**Full Paper**

**Chronopharmacology of Mizoribine in Collagen-Induced Arthritis Rats**

**Yuko Kanasaki**, **Mari Tomonari**\(^1,2\), **Hitoshi Sasaki**\(^1\), and **Hideto To**\(^1,2,*\)

\(^1\)Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki 852-8102, Japan
\(^2\)Department of Medical Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Toyama 930-0194, Japan

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**Abstract.** We previously reported that higher therapeutic effects were obtained in rheumatoid arthritis (RA) patients and RA model animals when the dosing-times of methotrexate and tacrolimus were chosen according to the 24-h rhythms of the inflammatory response. Mizoribine (MZR) is an immunosuppressive agent and is used against RA in the same manner as methotrexate and tacrolimus. In this study, we examined whether a dosing-time dependency of the therapeutic effect of MZR could be detected in collagen-induced arthritis (CIA) rats. To measure C-reactive protein (CRP) and tumor necrosis factor (TNF-\(\alpha\)) levels, blood was collected from CIA rats at different times. MZR was administered at two different dosing-times based on these findings and its effects and toxicity were examined. CRP and TNF-\(\alpha\) concentrations in blood showed significant 24-h rhythms. The exacerbation of arthritis and excessive increase in leukocytes in CIA rats were markedly lower in the group treated with MZR at the dark phase than those of the group treated with MZR at the light phase. These findings suggest that the therapeutic index of RA therapy may be improved by administering MZR at a time in the day when the inflammatory reaction begins to activate.

**Keywords:** mizoribine, rheumatoid arthritis, 24-h rhythm, collagen-induced arthritis, chronopharmacology

**Introduction**

Mizoribine (MZR), an imidazole nucleoside, is an immunosuppressive agent isolated from the culture filtrate of *Eupenicillium brefeldianum* (1). Since MZR is less toxic than azathioprine, MZR is considered to be a potential alternative agent in organ transplantation (2, 3). In recent years, MZR has been used as a drug against autoimmune diseases such as rheumatoid arthritis (RA) and lupus nephritis because it has immunosuppressive and anti-inflammatory effects (4, 5). In clinical studies, MZR shows antirheumatic effects in RA patients (6, 7). It was thought that MZR had higher safety than other antirheumatic drugs, whereas its efficacy was inferior. Therefore, it is necessary to find a therapeutic method through which a greater availability of MZR can be obtained.

Many organisms display 24-h rhythms in various factors such as heart rate, blood pressure, hormones, and DNA synthesis in cells (8 – 11). These 24-h rhythms are also associated with the risk or frequency of disease occurrence (12, 13). For example, asthma attacks get worse between midnight and early morning and are seldom observed in the daytime (14). Chronotherapy is defined as the administration of medication in accordance with biological rhythms in order to optimize therapeutic outcomes and/or control adverse effects, and it has been reported that many drugs such as antitumor drugs, antidepressants, and analgesic drugs show rhythm-dependent differences in their effects and pharmacokinetics (15 – 17). These effects arise from the 24-h rhythms found in elements of cellular physiology such as the cell cycle and expression of receptors, hormones, and enzymes (18, 19).

RA is an autoimmune disorder of unknown etiology and is a chronic progressive disease that reduces the quality of life (20, 21). Although many requirements must be met to establish a diagnosis of RA, morning
stiffness, which shows a 24-hour rhythm with a peak in the early morning, is a characteristic feature of RA (22). Chronotherapy and chronopharmacology in RA was used basically and clinically with glucocorticoids (23, 24), methotrexate (MTX) (25, 26), and tacrolimus (27). These results clarified that choosing an optimal dosing-time associated with the 24-h rhythm of RA symptoms could lead to an effective treatment for RA. It was suggested that the toxicity of MZR varies with the dosing-time (28, 29). Therefore, chronopharmacological efficacy of MZR is expected against RA therapy, as well as MTX and tacrolimus.

