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Antitussive principles of *Glycyrrhizae radix*, a main component of the Kampo preparations Bakumondo-to (Mai-men-dong-tang)

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Abstract

We attempted to elucidate the antitussive principles of *Glycyrrhizae radix*, a main component of Bakumondo-to (Mai-men-dong-tang). Although the 50% methanol-eluted fraction (100 mg/kg, p.o.) caused a more than 60% reduction in the number of capsaicin-induced coughs, neither the water-eluted nor 100% ethanol-eluted fractions of water extract of *G. radix* had antitussive effects. The water extract of *G. radix* contained high levels of liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside and glycyrrhizin. On the other hand, the 50% methanol-eluted fraction contained mainly liquiritin and liquiritin apioside, but not the other compounds. Liquiritin apioside (3–30 mg/kg, p.o.), but not liquiritin, isoliquiritin, isoliquiritin apioside or glycyrrhizin, dose-dependently inhibited the number of coughs. Methysergide, a serotonin receptor antagonist, antagonized the antitussive effect of liquiritin apioside. However, the antitussive effect of liquiritin apioside was not antagonized by naloxone. Pretreatment with glibenclamide (3 mg/kg, i.p.), an ATP-sensitive potassium channel blocker, also significantly reduced the antinociceptive effect of liquiritin apioside. These results suggest that *G. radix* contains a potent antitussive compound, liquilitin apioside, whose antitussive effect may depend on both peripheral and central mechanisms.

Keywords: *Glycyrrhizae radix*; Bakumondo-to (Mai-men-dong-tang); Antitussive effect; Cough; Kampo preparation

1. Introduction

Bakumondo-to (TJ-29: Mai-men-dong-tang) has been used to treat severe dry cough in patients with bronchitis and pharyngitis (Asano et al., 1993; Takahama and Miyata, 1995). *Glycyrrhizae radix* is a main component of Bakumondo-to. It has been used since ancient Egyptian times as a drug for the respiratory organs, and has long been used as a flavoring and sweetening agent as well as a drug for analgesic and expectorant in Europe. Furthermore, *G. radix* has been used as a very important crude drug in many Kampo preparations (traditional Chinese medicine) and acts as an antispasmodic, carminative and antidote, and is also taken for bronchial problems, coughs, mucous congestion, stomach problems, such as peptic ulcers, and for bladder and kidney ailments.

There has been no previous detailed study on the antitussive principle, since previous studies on *G. radix* have mostly focused on glycyrrhizin, one of its components. In the present study, we attempted to elucidate the antitussive principles of *G. radix*.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Tokyo Animal Laboratory, Tokyo, Japan), weighing about 300–350 g, were used. The animals were housed in groups of four per cage under a 12-h light–dark cycle with food and water available continuously. This study was carried out in accordance with the Declaration of Helsinki and/or with the guide for the care and use of laboratory animals as adopted by the

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committee on the care and use of laboratory animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

2.2. Antitussive assay

The cough reflex was induced as described previously (Kamei et al., 1989, Kamei and Kasuya, 1992). Briefly, animals were exposed to a nebulized solution of capsaicin (30 μM) under conscious and identical conditions using a body plethysmograph. Capsaicin was dissolved to a concentration of 30 mg/ml in a 10% ethanol and 10% Tween 80 saline solution. The solution was diluted with saline. The animals were exposed for 7 min to capsaicin 60 min before the injection of drugs to determine the frequency of control coughs (Cc). The animals were also exposed for 7 min to capsaicin 60 min after the injection of drugs. The number of coughs produced after antitussive drug injection (Ct) was compared with the number of control coughs (Cc). The antitussive effect was expressed as the % inhibition of the number of control coughs ((Cc − Ct)/Cc × 100).

