{2+}-flux assay in IMR-32 human neuroblastoma cells.[7,8] The L-type calcium channel inhibitory activities were estimated from the effects on high K^+-induced contraction in rat thoracic aorta ring by Magnus technique.[9,10] In our previous in vivo study, **1** can inhibit both the sympathetic N-type and vascular L-type calcium channels in antihypertensive doses (3 μg/kg, i.v. administration),[11] suggesting that its ratio of L-type/N-type blocking activity is about 1. Based on the information, the IMR model can be estimated to be about 1000 times less sensitive than the Magnus assay for detecting each calcium channel blocking action. Therefore, the IC_{50} values of the method against L-type calcium channel (Magnus method) could not be simply compared with the IC_{50} values of the cell-based IMR-32 method against N-type calcium channel.

In the present study, the structural optimization was started on the 2,6-dimethyl-1,4-dihydropyridine-3-carboxylate derivative **12a**, which had chlorine at 3′-position in place of nitro group in **2**, since **12a** showed higher inhibitory activity for N-type calcium channels than that of **2** (IC_{50} = 1.0 and 0.020 μM for N- and L-type calcium channels, respectively).[4g] The substitution of 6-methyl group in **12a** with ethyl group showed increased activities both for the N- and L-type calcium channels (**12b**, IC_{50} = 0.81 and 0.007 μM for N- and L-type calcium channels, respectively). While the introduction of cyano group (**11**) led to 5.8-times lower inhibitory activity at the N-type calcium channel than that of **12a**, but its blockage activity for L-type channel showed far larger decrease (100-fold less activity than that of **12a**), resulting in increased selectivity for N-type channel (IC_{50} = 5.8 and 2.0 μM for N- and L-type calcium channels, respectively). Furthermore, **7**, possessing dimethoxyacetal group at the 6-position, showed 400-fold decreased inhibitory activity for L-type calcium channel with only 3-times lower activity at N-type channel compared to those of **12a** (IC_{50} = 3.0 and 8.1 μM for N- and L-type calcium channels, respectively). This inhibitory activity for N-type calcium channel of **7** was enough to show the analgesic effect in vivo based on our previous result.[4h] However, the introduction of dimethoxyacetal group into 2-position (**12c**) was less effective than the substitution at the 6-position (IC_{50} = 3.9 and 1.3 μM for N- and L-type calcium channels, respectively). This result indicated that the dimethoxyacetal group at the 6-position strongly affected on the activity against L-type calcium channels but had fewer influence on the N-type activities, leading to the better selectivity for N-type channel with the preserved potency. While the introduction of dimethoxyacetal group at the 2-position of 1,4-dihydropyridine-3-carboxylate structure resulted in a smaller decrease in the activity for L-type calcium channel.

Further optimization of **7** was made on the phenyl ring of cinnamyl group (Table 2). The introduction of chlorine atom resulted in the higher inhibitory activities for N-type calcium channel than those of **7** (IC_{50} = 1.8, 1.4 and 1.7 μM for **13a**, **13b** and **13c**, respectively). However, their activities for L-type channel showed larger improvement than for N-type channel (IC_{50} = 0.7, 1.0 and 1.4 μM for **13a**, **13b** and **13c**, respectively). The derivatives with fluorine and trifluoromethyl group (**13d** and **13e**) showed similar activity

Table 2
Activity table of dihydropyridine derivatives



Compound	R	N-type IMR-32 IC_{50}, μM	L-type Magnus IC_{50}, μM
7	H	3.0	8.1
13a	2-Cl	1.8	0.7
13b	3-Cl	1.4	1.0
13c	4-Cl	1.7	1.4
13d	4-F	1.8	2.0
13e	4-CF3	2.0	3.2
13f	2-OMe	2.5	5.9
13g	3-OMe	3.0	4.4
13h	4-OMe	2.1	0.54

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

trends to **13a–c** with improved inhibitory activities at L-type calcium channel (IC_{50} = 2.0 and 3.2 μM for **13d** and **13e**, respectively). Therefore, all of these derivatives with halogen atoms (**13a–e**) showed larger improvement in the activities for L-type calcium channel rather than for N-type channel, resulting in the decreased selectivity for N-type calcium channel compared to those of **7**. The 4-methoxy derivative (**13h**) showed similar result to those of halogenated derivatives (**13a–e**) with 15-fold improvement in the inhibitory activity for L-type channel and 1.4-fold higher activity for N-type channel compared to those of **7** (IC_{50} = 2.1 and 0.54 μM for N- and L-type calcium channels, respectively). However, the blockage activity for L-type channel of 3-methoxy derivative (**13g**) was only two times higher than that of **7**. Moreover, this activity shift for the L-type calcium channel was 1.4-fold for the 4-methoxy derivative (**13f**), whose inhibitory activity for N-type channel showed 1.2-fold improvement with nearly equivalent selectivity for N-type channel to that of **7** (IC_{50} = 2.1 and 5.9 μM for N- and L-type calcium channels, respectively).

