Use of an electrophysiological technique for stepwise detection of trace agonist constituents of Hochuekkito in Xenopus oocytes injected with serotonin 2C receptor mRNA

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SUMMARY
An electrophysiological bioassay was used to isolate the active compound from Hochuekkito (HET), which the current authors previously described as having potent agonist action against serotonin 2C receptors (5-HT2CR). Synthetic 5-HT2CR mRNA was injected into Xenopus oocytes to specifically express these receptors. Crude extracts and purified products were subjected to an electrophysiological bioassay using the voltage clamp method. HET stimulated a 5-HT2CR-induced current response, whereas Juzentaohoto (JTT), which has anti-depressive action similar to that of HET, did not. Current responses were not observed with an extract mixed with five types of herbal medicines common to HET and JTT but were detected with an extract with the five types of herbal medicines found in HET alone (Hoc5). When the responses to each of the five types of Hoc5 were examined, current responses were noted with Cimicifugae rhizoma (CR) and Citrus unshiu Markovich extracts. Since efficacy and the EC50 value were higher for CR, its constituents were separated using three-dimensional high-performance liquid chromatography and the current response at each of the isolated peaks was examined. One constituent displayed a strong response and was identified as a single substance with a molecular weight of 283.1393 based on liquid chromatography/mass spectrometry. These results will contribute to the isolation of 5-HT2CR-stimulating constituents in HET and the identification of trace constituents with agonist action.

Keywords
Kampo medicine, current, Xenopus oocyte, mRNA, 3D-HPLC, LC/MS

1. Introduction
Kampo medicine is a Japanese variant of Chinese traditional medicine that involves the extensive use of herbs. Since Kampo medicines are prescribed depending on the symptoms of a patient and differences in the individual's condition, they are considered to be a forerunner in "tailor-made medicine". Studies on the mechanisms of action of each Kampo prescription are based on certain theories, and not on an analytical method that prioritizes determination of its structure, so those findings will contribute to the discovery of novel research concepts and ultra-low-dose therapeutics (1).

The current authors and others have reported that many antidepressants act as blockers of the serotonin 2C receptor subtype (5-HT2CR) (2). Many Kampo prescriptions affect "mood", such as "Hochuekki-to (HET): Bu Zhong Yi Qi Tang" and "Juzentaiho-to (JTT): Shih Quan Da Bu Tang". Accordingly, the current authors previously examined the effects of HET and found that it has antidepressant action on behavioral pharmacology (3) and specifically the 5-HT2CR response. Contrary to expectations, results indicated that HET potently activated 5-HT2CR (4). Activation of the digestive system is also important as a therapeutic action of HET. The serotonin receptor system is also known to perform important functions in the digestive system. To the extent known, no study of the 10 crude drugs in HET has identified a constituent that stimulates a serotonin receptor. These facts lead to 3 questions: 1) which crude drug in HET is responsible for stimulating 5-HT2CR, 2) what are its constituents, and 3) what are the functional
differences between HET and other Kampo medicine prescriptions that have antidepressant action? Therefore, the current study is the first to attempt to identify this active ingredient via bio-assays.

To date, studies on development of new drugs from Kampo medicines have been based on analytical methods from modern science; they have resulted in significant advances and they have provided extensive evidence of the effectiveness of each Kampo prescription. The main approach is to analyze and identify the constituents of an effective crude drug, to examine its physiological activity, and to consider the result to be the action of the crude drug. However, the effectiveness of a herbal medicine is sometimes markedly reduced or its activity is lost during the process of fractionation. A trace constituent may have activity that may be overlooked when prioritizing the identification of constituents. Fortunately, electrophysiological techniques are very sensitive and suitable for high-fidelity bioassays. In addition, the response of a targeted agonist is generally observed even at a concentration lower than that of an inhibitor/blocker, and a transient stimulus is sufficient.

In light of these facts, the current study used an electrophysiological technique to bioassay HET for a potent 5-HT2CR-activating constituent. This study also briefly discusses the specific concepts of Kampo medicine.

2. Materials and Methods

2.1. Extraction of Kampo prescriptions and galenicals

Approximately 50 g of each prescription or galenical was added to 900 mL of boiling water for 60 min. Galenicals were removed while the solution was still hot, and the extract was frozen using liquid nitrogen. The frozen extract obtained was freeze-dried.

All galenicals were purchased from Tochimoto Tenkai-do (Osaka, Japan). The contents of extracted prescriptions and galenicals and the lot number of each galenical and extracted yields are shown in Table 1. The weight of each galenical in a prescription is listed on the left side of the table.

