Communications to the Editor

(*S*)-4-Methyl-2-(methylamino)pentanoic Acid [4,4-Bis(4-fluorophenyl)butyl]amide Hydrochloride, a Novel Calcium Channel Antagonist, Is Efficacious in Several Animal Models of Pain

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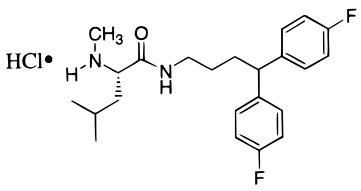
Introduction. There is considerable unmet medical need for treatments of pain and stroke. Voltage-dependent ion channels are attractive drug targets for analgesia and neuroprotection.[1,2] Voltage-dependent calcium channels (VDCC) are involved in regulation of many physiological functions of excitable cells, such as neurotransmitter and hormone release, gene expression, and muscle contraction.[1] Molecular cloning and pharmacological studies have shown that there are six known subtypes for VDCC: T, L, N, P, Q, and R.[1,3,4] Recently, the N-type calcium channel has attracted much attention as a drug target for analgesia and neuroprotection. Such interests stem in part from the following observations: First, N-type calcium channels are located at presynaptic termini of neurons, where they are directly involved in the regulation of neurotransmitter release. It is thought that N-type calcium channels play a key role in a pathological process called excitotoxicity due to ischemia or hypoglycemia.[5] Second, the transmission of pain signals from periphery to the central nervous system (CNS) is mediated by N-type calcium channels located in the spinal cord.[5] Third, ziconotide (SNX-111), a selective N-type calcium channel blocker, was found to have analgesic activity in animal models[6,7] and neuroprotective activity in focal and global ischemia models.[8−10] It has also shown marked effects in analgesia during clinical trials,[6,7] strongly supporting the above-mentioned concepts. Much has been learned about L-type calcium channels through the studies with dihydropyridines (DHPs), a class of L-type calcium channel antagonists.[11] It has been shown that inhibition of neuronal L-type VDCCs is beneficial for neuroprotection. However, inhibition of cardiac L-type calcium channel can lead to hypotension. It is believed that a rapid and profound lowering of arterial pressure tends to counteract the neuroprotective effects of L-type calcium channel antagonists. Therefore, it is desirable to have an antagonist that is selective for N-type calcium channels over L-type calcium channels to avoid the potential hypotensive effects.

Chart 1. Structure of Compound **1**



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Neuronal sodium channel antagonists have also been shown to have neuroprotective and analgesic effects.[12] To avoid side effects, it is usually desirable to have channel modulators that are selective for one subtype of certain ion channel over other subtypes of the same ion channel and other ion channels. However, in practice, it has been difficult to achieve selectivity at a meaningful level in vitro for small molecule ion channel modulators. Furthermore, in terms of in vivo efficacy, it could be beneficial to have a balanced ion channel antagonist that blocks several type ion channels such as Na^+ and Ca^{2+} channels.

A number of small-molecule N-type calcium channel antagonists have been reported in the literature.[1] The studies with those antagonists have been focused on the aspect of neuroprotection; some of them have been shown to be neuroprotective in stroke models.[1] Systematic studies on analgesic properties of small-molecule N-type calcium channel antagonists have not been reported to our knowledge.

We embarked on a project searching for small molecules that have potent activity against neuronal N-type calcium channels and show analgesic and neuroprotective effects in vivo without cardiovascular side effects. The SAR studies of several chemical series were primarily guided by IMR32 assay, and all synthetic analogues were also routinely tested in a L-type channel calcium flux assay using A10 cells (A10 assay), monitoring the activity against L-type calcium channels found in smooth muscle cells. Selected compounds were evaluated in electrophysiology to confirm that potent compounds identified in a functional assay are interfering with the channels' activity. To assess the CNS bioavailability the compounds were then tested in the audiogenic seizure model in DBA/2 mice (DBA/2 assay). Compounds with the best in vitro and in vivo profiles and desirable physical properties were evaluated in the formalin assay, Chung model, and other pain models for analgesic activity and in a head trauma model for neuroprotection. (*S*)-4-Methyl-2-(methylamino)pentanoic acid [4,4-bis(4-fluorophenyl)butyl]amide hydrochlo-

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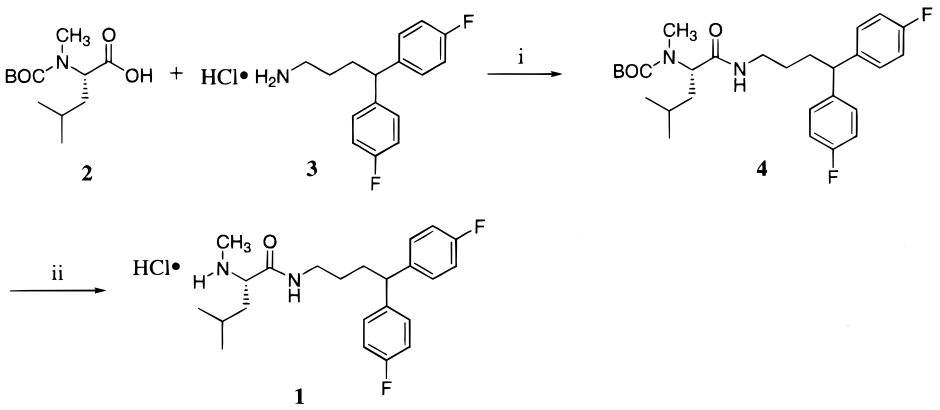
Scheme 1. Synthesis of Compound **1**[a]



