**Current Topics**

Pharmaceutical Research for Quality Evaluation and Characterization of Foods and Natural Products

**Review**

Isolation and Characterization of Neuroprotective Components from Citrus Peel and Their Application as Functional Food

Yoshiko Furukawa, Satoshi Okuyama, Yoshiaki Amakura, Atsushi Sawamoto, Mitsunari Nakajima, Morio Yoshimura, Michiya Igase, Naohiro Fukuda, Takahisa Tamai, and Takashi Yoshida

*Department of Pharmaceutical Pharmacology, College of Pharmaceutical Sciences, Matsuyama University; 4–2 Bunkyo-cho, Matsuyama, Ehime 790–8578, Japan: †Department of Pharmacognosy, College of Pharmaceutical Sciences, Matsuyama University; 4–2 Bunkyo-cho, Matsuyama, Ehime 790–8578, Japan: ‡Department of Geriatric Medicine and Neurology, Ehime University Graduate School of Medicine; 454 Shizugawa, To-on, Ehime 791–0295, Japan: §Ehime Institute of Industrial Technology; 487–2 Kamekubota, Matsuyama, Ehime 791–1101, Japan: and ¶Department of Pharmaceutical Sciences, Okayama University; Okayama 701–1152, Japan.

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The elderly experience numerous physiological alterations. In the brain, aging causes degeneration or loss of distinct populations of neurons, resulting in declining cognitive function, locomotor capability, etc. The pathogenic factors of such neurodegeneration are oxidative stress, mitochondrial dysfunction, inflammation, reduced energy homeostasis, decreased levels of neurotrophic factor, etc. On the other hand, numerous studies have investigated various biologically active substances in fruit and vegetables. We focused on the peel of citrus fruit to search for neuroprotective components and found that: 1) 3,5,6,7,8,3’-heptamethoxyflavone (HMF) and auraptene (AUR) in the peel of Kawachi Bankan (*Citrus kawachiensis*) exert neuroprotective effects; 2) both HMF and AUR can pass through the blood–brain barrier, suggesting that they act directly in the brain; 3) the content of AUR in the peel of K. Bankan was exceptionally high, and consequently the oral administration of the dried peel powder of K. Bankan exerts neuroprotective effects; and 4) intake of K. Bankan juice, which was enriched in AUR by adding peel paste to the raw juice, contributed to the prevention of cognitive dysfunction in aged healthy volunteers. This review summarizes our studies in terms of the isolation/characterization of HMF and AUR in K. Bankan peel, analysis of their actions in the brain, mechanisms of their actions, and trials to develop food that retains their functions.

**Key words** auraptene; heptamethoxyflavone; Kawachi Bankan; neuroprotection; extracellular signal-regulated kinase 1/2; antiinflammation

1. Introduction

Citrus is one of the most familiar types of fruit, and is cultivated and eaten worldwide. It has long been known that citrus fruit are a rich source of nutrients and bioactive compounds including vitamin C, folic acid, dietary fiber, pectin, and minerals.1,2) Abundant epidemiological data and experimental studies revealed that the intake of citrus fruit is beneficial to health.3,4) In the last two to three decades, intensive investigations have clarified that citrus is abundant in bioactive secondary metabolites such as flavonoids, coumarins, limonoids, and carotenoids.5) The biological activities of these citrus compounds are known to cover a wide range including antibacterial, antitumor, antiinflammatory, antioxidant, antidiabetic, and antimetabolic disorder activity.3)

There are three reasons why we tried to identify neuroprotective compounds in citrus peel. First, many bioactive secondary metabolites in citrus fruit have low molecular weight with lipophilicity, suggesting the possibility that they relatively easily pass through the blood–brain barrier (BBB) and act directly in the brain. However, the biological abilities of citrus components have been conventionally revealed using peripheral tissues or cells, and there have been few studies on their effects on the central nervous system (CNS). Second, the various bioactive components are present in all parts of citrus plants, but the peel is an especially rich source.6,7) Citrus peel is generally discarded along with the seeds because the fruit is usually consumed as fresh products or juice. The use of citrus peel as a neuroprotectant might therefore represent an effective use of waste. Third, citrus species are highly variable.8) The number of species in Tanaka’s classification systems is 156, although it is generally accepted that there were only 3 (or possibly as many as 5) original citrus species. The members of the citrus family can be readily hybridized and grafted, that is, most citrus species were created as hybrids. In Ehime prefecture, Japan, an eminent citrus-growing district, more than 40 types of citrus fruit are harvested. These facts

* To whom correspondence should be addressed. e-mail: furukawa@g.matsuyama-u.ac.jp

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suggested the high possibility of identifying new neuroprotective components in citrus plants.