HET and JTT are combinations of 10 galenicals, with five out of the 10 galenicals being common to both (Com5). Com5 and the 5 other galenicals in HET (Hoc5) were extracted and freeze-dried. The gross galenical weight of the extract of HET, JTT, Com5, and Hoc5 was 49, 57, 46.5, and 45 g, respectively. The weight of Com5 was assessed based on its constituent ratio in HET.

Before the extract was administered to the oocytes that expressed 5-HT2CR on their surface, the freeze-dried extract was resolved using the buffer for the electrophysiological experiment and then centrifuged to remove insoluble matter.

2.2. Three-dimensional high-performance liquid chromatography (3D-HPLC) analysis of extracts

Each extract solution (10 mg/mL) of the supernatant obtained by centrifugation followed by filtration through a 0.22-μm membrane filter was subjected to 3D-HPLC analysis (20 μL). The HPLC apparatus (Hitachi Ltd., Japan) consisted of a pump (L-2130) with analysis system software (Elite LaChrom), a photodiode array detector (UV 230-400 nm, L-2455), a system controller, an auto injector (L-2200), and a column oven (L-2300). Atractylodes Rhizoma (ZR) was a potent 5-HT2CR-activating constituent. This study also briefly discusses the specific concepts of Kampo medicine.

Table 1. List of galenicals in the Kampo prescription used in the present study

<table>
<thead>
<tr>
<th>g</th>
<th>g</th>
<th>Name</th>
<th>Lot. No</th>
<th>Yield (%)</th>
<th>Production Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>Citrus unshiu Markovich (CM)*</td>
<td>007807004</td>
<td>34.5</td>
<td>Japan Shikoku</td>
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<td>007108004</td>
<td>56.0</td>
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<td>2</td>
<td>2</td>
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<tr>
<td>2</td>
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<td>Bupleuri Radix (BR)</td>
<td>004208001</td>
<td>12.8</td>
<td>Japan Nara</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
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<td>006007001</td>
<td>26.1</td>
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</tr>
<tr>
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<td>008607037</td>
<td>20.0</td>
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</tr>
<tr>
<td>4</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<td>3</td>
<td>Cinnamomi Cortex</td>
<td>002807017</td>
<td>49.3</td>
<td>Vietnam</td>
</tr>
</tbody>
</table>

Lot numbers for the site of production and source as well as recovery rates are listed. All extract concentrations are shown in herbal equivalents based on these recovery rates. Individual extraction of the five herbal medicines specific to JTT was not performed. Citrus unshiu Markovich (CM) is a mixture of CM and Citrus reticulate Bianoco. This table will also facilitate an understanding of the abbreviations used in the text.

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Ultra C18 (5 μm, lot no. 21D5-011; Hitachi Ltd., Japan) with 150 × 4.6 mm i.d. column, eluant: (A) H₂O containing 0.1% formic acid and (B) CH₃CN containing 0.1% formic acid. A linear gradient was used from 95% A and 5% B' to 30% A and 70% B' for 90 min. The temperature of the column was controlled at 20°C. The flow rate was 0.2 mL/min.

2.3. Synthesis of 5-HT2CR mRNA and injection into Xenopus oocytes.

pBluescript II KS(-) vectors (Stratagene, La Jolla, CA, USA) with a rat 5-HT2CR cDNA insert (approximately 3.0 kb) were used as a template to make in vitro synthesized mRNA (5). The vector was transformed into DH5α Escherichia coli (Nippon Gene, Toyama, Japan) to increase the amount of mRNA via estimation. The vector obtained with 5-HT2CR cDNA was linearized with the restriction enzyme XhoI (Nippon Gene, Toyama, Japan) at 37°C for 60 min. The linearized vector (250 ng) was incubated with T7 RNA polymerase and the mCAP analog in the reaction buffer of the transcription kit (Stratagene, La Jolla, CA, USA) to make 5-HT2CR mRNA in vitro. Products were extracted with a phenol/chloroform solution (Nippon Gene, Toyama, Japan) and precipitated in ethanol and sodium acetate.

Synthesized 5-HT2CR mRNA (100 ng) was injected into Xenopus oocytes isolated from female Xenopus laevis. X. laevis (Hamamatsu Seibutsu Kyozai, Shizuoka, Japan) were anesthetized in ice water, and a lobe of the ovary was dissected and placed in sterile modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, and 7.5 mM HEPES-NaOH, pH 7.6). Oocytes were then isolated manually and defolliculated by incubation in 1.5 mg/mL collagenase (type IA; Sigma, St. Louis, MO, USA) at 20°C in calcium-free MBS solution. Synthetic mRNA was injected into oocytes using a microinjector (Drummond, Broomall, PA, USA), and oocytes were then incubated in MBS containing 2.5 units/mL penicillin and 2.5 μg/mL streptomycin at 18°C. Xenopus oocytes do not naturally express 5-HT2CR, ion channels, or many receptors (6). Muscarinic receptors are only expressed in the follicular cells and if the follicle cannot be removed, oocytes react to acetylcholine (7). In addition, Xenopus oocytes are traditionally used for cloning and functional analysis of ion channels and receptors because they efficiently translate injected mRNA (8). In the case of 5-HT2CR expression, responsiveness to 5-HT is evident about 18 h after injection of mRNA, and that responsiveness usually continues for 3 to 4 d (6).

