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Structure–Activity Study of L-Cysteine-Based N-Type Calcium Channel Blockers: Optimization of N- and C-Terminal Substituents

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Abstract—Synthesis and structure–activity relationship (SAR) studies of L-cysteine-based N-type calcium channel blockers are described. In the course of exploring SAR of the N- and C-terminal substituents, the L-cysteine derivative **4b** was found to be a potent N-type calcium channel blocker with an IC_{50} value of 0.14 μM on IMR-32 assay. Compound **4b** showed 12-fold selectivity for N-type over L-type calcium channels on AtT-20 assay.

Introduction

Voltage-sensitive calcium channels play crucial roles in various biological processes, and are classified into several subtypes on the basis of pharmacological and electrophysiological properties as L-, N-, P-, Q-, R- or T-type calcium channels. Among these calcium channels, the N-, P-, Q-, and R-type channels have all been shown to play key roles in neurotransmitter release.[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] Over the last decade, synthetic efforts have focused on small-molecule, non-peptide N-type calcium channel blockers for analgesia or neuroprotection, since clinical observations were reported for ω-Conotoxin MVIIA.[2] A number of small-molecule blockers of N-type calcium channels have been reported,[3,4] some of which have been shown to be active in analgesic models.[4] However, most of these compounds also block the other calcium channel subtypes, including L-type channels. Although blocking of neuronal L-type calcium channels shows neuroprotective effects,[5] inhibition of cardiac L-type calcium channels probably causes hypotensive side effects. Previously, we reported the discovery of a novel series of N-type calcium channel blockers.[6] *N*-(*t*-Butoxycarbonyl)-L-cysteine derivative **2** was identified as a lead compound for L-cysteine-based N-type calcium channel blockers, which inhibited calcium influx into IMR-32 human neuroblastoma cells[7] with an IC_{50} of 0.61 μM. Here, we report the synthesis and in vitro structure–activity relationship (SAR) studies of modification of N- and C-terminal substituents of L-cysteine.

The compounds synthesized were evaluated for inhibitory activity against both N-type (IMR-32 assay[7,9]) and L-type (AtT-20 assay[8,9]) calcium channels, focusing on selectivity to reduce cardiovascular side effects due to blocking of L-type calcium channels (Fig. 1).

Chemistry

The synthesis of L-cysteine-based N-type calcium channel blockers is outlined as shown in Scheme 1. Treatment of L-cysteine with cyclohexylmethyl bromide in the presence of aqueous sodium hydroxide in ethanol

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afforded sulfide **5**. Subsequent reaction of compound **5** with di-*tert*-butyl dicarbonate gave *N*-Boc-protected compound **6** from L-cysteine by one-pot procedure. Reaction of carboxylic acid **6** with appropriately substituted benzylamines in the presence of EDC and HOBt and subsequent deprotection gave intermediates **7** for N-terminal modification. Treatment of compounds **7** with *N*-Boc-(*R*)-thiazolidine-4-carboxylic acid in the presence of EDC and HOBt afforded compounds **4**. Compounds shown in Tables 1 and 2 were synthesized by the same method as described for compounds **4**. This procedure can be easily applied to solution-phase combinatorial synthesis using polymer-supported condensation reagents.

Compounds shown in Table 3 were synthesized from compound **4b** by deprotection followed by condensation with carboxylic acids or reductive amination with aldehydes.

Results and Discussion

The starting point in this study was an aspartic acid-derived compound **1**, which was identified as an initial lead for a novel series of N-type calcium channel blockers.[6] Compound **1** inhibited calcium influx into IMR-32 cells with an IC_{50} of 3.4 μM. In the process of exploring the SARs of this series of N-type calcium channel blockers using L-amino acids as structural motifs, compounds **2** and **3** were discovered as lead compounds for further modification.[6] These two compounds have L-cysteine residues and showed improved in vitro N-type calcium channel blocking potency (IC_{50} of 0.61 and 0.64 μM, respectively) compared to the initial lead compound **1**. In this study, we investigated the SARs and further optimization of substituents at N- and C-termini using L-cysteine as a central scaffold.

