Chronotoxicity and Chronopharmacokinetics of Methotrexate in Mice: Modification by Feeding Schedule

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ABSTRACT—The circadian rhythms of the toxicity and the pharmacokinetics of methotrexate (MTX), as well as the effects of manipulation of feeding schedule on the rhythms, were investigated in mice. Male ICR mice were housed under a standardized light-dark cycle (12:12) with food and water ad libitum (ALF) or under the time-restricted feeding (TRF) schedule (8 hr during the light phase) for 1 day or 14 days before the drug administration. The animals received MTX (100 mg/kg, i.p.) once daily for 7 days in the toxicity studies and a single dose of MTX (100 mg/kg, i.p.) for the kinetic studies. Under the ALF, a significant dosing time dependency was demonstrated for the toxicity of MTX with a longer survival time for the mid-dark dosing and a shorter one for the mid-light dosing. The MTX kinetics also showed a significant rhythm, with the highest clearance at mid-dark and the lowest one at mid-light. The rhythm in MTX kinetics well coincided with that in the toxicity of the drug. The TRF had a marked influence on the rhythms of MTX kinetics and toxicity. Thus, the timing of dosing is important in the kinetics and the toxicity of MTX in mice, and the manipulation of feeding schedule can modify the rhythm of the toxicity by changing that of the MTX kinetics.

Keywords: Methotrexate, Chronotoxicity, Chronopharmacokinetics, Circadian rhythm, Feeding schedule

Methotrexate (MTX) is one of the most potent anticancer agents, particularly for human leukemia, severe psoriasis and some solid tumors. The dose and the duration of treatment have been severely limited by the toxicity of the drug, which ranges from the unpleasant to the fatal. The therapy with a high dose of MTX has become possible by the addition of leucovorin rescue regimens (1). However, the highly toxic effects of the drug are still a considerable problem (2).

Substantial evidence exists on circadian rhythms in the susceptibility to cytotoxic agents and the pharmacokinetics of chemotherapeutic drugs (3, 4). The toxicity of antimetabolic agents depends on the timing of drug administration (5–7). Although the circadian rhythm of MTX toxicity has been reported in rats (8), it is not clear whether the toxicity rhythm is due to the rhythm of MTX kinetics. Since the drug has a narrow therapeutic range, it is important to clarify the role of pharmacokinetics in the circadian rhythm of MTX toxicity.

Numerous factors affect the circadian rhythm of drug actions. Lighting schedule is undoubtedly an important determinant of the circadian rhythm of drug actions in rodents. Feeding condition can also modify the rhythm to a great extent. We have previously shown that the manipulation of feeding schedule modifies the rhythms of toxicity and kinetics of phenobarbital (9) and theophylline (10) in mice. Thus, the feeding schedule may influence MTX toxicity and kinetics.

This study was designed to examine the existence of MTX chronotoxicity in mice and to clarify the role of pharmacokinetics on the dosing-time-dependent toxicity of the drug in mice. The influence of manipulation of feeding schedule on the rhythms of toxicity and kinetics of the drug was also investigated.

MATERIALS AND METHODS

Animals

ICR male mice, 6-week-old with body weights from 35 to 40 g, were housed, from 4-week-old, 10 per cage on a

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standardized light-dark cycle of lights on during 0700–1900 (LD 12:12), at a room temperature of 24±1°C and a humidity of 60±10%. The animals were fed either on a schedule of ad libitum food (Oriental Yeast Co., Ltd., Tokyo) and water (ALF) or under a time-restricted feeding (TRF) schedule (feeding time: 0900–1700) for 1 day or 14 days before the drug administration. The treatment of the animals was based on the Guidelines for Animal Experiments at the Ehime University School of Medicine, Japan.

Experiment 1: Chronotoxicity of MTX and the effect of feeding schedule
Eighty mice were used for the chronotoxicity study. Groups of 20 mice each, 2 groups fed under the ALF schedule and the other 2 groups under the TRF schedule for 14 days before the drug administration. The feeding schedules were continued during the experiments. The animals received the drug either at midlight period (13:00) or at middark (01:00). Each animal was given MTX (Lederle, Ltd., Tokyo) at the dose of 100 mg/kg/day, i.p., in a total volume of 0.1 ml/10 g body weight, once daily for 7 days. After injection, the animals were returned to the home cages, and the dead animals were removed at each observation.