2. Screening of Neuroprotective Components from Citrus Peel

As test samples, we selected representative citrus species grown in Ehime prefecture and prepared ethanol extracts from the peel of both unripe and ripe fruit. As the screening method, we analyzed the ability of citrus ethanol extracts to activate (i.e., phosphorylate) extracellular signal-regulated kinase (ERK)1/2 of neurons cultured from the rat embryonic cortex.3 ERK1/2 is one of the most common signal transduction molecules by which extracellular stimuli are propagated from the cell surface to cytoplasmic and nuclear effectors. In the nervous system, activated (phosphorylated) ERK1/2 regulates synaptic plasticity and development of long-term potentiation (LTP)10 through cellular changes including the activation of CREB, which is a transcription factor located within the nucleus.11 Activated (phosphorylated) CREB appears to be a critical step in the signaling cascade that leads to the structural changes underlying the development of LTP.12

Table 1 shows the levels of 8 ripe samples to activate ERK1/2, indicating that the extracts prepared from the peel of Kawachi Bankan (Citrus kawachiensis) have the greatest ability. The ethanol extract of K. Bankan was then partitioned into n-hexane-, ethyl acetate-, and water-soluble portions. When the neurons were treated with each extract, the n-hexane extract had the ability to phosphorylate ERK1/2. These results suggest that some nonpolar compound(s) of K. Bankan is responsible for this activity. We then isolated and characterized the active compound(s) in the hexane extract of K. Bankan. Chromatographic separation of the hexane extract revealed 4 known compounds, which were characterized by spectroscopic analysis as a coumarin derivative and 3 polymethoxyflavones (PMFs). The coumarin derivative was auraptene (7-geranyloxycoumarin; AUR), a simple coumarin characterized by spectroscopic analysis as a coumarin derivative (Fig. 1). The PMFs are 5,6,8,4'-pentamethoxyflavone (tangeretin; TGT) with 5 methoxy groups at the 5,6,7,8,3' positions (Fig. 1b), 5,6,7,8,3',4'-hexamethoxy flavone (nobiletin; NBT) with 6 methoxy groups at the 5,6,7,8,3', and 4' positions (Fig. 1c), and 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF) with 7 methoxy groups at the 3,5,6,7,8,3', and 4' positions (Fig. 1d).

Before our research, the neuroprotective abilities in the CNS of TGT and NBT had been reported. The neuroprotective ability of TGT was investigated in Parkinson’s disease (PD) model rats induced by 6-hydroxydopamine; subchronic treatment (oral administration) with high doses of TGT markedly reduced the loss of tyrosine hydroxylase-positive cells in the substantia nigra (SN) and decreased the striatal dopamine content.13 The neuroprotective ability of NBT was investigated in Alzheimer’s disease (AD) model mice induced by olfactory bulbectomization.14 We focused on AUR and HMF thereafter, because their effects in the brain had not yet been reported. As expected, we confirmed that HMF and AUR had the ability to phosphorylate CREB in cultured neuronal cells.

3. Analysis of Citrus Components

When we analyzed the constituents in the peel of more than 20 species of citrus (Ougonkan, Unshu Mikan, Ponkan, Kanpei, Benimadonna, Harehime, Marihi, Setoka, Haruka, Harumi, Kiyomi, Iyokan, kan Terocco, blood orange (Moro), mandarin orange (Kara), Dekopon, Hassaku, Amanatsu, Anseikan, Buntan, Grapefruit, K. Bankan, Lemon, Harehime, and Buttekan) using HPLC, we observed that the peel of K. Bankan contains 15 constituents, including AUR, TGT, NBT and HMF.15 As shown in Table 1, AUR is detected only in the peel of a kind of Citrus maxima (Hassaku, Amanatsu, Anseikan, Buntan, K. Bankan), and its level in K. Bankan was about three-fold greater than that in Hassaku (Citrus hassaku), which until then was thought to be richest in AUR among citrus fruit.16 Table 1 also shows that HMF was detected in the peel of many species examined, and its level in the peel of K. Bankan was the highest among the citrus species we examined. The most abundant flavonoid in the peel of K. Bankan was naringin (NGIN; naringenin-7-O-neohesperidoside) (Fig. 1e). NGIN, the glycosidic form of naringenin, was shown to protect mouse bone marrow cells against radiation-induced DNA damage and decline in cell proliferation.17 The peel of K. Bankan also contained narirutin (NRTN; naringenin-7-O-rutinoside), an isomer of NGIN.