Since Xenopus is a poikilotherm, it is completely anesthetized when soaked in ice water. After the reflex reaction was confirmed to have disappeared, the base of the foot was opened about 1-2 cm and oocytes were removed. The opening was then sewn shut with suture and surgical adhesive was used to waterproof the wound. Xenopus awakens when placed in ice-free water overnight as the water temperature gradually returns to room temperature. After confirming that Xenopus behaved properly, it was returned to the aquarium. One Xenopus can be used several times. Based on the above facts, the burden of surgery on Xenopus is minimal, and normal behavior resumes immediately after surgery.

2.4. Electrophysiological recordings

Responses to 5-HT were recorded using a two-electrode voltage-clamp amplifier (Nippon Kohden, Tokyo, Japan) at a holding potential of −60 mV. Oocytes were positioned in a 50-μL chamber and continuously perfused with MBS solution at approximately 1 mL/min at room temperature (less than 25°C). For extracts of the Kampo prescription or galenicals, drugs were administered by changing the perfusing solution to a buffer containing the given drug. When an isolated compound was administered, one drop of the MBS solution (approximately 20 μL) was directly dropped from a micropipette into the chamber. Data were recorded and digitized for analysis (MacLab, AD Instruments, Castle Hill, NSW, Australia).

2.5. Liquid chromatography/mass spectrometry (LC-MS) analyses

LC-MS analyses were performed using a new type of mass spectrometer that combines ion trap and time-of-flight mass spectrometry and that is equipped with an electrospray ionization (ESI) interface (Shimazu, Kyoto, Japan). The following ESI parameters were used: source voltage: 3.5 kV (negative mode), capillary temperature: 200°C, and nebulizer gas: 1.5 L/min. The mass spectrometer was operated in negative ion mode by scanning the m/z range from 100 to 2,000. A Waters Atlantis dC18 column (2.0 mm i.d. × 150 mm) (Waters Corp, MA, USA) was used and the column temperature was maintained at 40°C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) CH₃CN under the following gradient conditions: 0-30 min linear gradient from 10 to 100% B and 30-40 min isotonic gradient at 100% B. The flow rate was 0.2 mL/min.

3. Results

The current authors previously reported that many types of antidepressants inhibit 5-HT2CR (2). Based on those findings, Kampo medicines that exhibit anti-depressive action, such as HET (3), are anticipated to exhibit similar inhibitory activity. Contrary to expectations, HET exhibited significant stimulating rather than inhibitory action (4). Similarly, in the current experiment, HET at 3 mg/mL potently activated Xenopus oocytes expressing...
5-HT2CR (Figure 1). HET consists of 10 types of herbal medicines, 5 of which are common to JTT. The five types specific to HET (Hoc5) had potent 5-HT2CR-stimulating action while Com5 and JTT did not (Figure 1). The stimulatory action of Hoc5 was concentration-dependent (Figure 1).

Since Hoc5 had potent 5-HT2CR-stimulating action, the activity of each of the five herbal medicines was examined. Figure 2 shows the current responses of each herbal medicine at 3 mg/mL. Cimicifugae rhizoma (CR) had potent stimulating action, with an EC50 value of 0.9 mg/mL and a confidence interval (CI) value of 0.48-1.65 mg/mL. Stimulating action was also observed with Citrus unshiu Markovich (CM), but it was weaker, with an EC50 value of 4.0 mg/mL and a CI value of 1.73-9.29 mg/mL. Significant activation was not observed with the three other crude extracts.

Since CR activated 5-HT2CR, its constituents were separated using 3D-HPLC and their activity was examined via direct administration to oocytes. The 3D-HPLC chart for CR is shown in Figure 3A, and that

![Figure 1. Effects of HET, JTT, and constituent crude drugs, Com5 and Hoc5, on 5-HT2CR-induced current responses in Xenopus oocytes injected with synthetic 5-HT2CR mRNA.](image1)

![Figure 2. Effects of each extract of Hoc5 constituent crude drugs on 5-HT current responses in Xenopus oocytes injected with synthetic 5-HT2CR mRNA.](image2)

![Figure 3. Constituent analysis and fractionation of the CR extract by 3D-HPLC and 5-HT2CR current responses by each fraction.](image3)
recorded at 274 nm is shown in Figure 3B. The fraction producing large peaks under these HPLC conditions was purified. After the HPLC solvent was removed by evaporation, CR was dissolved in a small amount of HBS buffer and administered to cells. Therefore, its exact concentration could not be quantified. Potent activation by fraction no. 12 was observed. There was no change in the large peak after an elution time of 20 min (data not shown).