In the present study, to detect the 24-h rhythms of C-reactive protein (CRP) and tumor necrosis factor (TNF)-α, their concentrations were measured at six different times in collagen-induced arthritis (CIA) rats. MZR was administered at two different dosing-times based on these findings and its efficacy and toxicity were then evaluated.

Materials and Methods

Animals

LEW rats (7-week-old) were purchased from Japan SLC, Inc. (Hamamatsu). Rats were housed under standardized light-dark cycle conditions (lights on and off at 7:00 and 19:00, respectively) with free access to food and water. Experiments were performed after formal approval by the Institutional Ethical Committee for Research on Animals.

Induction of CIA

Bovine type II collagen (CII) (Collagen Gijutsu Kenshukai, Tokyo) and Freund’s incomplete adjuvant (Difco Laboratories, Detroit, MI, USA) were mixed and the emulsion was prepared at a concentration of 1 mg/mL in CII. Rats were intradermally sensitized at ten sites (per 100 μL) on day 0 by administration of 1 mg CII. Seven and 11 days later, rats received an intradermal booster injection of one-tenth of the volume used for sensitization.

Preparation of MZR

MZR, which was supplied by Asahi Kasei Pharma Corporation (Tokyo), was dissolved in saline. Final concentrations were 5 (10 mg/kg) and 10 (20 mg/kg) mg/mL in each dosing group. MZR was perorally (p.o.) administered to rats by gavage at 2 mL/kg.

Experiment I: 24-h rhythm in serum CRP concentrations in CIA rats

To measure CRP levels, blood was taken at different times (2, 6, 10, 14, 18, or 22 h after the light was turned on (HALO) in CIA (n = 6) rats. Blood was collected from the tail vein on day 20 after the first immunization when arthritis showed in all rats. All blood samples were immediately centrifuged at 3,000 rpm for 15 min, after which serum was removed and frozen at −20°C until assay. Serum concentrations of CRP were determined by Rat CRP ELISA (Immunology Consultants Laboratory, Inc., Portland, OR, USA) Measurements were performed according to the manufacturer’s protocols.

Experiment II: MZR dosing-time dependent antirheumatic effect

MZR (10 mg/kg) was administered one day after the first immunization and was administered p.o. at 10 or 22 HALO every day for 28 days in CIA rats (n = 12, respectively). From 8 days after the first immunization, MZR (20 mg/kg) was administered p.o. at 10 or 22 HALO every day for 21 days in CIA rats (n = 6, respectively). Saline was administered in the control group (n = 6 or 12).

The arthritis score was recorded every day after the first immunization. A previously described arthritis scoring system (30) was used that evaluated individual joints and weighted the arthritis severity by joint size, as follows: a) for the interphalangeal joints, each of the 4 lateral digits in the hind legs was scored as 0 or 1 (0 = no arthritis and 1 = arthritis present); and b) for the ankle and midfoot joints, each was scored on a scale of 0 – 4 (0 = normal, 1 = minimal swelling, 2 = moderate swelling, 3 = severe swelling, and 4 = severe swelling and non-weight bearing). The macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 32.

Blood samples were collected from the tail vein of each rat at 4 HALO on days 0 and 14 after the initiation of administration and leukocyte counts were then measured.

Experiment III: MZR dosing-time dependent leukopenia and oligocytemia

To investigate leukocyte and erythrocyte counts, 8-week-old LEW rats were divided into 10 HALO– and 22 HALO–treated groups. MZR (10 and 20 mg/kg) was administered p.o. at 10 or 22 HALO every day for 28 days in LEW rats (n = 6, respectively). Saline was administered in the control group (n = 9). Blood samples were drawn from the tail vein of each rat at 4 HALO on days 14 and 28 after the initiation of administration and leukocyte and erythrocyte counts were then measured.