Table 1. Summary of ERK 1/2 Activation by the Peel Extracts and the Contents of Auraptene/3,5,6,7,8,3'-Heptamethoxyflavone in the Peel of Citrus Fruits

| Conventional name | ERK1/2 activation | Content of AUR (%) | Content of HMF (%) |
|-------------------|-------------------|-------------------|------------------|
| Ougonkan          | n.d.              | 0.008             |                  |
| Unshu Mikan       | +                 | 0.010             |                  |
| Ponkan            | +                 | n.d.              |                  |
| Kanpei            | n.d.              | 0.016             |                  |
| Benimadonna       | n.d.              | n.d.              |                  |
| Harehime          | n.d.              | 0.038             |                  |
| Marihi            | n.d.              | 0.007             |                  |
| Setoka            | n.d.              | n.d.              |                  |
| Haruka            | n.d.              | 0.013             |                  |
| Harumi            | n.d.              |                   |                  |
| Kiyomi            | n.d.              | 0.012             |                  |
| Iyokan            | n.d.              | 0.008             |                  |
| Blood Orange (Tarocco) | n.d.     | 0.011             |                  |
| Blood Orange (Moro) | +                 |                   |                  |
| Mandarin Orange (Kara) | +               |                   |                  |
| Dekopon           |                  | 0.006             |                  |
| Hassaku           | 0.028             | 0.011             |                  |
| Amanatsu          | 0.005             | n.d.              |                  |
| Anseikan          | 0.004             | n.d.              |                  |
| Buntan            | 0.002             | n.d.              |                  |
| Grapefruit        | 0.020             |                   |                  |
| Kawachi Bankan    | + +               | 0.078             | 0.025            |
| Lemon             |                   | n.d.              |                  |
| Hirami Lemon      | +                 |                   |                  |
| Buttekan          |                   | n.d.              |                  |

(a): Not detected. a) Fresh peels were homogenized in ethanol and concentrated in vacuo to give an ethanol extract. The cultured neurons were treated for 30 min with this ethanol extracts (100 μg/mL). The protein extracts were prepared from the cells and applied to Western blot analysis with antibody against phosphorylated ERK1/2 along with antibody against unphosphorylated ERK1/2. b, c) The contents of AUR and HMF were analyzed by HPLC.
4. Neuroprotective Effects of HMF in the Brain

In the 1990s and 2000s, a few groups reported on the antitumor and antiinflammatory activity of HMF, but there were no reports showing that HMF acted in the CNS. We showed in an *in vitro* study that HMF had the ability to promote neurite outgrowth from neuro2a cells, which is an immortalized murine nerve cell line used in studies of neuronal differentiation.

In that *in vivo* study, we peripherally administered HMF into various pathological mouse models and investigated whether HMF has neuroprotective effects in the brain (Table 2). Behavioral pharmacological analysis showed that HMF suppressed the memory dysfunction in MK-801-induced transient amnesia model mice, abnormal motility in global cerebral ischemia model mice, or those in MK-801-induced schizophrenia positive symptom-like model mice. Immunochemical analysis showed that HMF suppressed neuronal cell death in the hippocampus (the region associated with memory) of the ischemic brain.

One of the mechanisms of neuroprotection by HMF was suggested to be antiinflammatory activity, because immunohistochemical analysis showed that HMF suppressed the hyperactivation of microglia and/or astrocytes in the ischemic brain and in the brain of lipopolysaccharide (LPS)-induced systemic inflammatory model mice. Microglia, immunocompetent cells in the CNS, are known to play important roles in removing damaged neurons and infections in the CNS. Astrocytes, which play a regulatory role in brain functions, were also shown to play a crucial role in the progression of the inflammatory state. Inflammation is a protective response to harmful stimuli, such as pathogens, damaged cells, or irritants, but excessive inflammatory activity can have a negative impact on normal tissues and organs.