2.3. Isolation of compounds from G. radix

The air-dried root of *Glycyrrhiza uralensis* (5.0 kg) was crushed and extracted with H_2O under reflux two times. The water extract was suspended in 30 l of water, and chromatographed on Diaion HP20 (Mitsubishi Kasei, Tokyo, Japan), eluting with 20 l of water (fraction 1; 235 g), 20 l of 50% methanol (fraction 2; 138 g), and then 20 l of 100% methanol (fraction 3; 115 g).

The 50% methanol extract was further chromatographed on silica gel (eluted with $CHCl_3/CH_3OH/H_2O = 10:3:1$) to give three fractions as checked by TLC, and then subjected to C_{18} column chromatography (500 g, Cosmosil 140 C_{18}-OPN, Nakarai Chemicals, Japan), eluting with H_2O/CH_3CN (4:1) to obtain liquiritin (3.9 g) and liquiritin apioside (726 mg). The 100% methanol extract was further chromatographed on a silica gel column with gradient elution of $CHCl_3/CH_3OH$ (1:4 → 1:1), and then subjected to C_{18} column chromatography (500 g, Cosmosil 140 C_{18}-OPN, Nakarai Chemicals), eluting with H_2O/CH_3CN (4:1) to obtain isoliquiritin apioside (210 mg) and isoliquiritin (120 mg). Liquiritin, liquiritin apioside, isoliquiritin and isoliquiritin apioside were identified by comparison with the spectral data reported previously (Nakanishi et al., 1985; Aida et al., 1989; Kobayashi et al., 1995).

2.4. 3-Dimensional (3D) high performance liquid chromatographic (HPLC) analysis of the water extract of G. radix

2.4.1. Analysis of G. radix by 3D-HPLC

Crushed *G. radix* (0.25 g) was extracted with H_2O (50 ml) under sonication for 30 min. Twenty microliters of this eluate was subjected to HPLC analysis with an LC-10AD system (Shimadzu) using a TSKgel ODS-80T_S column (250 × 4.6 mm), eluting with a gradient [0.05 M CH_3COONH_4 (pH 3.6)/$CH_3CN = 9:1 \rightarrow 0:10$, for 60 min]. The flow rate was 1.0 ml/min. The eluate was monitored from 200 to 400 nm.

2.4.2. Analysis of 50% methanol-eluted fraction by HPLC

Fraction 2 (0.03 g) was extracted with 50% methanol (50 ml) under sonication for 30 min. Twenty microliters of this eluate was subjected to HPLC analysis with an LC-10AD system (Shimadzu), using a TSKgel ODS-80T_S column (250 × 4.6 mm), eluting with a gradient [0.05 M CH_3COONH_4 (pH 3.6)/$CH_3CN = 9:1 \rightarrow 0:10$, for 60 min]. The flow rate was 1.0 ml/min. The eluate was monitored from 200 to 400 nm.

2.5. Drugs

Glycyrrhizin was purchased from Alps Pharmaceutical Industry (Gifu, Japan). Dihydrocodeine hydrochloride was purchased from Sankyo (Tokyo, Japan). Bakumondo-to (TJ-29) was purchased from Tsumura and Co. (Tokyo, Japan). Methysergide maleate, glibenclamide and naloxone hydrochloride were purchased from Sigma (St. Louis, MO, USA). Methysergide and naloxone were dissolved in 0.9% saline. All other drugs were suspended in 0.5% carboxyl methylcellulose solution. Methysergide (3 mg/kg, i.p.) and naloxone (0.3 mg/kg, i.p.) were each injected 30 min before the administration of antitussive drugs. Glibenclamide (10 mg/kg, i.p.) was injected 5 min before the administration of antitussive drugs. Feeding probe was used when drugs and their vehicle were given p.o. The dose and schedule for methysergide, naloxone and glibenclamide in this study were determined as described previously (Kamei et al., 1991, 1996; Morita and Kamei, 2000).

