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The flavonoid myricetin reduces nocturnal melatonin levels in the blood through the inhibition of serotonin N-acetyltransferase

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ABSTRACT

Melatonin is secreted during the hours of darkness and is thought to influence the circadian and seasonal timing of a variety of physiological processes. AANAT, which is expressed in the pineal gland, retina, and various other tissues, catalyzes the conversion of serotonin to N-acetylserotonin and is the rate-limiting enzyme in the biosynthetic pathway of melatonin. The compounds that modulate the activity of AANAT can be used to treat patients with circadian rhythm disorders that are associated with specific circadian rhythm alterations, such as shift work disorder. In the present study, we screened modulators of AANAT activity from the water extracts of medicinal plants. Among the 267 tested medicinal plant extracts, Myricae Cortex (*Myrica rubra*), Perillae Herba (*Perilla sikokiana*), and Eriobotryae Folium (*Eriobotrya japonica*) showed potent inhibition of AANAT activity. Myricetin (5,7,3′,4′,5′-pentahydroxyflavonol), a main component of the Myricae Cortex, strongly inhibited the activity of AANAT and probably block the access to the substrate by docking to the catalytic residues that are important for AANAT activity. Myricetin significantly decreased the nocturnal serum melatonin levels in rats. In addition, the locomotor activity of rats treated with myricetin decreased during the nighttime and slightly increased throughout the day. These results suggest that myricetin could be used as a therapy to increase nighttime alertness by changing the circadian rhythm of serum melatonin and locomotor activity.

1. Introduction

Melatonin is thought to influence the circadian and seasonal timing of a variety of physiological processes including sleep, mood, reproduction, and aging. The timing of these processes is a result of melatonin rhythm, which is synchronized to a 12:12 light:dark cycle [1–4]. Melatonin imbalance has been implicated in various diseases, including delayed sleep phase syndrome, advanced sleep phase syndrome, jet lag syndrome, shift work disorder, depression, seasonal affective disorder, and premenstrual syndrome [5–8]. Shift work disorder has been characterized as insomnia during the hours allotted for sleep or excessive sleepiness during waking hours, resulting in an increased potential public health risk due to occupational or vehicular accidents. Some therapies, such as chronobiotics, ramelteon, planned sleep schedules or timed light exposure, are used to normalize the disrupted circadian cycle, whereas other therapies focus on alleviating the symptoms of the disorder. These therapies include reducing daytime insomnia with melatonin or sedative hypnotics and reducing nighttime excessive sleepiness with wakefulness-promoting agents such as modafinil, armodafinil, caffeine, and amphetamines [9].

The daily rhythm of melatonin is a unique characteristic of the pineal gland. Melatonin levels are low during the day and high at night. The switch between the day and night profiles of melatonin is predominantly regulated by the activity of serotonin N-acetyltransferase (AANAT, acetyl coenzyme A: arylalkylamine N-acetyltransferase; EC 2.3.1.87). AANAT catalyzes the conversion of serotonin to N-acetylserotonin, which is subsequently methylated by hydroxyindole-O-methyltransferase (HIOMT) to synthesize melatonin [3]. Previously, we have demonstrated that rhythmic AANAT mRNA decay and translation were essential to maintain circadian melatonin production [10,11]. Therefore, the modulation of

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AANAT activity could be used to treat patients with circadian rhythm disorders that are associated with specific circadian rhythm alterations, such as shift work disorder.

Flavonoids are a class of plant-derived polyphenolic substances that have various biological and pharmacological properties, such as anti-inflammatory, anti-allergenic, anti-viral, pro- or anti-mutagenic, pro- or anti-carcinogenic, antibacterial, and antioxidant effects [12,13]. Flavonoids are generally considered nontoxic and are found in many traditional herbal medicines [14] and in the human diet [15]. Flavonoids inhibit many enzymes, including protein kinase C, tyrosine kinase, thyroid peroxidase, NO-synthase, and glutathione S-transferase [16–19]. Therefore, naturally occurring flavonoids have been proposed to exert biological effects by inhibiting different enzymes. In this study, we have found that myricetin, a natural bioflavonoid, inhibits AANAT activity by binding to its substrate (Acetyl CoA) binding site. We also showed that myricetin reduces the serum melatonin level and locomotor activity in rats during the nighttime. Our results provide insightful information about the role of myricetin as a therapy to increase nighttime alertness.

