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Extractive from *Hypericum ascyron L* promotes serotonergic neuronal differentiation *in vitro*

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ABSTRACT

Background: Plant natural products have many different biological activities but the precise mechanisms underlying these activities remain largely unknown. *Hypericum longistylum* has long been recorded in Chinese medicine as a cure for depression and related disorders, but how it repairs neural lineages has not been addressed.

Methods: We extracted compounds from *Hypericum longistylum* and determined their effect on neural differentiation of embryonic stem cells (ESCs) *in vitro* by using the *Pax6-GFP* reporter system. The amount of serotonin released during differentiation was measured by HPLC. The tail suspension test and forced swimming test was performed for determining the effect of compounds on depression-like behaviors in mice.

Results: We found that one of the phloroglucinol derivatives not only facilitated differentiation of neural progenitor cells, but also increased the efficiency of differentiation into serotonergic neurons. This compound also improved the behaviors of mice placed in a stressful environment and reduced signs of depression.

Conclusions: This is the first use of Chinese herb derived-natural products to promote neurogenesis of ESCs, including the generation of serotonergic neurons, and the first attempt to identify the active compound in *Hypericum longistylum* responsible for its beneficial effects on depressive diseases.

1. Introduction

Since the discovery of artemisinin, Chinese medicine has attracted increasing global interest. Many natural products extracted from Chinese herbs show significant pharmacological activity and could potentially be used as medicines to cure diverse diseases (Xue and Roy, 2003; Cao et al., 2017). Depression is a type of mental illness that leads to severe and persistent low mood, which could lead to physical harm and increased risk of suicide (Steidtmann et al., 2012). The main cause of depression remains unclear and effective therapies are lacking. Three main types of drugs are currently used to treat depression: tricyclic antidepressants (Willner et al., 2013), monoamine oxidase inhibitors, and tetracyclic antidepressants. These drugs, however, only relieve the symptoms of depression and have severe side effects, including cardiovascular effects (Choi, 2003; Stahl, 2014). Chinese herbs are typically effective and have relatively few side effects, which is why scientists are increasingly trying to extract or synthetize the active components. Some Chinese herbs, such as *Hypericum longistylum*, have been shown to

Abbreviations: ESCs, embryonic stem cells; 5-HT, 5-hydroxytryptamine; MPLC, medium pressure liquid chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ECD, electronic circular dichroism; TDDFT, time-dependent density functional theory; EB, embryoid body; FACS, fluorescence-activated cell sorting; NPCs, neural precursor cells; RA, retinoic acid; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; NSC, neural stem cell; FGF4, fibroblast growth factor-4; SHH, sonic hedgehog; bFGF, basic fibroblast growth factor; FGF8, fibroblast growth factor-8; BDNF, brain-derived neurotrophic factor; SERT, serotonin transporter; TLC, thin layer chromatography; ODS, octadecylsilyl; LIF, leukemia inhibitory factor; TCA, trichloroacetic acid; MS, mass spectrometer

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have a positive effect on depression, but the active ingredient(s) and mechanism of action need to be further elaborated.

One possible cause of depression is the degeneration of serotonergic neurons, which would lead to a reduction in levels of 5-hydroxytryptamine (5-HT) in the central nervous system (Brambilla et al., 2010). Drugs that can increase levels of 5-HT have been shown to provide some benefits in depression (Li et al., 2006). *Hypericum longistylum* has many active chemical components, including phloroglucinol derivatives and, in the present study, we aimed to clarify whether these phloroglucinols can induce neural regeneration, especially of serotonergic neurons. Embryonic stem cells (ESCs) are pluripotent cells that can differentiate to neural lineages *in vitro* in the presence of growth factors (Li et al., 1998) and thus provide an ideal platform for the study of drug function and mechanisms. We therefore examined the effect of phloroglucinol derivatives, extracted from *Hypericum longistylum*, on serotonergic neuron differentiation in ESCs undergoing neural differentiation. We also tested the ability of one of these phloroglucinols to enhance the performance of mice in a stressed environment.

2. Material and methods

2.1. Plant materials

The aerial parts of *Hypericum longistylum* were collected from Changbaishan, Jilin Province, People's Republic of China, in August 2015. Botanical identification was carried out by one of the authors (X. Cao), and a voucher specimen (No. 20150803) was deposited at the laboratory of the Research Department of Natural Medicine, College of Pharmacy, Nankai University.

2.2. Mice

Specific pathogen-free (SPF)-grade mice were obtained from Beijing Vital River laboratory animal center and housed in the animal facilities of the Nankai University, China. Male and female mice of CD1 backgrounds at 8 week were used for tail suspension test and forced swimming test. The experiments were approved by the Animal Care and Use Committee of Nankai University, and all experiments conform to the relevant regulatory standards.

