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Neurotrophic Activity of Honokiol on the Cultures of Fetal Rat Cortical Neurons

Yoshiyasu Fukuyama,[a,*] Kousuke Nakade,[a] Yuka Minoshima,[a] Ritsuko Yokoyama,[a] Haifeng Zhai[a] and Yasuhide Mitsumoto[b]

[a]*Institute of Pharmacognosy, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*

[b]*Neurodegenerative Disease Research Group, Second Institute of New Drug Research, Otsuka Pharmaceutical Co. Ltd., Tokushima 771-0192, Japan*

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Abstract—Honokiol, a main biphenyl neolignan of the traditional crude medicine, *Magnoliae* cortex, was found to show neurotrophic activity on the cultures of rat cortical neurons at concentration from 0.1 to 10 μM. In the cortical neurons cultured in serum-free medium supplemented with B27, honokiol could promote neurite outgrowth. In addition, the survival and growth of neurons were significantly enhanced by adding honokiol to the primary cultures in serum-free medium supplemented with N2. Its neurotrophic activity was comparable to 40 ng mL^{-1} of bFGF at concentration of 10 μM.

Introduction

Simple biphenyl neolignans, honokiol (**1**) and magnolol (**2**), are the main constituents of the stem bark of *Magnolia obovata* Thunb[1] and *Magnolia officinalis* Rhed, which have been used as a traditional Chinese medicine for the treatment of thrombotic stroke, nervous disturbance, and gastrointestinal complaints.[2] Compounds **1** and **2** were shown to exhibit a central depressant, with successively higher doses eliciting muscle relaxation, sedation, sleeping, and anesthesia.[3,4] The dihydrogenated derivative of **1**, in particular, showed an significant anxiolytic-like activity.[5] In addition to these CNS activities, a number of biological activities of honokiol and magnolol have been documented.[6] In this paper, we report an intriguing neurotrophic and neuroprotective effects of honokiol (**1**) on the primary cultures of fetal rat cortical neurons in two different serum-free mediums, and thereby significance of **1** can be jumped up to another useful field of CNS (Fig. 1).

*Corresponding author. Tel.: +81-88-622-9611x5911; fax: +81-88-655-3051; e-mail: fukuyama@ph.bunri-u.ac.jp

Results and Discussion

In the previous paper,[7] we reported the structures of neurotrophic sesquiterpenes linked with magnolol and/or honokiol isolated from the stem bark of *M. obovata*. As the neuronal cells used for screening active compounds were cultured in the serum-containing medium,[8] the issue on indirect effects of unknown components in the serum remained to be unsolved. Thus, first, the present cell cultures were performed using 18-day fetal rat cortical neurons in the serum-free Neurobasal Medium (NBM) supplemented with B27.[9,10] Honokiol (**1**) had a striking effect on the morphological differentiation of

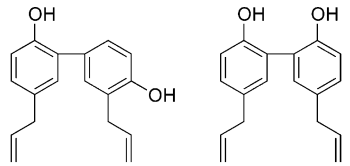

Figure 1. Structures of honokiol (**1**) and magnolol (**2**).

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cortical neurons as shown in Figure 2. After 7 days in cell cultures, neurons in cultures (c, d, and e) containing 0.1~10 μM of **1** were found to extend very long neurites, and more prominent and darker staining neuronal somata in comparison with those of control culture (a) containing 0.5% EtOH. In addition, one of the fibers was much longer and showed more neurite branches than those of the control cultures. Basic fibrobrast growth factor (bFGF) is well known as a trophic factor to enhance neurite extension and increase neuronal survival.[11] Adding 40 ng mL^{-1} of bFGF to this cell culture could significantly enhance neurite extension as shown in Figure 2(b). On the other hand, magnolol (**2**) was found to promote neurite extension at concentration of 1.0–10 μM as shown in Figure 2(f). However, its effect on neuronal morphology was not comparable to that of **1**.

