Structure-Antinociceptive Activity Studies of Incarvillateine, a Monoterpene Alkaloid from *Incarvillea sinensis*

Motoyuki Nakamura[1], Yu-Ming Chi[2], Wen-Mei Yan[3], Akihiko Yonezawa[4], Yumiko Nakasugi[2], Toyokichi Yoshizawa[2], Fumio Hashimoto[1], Junei Kinjo[1], Toshihiro Nohara[1,*], Shinobu Sakurada[4]

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan
[2] Seiwa Pharmaceutical Ltd, Ibaraki, Japan
[3] Beijing University of Traditional Chinese Medicine and Pharmacy, Beijing, China
[4] Department of Pharmacology, Tohoku College of Pharmacy, Sendai, Japan

Received: March 23, 2000; Accepted: July 16, 2000

Abstract: Incarvillateine (**1**), a new monoterpene alkaloid carrying a characteristic cyclobutane ring, has been found to show significant antinociceptive activity in a formalin-induced pain model in mice. To investigate the correlation between its structure and antinociceptive activity, and especially to study whether a cyclobutane ring is necessary or not for expression of activity, we evaluated the antinociceptive activity of two constructive units of incarvillateine, such as a monoterpene unit (incarvilline, **3**) and a phenylpropanoid unit (ferulic acid, **2**) in the formalin test, and compared activity of the units with that of incarvillateine. Furthermore, in order to obtain more information about the structure-activity relationships, monoterpene alkaloid derivatives, such as incarvine C (**5**, a precursor of incarvillateine), incarvine A (**4**, an ester compound comprised of two monoterpene alkaloids and a monoterpene) and 3,3′-demethoxy-4,4′-dehydroxyincarvillateine (**6**, a synthetic new compound), were examined. The antinociceptive effect of 3,3′-demethoxy-4,4′-dehydroxyincarvillateine was equal to that of incarvillateine. Meanwhile, the other compounds exhibited no or weak activity. These results suggested that the cyclobutane moiety of incarvillateine plays an important role in expression of antinociceptive action.

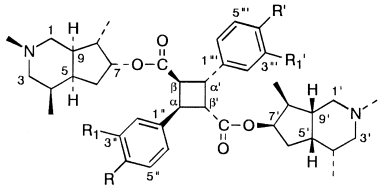
Key words: *Incarvillea sinensis* Lam., Bignoniaceae, incarvillateine, antinociceptive activity, monoterpene alkaloid, phenylpropanoid, cyclobutane ring, formalin-induced pain model.

Introduction

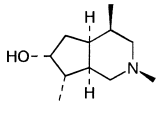
Incarvillea sinensis Lam. (Bignoniaceae) is a wild plant distributed in the northern area of China, and the dried whole plant has been traditionally used in treating rheumatism and relieving pain as an ancient Chinese crude drug designated as "Jiaohao (Kakko)". We previously reported the isolation and structural elucidation of many new alkaloids, such as the monoterpene alkaloid, incarvilline, and its derivative, incarvillateine, from the title plant (1), (2), (3), (4), (5), (6).

In the course of our investigation of its antinociceptive substance, we disclosed that incarvillateine (**1**) showed significant antinociceptive activity against a mouse pain model induced by formalin. Furthermore, we reported the antinociceptive activity of **1** and comparison of its action with morphine (7), (8). In a comparison of the antinociceptive effects of different doses of incarvillateine and morphine, the ED_{50} values of incarvillateine were about 1.06 (early phase) and 1.33 (late phase) times lower than those of morphine. In addition, the incarvillateine-induced antinociception was partially reversed by pretreatment with naloxone (a narcotic antagonist). These results suggested the possibility that its action was partially related to its influence on the central opioid pathway (8). However, studies of the structure-antinociceptive activity relationships had not been undertaken. Particularly, it is most important to know whether or not the cyclobutane ring essentially contributes to the effectiveness of **1**.

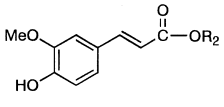
In order to examine the structure-antinociceptive activity relationships, we prepared several compounds, the minimum construction units of incarvillateine, such as (*E*)-ferulic acid (**2**) and incarvilline (**3**), and some monoterpene alkaloid derivatives, such as incarvine A (**4**), incarvine C (**5**) and 3,3′-demethoxy-4,4′-dehydroxyincarvillateine (**6**). The tested drugs were administered to mice, prior to inducer injection in a for-



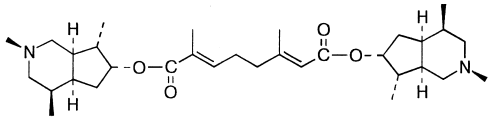
1 R=R′=OH, R_1=R_1′=OMe
6 R=R′=R^1=$R^{1'}$=H



3



2 R^2=H
5 R^2=**3**



4

Planta Med 67 (2001) 114 – 117

ISSN: 0032-0943

malin test, and the licking time of their pain reaction (paw-licking) was measured.

