Sanjoinine A Isolated from Zizyphi Spinosi Semen Augments Pentobarbital-Induced Sleeping Behaviors through the Modification of GABA-ergic Systems

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Received May 10, 2007; accepted June 20, 2007

Zizyphi Spinosi Semen (ZSS) has been widely used for the treatment of insomnia in oriental countries. This experiment was performed to investigate whether sanjoinine A, one of major alkaloid compounds of ZSS, has hypnotic effects and/or enhances pentobarbital-induced sleeping behaviors through the γ-aminobutyric acid (GABA)-ergic systems. Sanjoinine A itself did not induce sleeping at the higher dose used in this experiment. However, sanjoinine A prolonged sleeping time and reduced the sleeping latency induced by pentobarbital in a dose-dependent manner similar to muscimol, a GABA A receptor agonist. Sanjoinine A also increased sleeping rate and sleeping time when administered combined with pentobarbital at a sub-hypnotic dosage and showed synergistic effects with muscimol in potentiating sleeping onset and enhancing sleeping time induced by pentobarbital. In addition, both sanjoinine A and pentobarbital increased chloride influx in primary cultured cerebellar granule cells. Sanjoinine A also showed similar effects with muscimol in potentiating chloride influx inducing effects of low dose pentobarbital. Sanjoinine A decreased GABA A receptor α-subunit expression and increased γ-subunit expression, and had no effects on the abundance of β-subunits in primary cultured cerebellar granule cells, showing different subunit expression from pentobarbital. In addition, we found that sanjoinine A also enhanced expression of glutamic acid decarboxylase (GAD), but pentobarbital did not. In conclusion, sanjoinine A itself does not induce sleeping, but it augments pentobarbital-induced sleeping behaviors through the modification of GABA-ergic systems.

Key words Zizyphi Spinosi Semen (ZSS); sanjoinine A; sleep; pentobarbital; Cl⁻ influx; γ-aminobutyric acid (GABA) receptor subunit

Chronic insomnia affects a significant proportion of the population. Current pharmacological approaches focus primarily on the γ-aminobutyric acid (GABA)-ergic system, the major inhibitory neurotransmitter system of the central nervous system (CNS). Drugs that enhance synaptic GABA-ergic neurotransmission are widely utilized in the clinical setting for their sedative or hypnotic effects. Several classes of compounds—GABA, benzodiazepines, barbiturates, steroids and alcohol—act at different sites of the GABA/benzodiazepine receptors-chloride channel complex to increase the opening of the channel. In this way, they increase chloride currents through the GABA A receptors and enhance inhibitory synaptic transmission. GABA A receptors have been known to play an important role in the modulation of barbiturate-induced sleeping through the interaction with GABA-ergic system.1)

Zizyphi Spinosi Semen (ZSS), the dried seed of Zizyphus jujuba Mill. var. spinosa (Rhamnaceae), has been used as a tranquilizer, an analgesic and an anticonvulsant in oriental countries such as China, Japan and Korea for centuries,2–4) and also has been prescribed for the treatment of insomnia and anxiety in Asia.5) In modern pharmacological studies, ZSS is known to contain many pharmacologically active components such as alkaloids, triterpenes and flavones.5–12) These compounds have been shown to have central inhibitory activity.4,13–17) Cyclopeptide alkaloids from ZSS were demonstrated to be the major hypnotic components of ZSS.18) Therefore, we were interested to investigate whether sanjoinine A (Fig. 1), one of major alkaloid compounds, exerts hypnotic effect and/or enhances pentobarbital-induced sleeping behaviors through GABA-ergic systems.

Animals Male ICR mice (Samtako, Korea) weighing 18–22 g, in groups of 10–15, were used for all the experiments. Animals were housed in acrylic cages (45 × 60 × 25 cm) with water and food available ad libitum under an artificial 12-h light/dark cycle (light on at 7:00 am) and at a constant temperature (22±2 °C). To ensure adaptation to the new environment, the mice were kept in the departmental holding room for 1 week before testing. All the mice were maintained in accordance with the National Institute of Toxicological Research on the Korea Food and Drug Administration guidelines for the care and use of laboratory animals.