[a] (i) HBTU, DMF, (*i*-Pr)$_2$NEt, rt; (ii) (a) TFA, DCM, (b) HCl/Et$_2$O.

ride, compound **1** (Chart 1), was identified as one of the most interesting compounds in terms of its overall profile, especially its pharmacological properties. In this Communication, we report the in vitro studies and the pharmacological properties of compound **1**.

Chemistry. Leucine derivative **2** (Scheme 1) was coupled with primary amine **3** to gave amide **4**. Deprotection followed by treatment of ethereal HCl provided compound **1** in 89% yield over two steps.

Biological Methods. 1. IMR32 Assay.[13] IC_{50} values for N-type calcium channel blockade were measured using the fluorescent Ca^{2+} indicator Indo-1 in IMR32 human neuroblastoma cells in the presence of 5 μM nitrendipine to block L-type channels. Fluorescence measurements were carried out on a Photon Technology International (PTI) spectrofluorometer.

2. A10 Assay.[14] IC_{50} values for L-type calcium channel blockade were measured using Oregon Green 488 Bapta-1 dye in the A10 smooth muscle cell line. Fluorescence measurements were carried out on a fluorescent image plate reader (FLIPR).

3. Electrophysiology.[15] Percent inhibition or EC_{50} values were determined by the voltage clamp assay using superior cervical ganglion neurons (SCG neurons).

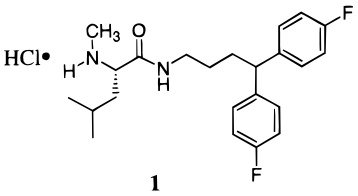
4. Acetic Acid-Induced Writhing Model.[16] Male CF-1 mice (23−30 g) were injected intraperitoneally with acetic acid (0.6%) to induce writhing behavior. The number of writhing movements in 5 min (starting 7 min after injection) was recorded.

5. DBA/2 Audiogenic Seizure Model.[17] Male DBA/2J mice 21−26 days old were exposed to a sinusoidal tone (SPL = 100 dB), swept in frequency between 8 and 16 kHz once each 10 ms for a total of 60 s. Vehicle-treated mice experienced wild running behavior, followed by clonic seizures and a tonic extension seizure of forelimbs and hindlimbs within 20 s. Absence of tonic extension was scored as an anticonvulsant effect.

6. Footpad Incision Model of Hyperalgesia.[18] Rats were anesthetized, and the footpads and plantaris muscles were incised on one hindlimb. After the wound was sutured and the mice had recovered from anesthesia, withdrawal threshold was measured with vonFrey filaments and heat hyperalgesia was measured by the method of Hargreaves (score in seconds, 12 s under control conditions).

7. Formalin Footpad Test.[19] Rats were injected with 50 μL of 5% formalin into a single hindpaw and housed individually. The number of flinching behaviors was counted for 60 s during each 10-min interval afterward for a total of 90 min.

Table 1. In Vitro Properties of Compound **1**



IMR32 (N-type) $IC_{50} \pm$ SEM (μM)	A10 (L-type) $IC_{50} \pm$ SEM (μM)	electrophysiology IC_{50}[a] (μM)
1.5 ± 0.071	0.40 ± 0.080	SCGCa: 1.3 SCGNa: 5.1 SCGK: 9.9

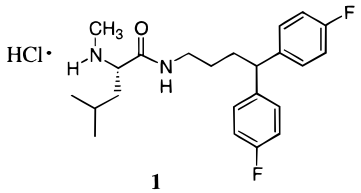
[a] The IC_{50} values are the average of two determinations.

8. Kim & Chung Model of Neuropathic Pain.[20] Rats were anesthetized, and a tight ligature was placed on the L5 and L6 dorsal roots distal to the dorsal root ganglion. 14−21 days later, 50% withdrawal thresholds were measured using calibrated vonFrey filaments.

Results and Discussions. Compound **1** inhibited the function of neuronal N-type calcium channels with an IC_{50} value of 1.5 μM as measured in the IMR32 assay (Table 1). Its antagonistic activity toward neuronal N-type calcium channels was confirmed by electrophysiology studies. It had an IC_{50} value of 1.3 μM against N-type calcium channels in SCG neurons. It was also active, to a lesser extent, against Na^+ channels (IC_{50} = 5.1 μM) and K^+ channels (IC_{50} = 9.9 μM) in SCG neurons. Therefore, compound **1** is a balanced ion channel antagonist.