The morphometric analysis of the neurotrophic effect of **1** was carried out by measuring the longest neurite length of each neuron using Lumina Vision and MacSCOPE software.[12] The results were shown in Figure 3. The length of neurites were found to be increased in the cultures treated with **1**. The neurite length was increased in a dose-dependent manner, and reached to the maximum peak at 10 μM.[13] It was striking that the neurite length was 1.8 times greater than in control and the effect of **1** was much more potent than bFGF. On the other hand, magnolol (**2**) was found to have weak effect on neurite extension according to the morphometric analysis as shown in Figure 3. This prominent difference of trophic effects caused by **1** and **2** is presumably responsible for the position of aryl–aryl bond formed between two units of allylphenol, and is consistent with the fact that **1** shows other CNS related activities more effectively than **2**. [14,15]

In general, the culture conditions without serum are not enough for most neurons to survive for a long period. NBM/B27, however, supplies a high neuronal survival under serum-free condition. The survival of cortical neurons can be maintained in this medium within 7 days even if a final cell density is decreased up to 9000 cells cm^{-2}.[10,16] Thus, NBM/B27 is useful for in vitro studies of neuronal development, pharmacology, and effects of growth factors. In contrast, it is not good at screening neuroprotective effects as measured by neuronal survival and viability. We observed that most neurons died within 5 days in the primary cultures plated at cell density less than 2×10^5 cells cm^{-2} in the case of using DMEM/N2 medium as shown in Figure 4(a). Therefore, we have decided that this serum-free medium appropriates to the cell cultures to see whether honokiol can protect neuronal death. In the experiment, cell suspensions were initially plated at a density of 2×10^5 cells cm^{-2} and cultures were fixed 3 days after addition of honokiol (**1**) (0.01, 0.1, 1.0, 10, and 100 μM). The results were shown in Figures 4 and 5. The control culture (a) in the absence of **1** carried only a small number of survival neurons. In contrast, the cultures (b) and (c) in the presence of **1** not only increased the number of survival neurons but also enhanced neurite extension and wide neurite branching in a concentration-dependent manner. *In particular, the culture (c) containing 10 μM of **1** showed fantastic neuronal survival and growth.*

