Chronopharmacology of Melatonin in Mice to Maximize the Antitumor Effect and Minimize the Rhythm Disturbance Effect

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ABSTRACT

The influence of dosing time on the antitumor effect and the rhythm disturbance effect of melatonin (MLT) was investigated in ICR male mice under a light/dark (12:12) cycle. In tumor-bearing mice, the antitumor effect of MLT (1 mg/kg intraperitoneal) was most effective in the dark phase, and the rhythm disturbance effect of MLT on the locomotor activity was more serious in the light phase than in the dark phase. The antitumor effect and the rhythm disturbance effect of MLT increased when the specific binding of MLT receptor in target tissues, tumor or suprachiasmatic nucleus, increased and they decreased when the level decreased. Furthermore, because luzindole, an MT<sub>1</sub> and MT<sub>2</sub> blocker, caused the antitumor effect or rhythm disturbance effect of MLT to decrease, it is suggested that the time-dependent change of the pharmacological effects of MLT were influenced by that of MLT receptor(s) function. On the other hand, there was no significant dosing time-dependent change of MLT concentration in tumor or brain after injection. Thus, the pharmacokinetic factor does not seem to contribute to the dosing time-dependent effect of MLT. These results suggest that by choosing the most suitable dosing time for MLT, the efficacy of the drug can be increased, and the toxicity of the drug can be decreased in certain experimental and clinical situations.

A large number of rhythmic variables are influenced by environmental factors such as light, temperature, and social communication that vary cyclically in nature and serve to synchronize biological rhythms to the daily rotation of the earth. Responses to a variety of drugs show 24-h rhythmicity (Ohdo et al., 1991, 1996; Watanabe et al., 1992, 1998). Use of a chronopharmacological strategy can improve the outcome of pharmacotherapy. However, several drugs cause alterations in the 24-h rhythms of biological, physiological, and behavioral processes (Duncan, 1996; Horikawa et al., 2000; Ohdo et al., 2001). The alteration of rhythmicity is sometimes associated with therapeutic effects or may lead to illness and altered homeostatic regulation.

MLT is an indoleamine synthesized from serotonin in the pineal gland. MLT has many pharmacological and physiological effects. One of them is the antitumor effect. Both in vitro and in vivo, MLT has been found to inhibit neoplastic growth and to delay tumor progression (Cini et al., 1998). Moreover, MLT is well known to show no toxic effects (Lissoni et al., 1993a,b), and, therefore, serves as a nontoxic anticancer agent. In phase II-type clinical trials, the effectiveness of MLT was shown either alone or in combination with other chemotherapeutic drugs, as an anticancer agent (Panzer and Viljoen, 1997). Although very little is known about the exact mechanisms by which MLT inhibits tumor growth, it has been shown that MLT has both a direct oncostatic effect on tumor cells (Blask, 1993, 1997) and an anticancer action via stimulation of the immune system (Conti and Maestroni, 1995). It was suggested that both direct and indirect antitumor effects are via its specific binding site in tumor cells or immune cells. Some drugs, which elicit their actions via their specific binding sites, have been shown to have a dosing time-dependent effect in relation to their receptor sensitivity (Takane et al., 2000). Therefore, it is probable that the antitumor effect of MLT changes according to the time of admin-
istration. However, the influence of MLT dosing time on tumor growth has not been revealed.

MLT has an effect on biological rhythms similar to light. The effects of MLT treatment or light exposure on biological rhythms follow a phase-response curve. Light exposure in the dark phase causes a phase advance in the sleep-wake cycle, whereas light exposure in the early dark phase causes a phase delay. However, MLT treatment produces the opposite effects on biological rhythms to light (Lewy et al., 1992).