2.6. Statistics

Data are expressed as means ± S.E. The statistical significance of differences was assessed by the Mann–Whitney *U*-test to evaluate the antitussive effect. A level of probability of 0.05 or less was considered significant. ED_{50} values for the antitussive effect and 95% confidence limits (95% CL) were determined using linear regression techniques.

3. Results

3.1. Antitussive effects of the eluted fraction of the water extract of G. radix

Exposed for 7 min to capsaicin 60 min before and after the injection of vehicle of drugs induced 23.3 ± 3.0 coughs/7 min and 19.3 ± 1.6 coughs/7 min, respectively. The effect of vehicle on the number of capsaicin-induced coughs was not significant.

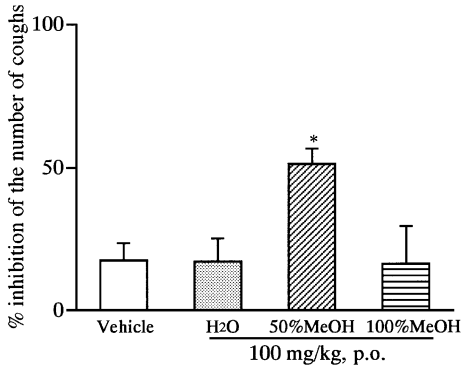

Fig. 1. Antitussive effects of water (H_2O)-, 50% methanol (50% MeOH)- and 100% methanol (100% MeOH)-eluted fraction of the water extract of *G. radix*. The antitussive effects were assessed 60 min after p.o. administration of each fraction (100 mg/kg). The effects of each fraction on the number of capsaicin-induced coughs were determined. Each column represents the mean ± S.E. ($n = 5–8$). *$P < 0.05$ vs. vehicle-treated group.

The antitussive effects of the eluted fraction of the water extract of *G. radix* are shown in Fig. 1. Fifty percent methanol-eluted fraction (fraction 2) had a potent antitussive effect when administered orally (p.o.) in guinea pigs (Fig. 1). Although the 50% methanol-eluted fraction (fraction 2), at a dose of 100 mg/kg, p.o., caused a more than 50% reduction in the number of capsaicin-induced coughs, similar effects were not seen with the same dose (100 mg/kg, p.o.) of water-eluted (fraction 1) and 100% ethanol-eluted fraction (fraction 3).

3.2. Antitussive effects of liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside and glycyrrhizin

Liquiritin apioside, at a dose of 30 mg/kg, p.o., caused a more than 70% reduction in the number of capsaicin-induced coughs (Fig. 2). However, at the same dose (30 mg/kg, p.o.), neither liquiritin, isoliquiritin, isoliquiritin

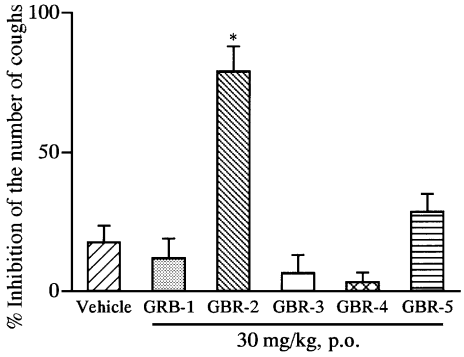

Fig. 2. Antitussive effects of liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside and glycyrrhizin. The antitussive effects of liquiritin (GBR-1), liquiritin apioside (GBR-2), isoliquiritin (GBR-3), isoliquiritin apioside (GBR-4) and glycyrrhizin (GBR-5) were assessed 60 min after p.o. administration of each drug. The effects of liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside and glycyrrhizin on the number of capsaicin-induced coughs were determined. Each column represents the mean ± S.E. (n = 5–8). *$P < 0.05$ vs. vehicle-treated group.

