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L-Cysteine Based N-type Calcium Channel Blockers: Structure–Activity Relationships of the C-Terminal Lipophilic Moiety, and Oral Analgesic Efficacy in Rat Pain Models

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Abstract—This study was performed to determine the structure–activity relationships (SAR) of L-cysteine based N-type calcium channel blockers. Basic nitrogen was introduced into the C-terminal lipophilic moiety of L-cysteine with a view toward improvement of its physicochemical properties. L-Cysteine derivative **9** was found to be a potent and selective N-type calcium channel blocker with IC_{50} of 0.33 μM in calcium influx assay using IMR-32 cells and was 15-fold selective for N-type calcium channels over L-type channels. Compound **9** showed improved oral analgesic efficacy in the rat formalin induced pain model and the rat chronic constriction injury (CCI) model, which is one of the most reliable models of chronic neuropathic pain, without any significant effect on blood pressure or neurological behavior.

Voltage-dependent calcium channels play crucial roles in various biological processes. Based upon their pharmacological and electrophysiological properties, these calcium channels are classified into several subtypes as L-, N-, P-, Q-, R- or T-type.[1] N-Type calcium channels are located at presynaptic terminals throughout neurons and directly mediate spinal transmission of pain signals from the peripheral to the central nervous system.

ω-Conotoxin MVIIA, a 25-amino acid peptide, is a selective blocker of N-type calcium channels that shows analgesic activity when administered intrathecally.[2] These observations strongly suggested that selective and orally active N-type calcium channel blockers could be useful for the treatment of neuropathic pain.[3]

We have recently described a discovery of lead compound for a novel series of L-cysteine-based N-type calcium channel blockers.[4] In the course of modification of N- and C-terminals of compound **1**, the phenoxybenzyl moiety at the C-terminal of L-cysteine was found to be an excellent functional group for both activity and selectivity (Chart 1). Thus, compound **2** was identified as the best compound among this series, showing improved N-type inhibitory activity (IC_{50} 0.14 μM) and selectivity (L/N = 12).[5] However, despite its good activity in vitro, **2** did not show oral analgesic efficacy in the rat formalin-induced pain model. It was speculated that this poor oral efficacy was a consequence of unfavorable physicochemical properties such as high lipophilicity (LogP = 5.0).[6] In addition, compound **2** showed weaker activity (25% inhibition at 3 μM) in electrophysiological studies than that expected from the results of calcium influx assay (IMR-32 assay) using IMR-32 human neuroblastoma cells.[7,8]

As part of our continuing efforts to identify orally active N-type calcium channel blockers, we report here modifications of the C-terminal lipophilic moiety to improve their physicochemcal properties using polar

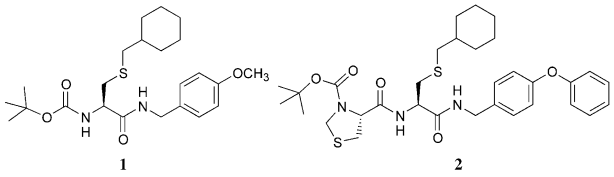

Chart 1.

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functional groups. In particular, basic nitrogen was introduced with the aim of modifing the lipophilicity of the compounds, and to maintain the same N-type blocking activity and selectivity. These modifications should be able to increase water solubility of compounds that could improve their in vivo efficacy.

Results and Discussion

The synthesis of L-cysteine based compounds were carried out by sequential condensation as reported previously.[5] The SAR study was performed based on the results of IMR-32 assay,[7,8] as well as in vitro electrophysiological experiments.[9] To estimate the selectivity for N-type calcium channels versus L-type channels, we also evaluated L-type blocking activity in terms of calcium influx assay using AtT-20 mouse pituitary tumor-derived cells (AtT-20 assay),[8,10] as inhibition of cardiac L-type calcium channels causes hypotensive side effects. In addition, in vivo analgesic efficacy was investigated using the rat formalin-induced pain model[11] and rat chronic constriction injury (CCI) model.[12] In parallel, we assessed the cardiovascular side effects of the most selective N-type calcium channel blocker.