Another mechanism of neuroprotection by HMF was suggested to be inducing the expression of brain-derived neurotrophic factor (BDNF), a representative neurotrophic factor (NTF) in the brain. Immunohistochemical analysis of these brain samples showed that the most BDNF-positive cells were merged with antiglial fibrillary acidic protein (GFAP; an intermediate filament protein of mature astrocytes) antibody staining, suggesting that HMF stimulated BDNF synthesis in astrocytes. We then investigated the mechanism of BDNF induction by HMF *in vitro* using rat C6 astroglialoma cells and found that HMF induces BDNF via cAMP/ERK/CREB signaling and reduction of phosphodiesterase activity.

The permeation of HMF into the mouse brain was confirmed after its intraperitoneal or oral administration. When HMF was given to mice intraperitoneally, it was immediately detected in the brain and then promptly disappeared, suggesting that it passes rapidly through the BBB and undergoes metabolism in the brain. When HMF was given orally to mice, it was also immediately detected in the brain. These findings suggested that HMF acts directly in the brain.
Table 2. Summary of Our Studies Reporting Neuroprotective Effect of HMF in Brain

| Experimental model                          | Administration method | Effect                                                                                           | Ref. |
|---------------------------------------------|-----------------------|--------------------------------------------------------------------------------------------------|------|
| Transient global cerebral ischemic model mice | s.c. (continuous) 25 or 50 mg/kg/d 8d 5d before surgery 3d after surgery                         | 1) Induction of phosphorylation of ERK1/2 and CREB and 2) induction of BDNF expression in astrocytes in hippocampal DG; 3) induction of neurogenesis in subventricular zone or DG subgranular zone in hippocampus. | 33   |
|                                             | s.c. (continuous) 25 or 50 mg/kg/d 3d (start just after surgery)                              | 1) Protection against memory dysfunction in Y-maze test; 2) rescue of neuronal cell death in the CA1 cell layer; 3) induction of BDNF expression; 4) stimulation of autophosphorylation of CaMK II and 5) suppression of hyperactivation of microglia in hippocampus. | 26   |
| MK-801-induced transient amnesia model mice  | s.c. (continuous) 50 mg/kg/d 7d                                                           | 1) Restoration of deterioration of spatial learning performance in Morris water maze test. | 9    |
| MK-801-induced schizophrenia positive symptom-like model mice | s.c. (once a day) 25 or 50 mg/kg/d 7d i.p. (once a day) 50 mg/kg/d 7d | 1) Protection of locomotor hyperactivity in Y-maze test and open field test. | 27   |
| LPS-induced systemic inflammatory model mice | s.c. (once a day) 100 mg/kg/d 10d                                                         | 1) Suppression of body weight loss; 2) suppression of hyperactivation of microglia and 3) suppression of IL-1β mRNA expression in hippocampus. | 29   |
| Corticosterone-induced depression model mice | s.c. (once a day) 50 mg/kg/d 9, 16, 25d                                                   | 1) Suppression of body weight loss; 2) amelioration of depression-like behavior in forced swim test; 3) amelioration of the reduced expression of BDNF in hippocampus; 4) amelioration of the reduced neurogenesis in DG subventricular zone; 5) amelioration of the reduced expression of phosphorylated CaMK II and ERK1/2. | 41   |
| Chronic unpredictable mild stressed (CUMS) mice | p.o. (once a day) 50 or 100 mg/kg/d 15d                                                   | 1) Suppression of body weight loss; 2) amelioration of depression-like behavior in forced swim test; 3) amelioration of the reduced expression of BDNF/neurogenesis/phosphorylated CaMK II in hippocampus. | 35   |

