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Effect of Zaleplon, a Non-benzodiazepine Hypnotic, on Melatonin Secretion in Rabbits

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Abstract. Melatonin, a major hormone secreted by the pineal gland, is known to play an important role in regulation of the circadian rhythm. (*N*-[3-(3-cyanopyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl]-*N*-ethylacetamide (zaleplon) is a non-benzodiazepine hypnotic that acts via the benzodiazepine site of the $GABA_A$ receptor. In the present study, we investigated the effect of zaleplon on melatonin secretion in rabbits using RIA and compared the effect to triazolam and zopiclone. Zaleplon increased a dose-dependent concentration of melatonin in rabbit plasma collected at 30 min after intravenous administration at doses of 1 and 2 mg/kg. The zaleplon-induced increase in plasma melatonin level was not blocked by flumazenil, a benzodiazepine-receptor antagonist. In contrast, triazolam and zopiclone failed to affect the plasma melatonin level. We also investigated the effect of zaleplon on intracellular cAMP in rat pinealocytes. Consequently, zaleplon had no effect on the intracellular cAMP levels in rat pinealocytes. These results of the present studies suggest that zaleplon may promote melatonin secretion and the elevation of plasma levels of melatonin may suggest an influence of zaleplon on chronobiology.

Keywords: zaleplon, melatonin, pinealocyte, cAMP

Introduction

Zaleplon (*N*-[3-(3-cyanopyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl]-*N*-ethylacetamide, CL284,846), a pyrazolopyrimidine derivative, displaces [^{3}H]flunitrazepam from neuronal binding sites in vitro and enhances the binding of *t*-butylbicyclophosphorothionate, a compound known to bind to a site affiliated with the benzodiazepine /GABA receptor complex (1, 2). Pharmacokinetic studies on zaleplon indicate a plasma t_{max} and an elimination $t_{1/2}$, both, of about 1 h (3). Very low plasma concentrations of the desethyl metabolite are detected, and three major metabolites do not exhibit pharmacological effects (4). These pharmacokinetic profiles predict a rapid-acting sedative activity of relatively short duration. Moreover, zaleplon has the ability to shorten sleep latency, promote the maintenance of sleep, and increase slow wave sleep in the early phases as seen in polysomnographic studies in humans (5). Therefore, zaleplon is especially licensed for the treatment of insomnia, including disruptions in sleep chronobiology. On the contrary, disruptions in sleep chronobiology when under a non 24-h sleep/wake cycle, which may occur in shift workers or travelers experiencing jet lag, and delayed sleep phase syndrome often respond to the exogenous administration of melatonin (6 – 8). Melatonin, a major hormone secreted by the pineal gland, is known to play an important role in regulation of the circadian rhythm (9). For example, plasma melatonin concentration is significantly lower in elderly sufferers of insomnia than in age-matched controls without insomnia (10). Thus, the attenuated melatonin secretion may be the cause of sleep disturbances in elderly people, which imply that an increasing plasma melatonin level may trigger the onset of sleep and synchronization of the endogenous circadian rhythm (10).

Many frequent travelers have a specific routine for coping with jet lag that often includes the use of sleep inducing drugs. It has been reported that short-acting benzodiazepines such as triazolam and midazolam facilitate sleep for several nights after a transcontinental flight (11, 12). Since benzodiazepines are more fre-

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quently used to treat these circadian rhythm disorders, zaleplon may also be useful.

Recently, we reported that intravenous administration of zaleplon to rabbits showed a remarkable increase in the delta frequency band indicative of a hypnotic-like state on EEG. Spectral analysis indicated that both triazolam (0.1 mg/kg, i.v.) and zopiclone (2 mg/kg, i.v.) demonstrate equivalent potency with zaleplon (1 mg/kg, i.v.) with regard to increasing the delta wave activity (2). Thus, in the present study, we investigated the effect of zaleplon on melatonin secretion in rabbits in comparison with the effect of triazolam and zopiclone under the same conditions. Since it is widely known that cAMP accumulation in pinealocytes induces arylalkylamine-*N*-acetyltransferase activity, which results in the elevated production and release of melatonin (13), we also studied the effect of zaleplon on intracellular cAMP in rat pinealocytes.

Materials and Methods

All animals used in the present study were treated according to the guidelines of the National Institutes of Health on the welfare of laboratory animals.