Extraction and Isolation of Sanjoinine A ZSS was purchased from an oriental drug store in Seoul, Korea. ZSS (300 g) was extracted three times with 11 of hexane in a reflux condenser for 24 h. The residues were extracted with methanol for 24 h, and this methanol extraction was evaporated to dryness by rotary evaporation; the solvent was removed by rotary evaporation and partitioned between 5%

Fig. 1. Chemical Structure of Sanjoinine A

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hydrochloric acid (60 ml) and ether (50 ml). This aqueous acid solution was extracted three times more with ammonia hydrate (pH 9.0) and chloroform (60 ml×3). The alkaloid fractions in chloroform solution were combined, filtered through Whatman No. 1 filter paper, and concentrated using a rotary vacuum evaporator followed by lyophilization. The yield of the alkaloid fraction was about 0.03% (w/w). Using combined flash column chromatography and preparative thin layer chromatography, fourteen alkaloids were isolated in a crystalline state from the alkaloid fraction of ZSS. The alkaloids were labeled as Sanjoinine-A, B, C, etc. in the order of increasing polarity. The isolation yields were highly varied ranging from $1.4\times10^{-3}$ to $5\times10^{-3}$%. The chemical structures of sanjoinine A were established by a combination of chemical correlation methods and spectral analysis (Fig. 1).19

**Pentobarbital-Induced Sleeping** Pentobarbital sodium (Hanlim Pharm. Co., Ltd., Korea) was diluted in physiologically saline and administered to each mouse intraperitoneally (i.p.) to induce sleep. Test samples suspended in 1% CMC in physiological saline were administered orally (p.o.) to animals (10 ml/kg). 10—15 mice were used for each treatment group. Muscimol (Sigma, U.S.A.) was administered as a reference drug 15 min prior to administration of pentobarbital. All experiments were carried out between 1:00 and 5:00 pm. Animals were fasted for 24 h prior to the experiment. Pentobarbital was given to animals placed in a box 30 min after the oral administration of test samples. Those animals that stopped moving in the box within 15 min after pentobarbital injection were immediately transferred to another box. Those individuals that stayed immobile for more than 3 min were judged to be asleep. The time that elapsed from receiving pentobarbital until an animal, positioned delicately on its back, lost its righting reflex represented the latency to onset of sleep. The animals were observed constantly, and the time of awakening, characterized by righting of the animal, was noted. The sleeping time was defined as the time taken for the animal to regain spontaneous movements after having been transferred to the second box. Animals that failed to fall asleep within 15 min after pentobarbital administration were excluded from the experiments.20,21

**Cell Culture** Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8 d old Sprague-Dawley rats (Samtako, Korea) as previously described.22 After culture for 8 d, these cells express functional GABA$_A$ receptors, with an expression pattern similar to that apparent in the cerebellum during postnatal development but different from that observed in the adult rat cerebellum. Briefly, cells were plated (1×10$^5$ cells per 0.2 ml) in 96 microplates or (2×10$^6$ cells per 2.0 ml) in 60-mm dishes that had been coated with poly-$\alpha$-lysine (10 $\mu$g/ml) (Sigma, U.S.A.). The cells were cultured in basal Eagle's medium (Life Technologies, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, U.S.A.), 2.0 mM glutamine, gentamicin (100 $\mu$g/ml), antibiotic--antimycotic solution (100 unit/ml; Sigma, U.S.A.) and 25 mM KCl. This high concentration of potassium was necessary to induce persistent depolarization, which promotes the survival of granule cells. Cytosine arabinofuranoside (10 $\mu$m final concentration; Sigma, U.S.A.) was added to cultures 18—24 h after plating to inhibit the proliferation of nonneuronal cells.