Experiment IV: chronopharmacokinetics of MZR

To investigate pharmacokinetics, 8-week-old LEW rats were divided into 10 HALO– and 22 HALO–treated
groups (n = 4 or 5). Blood samples were collected from the tail vein of each rat at 0.25, 0.5, 1, 1.5, 2, 3, 4, and 5 h after MZR (10 mg/kg) was p.o. administered. Samples were immediately centrifuged at 3,000 rpm for 15 min. Plasma was stored at −20°C until analysis.

**Measurement of MZR plasma concentrations**

Plasma (0.1 mL) was centrifuged at 14,000 × g for 110 min at 25°C with the Microcon (Ultracel YM-3; Millipore, Tokyo). Thirty microliters of the filtrated solution was injected into the high-performance liquid chromatographic system that comprised a pump (LC-10AD) (Shimadzu, Kyoto), a detector (SPD-10A) (Shimadzu), and an analytical column [Unison UK-Amino, 150 × 6 mm (3 μm); Intakt Corporation, Kyoto]. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B), which was delivered at a flow-rate of 1.3 mL/min. In the initial condition, the mobile phase consisted of 5% A and 95% B and changed from 8 – 13 min with a linear gradient to 50% A and 50% B. MZR was detected and quantified by ultraviolet absorption at 280 nm.

**Experiment V: 24-h rhythms in serum TNF-α concentrations**

To measure the concentrations of TNF-α, blood was collected at different times (2, 6, 10, 14, 18, or 22 HALO) from CIA rats (n = 4 or 5) on day 14 after the first immunization. All blood samples were immediately centrifuged at 3,000 rpm for 15 min, after which serum was removed and frozen at −80°C until the assay. Serum TNF-α concentrations were measured using the Luminex-100 system (Luminex Corporation, Austin, TX, USA). Acquired fluorescence data were analyzed using MasterPlex™ QT software (Ver. 1.2). All analyses were performed according to the manufacturer’s protocols.

**Statistical analyses**

Data were recorded as the mean ± standard deviation (S.D.). Groups were compared by a one-way analysis of variance (ANOVA) and repeated ANOVA and differences between groups were determined using Scheffe’s test. Differences in MZR concentrations between two groups were analyzed by a Student’s t-test. We defined 24-h rhythmicity as significant when both cosinor analysis and one-way ANOVA were significant. A probability level of less than 0.05 was considered to be significant.

**Results**

**Twenty-four-hour rhythm of CRP concentrations in CIA rats**

On day 20 after the first immunization, a significant 24-h rhythm was demonstrated for CRP concentrations in CIA rats (F from ANOVA = 11.54, \( P < 0.001 \); \( P \) from cosinor < 0.001, Fig. 1) and levels were higher at the late dark phase to the early light phase.

**Influence of dosing-time on the arthritis score during MZR administration in CIA rats**

When MZR (10 mg/kg) was administered, the arthritis score was significantly lower in the MZR-treated groups than the control group (\( P < 0.001 \), respectively; Fig. 2). The 22-HALO group showed a significant inhibition of increase in the arthritis score over that of the 10-HALO group (\( P < 0.001 \), Fig. 2).

MZR (20 mg/kg)-treated groups showed significant suppressions of arthritis over the control group (\( P < 0.001 \), respectively; Fig. 3). On day 28, the mean arthritis score was 18.3 in the 10-HALO group and 10.2 in the 22-HALO group, and the arthritis score in the 22-HALO group was 55.5% lower than that of the 10-HALO group.

**Influence of dosing-time on inhibition of increased leukocyte counts during MZR administration in CIA rats**

Leukocyte counts on day 14 were markedly higher than those on day 0 in the control group after the first immunization (\( P = 0.063 \), Table 1). Leukocyte counts in the 10-HALO group were 1.6 – 1.7-fold higher on day 14 than those on day 0 when 10 or 20 mg/kg of MZR was administered (Table 1). On the other hand, the 22-HALO group maintained a normal level of leukocytes on day 14.