Namely, MLT treatment in the light phase causes a phase advance in the sleep-wake cycle, whereas MLT treatment in the early light phase causes a phase delay. This effect of MLT seems to act via the SCN, the principal circadian clock. Due to this effect, in clinical situations, MLT is used to cure sleep disorders, jet lag, and so on. However, a low dose of MLT (0.1–10 mg) given in the middle of light phase when endogenous levels are lowest causes decreased sleep-onset latency, decreased oral temperature, and a reduction in the number of correct responses on the Wilkinson auditory vigilance task (Dollins et al., 1993). Therefore, if dosing time is not appropriate, this effect of MLT may cause rhythm disorders.

The purpose of this study was to investigate the influence of MLT dosing time on tumor growth in tumor-bearing mice to maximize the antitumor effect and to investigate the influence of MLT dosing time on the locomotor activity to minimize the risk of rhythm disturbance caused by exogenous MLT. The mechanism underlying the dosing time-dependent difference was elucidated from the viewpoints of MLT pharmacodynamics or pharmacokinetics.

Materials and Methods

Animals and Cell Culture. Four-week-old male ICR mice (Charles River Japan, Inc., Kanagawa, Japan) were housed 3 to 10 per cage under standardized light/dark cycle conditions (lights on at 7:00 AM and off at 7:00 PM) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water ad libitum. All mice were adapted to their light/dark cycle for 2 weeks before experiments. Sarcoma 180 (S-180) cells (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 0.05% kanamycin, penicillin, and streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. S-180 cells were transplanted with 5 × 105 cells into the hind footpads of mice at the day before drug injection.

Preparation of Dosing Solutions. MLT (Wako Pure Chemicals, Osaka, Japan) dissolved in saline to yield an appropriate concentration of 0.1 mg/ml. Luzindole (LZD, 2-benzyl-N-acetyltryptamine; Wako Pure Chemicals) was prepared in 50% ethanol and then diluted in Krebs’ solution to appropriate concentrations. The final ethanol concentrations in the tissue bath did not affect contractility. MLT and LZD were used at an intraperitoneal (i.p.) dose of 1 mg/kg or 10 mg/kg by means of 23-gauge needle connected to a 1-ml syringe. The volume of drug solutions administered was 10 ml/kg. Other reagents, purchased from Wako Pure Chemicals, were of analytical grade and used without further purification.

Influence of MLT Dosing Time on Growth of Tumor Weight. First, groups of 9 to 10 mice were intraperitoneally injected with MLT (1 mg/kg) at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM to investigate the influence of dosing time on the antitumor effect of MLT. Second, we selected two different dosing times such as peak and trough. Groups of 9 to 10 mice were intraperitoneally injected with MLT (1 mg/kg), MLT and LZD (10 mg/kg), or saline at 1:00 PM or 1:00 AM. Drug or saline was administered daily for 8 days. On the 8th day, the growth of S-180 cells was monitored by calculating the tumor volume from caliper measurements. The tumor weight was calculated according to the following equation: tumor weight = A × B/2, where A is the longer diameter and B is the shorter one (millimeters).

Influence of MLT Dosing Time on Locomotor Activity. After groups of three mice were intraperitoneally injected with saline for 5 days, they were intraperitoneally injected with MLT (1 mg/kg), MLT and LZD (10 mg/kg), or saline at 1:00 PM or 1:00 AM. Drugs were administered daily for 7 days. Locomotor activity was measured using scaset SV-10LD (ToyO Sangyo Co. Ltd., Toyama, Japan), and the activity count (number of movements) was recorded at 1-h intervals. For visualization of locomotor activity rhythm, hourly the activity counts were calculated using a moving average with a 4-h window. The average counts were plotted at 9:00 AM, 1:00 PM, 7:00 PM, 9:00 AM, 1:00 AM, or 5:00 AM.