MK-801: an antagonist of a NMDA receptor (one of the glutamate receptor which plays an important role in synaptic plasticity and synapse formation underlying memory, learning and formation of neural networks during development in the CNS). LPS: lipopolysaccharide (inflammatory agent). DG: dentate gyrus (the site which plays critical roles both in cognitive processing, and in regulation of the induction and propagation of pathological activity). CA1: the first region in the hippocampal circuit. CaMK II: calcium-calmodulin-dependent protein kinase II (serine/threonine protein kinase). s.c. (subcutaneous). i.p. (intraperitoneal). p.o. (per os). Transient global cerebral ischemic model mice were prepared by a bilateral common carotid artery occlusion (2VO) surgery. LPS-induced transient amnesia model mice were prepared by i.p. injection of MK-801 at the concentration of 0.05 mg/kg, 30 min before the behavior test.35) MK-801-induced transient amnesia model mice were prepared by i.p. injection of MK-801 at the concentration of 0.2 mg/kg; thirty minutes after the last sample administration on Day 7.28) Corticosterone-induced depression model mice were prepared by s.c. administration of corticosterone at the dose of 20 mg/kg/d once a day during the experimental period.8) CUMS procedure (14d) was consisted of six stressors including a forced swim stress (15 min), wet sawdust stress (24 h), food deprivation stress (24 h), restraint stress (2 h), cage tilt stress (30°, 24 h), foot-shock stress (intensity 0.5 mA, duration 5 s., inter shock interval 30 s., 20 shocks).7) BDNF is known to promote neurogenesis.36) Our immunohistochemical analysis of ischemic brain samples showed that HMF treatment markedly increased the number of neuronal precursor cells.37) BDNF was also shown to be involved in stress-induced depressive behavior, because decreases in hippocampal BDNF levels are correlated with that behavior, and treatment with antidepressants was suggested to restore BDNF levels.37–40) We thus evaluated the antidepressive effects of HMF in corticosterone-induced depression model mice31) and chronic unpredictable mild stressed (CUMS) mice.35) The results showed that HMF effectively suppressed the depressive-like behavior of stress-induced neurochemical changes in these mice such as reduced BDNF expression, decreased neurogenesis, and decreased level of phosphorylated calcium-calmodulin-dependent protein kinase (CaMK) II (the enzyme thought to be an important mediator of learning and memory) in the hippocampus. These effects of HMF were inhibited by the preadministration of U0126, a mitogen-activated protein (MAP) kinase inhibitor. Those results suggested that HMF is beneficial for the upregulation of BDNF in the hippocampus via the ERK1/2/MAP system, which may account for its anti-depressive effects.

5. Neuroprotective Effects of AUR in the Brain

In the 2000s, a huge body of evidence accumulated indicating that AUR has various biological functions in the peripheral tissues, such as antiinflammatory activity, anticarcinogenic activity, and regulation of hepatic lipid metabolism.7) One in vitro study found that AUR exerts protective effects against NMDA-induced excitatory neurotoxicity in mixed cortical cell cultures (composed of neurons and astrocytes).48) We showed in an in vitro study that AUR had the ability to promote neurite outgrowth from PC12 cells,7) a model system for studies on neuronal differentiation.

We peripherally administered AUR into various pathological model mice and found that it exerted neuroprotective effects in the brain, as shown in Table 3. Immunohistochemi-
cal analysis showed that AUR suppressed neuronal cell death in the hippocampus of the ischemic brain and the brain of LPS-induced PD model mice. The neuroprotective effects of AUR may be mediated by the suppression of the inflammatory response, as it effectively inhibits hyperactivation of the microglia and astrocytes and hyperexpression of cyclooxygenase (COX)-2 (an inducible enzyme in inflammation) in the brain. In in vitro studies using primary cultured astrocytes, AUR effectively suppressed the LPS-induced expression of COX-2 mRNA and IL-1β mRNA.

Our latest studies showed that AUR stimulated gdnf gene expression via the upregulated protein kinase A (PKA)/ERK/CREB pathway in C6 cells. GDNF, which was originally found as a trophic agent for midbrain dopamine neurons, has pronounced neuroprotective effects in various nervous system pathologies, including ischemic brain damage and neurodegenerative diseases. AUR also slightly but significantly induced bdnf gene expression via the upregulated ERK/CREB pathway in neuro2a cells. Those in vitro study results suggested that the induction of neurotrophic factor in astrocytes and neurons may be one of the mechanisms partially accounting for the neuroprotective effects of AUR.

Recently, another group has shown that intraperitoneal or subcutaneous administration of AUR can reverse scopolamine-disrupted memory in mice, and oral administration of AUR was found to exert neuroprotective activity and memory-enhancing effects in ischemic model rats. We also found that intraperitoneal administration of AUR increased oligodendroglial lineage precursor cells in a cuprizone-induced mouse model of demyelination, probably via monocyte/macrophage functions.

The permeability of AUR into the mouse brain was confirmed after intraperitoneal administration. As shown in Fig. 2, the AUR level in the brain increased gradually at 5 and 10 min, with the highest concentration detected 30 min after administration, and was detected even after 60 min. These results revealed the permeability of AUR through the BBB and suggested that AUR acts directly in the brain.