Animals

Male Sprague-Dawley rats (250 – 300 g) and Japanese white rabbits (2.43 – 3.58 kg) were obtained from Charles River Japan (Atsugi) and Kitayama Labes Co., Ltd. (Nagano), respectively. Rats were housed five to a cage and rabbits were individually housed with free access to food and water in a controlled environment (23 ± 1°C, 50 ± 5% humidity), with a 12-h light-dark cycle (lights on 7:00 AM, off 19:00 PM). Experiments were conducted following adaptation to laboratory conditions for at least 7 days (rats) and 14 days (rabbits), respectively. The present studies were carried out between 11:00 AM and 14:00 PM.

Effect of zaleplon on melatonin secretion

Blood was collected from the auricle artery of the rabbits 30 min after intravenous administration of each drug. Flumazenil was injected 10 min prior to administration of zaleplon. Plasma was separated by centrifugation ($1{,}800 \times g$ for 10 min at 4°C) and stored at −80°C until the melatonin assay. The plasma melatonin was detected using the RIA (14). Briefly, 1 ml of plasma was extracted with 5 ml dichloromethane and the phase separated by centrifugation (4°C, $2500 \times g$, 30 min). The organic phase was dried with N_2 gas. To the residue were added [*O*-methyl-^{3}H]melatonin and a specific antiserum for melatonin (CIDtech Research, Cambridge, Canada) in a 0.05 M phosphate buffer containing 0.1% gelatin. The mixture was incubated for 19 h at 4°C and bound and free ligand were separated by the addition of saturated ammoniun sulfate. Then the mixture was incubated for 1 h in 4°C. The residue was collected by centrifugation ($4{,}000 \times g$ for 20 min at 4°C) and resuspended in 550 μl of distilled water. The radioactivity of 500 μl of this suspension was determined by a liquid scintillation counter (LSC-1000; Aloka, Tokyo).

Effects of zaleplon on intracellular cAMP level

A modification of the method of Olcese (15) was used. Briefly, male rats were killed by decapitation and their pineal glands were immediately collected in M199 medium containing 0.3% bovine serum albumin, 10 mM HEPES, 0.01% streptomycin, and 100 U/ml penicillin. The glands were minced, and dissociated with collagenase (1 mg/ml), hyaluronidase (500 μg/ml), and Dnase (10 μg/ml) at 37°C for 40 min. The suspension of cells was filtered through nylon mesh. The cells were collected by centrifugation ($230 \times g$ for 5 min at 4°C) and resuspended in M199 medium containing 10% fetal calf serum. The cells (7×10^4 cells/500 μl per well) were plated on 48-well plates and grown in monolayer culture for 4 days at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. The pinealocytes were washed with prewarmed M199 medium, and incubated with M199 medium containing zaleplon for 10 min. After removing the culture medium, the pinealocytes were washed with ice-cold PBS(−), and immediately after, ethanol at a concentration of 65% was added to extract intracellular cAMP (16). The supernatant was collected, and then dried under a stream of nitrogen at 60°C. Intracellular cAMP content in the residue, dissolved in assay buffer, was measured using a cAMP enzyme-immunoassay.

Drugs

Zaleplon and flumazenil were supplied by Wyeth Ayerst Laboratories, Philadelphia, PA, USA. Triazolam (purity: 99.9%) and zopiclone (purity: 99.9%) were extracted from commercially available tablets, Halcion® (Upjohn Japan Co., Ltd., Osaka) and Amoban® (Chugai Pharmaceutical Co., Ltd., Tokyo), respectively. [*O*-Methyl-^{3}H]melatonin (85 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, USA). Dimethyl sulfoxide ethanol, ammoniun sulfate (Wako Pure Chemicals, Osaka), and cAMP enzyme-immunoassay kit (*TiterFluor*® Dual Range cAMP EIA; PerSeptive Biosystems, Inc., Foster City, CA, USA) were purchased from the suppliers indicated in parentheses. Other chemicals and reagents of an analytical grade were obtained from commercial suppliers. Zaleplon, flumazenil, and the other hypnotic drugs were dissolved in physiological saline containing 5% dimethyl sulf-

oxide (DMSO) and intravenously administered for the melatonin secretion study in rabbit. In c-AMP assay, zaleplon was applied into M199 medium as a DMSO solution. Norepinephrine (Sankyo Co., Ltd., Tokyo), isoproterenol (Nikken Kagaku Co., Ltd., Tokyo), and 3-isobutyl-1-methylxanthine (IBMX) (Wako Pure Chemicals) were dissolved in M199 medium. The final concentration of DMSO in each well was less than 0.01%.

Statistical analyses

Data are expressed as the means ± S.E.M. Inter-group differences were analyzed statistically using Dunnett's multiple range tests. To determine the antagonism of flumazenil and the effect on cAMP accumulation, Tukey's multiple range test was used.

