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# BNIP-3: A novel candidate for an intrinsic depression-related factor found in NG108-15 cells treated with Hochu-ekki-to, a traditional oriental medicine, or typical antidepressants

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**Abstract**

Wakan-yaku is a type of Japanese and Sino traditional, systematized medical care that has been practiced for hundreds of years. To search for novel intrinsic factors related to the action of antidepressants, we used Hochu-ekki-to (HET), a Wakan-yaku medicine with antidepressive effects. First, we verified the quality of the HET by three-dimensional high-performance liquid chromatography and a cytotoxicity check in NG108-15 cells. We performed a DNA microarray analysis of the gene expression in cells treated with 50 μ/ml HET for more than 20 days. HET enhanced the expression of 125 (2.9%) genes and decreased the expression of 255 (6.0%) genes among the 4277 genes that were tested. The concentration-dependent increase in the expression of BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP-3) mRNA was particularly remarkable. A concentration-dependent increase in the expression of BNIP-3 mRNA was also observed when cells were treated with imipramine, mianserin, or milnacipran. These results suggest that BNIP-3 is a candidate for an intrinsic factor related to antidepressive effects and that Wakan-yaku theory may be useful for the identification of other intrinsic functional molecules.


*Keywords:* DNA array; Semi-quantitative RT-PCR; Depressive disorder; Sino-Japanese traditional medicines; NG108-15 cells; BNIP-3

## 1. Introduction

Since the discovery of the antidepressive effect of imipramine, which was developed as an anti-histamine drug in the 1950s, clinical antidepressants have been developed with monoamine transporters as the target molecules (Nestler et al., 2002). The first-generation tricyclic antidepressants such as imipramine have a strong side effect on anti-choline; therefore, the second-generation tetracyclic antidepressants such as mianserin were generated. However, the potency of the inhibitory effects of the second-generation drugs on transporters is also weaker than that of the first-generation drugs. The third-generation antidepressants were developed in the 1990s based on the monoamine hypothesis. These selective serotonin re-uptake inhibitors (SSRI), such as fluoxetine, have controlled the side effects. The fourth-generation antidepressants, which consist of selective noradrenaline/serotonin re-uptake inhibitors (SNRI) such as citalopram, were developed to overcome the serotonin syndrome. These developments strongly premise that a monoamine transporter participates in depression onset. However, the monoamine hypothesis cannot completely explain the action mechanism of antidepressants, since long-term treatment with antidepressants is necessary for therapeutic effects. Unfortunately, the popularity of the monoamine hypothesis may have delayed the elucidation of the molecular mechanisms of depression onset, in comparison with the progress made in Alzheimer's disease, Parkinson's disease, and other psychiatric disorders.

Wakan-yaku, Sino-Japanese traditional medicine, is systematized medical care that has been practiced for several hundred years or more in China and Japan. We thought that it would be

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useful to apply this systematized concept of Wakan-yaku to the search for intrinsic molecules that are involved in the depressive disorder. A lot of medical prescriptions that act on the "mind" are included in Wakan-yaku; a famous Wakan-yaku prescription that acts on the mind is Hochu-ekki-to (HET: Bu-Zhong-Yi-Qi-Tang). HET has antidepressant-like effects in the forced swimming test (Watanabe et al., 1990). A description of HET first appeared in Neiwaishang Bianhuolun, which was written by Dong Yuan Li in 1247. At that time, people living around Li had lost their physical strength because of malnutrition during war. Therefore, Li developed HET for the purpose of "improvement of the digestive system ("*hochu*" in Japanese) and profiting vigor ("*ekki*")" (Mayanagi, 1995).

We previously found that HET evokes a metabotropic current response though serotonin 2C receptor (5-HT2CR) stimulation in Xenopus oocytes injected with rat brain mRNA and that HET administration enhances the 5-HT2CR mRNA expression in the rat brain (Tohda et al., 2000). In addition to serotonin transporters and/or noradrenaline transporters, 5-HT2CR also plays an important role in the action of antidepressants. Indeed, many antidepressants have an affinity for 5-HT2CR (Palvimaki et al., 1996) and 5-HT2CR-mediated signal transduction (Tohda et al., 1989). We previously hypothesized that increased levels of 5-HT2CR mRNA work not only to synthesize the receptor protein but also to stimulate the expression of other genes, such as genes that encode anti-stress proteins.

In the present study, we observed the effects of HET on the morphology of NG108-15 cells, which possess 5-HT2CR mRNA (Tohda et al., 2002, 2004; Sukma et al., 2005). We used DNA array methods to determine the effect of long-term treatment with HET on gene expression at a pharmacological concentration, which was determined from morphological studies. Several factors that were shown to have enhanced expression by the DNA array assay were examined in detail for a concentration-dependent response to HET and typical antidepressants.