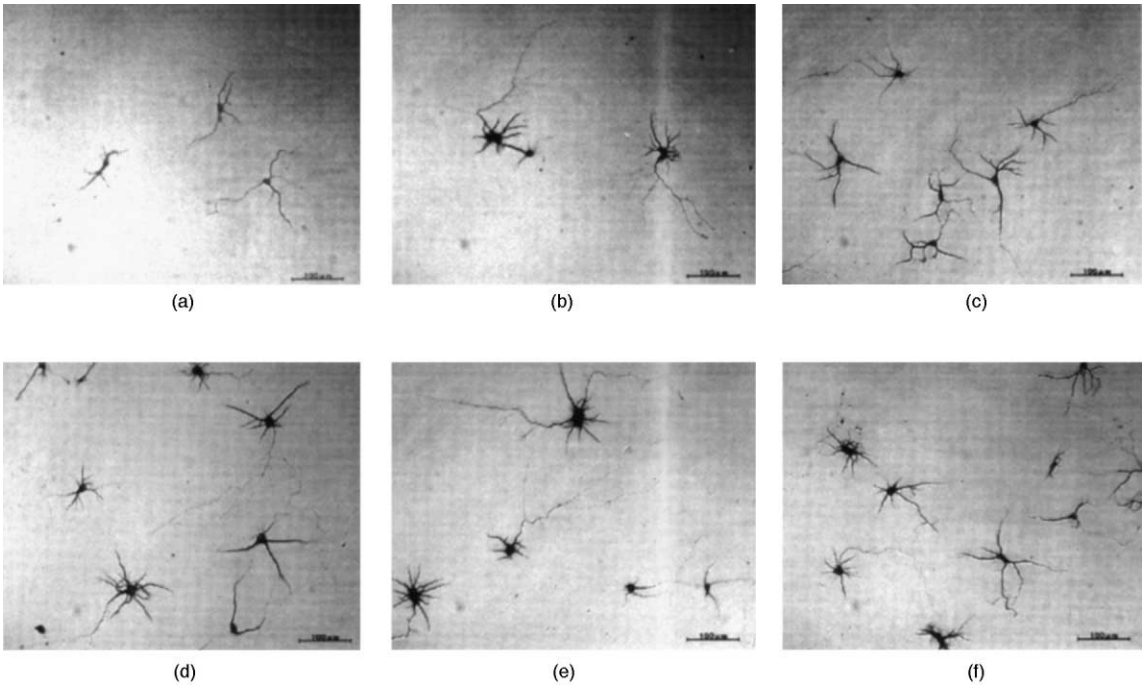

Figure 2. Enhancement of neurite outgrowth by honokiol (**1**) and magnolol (**2**) in primary cultures of E18 SD rat cortical neurons in NBM/B27. After the neuronal cells (9000 cells cm^{-2}) cultured for 6 days in the presence of 0.5% EtOH, **1**, and **2** were fixed by 4% paraformaldehyde-PBS, the immunohistochemical staining for the microtuble associated protein-2 (MAP-2) were performed. Pictures: (a) 0.5% EtOH, (b) 40 ng mL^{-1} bFGF, (c) 0.1 μM honokiol, (d) 1.0 μM honokiol, (e) 10 μM honokiol, and (f) 10 μM magnolol.

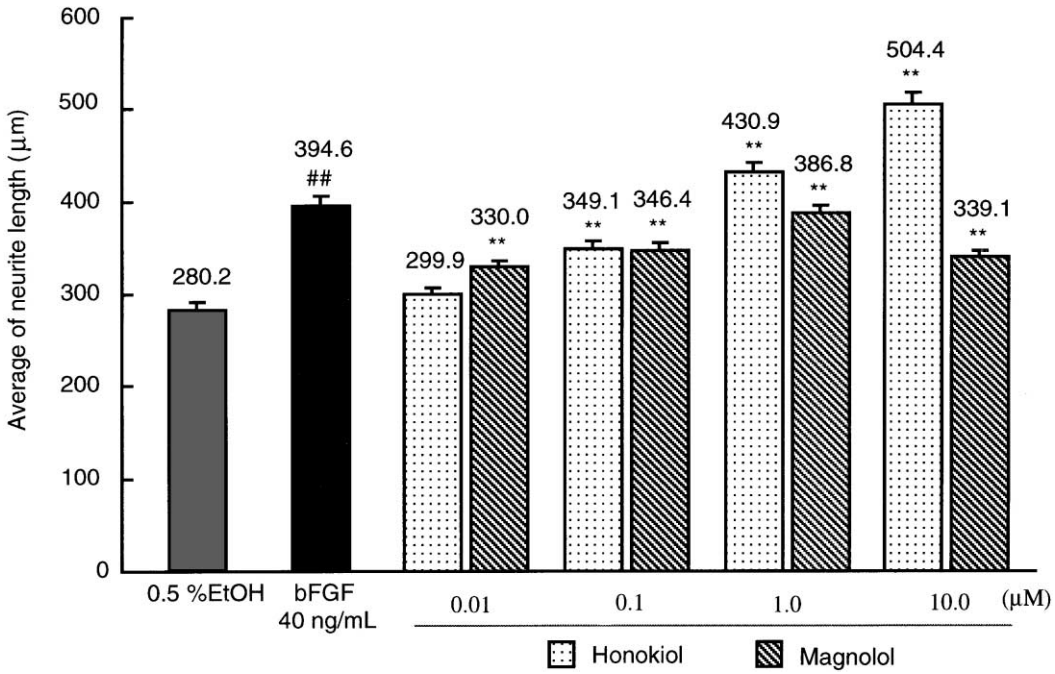

Figure 3. Morphometric analysis of the neurons effected by honokiol. After the neuronal cells (9000 cells cm^{-2}) cultured for 6 days in the presence of 0.5% EtOH, bFGF, honokiol, and magnolol were fixed by 4% paraformaldehyde-PBS, the immunohistochemical staining for MAP-2 were performed. Morphometric analysis was carried out on these neurons according to the criteria.[12] The data are expressed as means ± SE ($n = 80$); Student's t-test: $^{\#\#}p < 0.01$ versus control; Dunnet's t-test: $^{**}p < 0.01$ versus control.