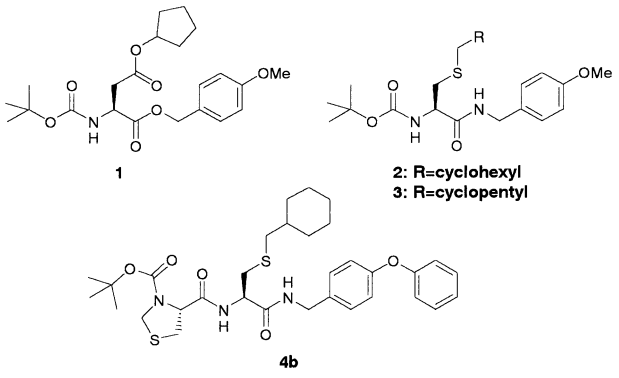
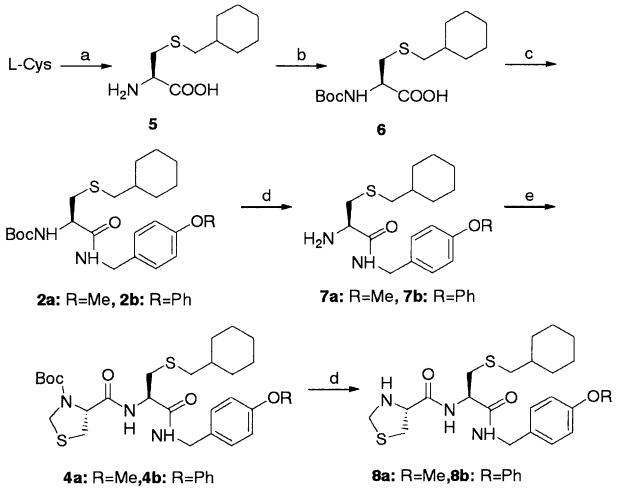


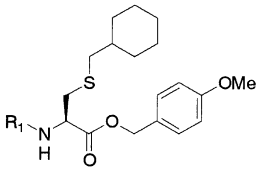
Figure 1.



Scheme 1. Reagents: (a) cyclohexylmethyl bromide, 2N NaOH, EtOH; (b) Boc_2O; (c) 4-methoxybenzylamine or 4-phenoxybenzylamine, EDC, HOBt, dichloromethane; (d) 4N HCl/dioxane; (e) *N*-Boc-(*R*)-thiazolidine-4-carboxylic acid, EDC, HOBt, dichloromethane.

The SAR of L-cysteine-based N-type calcium channel blockers was initially investigated for N-terminal substituent R_1 (Table 1). While replacement of the *tert*-butoxycarbonyl group with an *iso*-butoxycarbonyl group resulted in slight loss of activity (**9**, IC_{50} 0.72 μM), removal of the *tert*-butoxycarbonyl group resulted in 10-fold loss of inhibitory activity for N-type calcium

Table 1. Modification of the N-terminal acyl group R_1 of L-cysteine: in vitro inhibition of calcium influx in IMR-32 and AtT-20 assays



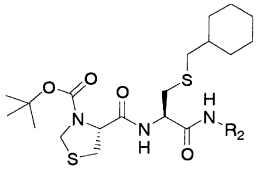
Compd	R_1	IC_{50} (μM)[a]	
		N-type (IMR-32)	L-type (AtT-20)
2		0.61	1.7
7a	H	5.8	—
9		0.72	1.5
10		1.2	2.7
11		0.92	2.3
12		1.3	1.9
4a		0.39	0.68
13		0.88	1.9
8a		2.7	3.5
14		1.9	2.7

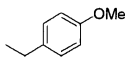
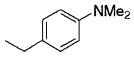
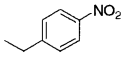
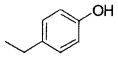
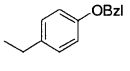
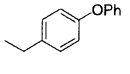
[a]Values represent means of multiple determinations performed in duplicate.

channels in IMR-32 assay (**7a**, IC_{50} 5.8 μM). This observation suggested that the lipophilic substituents enhance potency; however, substitution of the carbamate moiety with an acyl group showed decreased potency by 2-fold (**10**, IC_{50} 1.2 μM) compared to compound **2a**. Similarly, substitution with cycloalkyl group (**11** and **12**) also reduced N-type blocking activity. Screening of *N*-acyl substituents indicated that *N*-*tert*-butoxycarbonyl-thiazolidine-4-carbonyl increased N-type inhibitory potency (**4a**, IC_{50} 0.39 μM). Interestingly, compound **13**, which has an L-proline residue instead of a thiazolidine-4-carbonyl group on the N-terminal, showed lower potency (IC_{50} 0.88 μM). Comparison of **4a** and **13** showed that the thiazolidine ring led to a further enhancement of potency. Further modification of the sulfur-containing ring structure was considered, but compound **8a** (IC_{50} 2.7 μM), which did not have a *t*-butoxycarbonyl group on the thiazolidine ring, and compound **14**, which had thiazole-4-carbonyl moiety, showed very weak inhibitory activity for N-type calcium channels.