![Fig. 1.](image-url) The 24-h rhythm in CRP levels on day 20 after the first immunization. Each value represents the mean ± S.D. of 6 CIA rats. There was a significant 24-h rhythm in the CRP concentrations in CIA rats (F from ANOVA = 11.54, \( P < 0.001 \); \( P \) from cosinor < 0.001).
Fig. 2. Influence of the dosing-time of MZR (10 mg/kg) on the arthritis score in CIA rats. MZR (10 mg/kg) was administered orally at 10 (open circle) or 22 (closed circle) HALO from the day after the first immunization. Saline was given in the control group (square). Each value represents the mean ± S.D. of 12 rats. The group treated at 22 HALO had a significantly greater inhibitive action on the increase in arthritis score than that treated at 10 HALO and the control ($P < 0.001$, respectively).

Fig. 3. Influence of the dosing-time of MZR (20 mg/kg) on the arthritis score in CIA rats. MZR (20 mg/kg) was administered orally at 10 (open circle) or 22 (closed circle) HALO from day 8 after the first immunization. Saline was given in the control group (square). Each value represents the mean ± S.D. of 6 rats. The arthritis score was significantly lower in the MZR-treated groups than the control group ($P < 0.001$, respectively).

Table 1. Influence of the dosing time of MZR on leukocyte counts in CIA rats

<table>
<thead>
<tr>
<th>Leukocyte counts ($/\mu$L)</th>
<th>Day 0</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9,783 ± 2,508</td>
<td>13,700 ± 2,915</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>9,567 ± 2,138</td>
<td>14,450 ± 2,166</td>
</tr>
<tr>
<td>22 HALO</td>
<td>10,117 ± 1,977</td>
<td>9,850 ± 2,966</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>9,050 ± 3,081</td>
<td>13,167 ± 1,227</td>
</tr>
<tr>
<td>22 HALO</td>
<td>10,200 ± 2,029</td>
<td>10,317 ± 1,779*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6 rats. *$P < 0.05$ vs. control. MZR (10 mg/kg) was administered from the day after the first immunization. MZR (20 mg/kg) was given from 8 days after the first immunization.
Influence of dosing-time on leukocyte and erythrocyte counts during MZR administration in non-CIA rats

When administration occurred in the MZR (10 or 20 mg/kg)-treated group at 10 HALO, there was no significant difference in leukocyte counts between the control and drug-treated groups (Table 2). Leukocyte counts in the 22-HALO group were significantly lower than those of control and 10 HALO–treated groups ($P < 0.05$ and 0.01, respectively).

Groups treated with 10 mg/kg MZR maintained normal levels of erythrocytes throughout this study (Table 3). On the other hand, 20 mg/kg MZR–treated groups showed a significantly lower erythrocyte count than that of the control group ($P < 0.001$, respectively). On day 28, erythrocyte counts in the 22-HALO group were about 10% lower than those of the 10-HALO group ($P < 0.001$).

Influence of dosing-time on pharmacokinetics after MZR administration in rats

Plasma MZR concentrations at 0.25 h after MZR injection in the 22-HALO group were significantly higher than those in the 10-HALO group ($P < 0.05$, Fig. 4).

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Table 2. Influence of the dosing time of MZR on leukocyte counts in LEW rats (non-CIA)

<table>
<thead>
<tr>
<th>Erythrocyte counts ($\times 10^3/\mu L$)</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11,456 ± 1,319</td>
<td>10,567 ± 1,284</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>10,600 ± 1,927</td>
<td>9,050 ± 1,477</td>
</tr>
<tr>
<td>22 HALO</td>
<td>10,533 ± 2,372</td>
<td>8,333 ± 1,454*</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>10,867 ± 1,483</td>
<td>$9,400 \pm 1,553$</td>
</tr>
<tr>
<td>22 HALO</td>
<td>8,583 ± 1,222**</td>
<td>9,800 ± 1,117</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6–9 rats. *$P < 0.05$, **$P < 0.01$ vs. control.