Time-Dependent Change in Specific Binding of MLT to Tumor or SCN Cells. The tissues were removed from six mice at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM. To obtain three SCN samples, coronal brain slice were prepared from 18 mice using rodent brain matrix (RBMI-2000C; ASI Instruments Inc., Warren, MI) at 1:00 PM or 1:00 AM. Tumor or SCN was homogenized in 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenates were then centrifuged at 15,000 rpm for 15 min at 4°C. The obtained pellet was resuspended in 4 or 1 tumor, 4 SCN 1 ml of Tris-HCl buffer. The protein concentration was approximately 3 or 1 (tumor, SCN) mg/ml using Lowry’s method (DC protein assay; Bio-Rad, Hercules, CA). The binding assay was done in the method described previously (Takine et al., 2000). The binding assay was performed with a reaction mixture (total volume, 200 μl) containing 100 μl of aliquot of tumor homogenate and 50 nM [O-methyl-3H]MLT (Amersham Bio- sciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) or containing 100 μl of aliquot of SCN homogenate and 12.5 to 50 nM [O-methyl-3H]MLT. Nonspecific binding was determined in the presence of 100 μM MLT. After incubation at 4°C for 5 h, the reaction mixture together with 100 μl of Tris-HCl buffer for washing the tube was laid over the 300 μl of ice-cold fetal bovine serum, and centrifuged at 10,000 rpm for 3 min at 4°C. The supernatant was removed and then the pellet transferred to scintillation vials with 10 ml of scintillation cocktail and counted using a liquid scintillation counter (LSC-1000; Aloka Co., Mitaka, Tokyo Japan) after keeping for 6 h. Specific binding is the difference between binding determined in the absence of ligand and in the presence of ligand and was calculated as follows: specific binding (fmol/mg per milligram of protein) = (total binding [fmol per milligram of protein]) − (nonspecific binding [fmol per milligram of protein]). The data were plotted according to the method of Scatchard. The number of MLT receptors and the dissociation constant (Kd) were calculated by the method of least-squares to fit the data, and this experiment was repeated three times.

Influence of MLT Dosing Time on Pharmacokinetics. Groups of six mice were intraperitoneally injected with 1 mg/kg melatonin at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM. Blood samples (approximately 50 μl for each sample) were drawn by orbital sinus collection using micropipettes at 10, 30, 60, or 120 min after MLT injection. Blood samples were immediately centrifuged at 3000 rpm for 3 min. A mixture of plasma sample (20 μl), internal standard (3,4-dimethoxybenzoic acid; veratric acid, 10 mg/ml, 20 μl), and methanol (400 μl) was mixed with an automatic mixer (S-100; Taitec, Saitama, Japan) for 20 s and centrifuged at 10,000 rpm for 120 min. The residue was dissolved in 30 μl of methanol. The insoluble substance was removed by centrifugation at 10,000 rpm for 3 min. Then, 20 μl of the solution was injected into the HPLC system, which comprised a pump (LC-10AD liquid chromatograph; Shimadzu, Kyoto, Japan), a detector (RF-10A spectrofluorometric detec-
tor; Shimadzu), a chromatopac (C-R1B; Shimadzu), a guard column (TSK-GEL ODS-80TS, 5 mm, 3.5 mm i.d. × 15 mm; Toyo Soda, Tokyo, Japan), and an analytical column (TSK-GEL ODS-80TS, 5 mm, 4.6 mm i.d. × 15 mm; Toyo Soda). The mobile phases consisted of 0.01 M acetate buffer (pH 4.25) solution and methanol (75:25 (v/v)). The peak areas were integrated using a data processor. Tumor or brain was removed at 30 min after MLT injection and homogenized in 1 ml of methanol. The homogenate was centrifuged at 15,000 rpm for 15 min. The sample was prepared and determined using HPLC in the same way as the blood samples described above.

**Statistical Analysis.** The statistical significance of differences between groups was validated by analysis of variance, Tukey’s test, and Student’s t test. Statistical moment analysis was used to calculate the pharmacokinetic parameters such as area under the time-concentration curve (AUC) and mean residence time (MRT). A probability level of \( P < 0.05 \) was considered to be significant.