## 2. Materials and methods

### 2.1. Extraction of HET

HET is a combination of 10 galenicals. All galenicals were purchased from Tochimoto Tenkai-do (Osaka, Japan), from the same lot used in our previous report (Tohda et al., 2000). The daily dose of each galenical when used in HET by humans, the location where the plant source was grown, and the lot numbers are as follows: 4 g Ginseng Radix (Korea, No. 300190); 4 g Atractylodes Rhizoma (Zhejiang Sheng, China, No. 220295); 3 g Astragali Radix (Shan Xi Sheng, China, No. 100395); 3 g Angelicae Radix (Yamato, Japan, No. 120695); 2 g Citri Leiocarpae Exocarpium (Shikoku, Japan, No. 220894), 2 g Zizyphi Fructus (He Nan Zheng, China, No. 300395); 2 g Zingiberis Rhizoma (Yun Nan Sheng, China, No. 290788QC); 2 g Bupleuri Radix (Nara, Japan, No. 220290); 1.5 g Glycyrrhiza Radix (Xi Bei, China, No. 071194); and 1 g Cimicifugae Rhizoma (Ji Lin Sheng, China, No. 100288QC). A total of 98 g were combined and used for extraction with 900 ml of boiling water for 90 min. Galenicals were removed while the solution was still hot, and the extract was frozen. Part of the frozen extract was lyophilized to estimate the yield (34%). Before the HET extract was applied to cultured cells, the extract was centrifuged to remove the insoluble matter and filter-sterilized.

### 2.2. The three-dimensional high-performance liquid chromatography (3D-HPLC) pattern of HET

The 3D-HPLC pattern of HET is shown in Fig. 1. HET extract (100 μg/ml) was added to the same volume of methanol. The solution was filtrated with a membrane filter (0.22 μm) and then submitted for HPLC analysis (10 μl). The HPLC apparatus consisted of a Shimadzu LC-10ADvp pump (analysis system software: CLASS-M10A) equipped with a photodiode array detector (UV 230–400 nm) (Shimadzu SPD-M10Avp), system controller (Shimadzu SCL-10Avp), auto injector (Shimadzu SIL-10Avp), and column oven (Shimadzu CTO-10ACvp). HPLC conditions were as follows: column, YMC-Pack Pro C18 2 mm i.d. × 150 mm; elutant, (A) $H_2O$ containing 0.1% formic acid and (B) $CH_3CN$ containing 0.1% formic acid (a linear gradient of 95% A and 5% B that changed over 90–100 min to 30% A and 70% B was used); temperature, 20 °C; and flow rate, 0.2 ml/min.

### 2.3. LC–MS analyses

LC–MS analyses were performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The ESI parameters were as

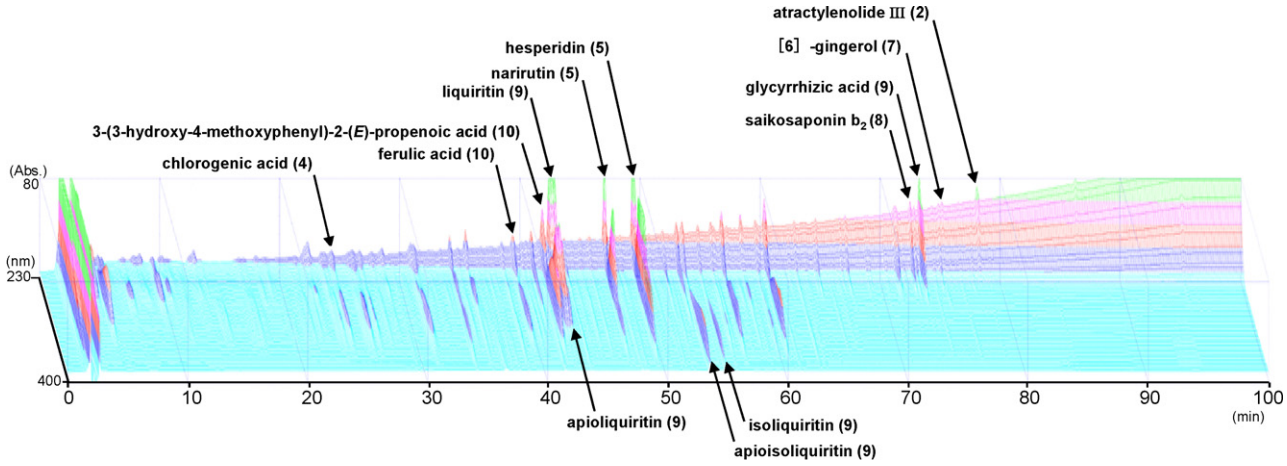


Fig. 1. The 3D-HPLC pattern of HET. HET extract (100 μg/ml) was added to the same volume of methanol. The solution was filtrated with a membrane filter (0.22 μm) and then submitted for HPLC analysis (10 μl). The HPLC apparatus and the analysis conditions are described in Section 2. The numbers in parentheses after names of the components indicate the source galenicals as follows: (2) Atractylodes Rhizoma, (4) Angelicae Radix, (5) Citri Leiocarpae Exocarpium, (7) Zingiberis Rhizoma, (8) Bupleuri Radix, (9) Glycyrrhiza Radix, and (10) Cimicifugae Rhizoma. As expected, the components originating from Ginseng Radix (1), Astragali Radix (3), and Zizyphi Fructus (6) were not detected under these conditions.