Modification of the phenoxybenzyl moiety of compound **2** was performed to reduce lipophilicity by introduction of basic nitrogen. Compound **3**, which had an ethylenediamine moiety instead of the benzylamine moiety of **2**, was synthesized. Compound **3** exhibited improved lipophilicity (LogP = 2.8), but both its inhibitory activity for N-type calcium channels and selectivity were decreased (IC_{50} 0.38 μM, L/N = 6.3).

As **3** had a conformationally flexible ethylene–diamine moiety, we designed a conformationally restricted compound with a ring structure between the amide and terminal benzene ring to improve its selectivity (Chart 2, Table 1).

Introduction of a piperazine ring (**4**) maintained the same N-type inhibitory activity (IC_{50} 0.37 μM) as compound **3**, but L-type inhibitory potency was increased (IC_{50} 1.0 μM). Conversion of the terminal benzyl group to phenyl group (compound **5**) resulted in a further increase in L-type activity (IC_{50} 0.58 μM), although N-type activity was maintained (IC_{50} 0.39 μM).

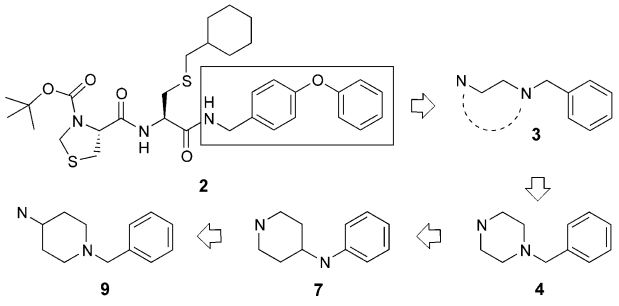
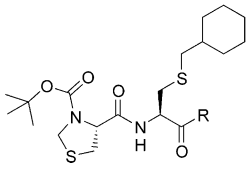


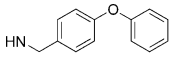
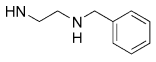
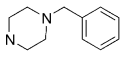
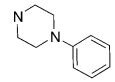
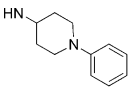
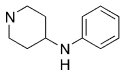
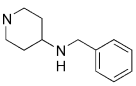
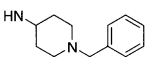
Chart 2.

Improvement of N-type inhibitory activity in IMR-32 assay was achieved by conversion of piperazine into 4-aminopiperidine (**6**). Compound **6** showed potent inhibitory activity (IC_{50} 0.15 μM) for N-type calcium channels; however, it showed the same L-type activity as compound **5**.

Consideration of the influence of basic nitrogen on inhibitory activity for L-type calcium channels revealed an interesting structure–activity relationship. Compounds with weaker basicity showed more potent L-type inhibitory activity. In contrast, the basicity did not significantly affect N-type activity in IMR-32 assay. To confirm this relationship between basicity of nitrogen and L-type inhibitory potency, we synthesized compound **7**, in which the nitrogen was shifted toward the benzene ring as compared to compound **4**. The L-type inhibitory activity of compound **7** was increased and showed the same potency as compounds **5** and **6**, which had an aniline moiety. In addition, electrophysiological experiments showed interesting results. These aniline derivatives (**5**–**7**), which had weaker basicity, showed weaker inhibitory effects on

Table 1. In vitro inhibition of calcium influx in IMR-32 and AtT-20 assays, and electrophysiological experiments using IMR-32 cells



Compd	R	Ca^{2+} influx IC_{50} (μM)[a]		Selectivity IC_{50} ratio L/N	Electrophysiology inhibition% at 3 μM (n = 3)
		N-type (IMR-32)	L-type (AtT-20)		
2		0.14	1.7	12	25
3		0.38	2.4	6.3	69
4		0.37	1.0	2.7	55
5		0.39	0.58	1.5	44
6		0.15	0.54	3.6	51
7		0.66	0.54	0.82	35
8		0.50	2.6	5.2	61
9		0.33	5.0	15	76

[a]Values represent means of multiple determinations performed in duplicate.