Materials and Methods

Plant materials

The aerial parts of *I. sinensis* were collected at Wuan County, Hebei Province, China, in August 1986. A voucher specimen (No. 86021) has been deposited in the herbarium of the Department of Kumamoto University, Kumamoto, Japan.

Isolation of monoterpene alkaloids

Incarvillateine (**1**), incarvilline (**3**), incarvine A (**4**), and incarvine C (**5**) were isolated from *I. sinensis* according to the previous reports (1), (3), (4), (5).

Chemicals

(*E*)-Ferulic acid (**2**) and (*E*)-cinnamic acid were purchased from Tokyo Kasei (Tokyo, Japan). Thionyl chloride was obtained from Kanto Chemicals (Tokyo, Japan). Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Nacalai Tesque (Kyoto, Japan). Ringer's solution was obtained from Fuso Pharmaceutical, (Osaka, Japan).

General

^{1}H-NMR: JEOL JNM-GX 500 NMR, int. standard (TMS); The other instruments and reagents used in this study were the same as those described in a previous paper (6).

*Synthesis of 3,3′-demethoxy-4,4′-dehydroxyincarvillateine (**6**)*

(*E*)-Cinnamic acid was irradiated in the solid state. (*E*)-Cinnamic acid crystals (1.0 g) were dispersed in hexane in a Pyrex flask. The flask was then irradiated with a 400-W high-pressure mercury lamp (Riko, Japan, UVL-400HA) at room temperature. After 2 days, the product was filtered off, washed with ether, and recrystallized twice from ethanol to give 750 mg of white crystals as α-truxillic acid, m.p. 274 – 275 °C (Lit. 274 – 278 °C) (9).

α-Truxillic acid (435 mg, 1.47 mmol) was refluxed with thionyl chloride (2.47 g) and *N,N*-dimethylformamide (one drop) for 3 h. Evaporation of the excess of thionyl chloride under reduced pressure left the solid α-truxillic acid dichloride. Yield: 490.0 mg (quantitative). The product (150 mg, 0.45 mmol) was dissolved in tetrahydrofuran (1 ml). Meanwhile, incarvilline (120 mg; 0.66 mmol) was dissolved in tetrahydrofuran (3 ml) and pyridine (0.5 g). The solution of dichloride was slowly instilled into the solution of incarvilline at 0 – 5 °C. Then the solution was stirred at 60 °C for 12 h. the reaction mixture was poured into ether (50 ml) and the precipitation was filtered off and dried. Compound **6** was derived from the precipitation as a yellowish-white powder performed by silica gel chromatography and eluted with cyclohexane-diethylamine-EtOH (10 : 1 : 1). Yield: 67.3 mg (32.7 %); $[\alpha]_D^{17}$: – 7.56 ° (*c* 0.40, $CHCl_3$). EIMS: *m/z* (rel. int.) = 626 $[M]^+$ (51), 418 (24), 417 (27), 384 (10), 183 (13), 182 (100), 177 (40), 167 (15), 166 (65), 165 (11), 164 (11), 163 (14), 139 (62), 131 (21), 110 (12), 109 (11), 65 (14), 58 (22). ^{1}H-NMR ($CDCl_3$): δ = 0.48 (1H, m, 6-Ha], 0.61 (3H, d, *J* = 7.1 Hz, 8-Me), 0.70 (3H, d, *J* = 6.8 Hz, 4-Me), 0.75 (3H, d, *J* = 6.8 Hz, 4′-Me), 0.81 (3H, d, *J* = 7.1 Hz, 8′-Me), 0.99 (1H, m, 6′-Ha), 1.38 (2H, m, 1, 1′-Ha), 1.53 (4H, m, 3, 3′-Ha, 6, 6′-Hb), 1.67 (2H, m, 8, 8′-H), 1.80 (2H, m, 9, 9′-H), 1.96 (2H, m, 4, 4′-H), 2.09 (2H, m, 5, 5′-H), 2.15 (6H, s, N, N′-Me), 2.40 (2H, m, 3, 3′-Hb), 2.51 (2H, m, 1, 1′-Hb), 3.89 – 4.00 (2H, m, β, β′-H), 4.36 – 4.50 (2H, m, α, α′-H), 4.86 (2H, m, 7, 7′-H), 7.21 – 7.33 (10H, aromatic-H).