Results

Effect of zaleplon on melatonin secretion in rabbit

When vehicle (physiological saline containing 5% DMSO) was intravenously administered to rabbits, the concentration of melatonin in plasma at 30 min after administration was 22.7 ± 3.5 pg/ml. Rabbits injected intravenously with 1 mg/kg of zaleplon had plasma melatonin levels of 63.8 ± 9.4 pg/ml. The effect of zaleplon was significant compared to that of the vehicle-treated group ($P<0.01$). As shown in Fig. 1a, when the dose of zaleplon was increased to 2 mg/kg, the plasma melatonin level was increased in a dose dependent-manner (79.1 ± 9.3 pg/ml). The zaleplon-induced increase in plasma melatonin level was not blocked by flumazenil (Fig. 2). In addition, flumazenil alone produced a slight increase in plasma melatonin level, but the alteration was not significant as compared to the vehicle control group (Fig. 2). Neither triazolam nor zopiclone affected plasma melatonin levels at 30 min after administration, even at doses of 0.2 and 4 mg/kg, i.v. (Fig. 1: b and c). Behaviorally, the zaleplon-, triazolam-, and zopiclone-treated animals showed notable sedation, while the animals treated with flumazenil exhibited no such behavior. Moreover, there were no behavioral abnormalities or sedative actions observed in the vehicle-treated group.

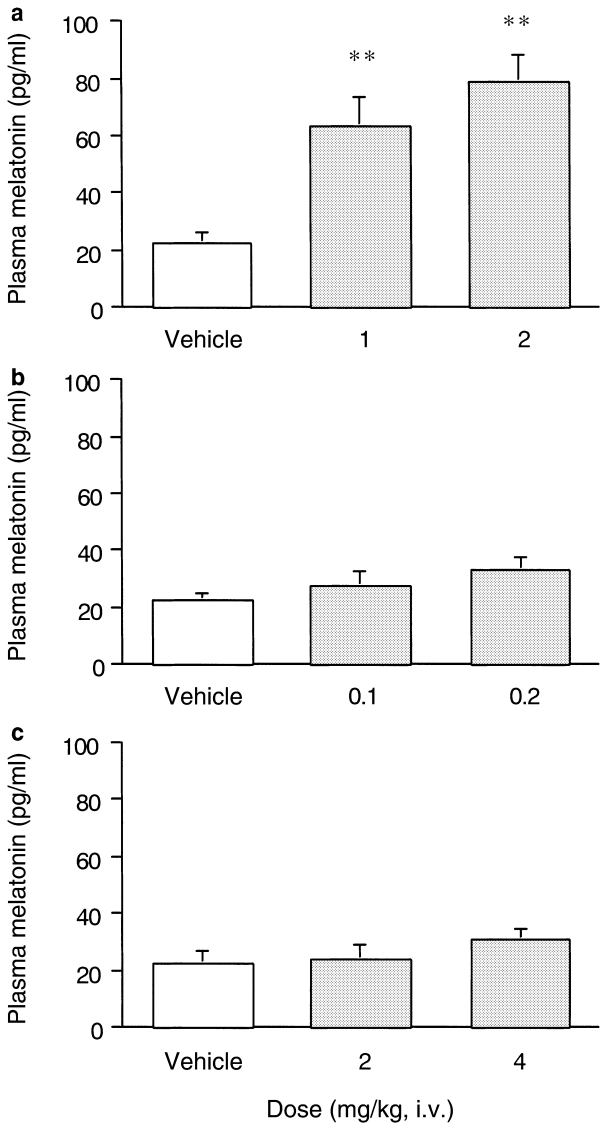

Fig. 1. Effects of zaleplon (a), triazolam (b), and zopiclone (c) on plasma melatonin level in rabbits. The experiments were carried out during 11:00 to 14:00. Blood was collected at 30 min after intravenous administration of test compound. Plasma melatonin level was determined by radioimmunoassay. Each column and bar represents the mean ± S.E.M. of 5 animals. **Significantly different from the vehicle control group at $P<0.01$ using Dunnett's multiple comparison test. One-way ANOVA; $F(2,12) = 13.640$, $P = 0.0008$.

Effects of zaleplon on Intracellular cAMP

The intracellular cAMP in the group treated with 1×10^{-5} M zaleplon for 10 min was not significantly different from that in the control group (Table 1). Then the effect of zaleplon on intracellular cAMP level under β-adrenoceptor stimulation by isoproterenol was studied. The intracellular cAMP level in the group treated with 1×10^{-7} M isoproterenol alone for 10 min was increased to 777.9 ± 38.3 fmol/well, which was significantly higher than that of the control group (319.1 ± 41.7 fmol /well). On the other hand, the combination treatment with isoproterenol (1×10^{-7} M) and zaleplon (1×10^{-5} M) produced no significant change as compared to the group treated with isoproterenol alone.