Experiment 2: Chronopharmacokinetics of MTX
To study the time course of plasma MTX concentrations in relation to the toxicity of the drug, 40 mice under the ALF schedule were used for the chronopharmacokinetic study. Separate groups of 10 animals each were given a single dose of MTX (100 mg/kg, i.p.) at one of four times: 07:00, 13:00, 19:00 and 01:00. Multiple plasma samples (approximately 60 μl for each sample) were drawn by orbital sinus collection with micropipets at 0.5, 1, 2, 4, 8 and 12 hr after drug administration.

Experiment 3: Effects of manipulation of feeding schedule on the chronopharmacokinetics of MTX
Eighty mice were used to examine the effects of feeding schedule manipulation on the rhythm of MTX kinetics. The animals were fed under the TRF schedule for 14 days or for just 1 day before the drug administrations. The procedures of the experiment and the dose of the drug were the same as those in experiment 2.

Assay of MTX concentrations in plasma
Total MTX concentrations in the plasma were determined by homogeneous enzyme immunoassay (Syva Corporation's EMIT® system, Syva Corp., Sanjose, CA, U.S.A.) (11). The coefficient of variation of identical samples was 10% or less.

Data analyses
A two-compartment open model was employed to calculate the kinetic parameters: elimination rate constant (Kₑ), half life (T₁/2ₑ and T₁/2₁), total body clearance (CL), area under the curve (AUC₀→∞) and volume of distribution (Vₑ₀) (12). Statistical evaluation was performed by analysis of variance (ANOVA) and Tukey's test was used to determine the significance of clock-hour difference between different administration times. Data analyses for circadian rhythms were done by the cosinor method to yield evidence of 24-hr rhythmicity and to obtain indices of the mesor (Mₑ rhythm-determined average), amplitude (Aₑ, measure of the extent of a rhythmic change) and acrophase (Φₑ, peak time of a phase reference) (13). Student's t-test was employed for statistical evaluation of the survival time in mice.

RESULTS

Experiment 1: Chronotoxicity of MTX and the effects of feeding schedule
There was a significant dosing-time-dependent difference in the toxicity of MTX in mice under the ALF schedule (Table 1). The survival time was shorter in mice injected at midlight than at middark (P < 0.05). The TRF schedule for 14 days had a marked influence on the dosing-time-dependent change in the toxicity of MTX, showing a shorter survival time in mice injected at middark than at midlight (P < 0.05).

Experiment 2: Chronopharmacokinetics of MTX
There was a highly significant dosing-time-dependent difference in plasma MTX concentrations at 0.5 hr after dosing. The highest MTX concentrations were found in mice injected at 13:00, showing significantly higher values than those injected at other times (P<0.01, Tukey's test). The lowest ones were in mice injected at 07:00. Some kinetic parameters of the drug also showed dosing-time-dependent changes. CL was significantly smaller and AUC was larger in mice injected at 13:00 than at other time

| Group  | Time of drug administration | Statistical significance |
|--------|-----------------------------|-------------------------|
|        | midnight (13:00)            | middark (01:00)         |                             |
| ALF    | 7.6±0.2 days                | 8.3±0.2 days            | P<0.05                     |
| TRF    | 8.3±0.2 days                | 7.8±0.3 days            | P<0.05                     |

Mean±S.E.M. of 20 mice per group.
However, no significant difference was found for any other kinetic parameters of the drug, although $T_{1/2}$ tended to be longer at 13:00 than at other time points ($P < 0.1$). The pharmacokinetic parameters derived are shown in Table 2.

The plasma MTX concentrations at 0.5 hr after dosing showed a significant circadian rhythm ($P < 0.001$) with a mesor of $166.1 \pm 7.7 \mu g/ml$, amplitude of $47.5 \pm 10.9 \mu g/ml$ and acrophase of $-53.9 \pm 13.1^\circ$. The acrophase of the rhythm shifted approximately 10 hr, showing the highest plasma concentration at around middark (03:36). CL and AUC also showed significant circadian rhythms with best-fit single cosine curves of $Y_t=0.53+0.1 \cdot \cos(15^\circ/hr \cdot ti-255^\circ) \mu g/hr$ ($P < 0.001$) and $Y_t=192.0+31.4 \cdot \cos(15^\circ/hr \cdot ti-53^\circ) \mu g/hr/ml$ ($P < 0.01$), respectively (Table 3).