**Results**

**Influence of MLT Dosing Time on Growth of Tumor Weight.** The influence of MLT (1 mg/kg) dosing time (7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM) on tumor growth is shown in Fig. 1A. The tumor weight in mice injected with MLT at 7:00 AM or 1:00 AM was significantly suppressed compared with that in mice injected with MLT at 1:00 PM (7:00 AM, \( P < 0.05 \); 1:00 AM, \( P < 0.01 \)). The tumor weight in mice injected with MLT at 7:00 PM showed a tendency to be suppressed by MLT. Moreover, the influence of LZD (10 mg/kg) dosing on the antitumor effect of MLT is shown in Fig. 1B. The tumor weight in mice injected with MLT at 1:00 PM was not significantly different from that in control mice, and LZD did not affect the antitumor effect of MLT at 1:00 PM.

On the other hand, the tumor weight in mice injected with MLT at 1:00 AM was significantly lower than that in control mice (\( P < 0.05 \)), and LZD significantly blocked the antitumor effect of MLT at 1:00 AM (\( P < 0.05 \)).

**Time-Dependent Change in Specific Binding of MLT to Tumor Cells.** The specific binding of MLT to tumor cells is shown in Fig. 2. The specific binding of MLT to tumor cells prepared at 1:00 PM showed a minimum value. The specific binding of MLT to tumor cells prepared at 1:00 AM showed the maximum value and was significantly higher than that prepared at 7:00 AM, 1:00 PM, or 7:00 PM (7:00 AM, \( P < 0.05 \); 1:00 PM, \( P < 0.01 \)).

**Influence of MLT Dosing Time on Locomotor Activity.** The time course of the locomotor activity in mice after daily MLT treatment is shown in Fig. 3, A and B. The locomotor activity in mice injected with MLT at 1:00 PM was suppressed immediately after the initiation of MLT treatment. On the other hand, the locomotor activity in mice injected with MLT at 1:00 PM was gradually decreasing each day and finally, on day 5 after initiation of drug treatment, reached to the same level as mice injected with MLT at 1:00 PM. LZD (10 mg/kg) blocked the rhythm disturbance effect of MLT at both 1:00 PM and 1:00 AM, and the locomotor activity in mice injected with LZD and MLT at 1:00 PM or 1:00 AM was not significantly different from that in control mice injected with saline.

**Time-Dependent Change in Specific Binding of MLT to SCN Cells.** The specific binding of MLT to SCN cells is shown in Fig. 4. The specific binding data were replotted by the method of Scatchard. The parameter calculated from the Scatchard analysis is shown in Table 1. The specific binding of MLT to SCN cells prepared at 1:00 PM was significantly larger than that prepared at 1:00 AM (\( P < 0.01 \)). The apparent \( K_d \) value did not differ significantly between SCN cells prepared at 1:00 PM and at 1:00 AM.

**Influence of MLT Dosing Time on MLT Pharmacokinetics and MLT Concentration in Plasma, Tumor, or Brain.** The time course of plasma MLT concentrations in mice injected with MLT at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM is shown in Table 2 and their pharmacokinetic parameters are shown in Table 3. The plasma MLT concentration at 30 min after the drug injection was significantly different among mice injected with MLT at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM (\( P < 0.05 \)). The maximum or minimum plasma MLT concentration was observed when MLT was injected at 1:00 AM or 7:00 PM. AUC, CL, and \( V_d \) were significantly different among them (AUC, CL, \( P < 0.01 \); AUC, CL, \( P < 0.05 \); 1:00 PM, \( P < 0.05 \); and 7:00 PM, \( P < 0.01 \)). The tumor weight in mice injected with MLT at 1:00 PM was gradually decreasing each day and finally, on day 5 after initiation of drug treatment, reached to the same level as mice injected with MLT at 1:00 PM. LZD (10 mg/kg) blocked the rhythm disturbance effect of MLT at both 1:00 PM and 1:00 AM, and the locomotor activity in mice injected with LZD and MLT at 1:00 PM or 1:00 AM was not significantly different from that in control mice injected with saline.
MLT has a capacity to inhibit the proliferation of various tumor cell lines. The antitumor effect of MLT to S-180 cells was reported both in vitro (Sze et al., 1993) and in vivo (Li and Xu, 1997). In the present study, the growth of S-180 implanted in mice was significantly inhibited by MLT. Furthermore, the antitumor effect of MLT was significantly different according to dosing time. Namely, the antitumor effect of MLT was observed in mice injected with the drug at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM. MLT concentrations in the brain at 30 min after the drug injection at 7:00 PM or 1:00 PM. MLT concentrations in tumors at 30 min after the drug injection at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM are shown in Fig. 5. MLT concentrations did not differ significantly among mice injected with the drug at 7:00 AM, 1:00 PM, 7:00 PM, or 1:00 AM. The maximum or minimum MLT concentration in tumors was observed when the drug was injected at 7:00 PM or 1:00 PM. The maximum or minimum MLT concentration in tumors was significantly inhibited by MLT. Furthermore, the antitumor effect of MLT was significantly different according to dosing time. Namely, the antitumor effect of MLT was observed in mice injected with MLT at 7:00 AM, 7:00 PM, or 1:00 AM, but not at 1:00 PM.