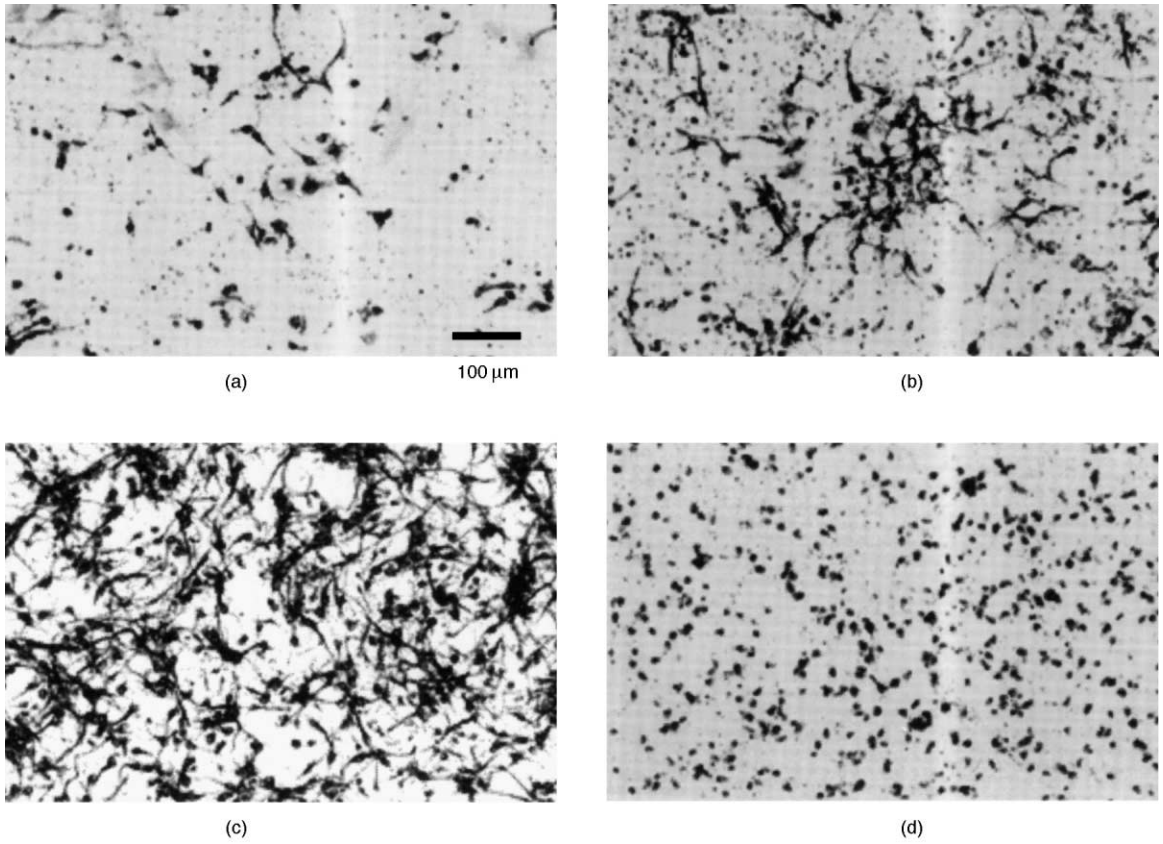

Figure 4. Enhancement of neurite survival by honokiol in primary cultures of E18 SD rat cortical neurons in DMEM/N2. After the neuronal cells (2×10^5 cells cm^{-2}) cultured for 4 days in the presence of 0.5% EtOH and honokiol were fixed by 4% paraformaldehyde-PBS, the immunohistochemical staining for MAP-2 were performed. Pictures: (a) 0.5% EtOH, (b) 1.0 μM honokiol, (c) 10 μM honokiol, and (d) 100 μM honokiol.