The rhythm of plasma MTX concentrations at 0.5 hr after dosing under the TRF schedule for just 1 day showed a significant rhythm with the highest plasma concentrations in mice injected with the drug at 07:00, which was significantly higher than those at any other time point ($P < 0.01$) (Table 2). The plasma MTX concentrations at 0.5 hr after dosing was also significantly higher at 01:00 than at 13:00 or 19:00 ($P < 0.05$). The circadian rhythm of MTX concentrations at 0.5 hr after dosing under the TRF schedule was markedly shifted in comparison to the rhythm under the ALF schedule. The characteristics of the rhythm showed a mesor of $156.7 \pm 4.6 \mu g/ml$, amplitude of $57.4 \pm 6.5 \mu g/ml$ and acrophase of $88.7 \pm 6.5^\circ$, showing the highest plasma concentrations of the drug at 05:55. CL was smaller and AUC was larger at 01:00 than at 13:00 or 19:00. The smallest CL and the largest AUC were found in mice injected at 07:00. The best-fit single cosine curves of CL and AUC were $Y_t=0.61 + 0.25 \cdot \cos(15^\circ/hr \cdot ti-257^\circ) \mu g/hr$ ($P < 0.001$) and $Y_t=193.5+97.9 \cdot \cos(15^\circ/hr \cdot ti-88^\circ) \mu g/hr/ml$ ($P < 0.001$), respectively.

**DISCUSSION**

The present results indicate that there is a marked dosing-time-dependent difference of MTX toxicity in mice fed under the ALF schedule. The MTX-induced mortality is considerably greater when the drug injected at midlight than at middark. This is consistent with the finding report-
ed by English et al. (8), who have shown that MTX is least toxic to the rat at about 6 hr after the light-off time (around middark). A similar significant circadian rhythm was shown for plasma MTX concentrations with the highest level at midlight and the lowest one at middark. The circadian-stage-dependent change in plasma MTX concentrations well corresponded to that in the toxicity of the drug. The dosing-time-dependent difference of MTX induced toxicity seems to be due to, at least in part, that of the MTX concentrations.

It is known that MTX is absorbed rapidly and completely from the intraperitoneal site of injection (14). Although the circadian rhythm in the blood flow rate is considered to be related to that in drug absorption (15), the rhythm is the reverse of that of the plasma drug levels, with lower values during the dark period in the present study. Therefore, there seems to be no possibility that the rhythm of drug absorption explains that of plasma MTX concentrations. A significant circadian rhythm was also demonstrated for MTX CL or AUC, which showed respectively a mirror image or a similar pattern with reference to the rhythm of the plasma MTX concentrations. However, no significant difference was found for any other pharmacokinetic parameters of the drug. Therefore, the circadian rhythm of CL seems to contribute to the mechanisms underlying the rhythm of plasma MTX