The inhibitory activity of compound **4a** for N-type calcium channels was also confirmed by electrophysiological study using IMR-32 cells (48% inhibition at 10 μM, $n=3$).[10] Therefore, the following SAR study was performed with compound **4a**, which has an *N*-*t*-butoxycarbonyl-thiazolidine-4-carbonyl at its N-terminus.

Table 2. Modification of the C-terminal amide group R_2 of L-cysteine: in vitro inhibition of calcium influx in IMR-32 and AtT-20 assays



Compd	R_2	IC_{50} (μM)[a]	
		N-type (IMR-32)	L-type (AtT-20)
4a		0.39	0.68
15		0.61	0.95
16		0.60	0.50
17		1.8	2.8
18		0.50	1.5
4b		0.14	1.7

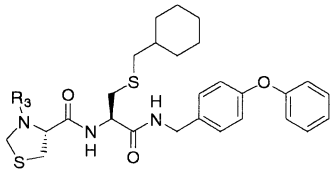
[a]Values represent means of multiple determinations performed in duplicate.

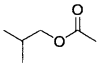
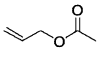
Modification of C-terminal substituent R_2 was next investigated (Table 2). Replacement of substituents on the benzene ring did not significantly affect the potency for compounds **15** ($R_2=NMe_2$; IC_{50} 0.61 μM) and **16** ($R_2=NO_2$; IC_{50} 0.60 μM). Although both of these compounds showed weaker activity than compound **4a**, there was no difference in the N-type inhibitory activity between compounds **15** and **16**. These results suggested that there was no influence of the electronic effect of the substituent on the benzene ring. On the other hand, compound **17** with a free phenol moiety showed 5-fold lower potency ($R_2=OH$; IC_{50} 1.8 μM). Compound **18**, which had benzyloxy moiety, was synthesized to estimate steric effects. Compound **18** showed slightly lower activity than compound **4a**.

As a consequence of modification of substituents on the benzene ring, compound **4b**, which had a phenoxy residue, was found to be the most potent N-type blocker among this series of compounds. Compound **4b** blocked N-type calcium channels with an IC_{50} of 0.14 μM and showed good selectivity over L-type calcium channels (selectivity ratio IC_{50} L-type/N-type = 12).

Finally, the SAR of modification of the substituents on the nitrogen atom of thiazolidine was investigated (Table 3). In contrast to the results with modification of

Table 3. Modification of substituents R_3 on the nitrogen atom of the thiazolidine ring: in vitro inhibition of calcium influx in IMR-32 and AtT-20 assays



Compd	R_3	IC_{50} (μM)[a]	
		N-type (IMR-32)	L-type (AtT-20)
4b		0.14	1.7
8b	H	0.29	2.0
19		0.12	0.87
20		0.36	1.2
21		0.33	0.44
22		0.12	0.93
23		0.20	0.60
24		0.35	1.1

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

the N-terminal acyl group R_1 (Table 1), no significant influence of substituents was observed on the N-type inhibitory activity. However, it should be noted that most of these compounds showed increased inhibitory activity and selectivity for N-type calcium channels compared to the compounds shown in Table 1. These results indicated that the phenoxy moiety played a crucial role in inhibitory potency and selectivity. Among the compounds shown in Table 3, compounds **19** (R_3 = methoxycarbonyl; IC_{50} 0.12 μM) and **22** (R_3 = 2-methoxyacetyl; IC_{50} 0.12 μM) were equipotent to compound **4b**.

In conclusion, the SAR study of a series of L-cysteine-based compounds led to the discovery of novel neuronal N-type calcium channel blockers. Compound **4b**, which had phenoxy residue on the C-terminal benzene ring, was a potent N-type calcium channel blocker and was 12-fold more selective for N-type over L-type calcium channels.

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7. IMR-32 cells were grown as described (Clementi, F.; Cabrini, D.; Gotti, C.; Sher, E. *J. Neurochem.* **1986**, *47*, 291. Carbone, E.; Sher, E.; Clementi, F. *Pflügers Arch.* **1990**, *416*, 170) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin.
8. (a) Xie, J.; Nagle, G. T.; Childs, G. V.; Ritchie, A. K. *Neuroendocrinology* **1999**, *70*, 1. Mouse pituitary tumor-derived cells (AtT-20/D16v-F2) were grown in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin–streptomycin.
9. $[Ca^{2+}]_I$ was measured in cell suspension. Cell suspensions were incubated with 5 mM fura-2/AM for 30 min at 37 °C. Cells were resuspended in Krebs–Ringer 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 suspensions were 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 AtT-20/D16v-F2 cells were used for N-type and L-type calcium channels, respectively.
10. The 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'.