

## Pharmacological Study on the Novel Antinociceptive Agent, a Novel Monoterpene Alkaloid from *Incarvillea sinensis*

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To determine the antinociceptive mechanism of incarvillateine (INCA), the opiate antagonists nor-binaltorphimine (nor-BNI),  $\beta$ -funaltrexamine ( $\beta$ -FNA) and naltrindole (NTI) were pretreated prior to its injection in a formalin test. The antinociceptive effect of INCA was antagonized by nor-BNI ( $\kappa$ -receptor antagonist) and  $\beta$ -FNA ( $\mu$ -receptor antagonist), while NTI ( $\delta$ -receptor antagonist) did not influence its effect. Furthermore, the antinociceptive effect of INCA was blocked by theophylline (THEO), an adenosine-receptor antagonist. These results suggested that the antinociceptive effect arose from the activation of  $\mu$ -,  $\kappa$ -receptors and adenosine-receptor.

**Key words** monoterpene alkaloid; incarvillateine; antinociceptive effect; opioid receptor; adenosine receptor

*Incarvillea sinensis* LAM. (Bignoniaceae) is a wild plant distributed in the northern area of China, and dried whole plants have traditionally been used in treating rheumatism and relieving pain as an ancient Chinese crude drug designated “Tougucao”.<sup>1)</sup>

In the course of our investigations of its antinociceptive substances, a number of novel monoterpene alkaloids and macrocyclic spermine alkaloids have been characterized.<sup>2–9)</sup> One of the monoterpene alkaloids, incarvillateine (INCA, Fig. 1), demonstrated a significant antinociceptive effect against the mouse pain model induced by formalin. We also reported the antinociceptive effect of INCA and comparison of its action with morphine (MOR). In comparison with antinociceptive effects of different doses of INCA and MOR, the ED<sub>50</sub> values of INCA were about 1.06 (early phase) and 1.33 (late phase) times lower than those of MOR. In addition, the antinociceptive effect of INCA in early phase was partially antagonized by pretreatment with naloxone (a narcotic antagonist, 5 mg/kg, s.c), while the effect of morphine was completely reversed. These results suggested the possibility that the action of INCA was partially related to its influence on the central opioid pathways.<sup>10)</sup> However, details on the pharmacological aspect of the mechanism have not, to our knowledge, been undertaken.

In order to examine the antinociceptive mechanism, some opiate antagonists and adenosine receptor antagonist were administered to mice prior to incarvillateine injection in a formalin test, and the licking time of their pain reaction (paw licking) was measured.

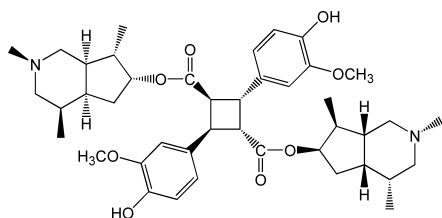


Fig. 1. Chemical Structure of Incarvillateine

## MATERIALS AND METHODS

**Chemicals** INCA was prepared according to the previous report.<sup>2)</sup> Nor-binaltorphimine (nor-BNI) and  $\beta$ -funaltrexamine ( $\beta$ -FNA) were purchased from Tocris Cookson (Bristol, U.K.). Naltrindole (NTI) was obtained from Sigma (St. Louis, U.S.A.). Theophylline (THEO) and Tween 80 (polyoxyethylene sorbitan monooleate) were obtained from Nacalai Tesque (Kyoto, Japan). Ringer solution was purchased from Fuso Pharmaceutical (Osaka, Japan).

**Formalin Test** This method represented a modification of that described by Dubuisson and Dennis.<sup>11)</sup> Male ddy mice (25 ± 5 g) were used. The tested drugs were prepared as suspensions with 0.5% Tween 80/saline. Since the test has a biphasic pain response with two peaks, from 0 to 10 min (early phase) and from 10 to 30 min (late phase), the time spent licking the injected paw was recorded and the data were expressed as total licking time in the early phase and late phase.

**Treatments of Antagonists** The experiments were performed according to a modified method described by Kamei *et al.*<sup>12)</sup> and Santos *et al.*<sup>13)</sup> In measurement of the early phase,  $\beta$ -FNA (40 mg/kg, s.c) and nor-BNI (20 mg/kg, s.c) were administered 24 h before the inducer treatment, while NTI (0.5 mg/kg, s.c) and THEO (5 mg/kg, s.c.) were treated 10 min prior to the inducer injection.

In measurement of the late phase,  $\beta$ -FNA and nor-BNI were administered 24 h before the inducer treatment. NTI and THEO were administered at the same time and 5 min later than the inducer treatment, respectively.

**Statistical Analysis** All values were expressed as mean ± S.E. ( $n = 10$ ). 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 Effect of INCA on Formalin-Induced

**Licking Response** The formalin-induced licking response has been used as a model for evaluating new analgesics.<sup>14,15)</sup>

The duration of the nociceptive response induced by formalin can be divided into two phases. The early phase is from 0 to 10 min after formalin injection, and the late phase is from 10 to 30 min 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.

In the previous study, we reported that intraperitoneal administration of INCA, at doses of 5 to 20 mg/kg, produced a marked and dose-dependent antinociceptive activity against both the neurogenic (early phase) and inflammatory (late phase) pain responses induced by formalin. The ED<sub>50</sub> values (mg/kg with 95% confidence limits) were 12.5 (9.08—17.2) and 5.6 (4.41—7.10) for early and late phases, respectively. The antinociception caused by INCA in the formalin test was found, at least partly, to be related to an opioid-like action. This observation was substantiated by the demonstration that INCA-induced antinociceptive activity against the neurogenic pain response was partly reversed by naloxone.<sup>10)</sup>

**Effects of Selective Opioid-Receptor Antagonists on the Antinociceptive Effect of INCA** The effects of  $\beta$ -FNA, a selective  $\mu$ -opioid receptor antagonist, NTI, a selective  $\delta$ -opioid receptor antagonist, and nor-BNI, a selective  $\kappa$ -opioid receptor antagonist, on the antinociceptive effect of INCA are summarized in Figs. 2 and 3. The antinociceptive effect of INCA was significantly antagonized by pretreatment of  $\beta$ -FNA and nor-BNI in both early and late phases. However, pretreatment with NTI did not affect the antinociceptive potency in either phase.