| Table 2. Pharmacokinetic parameters of methotrexate (MTX) in mice under the ad libitum feeding (ALF) and the time-restricted feeding (TRF) schedule after a single dose of 100 mg/kg, i.p. |
|-----------------|--------|--------|--------|-----------------|--------|--------|--------|-----------------|--------|--------|--------|
|                  | ALF    | TRF (1 day) | TRF (14 days) |                  | ALF    | TRF (1 day) | TRF (14 days) |                  |
|                  | 07:00  | 13:00 | 19:00 | 01:00 | Statistical significance | 07:00  | 13:00 | 19:00 | 01:00 | Statistical significance | 07:00  | 13:00 | 19:00 | 01:00 | Statistical significance |
| AUC_{i.o} (ng·hr/mL) | 156* | (13) | 177 | (11) | 155* | (12) | 177 | (7) | 155* | P < 0.01 | 324* | (16) | 128** | (6) | 137* | (8) | 185 | P < 0.01 | 193 | (16) | 185** | (8) | 155* | 237 | P < 0.01 |
| V_{d0} (l/kg) | 0.68 | (0.09) | 0.92 | (0.15) | 1.03 | N.S. | 0.55 | (0.11) | 0.56 | (0.08) | 0.62 | N.S. | 0.67 | (0.11) | 1.14 | 0.77 | N.S. |
| k_{1} (1/hr) | 2.57 | (0.10) | 3.66 | (0.21) | 12.2 | N.S. | 3.15 | (0.27) | 2.89 | (0.21) | 2.45 | N.S. | 3.23 | (0.15) | 2.71 | 3.95 | N.S. |
| T_{1/2} (hr) | 0.24 | (0.03) | 0.19 | (0.03) | 0.20 | N.S. | 0.26 | (0.03) | 0.25 | (0.03) | 0.25 | N.S. | 0.20 | (0.03) | 0.24 | 0.19 | N.S. |
| T_{1/2} (hr) | 3.73 | (0.83) | 4.28 | (0.59) | 3.54 | N.S. | 2.97 | (0.47) | 2.04 | (0.41) | 3.00 | N.S. | 3.79 | (0.45) | 4.59 | 4.26 | N.S. |
| CL (l/kg/hr) | 0.68* | (0.05) | 0.50 | (0.04) | 0.67* | (0.05) | 0.32** | 0.80** | 0.76* | 0.56 | P < 0.01 | 0.48 | (0.02) | 0.55* | 0.65** | 0.45 | P < 0.01 |

Mean values (S.E.M. in parentheses) of 10 mice per group. AUC_{i.o}; area under the curve, V_{d0}; volume of distribution at steady-state, K_{1}; elimination rate constant, T_{1/2} and T_{1/2}; half lives at α-phase and β-phase, CL; total body clearance, C_{0.5}; plasma drug level at 0.5 hr after dosing. *: P < 0.05, **: P < 0.01, when compared with the group dosing at 13:00 (ALF) or at 01:00 (TRF) by Tukey's test.

| Table 3. Rhythm characteristics of methotrexate (MTX) kinetics in mice under the ad libitum feeding (ALF) and the time-restricted feeding (TRF) 1 day or 14 days schedule |
|-------------------|--------|--------|--------|-------------------|--------|--------|--------|-------------------|--------|--------|--------|
| Mesor (M) (ng·hr/mL) | 173.7 | (5.7) | 192.0 | (7.1) | Mesor (M) (l/kg/hr) | 0.61 | (0.02) | 0.61 | (0.02) | 0.53 | (0.02) |
| Amplitude (A) (ng·hr/mL) | 28.1 | (8.0) | 31.4 | (10.1) | Amplitude (A) (l/kg/hr) | 0.10 | (0.03) | 0.25 | (0.03) | 0.10 | (0.02) |
| Acrophase (degree) | -217 | (16) | -88 | (7) | Acrophase (degree) | -46 | (16) | -257 | (7) | -255 | (7) |
| Acrophase (hr : min) | 14:29 | (1:10) | 05:53 | (0:27) | Acrophase (hr : min) | 03:00 | (1:03) | 17:06 | (0:28) | 17:00 | (0:49) |
| Rhythm detection | <0.01 | <0.001 | <0.05 | Rhythm detection | <0.01 | <0.001 | <0.001 |

Mean values (S.E.M. in parentheses) of 10 mice per group. Yi=M+A·cos (W·ti+ψ)+εi. W: fixed angular velocity, 360°/24 hr = 15°/hr; ε: errors associated with the measurement for unknown or unevaled reasons.
concentrations. It is well known in mice that MTX is metabolized to only a minor extent and is excreted largely unchanged in equal amounts in the urine and bile (14). The circadian change in plasma CL rate of the drug could be caused by that in the renal filtration rate and/or biliary output. The maximum glomerular filtration rate is closely related to the time-point with the most rapid plasma CL of drugs with low lipid solubility such as MTX (16). Therefore, higher renal blood flow and filtration during the active period of animals may contribute to the increased rate of renal MTX CL. Similar circadian rhythms have been demonstrated for the CL of lithium, amikacin and gentamicin, which are mainly eliminated by glomerular filtration (17–20).