**Measurement of Intracellular Cl$^-$/ Influx** The intracellular Cl$^-$ concentration of cerebellar granule cells was estimated using the Cl$^-$ sensitive fluorescence probe MQAE according to the method of West and Molloy with a slight modification.23 The buffer (pH 7.4) used contained the following: 2.4 mM HPO$_4^{2-}$, 0.6 mM H$_2$PO$_4$, 10 mM HEPES, 10 mM $\beta$-glucose and 1.0 mM MgSO$_4$. A variety of MQAE-loading conditions were assessed. The cells were incubated overnight in a medium containing 10 mM MQAE (Dojindo, Japan). After loading, the cells were washed three times in the relevant Cl$^-$ containing buffer. The buffer was replaced with buffer with or without the compounds or control. Repetitive fluorescence measurements were initiated immediately using a FLUOstar (excitation wavelength: 320 nm; emission wavelength: 460 nm; BMG LabTechnology, Germany). The data is presented as the relative fluorescence Fo/F, where Fo is the fluorescence without Cl$^-$ ions and F is the fluorescence as a function of time. The Fo/F values were directly proportional to [Cl$^-$]$^i$.$^23$

**GABA$_A$ Receptor Subunits and Glutamic Acid Decarboxylase (GAD)65/67 Expression** Cells were maintained for a total of 8 d in culture and then treatment of sanjoinine A was initiated. Sanjoinine A was dissolved in ethanol and diluted sequentially in culture medium to final concentrations of 1.25, 2.5 and 5.0 $\mu$M. Control cells were treated with solvent alone at the same dilution as that used for drug treatment (0.1% v/v). The culture medium was completely replaced every day with fresh medium containing the appropriate drug. After treatment of sanjoinine A and pentobarbital, cells were harvested and treated with lysis buffer. The extracts were centrifuged at 20000 $g$ for 20 min. Equal amounts of proteins were separated on a SDS 12% polyacrylamide gel, and transferred to a nitrocellulose membrane (Hybord ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.). The blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline solution (10 mM Tris, pH 8.0 and 150 mM NaCl) containing 0.05% Tween-20. The membrane was incubated with the specific rabbit polyclonal antibodies against GABA$_A$ receptor subunits (1 : 500; Santa Cruz Biotechnology Inc.) or GAD65/67, for 6 h at room temperature. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). The immunoreactive proteins were detected using the ECL Western blotting detection system.

**Statistical Analysis** The results are presented as the mean±S.E.M. The significance of the effects of the compounds was assessed using analysis of variance (ANOVA). In case of significant variation, the individual values were compared with Dunnett’s test. For the sub-hypnotic dosage of pentobarbital-treated experiment, a Chi-square test was used to compare the proportions of sleep onset between sub-hypnotic pentobarbital alone-treated group and each of the other groups.

**RESULTS**

**Hypnotic Effects of Pentobarbital, Muscimol and Sanjoinine A** Pentobarbital induced sleeping in a dose dependent manner. We investigated the hypnotic effects of muscimol and different doses of sanjoinine A in rodents without pentobarbital treatment, and found that sanjoinine A alone
could not induce sleeping even at a very high dose (100 mg/kg). These results were similar to those of muscimol (Table 1).

**Effects of Sanjoinine A on Sleeping Onset of Mice Treated by a Sub-hypnotic Dosage of Pentobarbital** Sanjoinine A increased the rate of sleep onset and the duration of sleeping time induced by a sub-hypnotic dosage of pentobarbital (28 mg/kg, i.p.). Pretreatment with muscimol also increased the rate of sleep onset and prolonged the sleep time due to a sub-hypnotic dosage of pentobarbital (Table 2). It was found that sanjoinine A had similar effects to muscimol on sleeping behavior induced by pentobarbital in both experiments.