2.3. Extraction and isolation

The air-dried aerial parts of *Hypericum longistylum* (9.1 kg) were extracted with MeOH (3 × 80 L) under reflux. After evaporating the solvent, the residue (1000.0 g) was dissolved in H_2O (2.0 L) and extracted with petroleum ether (3 × 2.0 L). The petroleum ether soluble portion (309 g) was chromatographed on a silica gel column (silica gel, 100–200 mesh, 1.0 kg; column, 9 × 70 cm), using a petroleum ether-acetone step gradient system (100: 0, 100: 1, 100: 2, 100: 4, 100: 6, 100: 8, 100: 11, 100: 16, 100: 21, and 100: 26; 21 L for each gradient elution). Ten fractions (F1 − F10) were collected, guided by thin layer chromatography (TLC) analysis. Fraction F4 was subjected to MPLC, using an octadecylsilyl (ODS) column with a step gradient (68–95% MeOH in H_2O), to give seven sub fractions F4–1–F4–7. Sub fractions F4–2 were further purified by preparative HPLC (93% MeOH in H_2O) to provide compound 1 (t_R = 22 min; 21.4 mg yield). Fraction F6 was fractionated using the same MPLC conditions to give eight sub fractions, F6–1 − F6–8. Fraction F6–4 was further purified using the same HPLC system (90% MeOH in H_2O) to provide compound 2 (t_R = 31 min; 14.7 mg yield). The same MPLC and HPLC conditions were used to further separate fraction F5 into sub fractions F5–1 − F5–13. Further purification of F5–5 then yielded compounds 3 (t_R = 26 min, 39.4 mg) and 4 (t_R = 39 min, 14.0 mg).

2.4. Culture of mouse ESCs

Mouse ESCs (pax6-GFP) were grown on a mitotically inactivated mouse embryonic fibroblast feeder layer in an optimized stem cell growth medium (Ying et al., 2003). Briefly, the cells were grown in 1:1 mixture of DMEM/F12 (Gibco) supplemented with modified N2 (Gibco), and neurobasal medium (Gibco) supplemented with B27 (Gibco), supplemented with 5% knock out serum replacement (KOSR) (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich), 100 μM β-mercaptoethanol (Sigma-Aldrich), 100 mg/mL Strep-Pen (Gibco), 1500 U/mL leukemia inhibitory factor (LIF) (Millipore), 3 μM CHIR99021 (Gene-operation), and 1 μM PD0325901 (Gene-operation) at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. CCK-8 assay and DRAQ7 staining

A CCK-8 assay was used to measure the cytotoxicity of different concentrations of Compound 3 (Yang et al., 2014). Briefly, EBs that had been formed for 4 days were collected, resuspended in ES differentiation medium, and then re-plated on a fibronectin-coated dish. On the next day, when the EBs were attached on the bottom, the medium was aspirated and replaced with fresh medium (100 μL) containing different concentrations (2.5, 5, 10, 20, and 40 μM) of Compound 3. The cells were then incubated for an additional 72 h. Control wells contained medium alone, and three replicate wells were used at each concentration. After 72 h incubation, CCK-8 (10 μL, 5 mg/mL) was added to each well and the cells were incubated for another 3 h. The plate was then read using a BioTek luminescence reader (BioRed) at a test wavelength of 450 nm. For DRAQ staining, the cells were resuspended with culture medium at a concentration of no $> 5 \times 10^5$ cells/mL. DRAQ7 (CST) (1:100, 5 μL) was added to the cell suspension (0.5 mL), to a final concentration of 3 μM. The cells were gently mixed, incubated for 10 min on ice, and then analyzed directly in the presence of DRAQ7.

2.6. RNA isolation & real-time PCR

Total RNA from ESCs, ESC-derived neural precursors, and ESC-differentiated serotonergic neurons were isolated using Trizol reagent (Invitrogen). The cDNA was isolated using a Prime Script™ RT Reagent Kit with gDNA Eraser (Takara) for RT-PCR. RT-PCR was carried out using an ABI QuantStudio TM 6 Flex (ABI) with a FS Universal SYBR Green Master system (Roche). Relative expression levels were normalized to *Gapdh*. Average and SD are the result of three independent experiments. All primers are shown in Additional file 1: Table S1.

2.7. Immunocytochemistry

Cells were fixed overnight at 4 °C with 4% formaldehyde, washed three times with phosphate-buffered saline (PBS), and then incubated with blocking buffer (3% BSA) for 1 h at room temperature. The cells were then incubated overnight at 4 °C with rabbit anti-SERT antibody (Abcam), rinsed three times with PBS, and incubated with the corresponding secondary antibodies (Abcam) for 1 h at room temperature. After incubation, the samples were washed three times with PBS and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. The cells were examined under a fluorescent microscope and positive cells were counted by an investigator blinded to the identity of the samples. The data are the result of three independent experiments.

2.8. Neural differentiation of ESCs with a pax6-GFP reporter system

To generate NPCs, 6-/-ESCs (pax6-GFP reporter system), with well-defined morphology, were selected for differentiation. To form EBs, 6-/-ESCs were cultured with EB medium in non-coated petri dishes (Falcon) for 3 days. Briefly, the EB medium contained DMEM/F12 (Gibco),