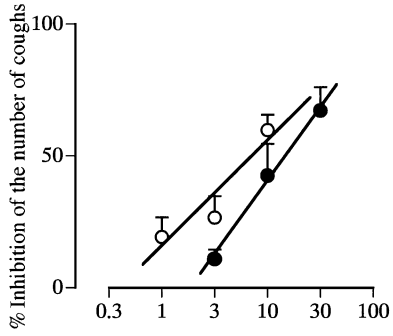

Fig. 3. Dose–response relationships of the antitussive effects of liquiritin apioside and dihydrocodeine in guinea pigs. The antitussive effects of liquiritin apioside (closed circles) and dihydrocodeine (open circles) were assessed 60 min after p.o. administration of each drug. The effects of liquiritin apioside and dihydrocodeine on the number of capsaicin-induced coughs were determined. Each column represents the mean ± S.E. ($n = 6–8$).

apioside nor glycyrrhizin had a significant effect on the number of capsaicin-induced coughs (Fig. 2). Furthermore, as shown in Fig. 3, liquiritin apioside and dihydrocodeine, at doses of 3, 10 and 30 mg/kg, p.o., dose-dependently inhibited the number of capsaicin-induced coughs when the antitussive effect was examined 60 min after injection. The antitussive ED_{50} values (95% confidence limit) of liquiritin apioside and dihydrocodeine were determined to be 14.5 (8.1–25.7) and 7.9 (5.1–10.7) mg/kg, respectively.

3.3. Effects of methysergide, naloxone and glibenclamide on the antitussive effect of liquiritin apioside

Pretreatment with methysergide (3 mg/kg, i.p.), a selective 5-HT receptor antagonist, significantly reduced the

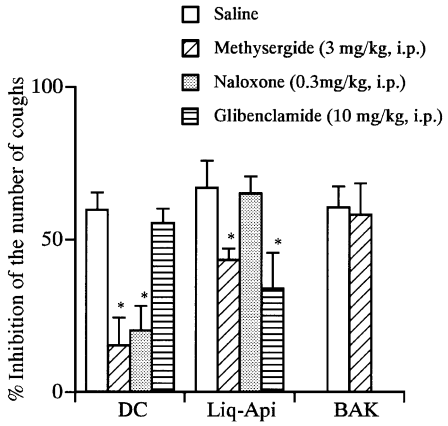

Fig. 4. Effects of methysergide, naloxone and glibenclamide on the antitussive effect of dihydrocodeine, liquiritin apioside and Bakumondo-to. Methysergide (3 mg/kg) and naloxone (0.3 mg/kg) were each injected i.p. 30 min before the administration of dihydrocodeine, liquiritin apioside and Bakumondo-to. Glibenclamide (5 mg/kg) was injected i.p. 5 min before the administration of liquiritin apioside and dihydrocodeine. The antitussive effect of dihydrocodeine (DC, 10 mg/kg), liquiritin apioside (Liq-Api, 30 mg/kg) and Bakumondo-to (BAK, 1 g/kg) was assessed 60 min after p.o. administration. Each column represents the mean ± S.E. ($n = 6–8$). *$P < 0.05$ vs. respective saline pretreated group (vehicle).

antitussive effect of dihydrocodeine, a centrally acting narcotic antitussive drug (with saline, 68.7 ± 8.6%, $n = 6$; with methysergide, 32.3 ± 5.2%, $n = 7$). Furthermore, methysergide partially antagonized the antitussive effect of liquiritin apioside (Fig. 4). However, the antitussive effect of Bakumondo-to (1 g/kg, p.o.) was not antagonized by pretreatment with methysergide (with saline, 60.7 ± 6.8%, $n = 7$; with methysergide, 58.3 ± 10.2%, $n = 7$). Moreover, naloxone significantly reduced the antinociceptive effect of dihydrocodeine, but not that of liquiritin apioside (Fig. 4).

On the other hand, pretreatment with glibenclamide (10 mg/kg, i.p.), an ATP-sensitive K^+ channel blocker, significantly reduced the antinociceptive effect of liquiritin apioside, but not of dihydrocodeine (Fig. 4).