Formalin test

This method represented a modification of that described by Dubuisson and Dennis (10). Male ddy mice (25 g ± 5) were used. The tested drugs (each 20 mg/kg) were prepared as suspensions with 0.5 % Tween 80/saline and intraperitoneally administered 10 minutes prior to the injection of an inducer (1 % formalin/saline, 20 μl). The mice were observed for 30 minutes and the time that the mice spent licking the injected right hindpaw was recorded. Since this test has biphasic pain response having two peaks, from 0 to 10 min (first phase) and from 10 to 30 min (second phase), the time spent licking the injected paw was recorded and the data were expressed as total licking time in the first phase and the second phase.

Statistical analysis

All values were expressed as mean ± SE (n = 10). For statistical analysis, we used one-way analysis of variance combined with Dunnett's multiple range test for multiple comparisons. Differences were considered significant at $p < 0.01$.

Results

*Antinociceptive effects on the formalin-induced licking response (Figs. **1** and **2**)*

The formalin-induced licking response has been used as a model for evaluating new analgesics (11). The duration of these nociceptive responses induced by formalin can be divided into two phases. The first phase is from 0 to 10 minutes after formalin injection, and the second phase is from 10 to 30 minutes after the injection. These phases have obvious differential properties. The pain of the early phase is evoked by the direct stimulation of the nerve fibers, and that of the late phase is due to inflammatory reaction. Centrally acting drugs such as morphine inhibited both phases equally. On the other hand, peripheral acting drugs such as aspirin inhibited only the late phase.

Intraperitoneal administration of compounds **1**, **3**, **4**, and **6**, at dose of 20 mg/kg, showed significant antinociceptive effect on the early (Fig. **1**) and late phases (Fig. **2**) of the formalin-induced licking responses. In comparison with the antinociceptive effects of **1**, that of **6** was as strong as **1**, while compounds **3** and **4** exhibited weak activity. Meanwhile, compounds **2** and **5** did not show any significant effect on both phases in the formalin test.

Discussion

Incarvillateine (**1**) is a novel monoterpene alkaloidal derivative obtained from *I. sinensis* and showed potent antinociceptive action. It presented a unique feature, namely, having a di-

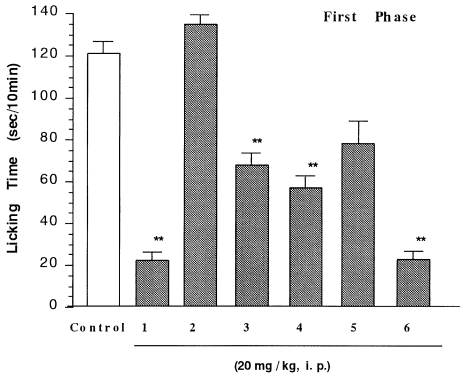


Fig. 1 The antinociceptive effects of various compounds on the first phase of formalin-induced pain response in mice. Samples are **1**: incarvillateine, **2**: ferulic acid, **3**: incarvilline, **4**: incarvine A, **5**: incarvine C and **6**: 3,3′-demethoxy-4,4′-dehydroxy-incarvillateine. Each value represents mean ± S.E. (n = 10). Significant differences between control and drug-treated groups are indicated by ** < 0.01.

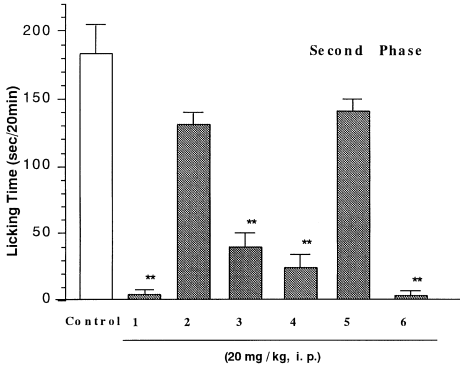


Fig. 2 The antinociceptive effects of various compounds on the second phase of formalin-induced pain response in mice. Samples are **1**: incarvillateine, **2**: ferulic acid, **3**: incarvilline, **4**: incarvine A, **5**: incarvine C and **6**: 3,3′-demethoxy-4,4′-dehydroxy-incarvillateine. Each value represents mean ± S.E. (n = 10). Significant differences between control and drug-treated groups are indicated by ** < 0.01.

meric structure in the molecule. We considered that incarvillateine (**1**) was biosynthetically derived through dimerization of incarvine C (**5**), which was regarded to be formed by esterification between incarvilline (**3**), a major monoterpene alkaloid of *I. sinensis*, and (*E*)-ferulic acid (**2**). In order to investigate the structure-antinociceptive activity relationships, we used these compounds and some other monoterpene alkaloids derivatives, such as incarvine A (**4**), incarvine C (**5**) and 3,3′-demethoxy-4,4′-dehydroxyincarvillateine (**6**) in the formalin test.