6. Neuroprotective Effects of K. Bankan Peel

As the peel of K. Bankan is a rich source of AUR with

![Fig. 2. Time Course of the Brain Concentration Profiles of AUR, HMF, NBT and TGT](image)

After i.p. administration of each sample (50 mg/kg), brain was removed at the indicated times for analysis by HPLC using a Shimadzu Prominence system. The results are presented as standard error of the mean (S.E.M.) (n = 3 at each time point). AUR ( ), HMF ( ), NBT ( ) and TGT ( ). (Color figure can be accessed in the online version.)

### Table 3. Summary of Our Studies Reporting Neuroprotective Effect of AUR in Brain

| Experimental model | Administration method | Effect | Ref. |
|--------------------|-----------------------|--------|------|
| Transient global cerebral ischemic model mice | s.c. (continuous) 25 or 50 mg/kg/d 8d (start just after surgery) | 1) Suppression of hyperactivation of microglia; 2) inhibition of COX-2 expression in astrocytes, and 3) inhibition of neuronal cell death in hippocampus. | 49) |
| | s.c. (continuous) 10 or 25 mg/kg/d 8d 5d before surgery 3d after surgery | 1) Suppression of hyperactivation of microglia and 2) inhibition of COX-2 mRNA expression in astrocytes in hippocampus. | 52) |
| LPS-induced systemic inflammatory model mice | s.c. (once a day) 10 or 25 mg/kg/d 7d | 1) Suppression of hyperactivation of microglia and 2) inhibition of COX-2 expression in astrocytes in hippocampus. | 51) |
| LPS-induced model mice of PD | s.c. (once a day) 25 mg/kg/d 21d | 1) Suppression of hyperactivation of microglia and 2) suppression of dopaminergic neuronal cell death in the SN. | 50) |
| Cuprizone-induced mouse model of demyelination | i.p. (twice a week) 17 or 50 mg/kg 3 weeks | 1) Increase in the number of oligodendrocyte lineage precursor cells; 2) suppression of hyperactivation of microglia. | 58) |

COX-2: cyclooxygenase-2. PD: Parkinson’s disease. SN: substantia nigra (a region in the midbrain). Transient global cerebral ischemic model mice were prepared by 2VO surgery. LPS-induced systemic inflammatory model mice were prepared by i.p. injection of LPS at the concentration of 1.0 mg/kg on Day 7. LPS-induced model mice of PD were prepared by the intranigral injection of LPS (3 µg/hemisphere for both hemisphere) on Day 1. Cuprizone-induced mouse model of demyelination were prepared by the feeding a diet containing 0.2% cuprizone for 4 weeks.
antinflammatory activity as mentioned above, we attempted to ascertain whether administration of the peel powder was effective against inflammation in the brain. To prepare the dried peel powder, the peels of the fruits (after having been squeezed to obtain the juice) were chopped into small pieces, dried in vacuo at 60°C for 40 h, ground to a fine powder with a mill mixer, and passed through a 150-mesh sieve. This peel powder contained AUR 4.07 ± 0.033 mg/g, HMF 0.27 ± 0.0039 mg/g, NGIN 44.02 ± 0.491 mg/g, and NRTN 4.46 ± 0.0563 mg/g.\(^{51}\) The dried peel of K. Bankan was administered orally to various pathological model mice, and we found that it exerted neuroprotective effects in the brain. As shown in Table 4, immunohistochemical analysis showed that the peel of K. Bankan suppressed neuronal cell death in the ischemic brain\(^{59}\) and the brain of LPS-induced PD model mice.\(^{60}\)

The K. Bankan peel preparation also ameliorated the suppressed neurogenesis in the brain of streptozotocin (STZ)-induced hyperglycemia model mice, type 2 diabetic db/db mice,\(^{61}\) and senescence-accelerated mice prone 8 (SAMP8).\(^{52}\) Those neuroprotective effects may be mediated via suppression of the inflammatory response, as the peel preparation effectively inhibits hyperactivation of microglia and astrocytes.\(^{51,59,60}\)

We investigated the relationship between the phytochemical concentration and antinflammatory activity of peel fractions in LPS-induced systemic inflammatory model mice. The results indicated that: 1) AUR but not NGIN was the main ingredient responsible for the antinflammatory activity of the dried peel of K. Bankan; and 2) flavedo tissue (the outer colored layer of the mesocarp of a citrus fruit) but not albedo (the inner layer) contained sufficient amounts of AUR to exert the antinflammatory activity.\(^{51}\) Although the peel of K. Bankan contains an abundance of NGIN, it was reported that it is difficult for NGIN to cross the BBB.\(^{53}\) However, when the BBB is disrupted by traumatic brain injury\(^{64}\) or cerebral ischemic injury,\(^{59,65}\) NGIN might be able to exert neuroprotective effects.