The molecular weight of the substance producing peak no. 12 was assessed using LC/MS. The LC results for CR at 280 nm are shown in Figure 4A. The absorption wavelength of peak P2 in Figure 4A is shown in Figure 4B, and it was similar to the spectrum in Figure 3. The molecular weight of the substance was estimated to be 283.1393; however, the substance could not be identified based on that value. Identification using nuclear magnetic resonance (NMR) was attempted, but it was unsuccessful because the required amount with sufficient purity was not obtained.

4. Discussion

The active constituents responsible for the 5-HT2CR-stimulating action of HET were examined, and a trace amount of a constituent with a molecular weight of 283.1393 was obtained. Since the constituent was obtained in a very limited amount, its structure could not be determined with NMR.

The effectiveness of herbal extracts has been successfully assessed to date by considering the main constituents to be active and contributing factors. However, the current results demonstrated that, similar to aromatic constituents, some agonist constituents may exhibit action even when present in only a small amount. While limited evidence is currently available to support this contention, some studies have indicated that the aroma emitted during the extraction of a Kampo medicine is also effective as a treatment. An assessment of electrophysiological activity is a highly sensitive method that may be used to identify active and stimulating trace constituents in the future. When an electrophysiological technique is used to evaluate active ingredients, it can detect both agonist and antagonist action; it is effective at evaluating agonists because it reveals a response by a constituent even in small amounts. In many cases, however, a large amount is needed to examine antagonist action. An electrophysiological technique is highly sensitive and is characterized by a dynamic reaction, so it is suitable for bioassay-like-purification and detection of agonists. In the future, agonist constituents will presumably be detected and analyzed using this technique.

The primary aim of the current study was to identify the active constituents in HET with anti-depressive action on 5-HT2CR. Anti-depressive action could not be directly attributed to a certain "trace constituent", but an active constituent that produced a strong response even when present in a small amount was successfully identified.

The active constituent seems to directly affect the function of the digestive system and not the brain and...
appears to be related to another action of HET, its enhancement of digestive function. Among the anti-depressive Kampo medicines, Rikkunshito (RKT): Hsiang Sha Lu Chun Tzu Tong is known to act on the digestive system (9,10). HET and RKT have similar herbal medicine compositions, but RKT does not contain CR. The stimulation of digestive function by RKT involves ghrelin and 5-HT2CR function (10-12). A point worth noting is the possibility that the trace constituent identified in the current study may involve the function of ghrelin via a mechanism unlike that of RKT. Further studies will be conducted to determine the constituent's structure and to subsequently synthesize that constituent.

In Kampo medicine, HET is an effective treatment for mild depression, and JTT is more effective than HET when mood is further depressed. JTT, which is considered to have potent anti-depressive action, did not affect 5-HT2CR in the current experimental system. HET and JTT consist of 10 herbal crude medicines, 5 of which are common to both. The 5 shared herbal medicines (Com5) do not have prescription names and are also included in "Kihito: Gui Pi Tang". Kihito is known to act on the brain and to have the potential to serve as an anti-dementia drug (13). Com5 may contain some constituents that are active in the brain and that reach their target site through absorption and metabolism. While the underlying mechanisms could not be fully elucidated in the current study, the current authors previously reported that the level of BNIP-3 mRNA expression increased as a result HET in another experimental system using cultured neuronal cells (14,15). This finding is currently being examined more intensively by the authors' group, including identification of the site of expression in the brains of small animals using MRI (16).

The current study used an electrophysiological bioassay to identify the constituents responsible for the 5-HT2CR-stimulating action of HET, and, as a result, it discovered an unknown constituent with a molecular weight of 283.1393. CM also acts on 5-HT2CR, so this constituent alone is unlikely to be solely responsible for the activation of 5-HT2CR by HET, but the current results indicate that ultra-trace constituents and not the main constituents of HET are involved in the responses generated by the Kampo medicine. The current study indicated that ultra-trace constituents of Kampo medicines also have potent "biofunctional action" and play a role in the specificity of those prescriptions. In the future, the identification of these "ultra-trace active ingredients" will promote research on Kampo and Wakan-yaku medicines.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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