3.4. 3D-HPLC analysis of the water extract of G. radix

As shown in Fig. 5A, the water extract of *G. radix* contained high levels of liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside and glycyrrhizin, and these actual contents were 3.9%, 3.7%, 0.6%, 0.4% and 10.8%, respectively. The 50% methanol-eluted fraction (fraction 2) of the water extract of *G. radix*, which showed potent antitussive activity, contained mainly liquiritin and liquiritin apioside, but not the other compounds (Fig. 5B). On the other hand, 100% methanol-eluted fraction (fraction 3) of the water extract of *G. radix*, which showed no antitussive activity, contained mainly isoliquiritin, isoliquiritin apioside and glycyrrhizin (Fig. 5C).

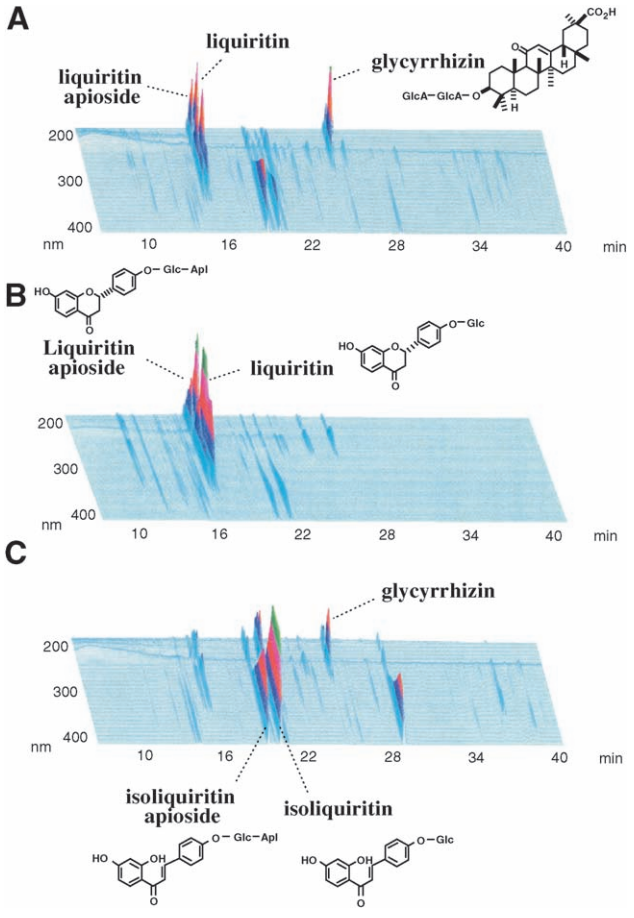

Fig. 5. 3D-HPLC analysis of the water extract of *G. radix* (A), 50% methanol-eluted fraction (B) and 100% methanol-eluted fraction of the water extract of *G. radix* (C).

4. Discussion

A traditional Chinese medicinal prescription, Bakumondo-to (Mai-men-dong-tang), consists of six herbs (*Ophiopogonis tuber*, *Pinelliae tuber*, *Zisyphi fructus*, *G. radix*, *Ginseng radix* and *Oryzaeae fructus*). Miyata and his coworkers previously reported that Bakumondo-to has potent antitussive effects in several experimental models of cough in guinea pigs (Miyata et al., 1989, 1999), including capsaicin-, citric acid-, or substance P-induced coughs and codeine-resistant coughs caused by the inhalation of neurokinin A (Takahama et al., 1993) or by treatment with an angiotensin-converting enzyme inhibitor (Miyata et al., 1999). In addition, Bakumondo-to inhibited the increase in the discharge of superior laryngeal nerve afferents in bronchitic animals, suggesting that the site of the antitussive action of Bakumondo-to may be in the airway (Miyata et al., 1989, 1999). These pharmacological characteristics of Bakumondo-to in antitussive actions are consistent with those of ophiopogonin-D, a steroidal glycoside that was originally isolated from *O. tuber*, suggesting that ophiopogonin-D may be one of the antitussive components in Bakumondo-to (Miyata et al., 1999).