### 7. Neuroprotective Effects of AUR-Rich K. Bankan Juice

K. Bankan is mainly eaten raw or drunk as juice. In our previous study,\(^{51}\) we observed that: 1) part of some bioactive constituents including AUR and NGIN transferred from the peel to the juice during squeezing; and 2) the dried juice powder contained sufficient levels of those bioactive constituents to exert antinflammatory effects. As the level of AUR in the raw juice was low (0.32 ± 0.0003 mg/g), we prepared test juice enriched in AUR (0.946 ± 0.0096 mg/g) by adding peel paste.\(^{66}\) When the dried powder of AUR-enriched K. Bankan juice was given orally to ischemic mice, ischemia-induced neuronal cell death in the hippocampus was significantly suppressed via inhibition of inflammatory processes (Fig. 3).

### 8. Randomized, Placebo-Controlled, Double-Blind Study of AUR-Enriched K. Bankan Juice in Healthy Volunteers

Accumulated evidence confirmed that inflammation is primarily a physiological response to infection/injury and a key contributor to the pathophysiological process of chronic diseases including asthma, age-related neurological disorders, and diseases including asthma, age-related neurological disorders, and senescence-accelerated mouse prone 8 (SAMP8)\(^{67}\) were purchased from Japan SLIC (Hamamatsu, Japan)

### Table 4. Summary of Our Studies Reporting Neuroprotective Effect of the Dried Powder of Kawachi Bankan in Brain

| Experimental model | Administration method | Effect | Ref. |
|-------------------|------------------------|--------|------|
| LPS-induced systemic inflammatory model mice | p.o. (once a day) 1.2 or 2.4 g/kg/d 7d | 1) Suppression of body weight loss; 2) suppression of hyperactivation of microglia/astrocytes and 3) suppression of COX-2 expression in hippocampus. | 51) |
| Transient global cerebral ischemic model mice | p.o. (once a day) 1.2 or 2.4 g/kg/d 8d 5d before surgery 3d after surgery | 1) Suppression of neuronal cell death; 2) reversed the reduction in the level of phosphorylated CaMK II; 3) the tendency to reverse the reduction in the level of glutathione; 4) suppression of hyperactivation of microglia/astrocytes. | 59) |
| STZ-induced hyperglycemia model mice | p.o. (once a day) 1.2 or 2.4 g/kg/d 7d | 1) Suppression of hyperactivation of microglia in the hippocampus; 2) suppression of hyper-phosphorylation of tau at 231 of Thr and 396 of Ser in hippocampal neurons; 3) the tendency to reverse the reduction in the level of glutathione; and 4) suppression of hyperactivation of microglia/astrocytes. | 61) |
| LPS-induced model mice of PD | p.o. (once a day) 1.2 or 2.4 g/kg/d 20d | 1) Suppression of hyperactivation of microglia and 2) protection against dopaminergic neuronal cell death in the SN. | 60) |
| Type 2 diabetic db/db mice | p.o. (free feeding) mixed diet containing 0.5% or 1% dried peel powder 14 weeks | 1) Suppression of hyperactivation of astrocytes in the hippocampus; 2) suppression of hyper-phosphorylation of tau at 231 of Thr and 396 of Ser in hippocampal neurons; 3) amelioration of the suppressed neurogenesis in DG of hippocampus. | 61) |
| Senescence-accelerated mice (prone 8) | p.o. (free feeding) mixed diet containing 1% dried peel powder 4 months | 1) Suppression of hyperactivation of microglia in hippocampus; 2) suppression of hyper-phosphorylation of tau at 231 of Thr in hippocampal neurons; 3) amelioration of the suppressed neurogenesis in DG of hippocampus. | 62) |

| Thr: threonine. Ser: serine. STZ: streptozotocin. LPS: lipopolysaccharide. NGIN: naringin. NRTN: naringenin. AUR: auraptene. COX-2: cyclooxygenase-2. CaMK II: calcium/calmodulin-dependent protein kinase II. BBB: blood-brain barrier. PD: Parkinson's disease. SAMP8: senescence-accelerated mouse prone 8 (SAMP8). |