Table 2. Activities of selected compounds in rat formalin test and chronic constriction injury (CCI) model

Compd	Minimum effective doses (mg/kg, po)	
	Rat formalin test ($n=8$)	Rat CCI model[a] ($n=10$)
2	>100	NT[b]
9	10[c]	30

[a]Antinociceptive effects on thermal hyperalgesia induced by CCI of the sciatic nerve.
[b]NT, not tested.
[c]$p<0.025$ versus vehicle (0.5% carboxymethyl cellulose sodium solution), Williams test.

N-type currents in electrophysiological experiments than those expected from their results of IMR-32 calcium influx assay.

To increase N-type inhibitory activity and selectivity, the aniline moieties of compounds **6** and **7** were converted to benzylamine. Both of these compounds (**8** and **9**) showed reduced L-type inhibitory activity (IC_{50} 2.6 and 5.0 μM, respectively). Interestingly, L-type activity of **9** was decreased by more than 9-fold compared to its aniline analogue **6**. Thus, compound **9** was identified as the most selective compound among this series of L-cysteine-based calcium channel blockers.

Compound **9** also showed the most potent blockade of N-type calcium channel current in electrophysiological experiments (76% inhibition at 3 μM) and improved lipophilicity (logP = 3.5).

Analgesic efficacy of compound **9** was evaluated using the rat formalin-induced pain model and rat CCI model (Table 2). Compound **9** inhibited paw flinching during the persistent nociceptive phase by oral administration (10 mg/kg, po) in the formalin test and also exhibited significant antinociceptive effects on thermal hyperalgesia induced by CCI of the sciatic nerve (30 mg/kg, po). It should be noted that **9** showed no significant effect on blood pressure,[13] general behavior, or locomotor activity[14] when administrated orally (rat, 100 mg/kg).

In conclusion, as a consequence of modification of the C-terminal lipophilic moiety, a series of L-cysteine derivatives with basic nitrogen were discovered as novel neuronal N-type calcium channel blockers. Improvement of physicochemical properties was achieved without losing N-type inhibitory potency or selectivity by introduction of a basic functional group and ring structure into the C-terminal lipophilic moiety. Compound **9** was identified as a potent N-type calcium channel blocker with 15-fold selectivity for N-type calcium channels over L-type channels and also exhibited oral analgesic efficacy in the rat formalin model and CCI model. This compound is a possible candidates for further development.

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6. logP was calculated using Pallas software (version 3.0, logD Prediction Module: PrologD 2.1, released for Windows CompuDrug Chemistry Ltd., 2000).
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8. $[Ca^{2+}]_I$ was measured in cell suspension. Cell suspension was incubated with 5 mM fura-2/AM for 30 min at 37 °C. Cells were resuspended in Krebs–Ringer's HEPES solution, and adjusted to 1.0×10^6 cells/mL (IMR-32), or 2.0×10^6 cells/mL (AtT20/D16v-F2). Fluorescence (λ_{Ex}: 340 and 380 nm, λ_{Em}: 500 nm) was detected with a fluorometer. Cell suspension was incubated with test compound and 10 μM nifedipine (IMR-32) or 3 μM ω-conotoxin MVIIC (AtT-20/D16v-F2) for 360 s before high-K^+ stimulus. To evaluate the inhibitory activities of test compounds, IMR-32 and AtT20/D16v-F2 cells were used for N-type and L-type calcium channels, respectively.
9. Electrophysiological recordings were performed in the conventional whole-cell configuration under voltage-clamp conditions. Pipettes had a resistance of 3–6 MΩ. Membrane currents were measured using a patch clamp amplifier (Axopatch 2B Axon Instruments). The test compounds were applied using a rapid application method designated as the 'Y-tube method'.
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13. No significant effect on mean blood pressure or heart rate was observed after single oral administration of **9** (100 mg/kg, conscious male SD rats, $n=3$, vehicle: 0.5% carboxymethyl cellulose Na solution).
14. Compound **9** did not affect circulatory and respiratory systems, gastrointestinal transit, and locomotor activity when administrated orally (single dose, up to 100 mg/kg).