In the present study, (*E*)-ferulic acid (**2**) and incarvine C (**5**) showed no antinociceptive activity in both phases in the formalin-induced pain response. Furthermore, incarvilline (**3**) and incarvine a (**4**) exhibited the antinociceptive effect, but the activity was lower compared to incarvillateine (**1**). Meanwhile, 3,3′-demethoxy-4,4′-dehydroxyincarvillateine (**6**), a synthetic new compound, showed strong antinociceptive effect in a dosage of 20 mg/kg on both phases and its activity was equal to that of incarvillateine (**1**).

An important finding in the present study is that incarvilline (**3**) might be rated as a basic antinociceptive substance, but its activity was not so strong. While incarvillateine (**1**) and incarvine A (**4**) have two units of incarvilline (**3**), respectively, the antinociceptive effect of **4** was lower than that of **1**. These results suggested that the number of incarvilline units was not so important in this case and the difference in activity between **1** and **4** was caused by their components. The former has a dimer formation of ferulic acid in the molecule, whereas the latter has a linear monoterpene unit (Hildebrandt's acid). Furthermore, this suggestion was supported by the result that incarvine C (**5**), an incarvilline 7-*O*-ferulate, considered to be the precursor of incarvillateine (**1**), showed no significant antinociceptive activity.

In conclusion, these results clearly indicate that the important factor of the antinociceptive effects of incarvillateine (**1**) was the presence of a monoterpene alkaloid and a dimeric structure carrying a cyclobutane ring in its component. Especially, the dimeric construction might play an important role in expression of its strong activity. This suggestion was supported by the result that the antinociceptive intensity of 3,3′-demethoxy-4,4′-dehydroxy-incarvillateine (**6**), a synthesized new incarvillateine derivative, was parallel to that of **1**. There is a possibility that stronger antinociceptive compounds could be synthesized using other types of incarvillateine derivatives. Further investigation is required to develop the potent antinociceptive substances.

References

[1] Chi Y-M, Yan W-M, Chen D-C, Noguchi H, Iitaka Y, Sankawa U. A monoterpene alkaloid from *Incarvillea sinensis*. Phytochemistry 1992; 31: 2930 – 2
[2] Chi Y-M, Yan W-M, Li J-S. An alkaloid from *Incarvillea sinensis*. Phytochemistry 1990; 29: 2376 – 8
[3] Chi Y-M, Hashimoto F, Yan W-M, Nohara T. Incarvine A, a monoterpene alkaloid from *Incarvillea sinensis*. Phytochemistry 1995; 40: 353 – 4
[4] Chi Y-M, Hashimoto F, Yan W-M, Nohara T. Two alkaloids from *Incarvillea sinensis*. Phytochemistry 1995; 39: 1485 – 7
[5] Chi Y-M, Hashimoto F, Yan W-M, Nohara T, Yamashita M, Marubayashi N. Monoterpene alkaloids from *Incarvillea sinensis*. VI. Absolute stereochemoistry of incarvilline and structure of a new alkaloid, hydroxyicarvilline. Chem. Pharm. Bull. 1997; 45: 495 – 8
[6] Chi Y-M, Hashimoto F, Yan W-M, Nohara T. Four monoterpene alkaloid derivatives from *Incarvillea sinensis*. Phytochemistry 1997; 46: 763 – 9
[7] Chi Y-M, Hashimoto F, Nohara T, Nakamura M, Yoshizawa T, Yamashita M et al.. Incarvilleae alkaloids and analgesic, sedative substance. 38th Symposium on the Chemistry of Natural Products. Sendai, Japan: October 14, 1996: Symposium Papers 43 – 8
[8] Nakamura M, Chi Y-M, Yan W-M, Nakasugi Y, Yoshizawa T, Irino N et al. Strong antinociceptive effect of incarvillateine, a novel monoterpene alkaloid from *Incarvillea sinensis*. J. Nat. Prod. 1999; 62: 1293 – 4
[9] Baracchi A, Chimichi S, Sio FD, Donati D, Nesi R, Sarti-Fantoni P et al. Preparation of Chloro-α-truxillic acid via 3-methyl-4-nitro-5-styrylisoxazole photodimers. Heterocycles 1986; 24: 2863 – 70
[10] Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effect of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977; 4: 161 – 74

[11] Hunskaar S, Hole K. The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. Pain 1987; 30: 103 – 14

Prof. Dr. T. Nohara, Ph. D.

Faculty of Pharmaceutical Sciences
Kumamoto University
5-1 Oe-honmachi
Kumamoto 862-0973
Japan
E-mail: none@gpo.kumamoto-u.ac.jp
Fax: +81-96-362-7799