**Enhancement of Sanjoinine A of the Latency and Duration of Sleeping in Pentobarbital-Treated Mice** Sanjoinine A decreased the latency of sleep and increased sleeping time induced by pentobarbital (42 mg/kg) in a dose-dependent manner. Pretreatment in mice with muscimol (0.2 mg/kg i.p.) as a positive control 15 min before the administration of pentobarbital also decreased the latency of sleep and increased the total sleeping time (Fig. 2).

**Synergistic Effects of Sanjoinine A with Muscimol on Sleeping in Pentobarbital-Treated Mice** To investigate the direct relationship between the effects of muscimol and sanjoinine A, sanjoinine A (0.5 mg/kg)-treated groups were pretreated with low doses of muscimol (0.05 mg/kg), which did not potentiate sleeping induced by pentobarbital. Muscimol and sanjoinine A alone did not affect the sleeping time induced by a hypnotic dosage of pentobarbital. However, co-administration of muscimol (0.05 mg/kg) significantly decreased sleep latency and increased sleeping time induced by pentobarbital in the sanjoinine A (0.5 mg/kg)-treated groups (Fig. 3).

**Increase of Sanjoinine A on Chloride Influx in Primary Cultured Cerebellar Granule Cells** Resting intracellular Cl\(^-\) concentrations were calibrated using standard Cl\(^-\) solutions of 0, 10, 20 and 40 mM Cl\(^-\), each containing 140 mM K\(^+\). Appropriate amounts of methylsulfate were used to replace Cl\(^-\) in these solutions. Tributyltin chloride (5.0 \(\mu\)M) and nigericin (5 \(\mu\)M) were present to artificially facilitate the balance between intracellular Cl\(^-\) and extracellular Cl\(^-\) concentrations. Resting [Cl\(^-\)]i in cultured cerebellar granule cells was 13.9 ± 2.4 mM, and treatment of granule cells with sanjoinine A (2.5 and 5.0 \(\mu\)M) increased [Cl\(^-\)]i to 41 and 47 mM respectively. Pentobarbital (10 \(\mu\)M) also increased the influx of Cl\(^-\) in primary cultured cerebellar granule cells. Sanjoinine A (1.25 \(\mu\)M) and muscimol (20 \(\mu\)M) treatment alone could not induce a significant Cl\(^-\) influx increase in cultured granule cells, while sanjoinine A (1.25 \(\mu\)M) potenti-

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**Table 1. Sleep-Inducing Effects of Pentobarbital, Sanjoinine A and Muscimol in Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>No. falling asleep/total</th>
<th>Sleeping time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentobarbital</td>
<td>28</td>
<td>5/15</td>
<td>13.8 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>15/15</td>
<td>55.4 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>15/15</td>
<td>197.6 ± 16.7</td>
</tr>
<tr>
<td>Muscimol</td>
<td>10</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>Sanjoinine A</td>
<td>100</td>
<td>0/15</td>
<td>0</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of 15–16 mice. \(p < 0.05\), \(p < 0.01\), compared to that of the control group.

**Table 2. Effects of Sanjoinine A on Sleeping Onset of Mice Treated by Sub-hypnotic Dosage of Pentobarbital (28 mg/kg, i.p.)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>No. falling asleep/total</th>
<th>Sleeping time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5/15</td>
<td>13.8 ± 5.4</td>
</tr>
<tr>
<td>Muscimol</td>
<td>0.2</td>
<td>12/15*</td>
<td>55.4 ± 11.4</td>
</tr>
<tr>
<td>Sanjoinine A</td>
<td>0.25</td>
<td>6/15</td>
<td>13.9 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>8/15</td>
<td>18.6 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>13/15**</td>
<td>65.3 ± 10.7**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. (\(n = 15–16\)). \(p < 0.05\), \(p < 0.01\), compared to that of the control group.
ated effects of 2.5 μM pentobarbital, which did not potentiate Cl− influx also, in increasing the influx of Cl−. Similarly, co-treatment of muscimol (20 μM) and pentobarbital (2.5 μM) significantly increased Cl− influx in primary cultured cerebellar granule cells (Fig. 4).