MLT exerts its proposed antitumor action through direct cellular effects and immune stimulatory effects. MLT's direct antitumor action was suggested to be through its receptor(s) MT₁ and/or MT₂ leading to receptor activation and association with an inhibitory G protein (Blask et al., 1999). In the present study, because LZD, both an MT₁ and MT₂ blocker, inhibited MLT's antitumor effect, MLT's antitumor action was suggested to be influenced by the MLT receptor(s) function. Moreover, the specific binding of MLT to tumor cells showed rhythmicity with the minimum value in cells prepared at 1:00 PM when the antitumor effect of MLT was not observed. In general, the rhythm of MLT receptor(s) density is affected by the rhythm of the MLT level in blood. Namely, prolonged exposure in high concentration to MLT causes MLT receptor(s) desensitization, and short time exposure at a low concentration of MLT does not cause MLT receptor(s) desensitization. In addition, it was reported that MLT directly suppresses the growth of cells by delaying their progression from the G1 phase to S phase of the cell cycle (Cos et al., 1991; Shiu et al., 1999; El-Missiry and Abd El-Aziz, 2000). In addition, the middle of the light phase is the time that S-180 implanted in mice has the highest percentage of cells in the later S phase until the beginning of the G2 phase (Akagi et al., 2003). Although it is not known whether the rhythm of the MLT concentration influences a cell cycle, it is suggested that the time-dependent change of MLT antitumor effects is related to the change in the MLT receptor in tumor cells.

Furthermore, MLT exerts indirect antitumor effects such as the immune stimulatory effect mediated by MLT. MLT enhances the activity of tumoricidal cells and potentiates the production and release of some cytokines, tumor necrosis factor, interleukin-2, and interferon-γ (Neri et al., 1995). MLT injection in the late light phase, but not in the early light phase, increases the serum interferon-γ level (Champney et al., 1998). The time-dependent effects of MLT on the immune system may contribute to time-dependent antitumor effects. Certainly, the specific binding sites of MLT are identified on immune cells (Calvo et al., 1995), and their expression may also show the 24-h rhythm.

We also examined the influence of MLT dosing time on sleep-wake rhythm, because MLT has the effect on biological rhythms, notably sleep-wake rhythm. If MLT dosing time is not appropriate, MLT might cause the rhythm disturbance as a side effect of MLT being a nontoxic anticancer agent. We chose 1:00 PM when the antitumor effect of MLT was not observed, and 1:00 AM when the effect was the highest. The effect of MLT on locomotor activity varied depending on dosing-time. The altered locomotor activity induced by MLT was more potent in mice injected with the drug at 1:00 PM than at 1:00 AM.