2. Materials and methods

2.1. Materials

[^{3}H]Acetyl CoA was obtained from Amersham Pharrnacia Biotech. Econofluor was purchased from NEN Life Science Products (Boston, MA). Acetyl CoA, tryptamine and myricetin were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Preparation of plant extract

Medicinal plants were obtained from a local oriental medicine shop in Pohang, Gyeongbuk, Korea. Three grams of air-dried medicinal plants were extracted in a double boiler in 30 mL of distilled water for 3 h. The aqueous extracts were collected and filtered with Whatman No. 1 filter paper. One milliliter of the eluent was lyophilized to determine the concentration of the extract. The remaining extract was stored at −20 °C until further experiments.

2.3. Animals and treatment

Sprague–Dawley male rats (5 weeks of age) were purchased from Koatech Co. Ltd (Gyeonggi-do, Korea). All animal experiments were approved by the Ethics Review Committee of the Pohang center for the evaluation of biomaterials, Republic of Korea. The rat were maintained under a 12 h light/12 h dark regime with lights off at 20:00 h and *ad libitum* access to food and water prior to the day of the experiment.

2.4. AANAT assay

AANAT activity using rat pineal glands was performed as we previously described elsewhere in detail [20]. Briefly, the pineal gland, which was obtained from rats sacrificed at midnight because the peak activity of AANAT occurs at 6 h after the onset of darkness [21], was disrupted by ultrasound in 200 L ice-cold phosphate buffer (50 mM, pH 6.8). The debris was removed by centrifugation (15,000*g*, 5 min, 4 °C), and the supernatant was transferred to a new tube. The reaction mixture included 13 L supernatant, 5 L tryptamine–HCl (10 mM), 1 L acetyl CoA (0.5 mM), and 1 L [^{3}H] acetyl CoA (3.6 Ci/mmol, 250 Ci/mL) and was incubated at 37 °C for 30 min. The reaction was stopped by dilution with 180 L ice-cold phosphate buffer (50 mM, pH 6.8). Econofluor was rapidly added to the reaction mixture, and the amount of radiolabeled acetyltryptamine was determined using a liquid scintillation counter. The activity values were normalized against the amount of total protein [22].

2.5. Molecular docking

As the three dimensional structure of rat AANAT were lacking, we have built the homology model of rat ANNAT using three dimensional structure of sheep AANAT (pdb 1KUX) as template via "*Build homology models*" module in Discovery studio 3.5. Modeled protein is prepared by constraining the heavy atoms, adjusting hydrogen or any missing atoms with CHARMM force field and energy minimized using conjugate gradients algorithm for 5000 steps. 3D coordinates of the ligand myricetin were sketched and energy minimized using smart minimizer algorithm for 2000 steps in Discovery Studio 3.5. Molecular docking simulations were carried out with the GOLD 5.0 program [23]. The simulation parameters were standardized using the crystal ligand conformation, and the same parameters were used for myricetin. Annealing parameters for the van der Waals interactions and hydrogen bond interactions within 4.0 and 2.5 Å and solutions within a RMSD of 1.5 Å were considered for the interaction analysis.

2.6. Measurement of melatonin concentration

Myricetin (20 mg/kg) and saline were injected intraperitoneally (i.p.) at 20:00 h. After 6 h, rats were sacrificed and the blood was collect blood. The blood was stored at −20 °C until analysis. The serum melatonin concentrations were quantified using an enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany), according to the manufacturer's instructions.

2.7. Locomotor activity test

Myricetin (50 mg/kg) and saline were given orally (*per os*) for two weeks. Animal activity was measured in locomotor chambers (16″ × 16″) in which the activity, both horizontal and vertical, was measured by infrared beam breaks over 32 h, and the activity was scored using Acti-Track software in PheCOMP system (Harvard Apparatus, Panlab s.l.u., Spain).

2.8. Statistics

Statistical analyses were performed using GraphPad Prism software (version 5.0 GraphPad Software, USA). An unpaired two-tailed Student *t*-test was used to evaluate the differences between two means, and values of $P < 0.05$ were considered to be statistically significant.