Expression of GAD65/67 by Pentobarbital and Sanjoinine A The effects of treatment of cerebellar granule cells for 5 d with 2.5 μM sanjoinine A or 10 μM pentobarbital on the abundance of GAD were examined. Chronic treatment of pentobarbital showed no effect on the abundance of GAD, treatment of sanjoinine A enhanced the amount of the GAD significantly in cultured cerebellar granule cells (Fig. 5).

Subunits Expression of GABA A Receptors by Pentobarbital and Sanjoinine A GABA A subunits expression was examined after treatment with 2.5 μM sanjoinine A or 10 μM pentobarbital in the cerebellar granule cells for 5 d. Sanjoinine A treatment induced a significant decrease in the amount of the α-subunit and increase in γ-subunit, but had no effect on the abundance of the β subunit. The amounts of α-subunit were reduced by chronic pentobarbital treatment (Fig. 6).

DISCUSSION

We investigated the effects of different doses of sanjoinine A, one of alkaloids from ZSS, and muscimol in rodents without pentobarbital treatment. Sanjoinine A alone could not induce sleeping even at a very high dose. These results were similar to those of muscimol. In addition, sanjoinine A increased the rate of sleep onset and the duration of sleeping time induced by sub-hypnotic dosage of pentobarbital. Changes in pentobarbital-induced sleep time can be a useful tool for examining stimulatory or inhibitory effects on the CNS, in particular for investigating influences on the GABA-ergic system.24) Barbiturates interact with GABA receptors and modulate GABA-ergic effects.25,26) Many hypnotic, anti-anxiety and anti-epilepsy drugs prolong pentobarbital-induced sleeping time. We were interested in whether sanjoinine A prolonged pentobarbital-induced sleeping behaviors via the GABA-ergic systems. It has been reported that alkaloids from ZSS have sedative effects.16,17,27) The present study showed that administration of sanjoinine A prior to pentobarbital injection was associated with a statistically significant decrease of sleep latency and prolongation of pentobarbital-induced sleeping time. Therefore, we suggest that the sanjoinine A may play an important role in the hypnotic effect of ZSS. Muscimol, a standard GABA A receptors agonist, also potentiated the onset and prolonged the sleeping time in mice. Furthermore, sanjoinine A showed synergistic effects with muscimol in pentobarbital-treated mice. This indicates the involvement of the GABA receptor complex.

GABA A receptors possess different binding sites such as GABA, benzodiazepine and barbiturate. GABA A receptors form heteromeric GABA-gated chloride channels are assembled from a large family of subunit genes. GABA A receptor channels open after binding GABA to give a net inward flux of negative chloride ions (outward current), hyperpolarizing the membrane and reducing neuronal firing.28,29) The pharmacological profile of a GABA A receptor depends upon subunit composition, and distinct GABA A receptor subtypes differ in subunit composition. Sanjoinine A alone could not induce sleep but enhanced pentobarbital-induced sleeping. This suggests that there are differences between sanjoinine A and pentobarbital in their pharmacological properties on GABA A receptors. Sanjoinine A in combination with muscimol showed synergistic effects on pentobarbital-induced sleeping and increased Cl− influx in a similar way as pento-
barbital, sanjoinine A also showed similar effects with muscimol in potentiating Cl⁻ influx inducing effects of low dose pentobarbital. This indicated that sanjoinine A might act on functions of GABA receptor to induce Cl⁻ channel opening, and modulate pentobarbital-induced pharmacological properties like a GABA receptor agonist. Because the activation of glycine receptors also increase chloride ion channels open that facilitate inhibitory neurotransmission in the mammalian in brain, it is also possible that sanjoinine A might increase Cl⁻ influx by glycine receptors.30,31)

We tried to find out the typical responding subunits of the effective dosage of sanjoinine A, which might have at least a close relationship with the acting site by which sanjoinine A acts on GABA_A receptors and exerts its sleeping potentiating effects. We also investigated the effects of sanjoinine A and pentobarbital on expression of GAD65/67 expression by western blot technique. GAD65/67, which is necessary for GABA synthesis, plays a major role in GABA transmission in normal physiological condition. Sanjoinine A increased the GAD expression significantly in cultured cerebellar granule cells. It is suggested that sanjoinine A might activate GAD, then increase GABA transmission.