Table 3. Influence of the dosing time of MZR on erythrocyte counts in LEW rats (non-CIA)

<table>
<thead>
<tr>
<th>Erythrocyte counts ($\times 10^3/\mu L$)</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9,423 ± 298</td>
<td>9,151 ± 242</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>9,067 ± 354</td>
<td>8,777 ± 448</td>
</tr>
<tr>
<td>22 HALO</td>
<td>9,120 ± 399</td>
<td>8,900 ± 337</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 HALO</td>
<td>8,877 ± 517*</td>
<td>8,053 ± 296***</td>
</tr>
<tr>
<td>22 HALO</td>
<td>9,160 ± 949</td>
<td>7,177 ± 288***</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 6–9 rats. *$P < 0.05$, ***$P < 0.001$ vs. control.
Other sampling points were not different between both dosing groups.

**Twenty-four-hour rhythm of TNF-α concentrations in CIA rats**

On day 14 after the first immunization, a significant 24-h rhythm was demonstrated for TNF-α concentrations in CIA rats (F from ANOVA = 3.64, *P* < 0.05; *P* from cosinor < 0.001, Fig. 5) and levels were higher at the light phase and lower at the dark phase.

**Discussion**

Pain, functional disability, and stiffness show 24-h rhythms, with a peak in the early morning in many RA patients (31, 32), and the rhythms of pain and stiffness may play a role in local and systemic inflammatory responses. Herold and Günther reported that plasma CRP levels, an indicator of inflammatory responses, showed a 24-h rhythm with a peak in the early morning in RA patients, which matched the rhythms of pain and stiffness (33). Using MRL/lpr mice, which are an RA model known to develop autoimmune disorders sharing similarities with human RA (34, 35), we estimated plasma serum amyloid A (SAA) concentrations, which is an acute-phase protein and a sensitive marker of acute inflammatory states, because CRP levels cannot be detected in mice. An obvious 24-h rhythm in plasma SAA concentrations, involving higher levels at 2 HALO, was observed in MRL/lpr mice that developed RA (data not shown). Moreover, SAA levels in CIA mice also showed a 24-h rhythm with a peak at 2 HALO in our previous study (27). We reported that therapeutic effects markedly improved in clinical and animal studies by administering MTX and tacrolimus in consideration of the rhythm of the inflammatory response (25–27).

In the present study, CRP concentrations showed a clear 24-h rhythm with a peak at 22 HALO in accordance with those in CIA and MRL/lpr mice (25, 27). Moreover, during MZR (10 mg/kg) given the next day after the first immunization, the arthritis score in the 22 HALO group was half that of the 10-HALO group, and MZR showed a significant dosing-time dependent difference in its antirheumatic effect. In most RA patients, leukocyte counts increase (36, 37) and RA symptoms markedly improved when increased leukocytes in RA patients were eliminated by leukocytapheresis (38). In a previous study, we measured leukocyte counts in MRL/lpr mice. Leukocytes significantly increased after RA onset and a greater anti-rheumatic effect was shown at 2 HALO when increasing leukocyte counts were inhibited when tacrolimus was administered at 2 or 14 HALO (27). It has been suggested that inhibiting increased leukocyte counts, which induces an inflammatory response, is one of the options available for the treatment of RA. In this study, leukocytes increased after RA onset. This result corresponds to the response of RA patients and MRL/lpr mice. The 22-HALO group, in which exacerbation of arthritis was improved, showed inhibition of the increase in leukocytes when 10 mg/kg of MZR was administered for 28 days starting from day after the first immunization. It, however, is presently difficult to perform preventive treatment for RA in clinical situations, although early diagnosis of RA has been advancing year after year. Thus, the dosing-time dependent effects may be obtained because increasing leukocyte counts and aggravated inflammation are inhibited by preventive MZR administration. After the first immunization, leukocyte counts, which are an inflammatory marker of RA, were markedly higher on day 7 than on day 0 in this study. Thus, we examined whether the dosing-time dependency of the therapeutic effect of MZR was evident in CIA rats showing an aggravated inflammatory response. During MZR (20 mg/kg) treatment given from 8 days after the first immunization, only the 22-HALO group suppressed the increase in leukocytes, although the leukocyte count markedly augmented in the control and 10 HALO–treated groups on day 14 compared with before immunization. Moreover, the 22 HALO–treated group which was treated with 20 mg/kg MZR showed remarkable inhibition of the exacerbation in arthritis in the same manner as 10 mg/kg MZR administration. These findings demonstrate that both the preventive and therapeutic effects may be evidently higher in groups treated MZR at 22 HALO compared with those at 10 HALO.