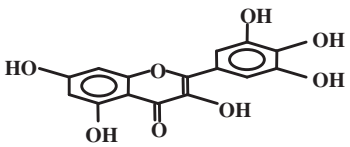
3. Results

3.1. Inhibition role of myricetin on AANAT activity

To search for a potent inhibitor of AANAT activity, we first examined how water extracts of medicinal plants affected AANAT activity. Among the 267 tested medicinal plant extracts, Myricae Cortex (*Myrica rubra* Siebold et Zuccarini), Zanthoxyli Fructus (*Zanthoxylum piperitum* De Candolle), and Perillae Herba (*Perillae sikokiana* Britton) potently inhibited AANAT activity (IC_{50} = 36.2 ng/mL, 67 ng/mL and 70 ng/mL, respectively) (Supplementary Table 1).

Next, we examined the effect of various chemical components of the effective medicinal plants on AANAT activity. Myricetin, a main component of Myricae Cortex, dramatically inhibited AANAT

Table 1
Inhibition of AANAT by myricetin.

Compounds	Structure	IC50 (μM)	Ki (μM)
Myricetin		1.64 ± 0.59	8.677 ± 3.122

with an IC_{50} of 1.64 ± 0.59 M (Fig. 1A and Table 1). Time-course experiments were also conducted with myricetin to measure the remaining activity of AANAT. AANAT activity gradually decreased as the incubation time with 100 M myricetin increased (Fig. 1B). At 15 min, the remaining activity of AANAT was 56.8%.

3.2. Predictive binding mode of myricetin with AANAT

Myricetin is deeply seated into the hydrophobic grooves formed by the residues from the loops between β1/β2, α6/α7 helices, β1, β4 sheets, and α4 helix. The chromen-4-one nucleus of myricetin possesses stacking hydrophobic interactions with residues such as F54, F186, P62, M157 and L184. The 3-hydroxyl moiety on the chromen-4-one ring formed the key hydrogen bonding contacts with L122 (Fig. 2A). The 5,7-hydroxyl groups on chromen-4-one and the 3′ are involved in interaction with A53 and S58 residues while the 3′,4′–5′ hydroxyl groups on 2-phenyl ring are engaged in hydrogen bonding with catalytically important H120, M157, L119, and Y166 residues respectively (Fig. 2A). Comparison of myricetin docked pose with the bi-substrate crystal ligand structure by superimposing both the structures reveals that myricetin docks to the catalytic site of AANAT in a manner similar to the bi-substrate analog and maintains key interactions at the catalytic site (Fig. 2B). Taken together, both the hydrophobic and the hydrogen bonding interactions with catalytically important residues stabilize the binding of myricetin and thereby inhibit the acetyltransferase activity of AANAT.

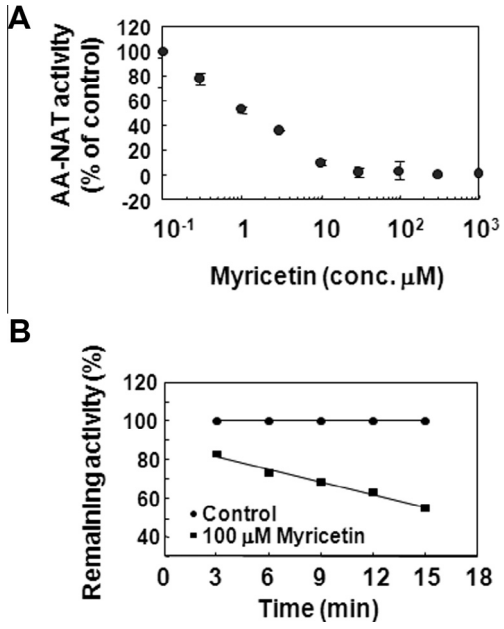


Fig. 1. The effect of myricetin on AANAT activity. (A) AANAT activity was measured at the indicated concentrations of myricetin. (B) Time-dependent inhibition of AANAT by myricetin. AANAT was incubated with 100 μM myricetin for the indicated periods of time in the assay mixture at 37 °C. The results are means ± SEM values from triplicate measurements.