The effect of MLT on 24-h rhythm of locomotor activity seems to be mediated by the direct action of MLT on the SCN, because MLT shifts the rhythmicity of electrical activity in the rodent SCN in vitro (McArthur et al., 1991, 1997; Starkey et al., 1995; Starkey, 1996). MLT seems to evoke its effects on SCN function through the G protein receptors with high affinity (Reppert and Weaver, 1995). In the present study, because LZD inhibited MLT’s rhythm disturbance effect, MLT’s rhythm disturbance effect was suggested to be decided by the MLT receptor(s) function. Moreover, the specific binding of MLT was significantly higher for SCN cells prepared at 1:00 PM than that prepared at 1:00 AM. This result supports findings of other studies in which the number of MLT binding sites in the rat SCN showed a significant 24-h rhythm with higher levels in the light phase (Vacas and Cardinali, 1979; Gauer et al., 1993; Tenn and Niles, 1993). In addition, not only the exposure to MLT but also the photoperiod plays an important role in regulating MLT receptor(s) density in SCN cells (Masson-Pevel et al., 2000). 2°[135]Indolmelatonin binding in the SCN increases in pinealectomized rats exposed to a 1-h light pulse during the night compared with rats kept in constant darkness. Namely, the time-dependent function of MLT receptor corresponded well to the finding
Fig. 4. Time-dependent change in specific binding of MLT to membranes of SCN cells prepared at 1:00 PM (○) or 1:00 AM (●). A, concentration dependence of [3H]MLT binding to membranes of SCN cells. The membranes were incubated with [3H]MLT (12.5–50.0 nM) at 4°C for 5 h. Nonspecific binding was determined in the presence of 100 μM MLT. Specific binding is defined as total binding minus nonspecific binding. Each value is the mean with S.E. of three observations. B, transformation of the saturation data using the Scatchard method. To fit, the data were carried out by the method of least-squares. Each value is the mean of triplicate.

Fig. 3. Locomotor activity in mice under daily saline (A), MLT (1 mg/kg i.p.) (B), or MLT and LZD (10 mg/kg i.p.) (C) treatment for 7 days at 1:00 PM (○) or 1:00 AM (●). The activity count (number of movements) of three mice was calculated using a moving average with a 4-h window after recorded at 1-h intervals. The average counts were plotted at 9:00 AM, 1:00 PM, 5:00 PM, 9:00 PM, 1:00 AM, or 5:00 AM. * P < 0.05, compared with 1:00 AM group using Student’s t test.
that the rhythm disturbance effect of MLT on locomotor activity was more serious in mice injected with the drug at 1:00 PM than 1:00 AM. Therefore, one of the reasons why the locomotor activity in mice injected with the drug at 1:00 AM decreased over time although the activity in mice injected with the drug at 1:00 PM was immediately decreased might be the difference of the function in MLT receptor (i.e., resolution and saturation in MLT receptor) between 1:00 AM and 1:00 PM. In addition, other reasons might be related to the hypnotic effect relevant to the function of MLT receptor because the hypnotic effect of MLT is stronger when MLT acts during the rest period (1:00 PM) than during the active period (1:00 AM). Moreover, an inverse relation between the melatonin binding sites in the tumor preparations and brain section was observed. The reason for this phenomenon might be that tumor cells are not controlled more strongly than SCN cells by light.

The AUC of MLT in plasma showed a significant dosing time-dependent change in specific binding of MLT to SCN cells prepared at 1:00 PM or 1:00 AM. 

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The AUC of MLT in plasma showed a significant dosing time-dependent difference with a maximum value at 7:00 AM and minimum value at 1:00 PM. The 24-h rhythm of blood flow in eliminative organs partially influences the time-de-
the dosing time of MLT, consideration of both the influence of biological rhythms on the pharmacological action of MLT and the influence of MLT on biological rhythms are required. Moreover, it is suggested that dosing time-dependent changes of MLT’s effects are caused by time-dependent differences in the sensitivity, particularly at the receptor level, of tumor cells or SCN cells to MLT, respectively. Therefore, the choice of dosing time based on the 24-h rhythm of MLT receptor expression in tumor cells and SCN cells may help establish a rational chronotherapeutic strategy, increasing the antimutagenic activity of the drug and decreasing the risk of the rhythm disturbance caused by MLT in certain clinical situations.

References