The adverse effects of MZR are myelosuppression
such as leukopenia and oligocytopenia. In the present study, severe leukopenia was not found in MZR-treated groups in non-sensitized rats, although leukocyte counts were temporarily lower in the 22-HALO group than the 10-HALO group. High doses (20 mg/kg) of MZR caused dosing-time dependent oligocytopenia, although low doses (10 mg/kg) did not reduce erythrocyte counts to lower than the level in the control group. These results were in agreement with those in our previous study (28). Because the half-life of erythrocytes is longer than that of leukocytes, risk generated oligocytopenia is lower than leukopenia caused by administering drugs such as antitumor drug administration (15, 42). MZR suppresses the proliferation of human T cells, blocking the transition from the G1 to S phase of the cell cycle (43). Inhibition of excessive increases in leukocytes and oligocytopenia was greater in the group treated at 22 HALO when DNA synthesis of bone marrow cells began to activate than that at 10 HALO. Although the mechanism is not sufficiently understood, it is thought that the 24-h rhythm of bone marrow contributes to the dosing time-dependency of efficacy and toxicity of MZR.

In our previous study, the MZR-treated group at 1 HALO had a significantly higher AUC than that at 13 HALO (28). Thus, daily variations in the pharmacokinetics of MZR may be concerned with the dosing-time dependency of antirheumatic effects. To establish the reason why the inhibitory effect of arthritis was affected by dosing-time, plasma concentrations were measured when MZR was given at 10 or 22 HALO. There were no significant differences in plasma levels between the two MZR-treated groups in this study. The difference between the present and past studies was the dosing-time. Further studies may be necessary to clarify the relationship between daily variations in the antirheumatic effects of MZR and its pharmacokinetics. However, the pharmacokinetics of MZR did not participate in the dosing-time dependency of the arthritis inhibitory effect seen in this study.

On the other hand, TNF-α levels showed a clear 24-h rhythm with higher levels at the light phase. This rhythm corresponded to those in CIA mice and MRL/lpr mice in our studies, and arthritis was reduced by administering MTX or tacrolimus at 22 to 2 HALO, when TNF-α concentrations began to increase (25, 27). In this study, arthritis was relieved after MZR administration at specific times in synchronization with the 24-h rhythm of TNF-α levels. Therefore, 24-h rhythms of the inflammatory reaction and cytokines involved in RA may have been important for selecting the optimal dosing-time of MZR.

In summary, we reported that MZR showed dosing-time dependent antirheumatic effects in this study. Low-dose MZR at 22 HALO especially prevents excessive increases in leukocyte counts caused by the onset of RA and does not provide myelosuppression at greatly lower than normal levels. It has been suggested that the reaction depends on the dosing-time corresponding to 24-h rhythms of the cell cycle in myelocyte cells and cytokines, and inhibition of the increasing inflammatory response may be one of the causes of the dosing-time dependency of inhibition of the exacerbation of arthritis. Although further investigation is necessary to elucidate these mechanisms in detail, choosing an optimal dosing-time and dosage are expected to lead to a more effective MZR therapy for RA. However, the usefulness of chronotherapy of MZR should be estimated in RA patients because the dose and dosing time of MZR in an RA model animal did not always correspond to that in RA patients.

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References