Finally, the effect of zaleplon was further studied under conditions in which the decomposition of intracellular cAMP could be ignored; IBMX, an inhibitor of intracellular phosphodiesterase, was used. Combination

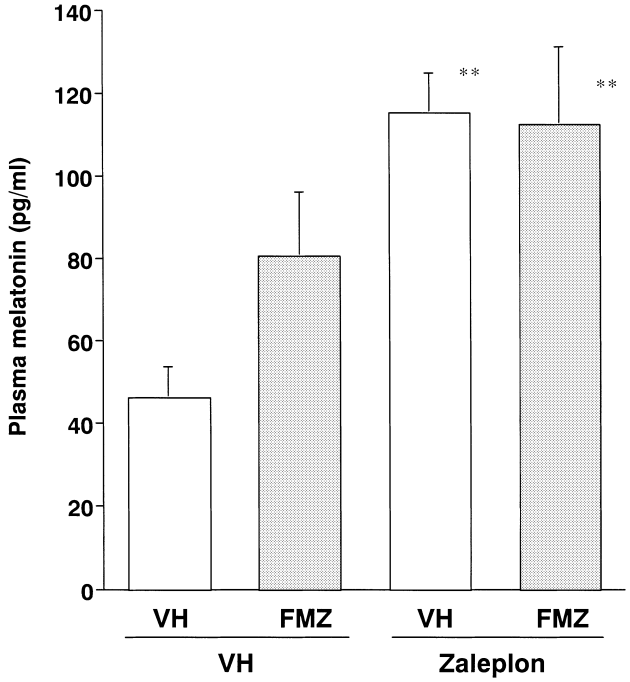

Fig. 2. Effect of flumazenil on melatonin secretion induced by zaleplon (1 mg/kg, i.v.) in plasma of rabbits. The experiment was carried out during 11:00 to 14:00. Blood was collected at 30 min after intravenous administration of zaleplon. Flumazenil was injected 10 min prior to administration of zaleplon. Plasma melatonin level was determined by radioimmunoassay. Each column and bar represents the mean ± S.E.M. of 10 animals. VH: Vehicle (5% DMSO), FMZ: flumazenil. **Significantly different from the VH + VH-treated group at $P<0.01$ using Tukey's multiple comparison test. One-way ANOVA; $F(3,36) = 5.720$, $P = 0.0026$.

Table 1. Effect on cAMP accumulation in cultured rat pinealocytes

Group	cAMP (fmol/well)
Control	319.1 ± 41.7
Zaleplon	229.0 ± 13.4
Isoproterenol	777.9 ± 38.3$^{\#\#}$
Isoproterenol + Zaleplon	819.1 ± 1.7$^{\#\#}$
IBMX	450.1 ± 27.2
IBMX + Zaleplon	384.5 ± 42.5

Pinealocytes (7×10^4 cells/well) were plated on 48-well plates and grown in M199 medium containing 10% FCS for 4 days at 37°C as described in Materials and Methods. Pinealocytes were stimulated with different combinations of drugs for 10 min. Values are the mean ± S.E.M. of 5 or 6 wells. Zaleplon: 1×10^{-5} M, Isoproterenol: 1×10^{-7} M, IBMX: 1×10^{-3} M. $^{\#\#}$Significantly different from the control group at $P<0.01$ using Tukey's multiple comparison test. One-way ANOVA; $F(5,22) = 50.741$, $P<0.0001$.

treatment with zaleplon (1×10^{-5} M) and IBMX (1×10^{-3} M) had no influence on the intracellular cAMP level compared with that of the IBMX alone group (Table 1).

Discussion

Zaleplon produced a dose-dependent increase of melatonin concentration in rabbit plasma collected 30 min after intravenous administration at doses of 1 and 2 mg/kg, whereas triazolam and zopiclone did not affect plasma melatonin levels. The zaleplon-induced increase in plasma melatonin level was not blocked by flumazenil, a benzodiazepine receptor antagonist, suggesting that the increase in melatonin secretion observed in the zaleplon-treated group may not involve a benzodiazepine receptor.