1.2 or 2.4 g/kg/d on Day 1.\(^{61}\) Type 2 diabetic db/db mice\(^{61}\) and senescence-accelerated mouse prone 8 (SAMP8)\(^{67}\) were purchased from Japan SLIC (Hamamatsu, Japan). LPS-induced model mice of PD were prepared by the intranigral injection of LPS (2 µg/hemisphere for both hemisphere) on Day 1.\(^{51}\)
and neurodegenerative diseases such as AD, PD, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and brain tumors.67,68) The elderly are likely to be constantly exposed to the risks of neuroinflammation, causing age-related dysfunctions including cognitive impairment.69) Our findings combined with those of others prompted us to investigate whether AUR-enriched K. Bankan juice could contribute to the prevention of cognitive dysfunction in elderly individuals.

We therefore conducted a randomized, placebo-controlled, double-blind clinical trial involving 84 healthy adult volunteers with normal cognitive function. Half received test juice containing AUR at the concentration of 0.1 mg/d for 24 weeks. The results of the Mild Cognitive Impairment (MCI) screening using the 10-word immediate-recall test at the end of the 24-week trial period revealed a significant difference in the percentage change in immediate-recall test at the end of the 24-week trial period vs. Sham (### p < 0.001, ** p < 0.01, * p < 0.05).70) Those results suggested the effectiveness of AUR-enriched K. Bankan juice in preventing cognitive decline in humans. Subsequently, AUR-enriched K. Bankan juice was approved for listing as a “Food with Functional Claims” by the Consumer Affairs Agency of Japan in 2018.

9. Conclusion

In our exploratory in vitro study using neurons or neuronal cells, citrus AUR and HMF induced the activation of the ERK/CREB pathway and neurite promotion, suggesting that AUR and HMF have neuroprotective ability.57,25) In subsequent in vivo studies using mouse models of various neuropsychiatric disorders, although direct neuronal effects of AUR and HMF were not observed, it appeared that AUR and HMF might exert neuroprotective effects via suppression of the excessive antiinflammatory response by microglia/astrocytes and the induction of neurotrophic factors in astrocytes. Are neurons the site of action of AUR and HMF in the CNS in vivo? In an in vitro study, AUR induced BDNF expression in neuro2a cells, although at a low level.55) While AUR and HMF may act on neurons, they exhibited greater activity on glial cells under our experimental conditions (disease states). The site of their antiinflammatory activity in the CNS is glial cells,30,31) instead of neurons, and NTFs in glial cells play important roles in the CNS in regulating neurodegeneration and injury.71)

K. Bankan peel, which contains markedly higher levels of AUR compared with the peel of other citrus fruit, exerted neuroprotective effects in mouse models of neuropsychiatric disorders by mediating antiinflammatory effects on microglia/astrocytes.51,59–62) AUR-enriched K. Bankan juice also exerts neuroprotective effects in the ischemic mouse brain66) and may be effective in preventing cognitive decline in humans.70) Studies to identify neurotrophic functions of phytochemicals which may provide neuroprotection in age-related neurodegenerative disorders continue to increase,22–24) and we believe that citrus peel might be a beneficial source of neuroprotective phytochemicals in the future.

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Conflict of Interest Y. Furukawa, S. Okuyama, Y. Amakura, M. Nakajima, M. Yoshimura, M. Igase, N. Fukuda, and T. Tamai were involved in the development of AUR-enriched K. Bankan juice (Food with Functional Claims) with Ehime Beverage Inc. (Ehime, Japan).

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Fig. 3. Effects of Dried AUR-Rich Juice of Kawachi Bankan on Ischemic-Induced Neuronal Cell Loss, Ischemic-Induced Hyperactivation of Microglia and Ischemic-Induced Hyperactivation of Astrocytes in the Mouse Brain

(A) The number of intact neurons in CA1 regions of the hippocampus were counted as Nissl staining-positive cells. (B) The levels of microglial activation in the stratum lacunosum-molecular/stratum radiatum of the hippocampal region were analyzed as the density of glial fibrillary acidic protein (GFAP; an astroglial marker) staining signal. (C) The levels of astroglial activation in the stratum lacunosum-molecular/stratum radiatum of the hippocampal region were analyzed as the density of ionized calcium-binding adaptor molecule 1 (IBA1; a microglial marker) staining signal.
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