In the present study, we demonstrated that *G. radix*, one of the main components of a Bakumondo-to, also contained a potent antitussive compound: liquiritin apioside. The antitussive potency of liquiritin apioside was as high as that of dihydrocodeine. In the present study, we also demonstrated that although methysergide completely antagonized the antitussive effect of dihydrocodeine, the antitussive effect of liquiritin apioside was only partially antagonized by pretreatment with methysergide. However, the antitussive effect of liquiritin apioside was not also antagonized by naloxone. We previously demonstrated that a reduction in the level of serotonin (5-HT) in the whole brain decreased the potency of antitussive drugs that acted at the central nervous system, but not peripherally (Kamei et al., 1987). Neonatal treatment with 5,7-dihydroxytryptamine, which is sufficient to reduce whole brain levels of 5-HT to 19% of control levels, resulted in supersensitivity to the cough-depressant effect of dihydrocodeine (Kamei et al., 1988). Furthermore, the potentiation of the antitussive effect of dihydrocodeine observed in 5,7-dihydroxytryptamine-treated rats was abolished by pretreatment with methysergide, a 5-HT receptor antagonist. Therefore, the marked increase in the antitussive effect of dihydrocodeine might have been due to changes in the sensitivity of 5-HT receptors. Thus, we proposed that 5-HT receptors play an important role in the cough-depressant

activities of centrally acting, but not peripheral acting, antitussive drugs (Kamei et al., 1987, 1988). On the other hand, we previously reported that the antitussive effect of subcutaneously administered or inhaled moguisteine, a peripherally acting non-narcotic antitussive drug (Gallico et al., 1994; Morikawa et al., 1997; Ishii et al., 1998; Sant'Ambrogio and Sant'Ambrogio, 1998), was reduced by pretreatment with glibenclamide, an ATP-sensitive K^+ channel blocker, in a dose-dependent manner (Morita and Kamei, 2000). However, pretreatment with glibenclamide had no effect on the antitussive effects of dihydrocodeine and dextromethorphan (Morita and Kamei, 2000). These results indicated that moguisteine, but not centrally acting antitussive drugs, may exert its antitussive effects through the activation of ATP-sensitive K^+ channels. Based on these results, we proposed that ATP-sensitive K^+ channels may be involved in the antitussive effect of peripherally acting non-narcotic antitussive drugs (Morita and Kamei, 2000). In the present study, we observed that the antitussive effect of liquiritin apioside was significantly reduced by the pretreatment with glibenclamide. This result suggests that liquiritin apioside exerts its antitussive effect partly through the activation of ATP-sensitive K^+ channels. Thus the antitussive effect of liquiritin apioside may depend on both peripheral (modulation of ATP-sensitive K^+ channels) and central mechanisms (modulation of serotonergic systems).

As mentioned above, the present study suggested that the antitussive effect of liquiritin apioside depends partially on central mechanisms. However, it is well recognized that the site of the antitussive action of Bakumondo-to is in the airway, since Bakumondo-to inhibits the increased discharges of the airway vagal afferents in bronchitic animals (Miyata et al., 1989; 1999). Indeed, in the present study, we observed that pretreatment with methysergide had no significant effect on the antitussive effect of Bakumondo-to (1 g/kg, p.o.). The central nature of the antitussive effect of liquiritin apioside disappeared when all of the components of Bakumondo-to were combined. Although the detailed mechanisms are not clear, this interesting change in action is a characteristic of Chinese medicines.

In conclusion, *G. radix*, one of the main components of a Bakumondo-to, contains a potent antitussive compound: liquiritin apioside. The antitussive effect of liquiritin apioside depends on both peripheral and central mechanisms.

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