Since known potent L-type calcium channel antagonists, such as nitrendipine, exhibited hypotensive properties in vivo which is undesirable for neuron protection in the case of stroke, compound **1** was evaluated for its inhibitory activity toward the function of L-type calcium channels found in smooth muscles. As measured in the A10 assay, it had an IC_{50} value of 0.4 μM indicating that compound **1** is active against L-type calcium channels but considerably less potent than nitrendipine under the assay conditions. To assess this compound's potential cardiovascular side effects in vivo, compound **1** was dosed in rat and it did not decrease blood pressure dramatically following doses up to 30 mg/kg.

Table 2. Pharmacological Properties of Compound **1**



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model	DBA/2[a]	antiwrithing[b]	Chung model[c]	formalin test[d]	formalin test[d]	incisional pain model[e]
route of admin	iv	iv	intrathecal bolus injection 12.5−100 μg	oral	iv infusion 15 and 30 mg/kg over 10 min	iv infusion 30 mg/kg over 10 min
ED_{50}	4.05 mg/kg (95% CL)[f]	4.46 ± 0.23 mg/kg[g]	23 μg (95% CL)[h]	87 mg/kg (95% CL)[i]	16 mg/kg (95% CL)[i]	active in mechanical allodynia

[a] 3 doses, 5 mice/dose. ED_{50} value is the dose of the compound estimated by statistical methods to prevent tonic seizures in 50% of the animals. Phenytoin was tested in this assay as a positive control and gave a similar result as reported in the literature.[22] [b] 3 doses, 6 mice/dose. ED_{50} value is the dose of the compound resulting in 50% reduction in the number of writhes. Morphine was tested in this assay as a positive control and gave a similar result as reported in the literature.[23] [c] 4 doses, 6 rats/dose. ED_{50} value is the dose of the compound resulting in 50% reduction in the amount of foot withdraw responding to repeated mechanical stimuli. Ziconotide was tested in this assay as a positive control and gave a similar result as reported in the literature.[24] [d] 2 doses, 8 rats/dose. ED_{50} value is the dose of the compound resulting in 50% reduction in the number of flinches/minute. Ziconotide was tested in this assay as a positive control and gave a similar result as reported in the literature.[24] [e] 8 rats/dose. Ziconotide was tested in this assay as a positive control and gave a similar result as reported in the literature.[25] [f] ED_{50} values were calculated by Probit analysis. [g] ED_{50} value was calculated by logistic fit of data using nonlinear regression analysis. [h] Statistical analyses were made with one-way and RM ANOVA and post hoc two-tailed *t*-tests. [i] ED_{50} (95% CL) was calculated by a nonlinear least-squares fit to the logistic equation.

Compound **1** has exhibited analgesic efficacy in several animal models of pain. It was bioavailable to CNS in mice as indicated by the anticonvulsant activity in the DBA/2 model (ED_{50} = 4.05 mg/kg). Compound **1** gave an ED_{50} value of 4.46 mg/kg in the antiwrithing assay, suggesting analgesic property for visceral inflammatory pain. Compound **1** also showed analgesic efficacy for neuropathic pain: intrathecal administration of compound **1** completely reversed mechanical allodynia in the Chung model (ED_{50} = 23 μg). When given by iv, compound **1** prevented the early phase and more importantly the late phase formalin-evoked nocifensive behavior in rats (ED_{50} = 16 mg/kg), suggesting efficacy particularly for delayed inflammatory pain. Furthermore, in an acute postsurgical pain model, compound **1** was shown as an efficacious postoperative analgesic agent in rats.

Both compound **1** and ziconotide have been shown to be efficacious in the Chung model, formalin test, and incisional pain model. Ziconotide was far more potent than compound **1** in these pain models.[6] However, compound **1**, being a small, nonpeptide molecule, would have some advantage over ziconotide in terms of physical and pharmacokinetic properties and access across the blood−brain barrier after systemic dosing.

Since compound **1** is a balanced ion channel inhibitor with activities against N-type and L-type calcium channels and Na^+ and K^+ channels, it is almost certain that the pharmacological properties observed in vivo are not solely due to its antagonistic activity toward calcium channels. It is difficult to assess how much its calcium channel blocking activity has contributed toward the overall in vivo pharmacological properties observed. However, comparison of compound **1** and known Na^+ channel antagonists such as phenytoin or lidocaine did reveal some major differences in their pharmacological profiles in pain models.[21] For example, compound **1** was active in the antiwrithing model, whereas phenytoin and lidocaine were inactive at a dose of 30 mg/kg (data not shown). Therefore, it is likely that some of the pharmacological properties observed with compound **1** are mainly due to N-type calcium channel blockade.

Summary. Compound **1** was identified as a small-molecule, nonpeptide, balanced Ca^{2+}, Na^+, and K^+ antagonist; it is bioavailable to CNS when given iv or orally. It is the first example of small-molecule N-type calcium channel antagonists showing potent analgesic properties in several animal models for different types of pain, and it did not exhibit hypotensive effects in rat. The in vivo activity profile of compound **1** differs from Na^+ channel blocking drugs such as phenytoin or lidocaine. The results shown in this paper extend those with ziconotide and suggest that a small molecule with specific neuronal N-type calcium channel blocking activity could be a novel and useful analgesic. Further evaluations of PK properties and neuroprotective properties are in progress.

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