As a result of the optimizations, **7** and **13f** were found as the selective and effective N-type calcium channel blockers. Moreover, **7** and **13f** showed high aqueous solubility with low lipophilicity and were proved to be good drug candidates which could be administered via intravenous injection (Table 3).[12,13]

Finally, the N-type calcium channel inhibitory activity of compound **7**, which showed better solubility than **13f**, was confirmed by an electrophysiological study on rat superior cervical ganglion (SCG) neuron (85% inhibition at 10^{-5} M).[14]

In summary, we performed the structural optimization on 1,4-dihydropyridine-3-carboxylate derivatives to find a selective blocker for the N-type calcium channel over the L-type channel. The pharmacological characterizations of synthesized derivatives were described and the 6-dimethoxymethyl derivatives **7** and **13f** were found as the potent, selective, and aqueous soluble N-type calcium channel blockers which could be novel candidates for injectable neuroprotective and analgesic drugs.

Table 3
Aquarius solubility and $PrologD_{7.4}$ value of selected compounds

Compound	Solubility (mg/ml)	$PrologD_{7.4}$
3	3.5	3.51
7	>15	1.29
13f	10.2	1.20

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12. The sodium salt of selected compound was suspended in saline, which was sonicated for 10 min followed by shaking for 2 h 30 min at 25 °C. The suspension was kept for overnight and then, centrifuged at 10,000 rpm for 5 min. The supernatant liquid was diluted and analyzed by HPLC assay.
13. PALLAS for Windows 3.0, CompuDrug Chemistry Ltd., was employed: Tayar, N. E.; Tsai, R. S.; Carrupt, P. A.; Testa, B. *J. Chem. Soc. Perkin Trans.* **1992**, *2*, 79.
14. The electrophysiological activity for N-type calcium channel of the test compound was estimated as follows: (a) Isolation of superior cervical ganglia (SCG) neurons: Male Wistar rats (2- to 4-week-old) were decapitated after an injection of sodium pentobarbitone (50 mg/kg, i.p.). The SCG were dissected, connective tissues were removed, and three to four cuts were made in each ganglion in Ca^{2+}-free Tyrode solution. The ganglia were incubated at 37 °C under 100% O_2 atmosphere for 30 min in 20 U/ml papain and then for 60 min in a mixture of 600 U/ml collagenase and 16 mg/ml dispase. The ganglia were rinsed with the Ca^{2+}-free Tyrode solution and triturated gently through a small-pore glass pipette. The isolated SCG neurones were plated on a polylysine coated recording chamber. (b) Electrical measurements: Conventional whole-cell recordings of Ba^{2+} current through voltage-dependent Ca^{2+} channels were made with voltage-clamped conditions. The pipettes were pulled from 1.5 mm capillary glass (Narishige) in two stages by a vertical pipette puller (Narishige, PB-7). The resistance between the recording electrode filled with pipette solution and the reference electrode in external solution was 2–5 MΩ. For whole-cell recording, an application of negative pressure ruptures the captured membrane, providing continuity between the pipette electrode and the cell's cytoplasm. The dissociated SCG neuron was held at a holding potential (VH) of −60 mV, and Na/K free Ba^{2+} solution was applied from a tube within 200 μm from the SCG neuron by the 'Y-tube' method, as described elsewhere (Nakagawa, T.; Komune, S.; Vemura, T.; Akaike, N. *J. Neurophysiol.*, **1991**, *65*, 715., Uneyama, H.; Munakata, M.; Akaike, N.; *Brain Res.*, **1993**, *604*, 24., Uneyama, H.; Uneyama, C.; Akaike, N. *J. Biol. Chem.*, **1993**, *268*, 168.). The test compound was applied at 10^{-5} M. Neuron was held at a VH of −60 mV and Ba^{2+} current was elicited by 200 ms depolarizing pulses to 0 mV. Each concentration was applied to the neuron for 2 min before subsequent five depolarizing pulses were applied every 10 s. The amplitude of Ba^{2+} current was measured at the peak. The N-type current was defined as that blocked by 10^{-6} M ω-conotoxin-GVIA after the application of the test

compound. All the experiments were performed as a duplicate at room temperature. (c) Solutions: The ionic composition of Ca^{2+}-free Tyrode solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, N-2-hydroxye-thylpiperazine-N′-2-ethanesulfonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris(hydroxymethyl)aminomethane (Tris)-OH. Na/K free Ba^{2+} solution was (in mM): tetraethylammonium chloride 130, CsCl 4, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10 and glucose 10. The pH was adjusted to 7.4 with Tris-OH. The conventional patch-pipette solution contained (in mM): CsCl 75, Cs-methanesulfonate 75, ATP-Mg 2, EGTA 5 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. (d) Positive control:(*S*)-4-methyl-2-(methylamino) pentanoic acid [4,4-bis(4-fluorophenyl)butyl]amide hydrochloride (Ref. 4a) was used as a positive control (83% inhibition at 10^{-5} M).