We recently reported that for rabbits chronically implanted with electroencephalogram recording electrodes, zaleplon (1 mg/kg, i.v.) induced a drowsiness reflected on spontaneous EEG (2). The pattern, indicative of hypnotic action, was characterized by high voltage slow waves in the cortical EEGs and desynchronization of the hippocampal theta waves. The hypnotic action, that is, the EEG pattern and behavioral sedation, was completely antagonized by flumazenil. Moreover, triazolam (0.1 mg/kg, i.v.) and zopiclone (2 mg/kg, i.v.) showed almost equal potency in increasing the delta wave activity with zaleplon (1 mg/kg, i.v.) in spectral analysis (2). The doses used in the study show a comparable effect as the therapeutic dosages. Therefore, although melatonin is an effective hypnotic agent both in animals (17, 18) and humans (19), these findings suggest that melatonin may not directly affect the hypnotic action of zaleplon.

Melatonin has been used to treat sleep disorders, and it has been reported that the melatonin level secreted in insomniacs is lower than in healthy people (10). Among elderly people, as well, even those who are healthy, the frequency of sleep disorder is high and there is an association with impairment of melatonin production (20). It has also been reported that melatonin promotes circadian rhythm synchronization and might be simpler and more effective in treating jet lag than the limited solutions that are currently available (6, 21). The elimination half-life of zaleplon is approximately 1 h, regardless of dose, and the absorption is rapid (3). Our findings indicate that zaleplon, unlike triazolam and zopiclone, induces an increase in melatonin secretion and this, in turn, may have an effect on sleep chronobiology.

Melatonin is a hormone that shows daily fluctuations. After the onset of darkness, the rate-limiting enzyme *N*-acetyltransferase activity increases to values that are 15- to 30- fold greater than those that occur during light periods (22). The melatonin level in the rabbit pineal gland was 25 times greater at night than in the day (23). Therefore, in the present study, the significant increase

in plasma melatonin concentration resulting from zaleplon administration was considered to be within the normal range of fluctuation of this physiological level of secretion.

Olcese (15) reported that melatonin secretion in culture medium occurred in a concentration-dependent manner when pinealocytes were treated with 1×10^{-9} – 1×10^{-6} M norepinephrine. Locally in the gland, noradrenaline acts through postsynaptic β- and α_1-adrenoceptors stimulating intracellular cAMP. The accumulation of cAMP in pinealocytes induces the activation of *N*-acetyltransferase, a rate-limiting enzyme in the biosynthesis of melatonin, which results in the elevated synthesis and secretion of melatonin (24). In the present study, zaleplon was found to have no effect on intracellular cAMP in the pinealocytes, and its action was completely different from the action of norepinephrine, which could change cAMP by itself. In the presence of a β-agonist to stimulate adenlyate cyclase, zaleplon did not change the intracellular cAMP level. Phosphodiesterase is an enzyme that converts cAMP into 5'-AMP and is known to have higher activity in brain tissues. To suppress the activity of phosphodiesterase and detect changes in cAMP more accurately, IBMX is used occasionally. In the present study, we used a concentration of 1×10^{-3} M IBMX, which has been reported to elevate NE-induced cAMP level in control cells (25) and is known to be an inhibitor of cAMP-PDE activity (26). Even with the addition of IBMX to inhibit the decomposition of cAMP, however, no effect of zaleplon was observed. The results suggest that the stimulating effect of zaleplon on melatonin secretion may not be attributed to the increase in cAMP in pinealocytes, that is, the acceleration of synthesis and inhibition of the decomposition of intracellular cAMP. In the literature, several receptors involved in the control of melatonin are reported. Beside receptors that negatively control melatonin synthesis and release, such as nicotinic acetylcoline (27) and glutamate receptors (28), there are also other receptors that potentially stimulate melatonin release, including, for example, ANP (29), VIP (30), opiate (31), A2b (32), NPY (33), and especially 5-HT_2 (34) receptors. Thus, zaleplon may induce its melatonin releasing effects by acting through one of these receptors. These findings suggested that the hypnotic effect of zaleplon and the adjustment of sleep chronobiology by zaleplon are mediated by different mechanisms.

In the present study, blood samples were collected from rabbits 30 min after drug administration and the plasma melatonin level was measured. This blood collection time coincided with the time when the delta wave was increased mostly electroencephalographically. Rabbits were used for the in vivo study but rats were used for the in vitro study because it is unusual to use rabbits for experiments involving pinealocytes. However, although species vary in the amplitude, timing, and shape of their pineal melatonin rhythm, the overall control mechanism for the rhythm and biosynthetic pathway seems to be similar (35).

In conclusion, these results suggest that zaleplon may promote melatonin secretion and the elevation of plasma levels of melatonin may suggest an influence of zaleplon on chronobiology.

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