3.3. The effect of myricetin on serum melatonin

To test the effects of myricetin on the nocturnal serum melatonin levels, myricetin (20 mg/kg) and saline were injected intraperitoneally (i.p.) before turning the lights off. After 6 h, the rats were sacrificed and the blood was collected. The levels of serum melatonin were measured with an enzyme-linked immunosorbent assay. Fig. 3 shows that the serum melatonin concentration in myricetin-treated rats was significantly lower (448 ± 46.7 pg/mL, $n = 6$; $P < 0.05$) than the concentration in saline-treated rats (537 ± 61.6 pg/mL, $n = 6$) during the nighttime.

3.4. Effect of myricetin on locomotor activity

An hour-by-hour analysis of the locomotor activity during a 36-h recording period showed that the activity in the myricetin-treated rats was consistently lower than the saline-treated rats during the nighttime and was slightly higher during the daytime (Fig. 4A). When the mean activity was compared separately for the dark and light periods, there was a significant decrease in the mean activity in the myricetin-treated rats and an increase in saline-treated rats during the nighttime (Fig. 4B).

4. Discussion

In this study we demonstrated the modulatory effects of myricetin on circadian rhythm. Here, we report that myricetin reduces the serum melatonin levels and locomotor activity through the inhibition of AANAT activity. Moreover, we found that myricetin is able to inhibit AANAT activity by overlaying on the tryptamine moiety of the bisubstrate analog, suggesting that myricetin might act as a pharmaceutical composition to increase nighttime alertness.

Myricetin is a widely distributed bioflavonoid, which has been reported to possess a variety of biological activities, such as anticancer [24], prevention of neurodegeneration [25], anti-diabetic [26], and anti-photoaging [27] activities. We have found that myricetin is a main ingredient in some medicinal herbs, such as *M. rubra* Siebold et Zuccarini cortex and St. John's Wort, which is used as an additive or a dietary supplement to prevent or treat emotional instability. Our results show that myricetin reduced the nocturnal melatonin levels in the blood serum (Fig. 3) in rats. Previous studies have shown that melatonin is an important component of the mammalian circadian timing system, which regulates the sleep–wake cycle. Melatonin can affect the circadian locomotor activity in rats [28–30], and sleep disruption has been linked to impaired melatonin secretion as a result of pinealectomy in humans [31–33]. Although electroencephalography is necessary to accurately differentiate between sleep and wakefulness, the locomotor activity gives an approximate estimation of the sleep–wake cycle. Accordingly, we observed that myricetin

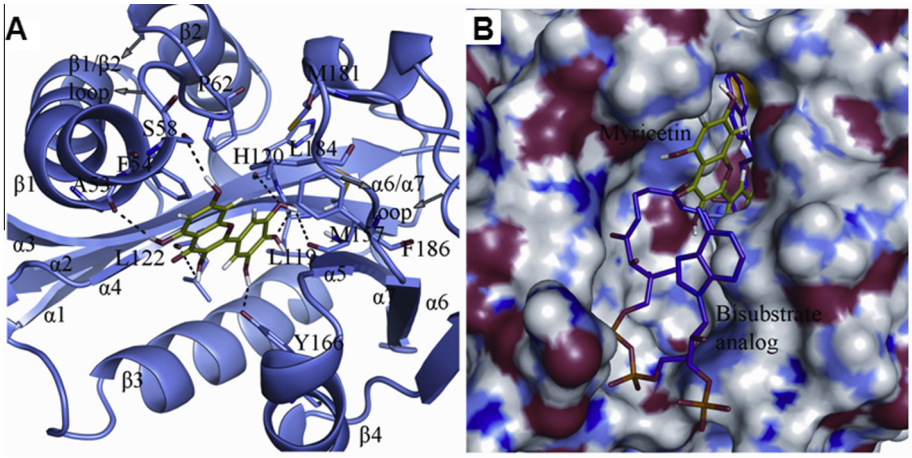


Fig. 2. The predictive binding mode of myricetin with AANAT. (A) Myricetin docks into the hydrophobic site, with strong hydrophobic interactions with the P62 F54, and F186 residues. The 3,5,7-trihydroxyl groups on chromen-4-one of myricetin are engaged in hydrogen bonding interaction with L122 and S58 and A53 residues whereas the 3′,4′–5′ trihydroxy substitutions on the 2-phenyl group interact with the catalytically important residues such as H120, M157, L119 and Y166 These interactions stabilize the binding of myricetin and thereby inhibit the AANAT acetyl-transferase activity. (B) Surface view of bisubstrate complex (magenta) and the myricetin docked complex (green).shows that myricetin docks to the substrate binding site and maintains key interactions with the critical residues (H120, Y166) that are involved in the AANAT catalysis.