Generally, the pharmacologic actions of a drug is thought to be determined by the sensitivity of the living organisms to the drug and the pharmacokinetics of the drug. Therefore, rhythmic change in the pharmacologic action is caused by that in the pharmacokinetics of drugs and/or the sensitivity to the drug as exemplified by sodium valproate (21, 22). The dosing-time-dependent difference in MTX toxicity may be due to the rhythm in the sensitivity to the drug in addition to the rhythm in the pharmacokinetics of the drug. MTX is generally thought to act specifically on the process of DNA synthesis and, therefore, is regarded as cell-cycle specific (23). The drug can kill cancer cells but also kills or injures cells of normal tissues. The outstanding features of fatal intoxication are anorexia, progressive weight loss, bloody diarrhea, leukopenia, depression, and coma. Especially, normal tissues that proliferate rapidly (bone marrow and intestinal epithelium) are seriously subject to damage. The susceptibility of the tissues may be rhythmically variable during the circadian cycle associated with the cell-cycle specific action of MTX.

Although the exact mechanism underlying the influence of the TRF schedule on the rhythms of the toxicity and the kinetics of MTX is not obvious from the present study, the manipulation of feeding schedule definitely modifies the circadian rhythms of MTX. Under the TRF schedule, higher mortality is found during the dark phase (nonfeeding period), which also coincides with a higher plasma concentration and lower CL of the drug. It is reported that the food and water intake of rodents are confined mostly to the dark phase (24) and that the feeding schedules share with L-D cycles the ability to control the overt phase of daily biological rhythms (25). Restricted daily food availability can substantially modify the circadian pattern of an animal’s behavioral and physiological activities, including drinking, activity, sleep states, enzyme activity, blood constituents and urinary excretion (26). One possible interpretation of the present findings is that under the TRF condition, the drive for food becomes paramount, causing the animal’s activity and sleep-wakefulness pattern to be altered so as to obtain adequate food intake (27). The rhythm of renal excretion is also influenced by feeding conditions (28) and cause the rhythm in MTX kinetics to be modified.

Attention is called to the dramatically high plasma MTX concentrations at 09:00 under the TRF schedule for 1 day. The increase in drug concentrations is too high to be accounted for by metabolic changes caused by feeding schedule. It could be due to the fasting of mice for 16 hr before dosing. However, the plasma MTX concentration at midnight (feeding period) is still significantly lower than that at middark (nonfeeding period). Under the schedule of TRF for 1 day, the amplitudes of MTX kinetic parameters are higher than those under the schedules of ALF or TRF for 14 days. The schedule of TRF for 1 day is not enough to reverse the rhythm of MTX kinetics.

In the present study, the time-restricted feeding schedule modifies the rhythm of MTX kinetics regardless of LD condition. The results suggest that the feeding schedule is capable of entraining the rhythmic variables, and it is even a stronger synchronizer than LD cycles for the rhythm of MTX kinetics. Furthermore, the rhythm of MTX kinetics quickly disassociates with changing of the feeding schedule and it takes time to reestablish the rhythm corresponding to the feeding schedule (29). The influence of feeding schedules on the rhythms is different between rodents and human beings. For rodents, feeding conditions relative to the LD cycles exert a major influence on the temporal rhythms, as we showed in the present study. For human beings, the overt phase and period of daily biological rhythms are more strongly controlled by the LD cycles even though the meal conditions also influence the rhythms to a great extent (30). Therefore, care must be taken when the experimental results from rodents are being evaluated and applied.

The maximization of the antineoplastic effects and the minimization of the toxicity to normal tissues are of great interest for cancer chemotherapy. One approach to increase the efficiency is administration of highly toxic drugs at times associated with their best tolerance. Since the lower MTX toxicity observed at the middark phase appears to be due to the higher total body clearance of the drug, the tumor cells of an affected organism would also be exposed to lower levels of MTX at that time. Although it might decrease the desired cytotoxic effects of MTX, the toxicity can be reduced definitely by the administration of MTX according to a circadian schedule. Manipulation of feeding schedule has a marked influence on the dosing time dependent difference in toxicity and kinetics of MTX. Thus, the choice of the most appropriate timing of drug administration in relation to feeding schedule may help to achieve rational usage of the drug.
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