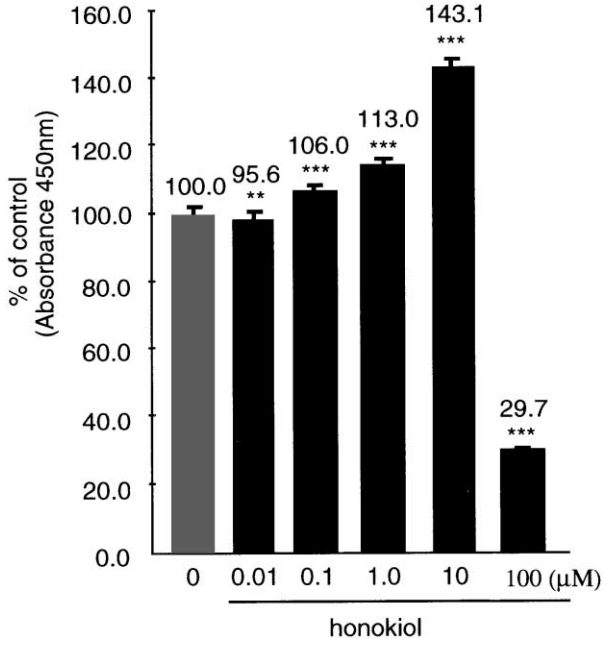

Figure 5. Cell viability of rat cortical neurons enhanced by honokiol in primary cultures. Effects of survival were assessed by the WST-8 reduction assay.[22] The data are expressed as means ± SE ($n=4$); $^{**}p<0.015$, $^{***}p<0.005$ versus control.

Next, the effect of **1** on neuronal survival was assessed using the WST 8 reduction assay.[17] The results (Fig. 5) showed that **1** enhanced cell viability in a dose-dependent manner, and its potency culminated in the summit at 10 μM. This was consistent with the morphological evaluation as shown in Figure 4.

In conclusion, we have found that honokiol (**1**), a simple biphenyl lignan, isolated from the bark of medicinal plant *Magnolia* species, can effect significantly neurite extension as well as neuronal survival in the primary cultures of rat cortical neurons. Honokiol appears to exert neurotrophic effects on neuronal cells and to affect neurite outgrowth. Although honokiol has been known to exhibit a number of biological activities related to CNS,[6] there is no report on neurotrophic activity of honokiol. Honokiol shows an anxiolytic-like activity in vivo,[18] and recently $GABA_A$ receptor was reported to be involved in this activity mediated by honokiol.[19] In addition, activation of $GABA_A$ receptor by the treatment of honokiol plays an important role in a variety of CNS related activities,[20] and thus trophic actions induced by honokiol are presumably closely related to $GABA_A$ receptors. Our studies along this line are now under way to clarify neurotrophic effects of honokiol.

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Cell culture in DMEM/N2: The procedure for cell cultures was essentially the same as NBM/B27 except for the followings: DMEM supplemented with N2[21] was used as a serum-free cultured medium; a cell density of 2×10^5 cells cm^{-2} was plated on; the cells were fixed 3 days after the addition of the test samples.
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12. The neurite outgrowths affected by samples as an average of neurite length were analyzed under microscope. 80 numbers of neurons, which did not grow on or near glial cells and made no network-formation to more than two cells, and were well stained with anti-MAP-2, were selected for measurements of each sample. Length of the longest neurite extended from a cell body was measured and calculated by using Lumina Vision and MacSCOPE software.
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