**Effect of Adenosine-Receptor Antagonist on the Antinociceptive Effect of INCA** The effect of THEO, an adenosine-receptor antagonist, on the antinociceptive effect of INCA is summarized in Figs. 2 and 3. This effect on INCA was significantly reversed by pretreatment of THEO in both early and late phases, and the action was stronger than that of any opiate antagonist.

## DISCUSSION

INCA is a representative of the novel monoterpene alkaloidal derivatives obtained from *I. sinensis*. It presented the unique feature of having a dimeric structure in the molecule. Furthermore, it displayed a significant antinociceptive effect which was partially blocked by pretreatment of naloxone, the narcotic antagonist, in the early phase of the formalin test.<sup>10)</sup> In the present study, we used the more selective opioid receptor antagonists nor-BNI,  $\beta$ -FNA and NTI in the formalin test. INCA-induced antinociception in both early and late phases was markedly reduced by s.c. pretreatment with  $\beta$ -FNA, a selective  $\mu$ -opioid receptor antagonist, and nor-BNI, a selective  $\kappa$ -opioid receptor antagonist. On the other hand, the antinociception was not antagonized by s.c. pretreatment with NTI, a selective  $\delta$ -opioid receptor antagonist, indicating that the antinociceptive effect of INCA resulted from the activation of  $\mu$ - and  $\kappa$ -opioid receptors. In conclusion, although INCA had a strong antinociceptive activity as well as MOR, the

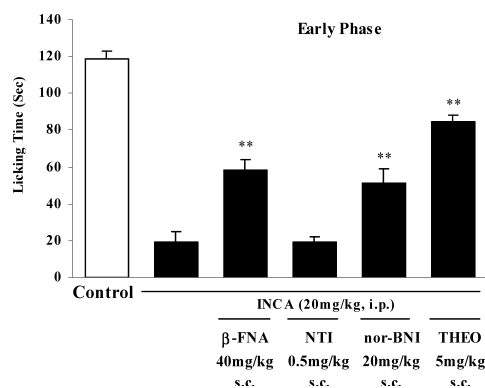


Fig. 2. Antinociceptive Effect of INCA (20 mg/kg, i.p.) and Reversed Effects of  $\beta$ -FNA (40 mg/kg, s.c.), NTI (0.5 mg/kg, s.c.), nor-BNI (20 mg/kg, s.c.) and THEO (5 mg/kg, s.c.) on the Early Phase of Formalin-Induced (1%, 20  $\mu$ l) Pain Response in Mice

Each column represents mean  $\pm$  S.E. ( $n=10$ ). Significant differences between INCA treated group and drug treated groups are indicated by \*\* $p<0.01$ .

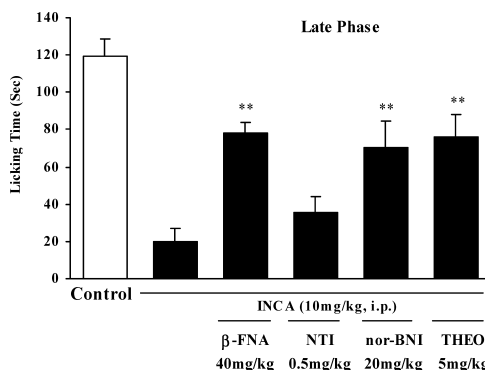


Fig. 3. Antinociceptive Effect of INCA (10 mg/kg, i.p.) and Reversed Effects of  $\beta$ -FNA (40 mg/kg, s.c.), NTI (0.5 mg/kg, s.c.), nor-BNI (20 mg/kg, s.c.) and THEO (5 mg/kg, s.c.) on the Late Phase of Formalin-Induced (1%, 20  $\mu$ l) Pain Response in Mice

Each column represents mean  $\pm$  S.E. ( $n=10$ ). Significant differences between INCA treated group and drug treated groups are indicated by \*\* $p<0.01$ .

mechanism of antinociception was different. MOR can act, to some extent, on all three of the opioid-receptor subtypes, however, the  $\mu$ -receptor is by far the most important.<sup>16)</sup> Meanwhile, INCA can equally bind to the  $\mu$ - and  $\kappa$ -receptors.

An important finding in the present study was that THEO, an adenosine receptor antagonist, could significantly decrease the antinociceptive effect of INCA. This result suggested that the antinociceptive effect of INCA also resulted from the strong activation of adenosine receptor. The administration of adenosine analogs and adenosine kinase inhibitors produced antinociceptions in behavioral studies using the formalin test. Furthermore, these antinociceptive effects were inhibited by adenosine receptor antagonists such as caffeine or THEO.<sup>17)</sup> The antinociceptive effect of MOR was likewise reversed by pretreatment of THEO.<sup>18)</sup>

In conclusion, these results clearly indicated that the antinociceptive effect of INCA was equally mediated *via*  $\mu$ - and  $\kappa$ -receptors. Furthermore, the mechanism of antinociception was mediated by adenosine receptor. These findings suggested that INCA might become a new type of antinociceptive agent having a different mechanism of action from that of morphine. Further investigation is required to eluci-

date the exact mechanism which underlies these effects.

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