On the other hand, variations in GABA_A receptor subunit composition confer unique pharmacological, biophysical, and electrophysiological properties to each receptor subtype and most native GABA_A receptors are composed of two α, two β and a γ subunit.32) Evidence suggests that subunit composition may determine the pharmacology of GABA_A receptors.32) The importance of subunit composition is reflected in recent evidence from mutant mouse literature suggesting that different GABA_A receptor subunits modulate different drug-induced behaviors.33) Multiple compounds (GABA, benzodiazepines, barbiturates, alcohol, neurosteroids, etc.) act at different sites of the GABA/benzodiazepine receptor-chloride channel complex to increase the opening of the channel, and hence enhance inhibitory synaptic transmission.34) The presence of any one of them influences the binding of the others. For example, barbiturates will bind more strongly to the receptor when GABA also is bound. Drugs that bind to the barbiturate site along with benzodiazepines and neurosteroids, act as allosteric modulators to enhance neuronal inhibition and result in sleep consolidation. We found that both sanjoinine A and pentobarbital decreased the abundance of the GABA_A receptor α-subunit, but only sanjoinine A increased the abundance of the γ-subunit. These different changes may be of the explanations for its different drug-induced sleeping behaviors.33)

Many herbal preparations and a rich chemical diversity of agents used to promote sleep is known acting on GABA_A receptor.35,36) Agents acting on GABA_A receptors mainly act to increase synaptic inhibition either by directly activating GABA_A receptors or, more usually, by enhancing the action of ligands on GABA_A receptors. This latter action is known as positive modulation and is considered to involve agents.

Fig. 6. Effects of Sanjoinine A on GABA_A Receptors Subunits in Primary Cultured Cerebellar Granule Cells

Each column represents the mean plus S.E.M. *p<0.05, **p<0.01, compared to that of the control group.
acting on allosteric sites on GABA_A receptors remote from the traditional ligand recognition sites.\(^3\) There is a significant diversity of GABA_A receptor subtypes composed of different protein subunits. The discovery of subtype specific agents is a major challenge in the continuing development of GABA_A receptor pharmacology and such allosteric sites are regarded as good targets for the development of subtype specific drugs.\(^3\)

Our results suggested that sanjoinine A might exert its sleeping potentiating effects by three pathways; 1st, increase GABA synthesis by GAD activation; 2nd, increase sensitivity of GABA receptor to pentobarbital or GABA by influence GABA receptor subunit compositions, especially by increasing \(\gamma\) subunit expression; 3rd, it is also possible that sanjoinine A activate GABA receptors directly. A considerable number of herbal constituents whose behavioral effects and pharmacological actions have been well characterized may be good candidates for further investigations that may ultimately lead to clinical use. An increasing number of herbal products have been introduced into psychiatric practice in the past decade. The potential benefits of herbal remedies such as St. John’s wort and kava-kava in psychiatric practice have been addressed.\(^3\) ZSS might be another good candidate for use in psychiatric illnesses such as sleeping disorders.

In conclusion, all the data presented here indicate that the sanjoinine A itself did not induce sleeping and only enhanced hypnotic effects in pentobarbital-treated mice. The GABA receptor–chloride channel complex might be involved in the mechanisms of these effects. It is also suggested that pentobarbital and sanjoinine A acts on GABA receptors pharmacologically differently. Further investigation is needed in order to understand the pharmacological actions of sanjoinine A.

**Acknowledgements** This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (The Regional Research Universities Program/Center for Healthcare Technology Development).

**REFERENCES**