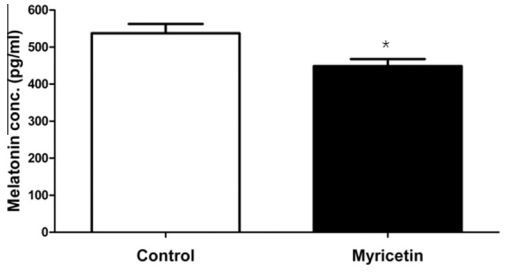


Fig. 3. Nocturnal serum melatonin concentrations of rats treated with saline or myricetin. Myricetin- (20 mg/kg, $n = 6$) and saline-treated ($n = 6$) rats were sacrificed 6 h after i.p. injection at 20:00 h. The blood was collected and the levels of serum melatonin were measured. Data are shown as the mean ± SEM. $^{*}P < 0.05$ versus control group by Student's *t*-test.

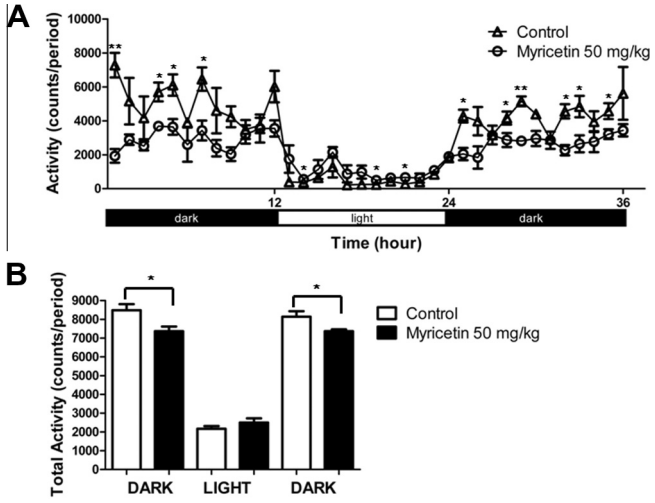


Fig. 4. The effect of myricetin on locomotor activity. (A and B) The total ambulatory locomotor activity of myricetin-(50 mg/kg, $n = 4$) and saline-treated ($n = 4$) rats was measured for 2 weeks. The locomotor activity was determined every hour for 36 h. The typical pattern of locomotor activity (A) and the mean AUC (B) were calculated. The horizontal axis shows the time, and the vertical axis shows the amplitude of locomotor activity (unit: counts). Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group by Student's *t*-test.

administration reduced the locomotor activity during the nighttime and slightly increased locomotion during the daytime in rats (Fig. 4). On the basis of these results, we suggest that the myricetin administration could influence the endogenous circadian rhythms by decreasing pineal melatonin secretion, causing the increase of nighttime alertness for shift workers.

The switch between the day and night profiles of melatonin is predominantly regulated by the activity of AANAT, which increases at night 10- to 100-fold [34]. The development of potent and specific inhibitors for AANAT is an important strategy for the pathophysiological therapeutics for various sleep–wake disorders. Although a variety of synthetic inhibitors of AANAT have been previously reported [35–37], the functions of these inhibitors are unknown. We attempted to identify AANAT inhibitors based on the enzyme activity of AANAT. We found that myricetin is potent and selective for AANAT ($K_i = 8.68$ μM, Table 1). The molecular docking study predicted that myricetin binds as a bisubstrate inhibitor through interactions with the acetyl-CoA and tryptamine binding sites (Fig. 2), suggesting that myricetin would be useful in the treatment of sleep–wake disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.076.

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