Chronotoxicity of Methotrexate in Mice and Its Relation to Circadian Rhythm of DNA Synthesis and Pharmacokinetics

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ABSTRACT The mechanisms underlying the circadian rhythm of methotrexate (MTX)-induced toxicity (body weight loss and leukopenia) were investigated from the viewpoints of the sensitivity of living organisms to the drug and the pharmacokinetics of the drug. ICR male mice were housed in a standardized light-dark cycle (lights on at 0700, off at 1900) with food and water ad libitum. The body weight loss after an intraperitoneal injection of MTX (400 mg/kg) was more serious in the late dark period and the early light period and milder in the late light period and the early dark period. The MTX-induced leukopenia was more serious in the late dark period and the light period and milder in the early dark period. Lower toxicity was observed when DNA synthesis, dihydrofolate reductase (DHFR) activity in bone marrow cells and folate level in plasma decreased, and higher toxicity was observed when they increased. There was a significant circadian rhythm in plasma MTX concentration, with a higher level in the light period and a lower level in the dark period. The circadian rhythm of plasma MTX concentration was associated with that of MTX-induced toxicity. The present study suggests that the circadian rhythm of MTX-induced toxicity is caused by that of the sensitivity of living organisms to the drug and the pharmacokinetics of the drug.

Keywords: Methotrexate, Chronotoxicity, Chronopharmacokinetics, Cell cycle, Circadian rhythm

The maximization of the antitumor effects to tumor cells and the minimization of the toxicity to normal cells are important in cancer chemotherapy because antitumor drugs can kill normal cells as well as tumor cells. The characteristic features of fatal intoxication are progressive weight loss, leukopenia, anorexia, bloody diarrhea, depression and coma. Especially, normal cells in bone marrow and intestine that proliferate rapidly are seriously subjected to damage. One approach for increasing the efficiency is administration of highly toxic drugs at times at which they are best tolerated. The use of a chronopharmacological strategy can improve tumor response to treatment and overall survival rates and reduce drug toxicities in rodents and humans (1–3). The mechanisms underlying the circadian rhythm of cytotoxicity induced by antitumor drugs have been investigated. One of them is considered to be circadian stage dependent change in cell cycle distribution (4, 5). However, the exact mechanisms involved are yet not clarified.

Methotrexate (MTX) is one of the most potent anticancer agents, particularly for human leukemia, severe psoriasis and some solid tumors (6, 7). The dose and 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. However, the highly toxic effects of the drug are still a considerable problem. There is a significant circadian rhythm in the toxicity and the pharmacokinetics of the drug (8–10). However, the mechanisms underlying the circadian rhythm have not been fully investigated. Especially, the relationship between the circadian rhythms of MTX cytotoxic activity and DNA synthesis is not clear, although MTX is considered to kill cells in the S phase by means of its irreversible binding to the enzyme dihydrofolate reductase (DHFR) (6, 7).

This study was designed to examine the existence of MTX chronotoxicity in mice. The mechanisms underlying the circadian rhythm of MTX-induced toxicity were investigated from the viewpoints of the sensitivity of living organisms to the drug and the pharmacokinetics of the drug. Whether the circadian rhythm of MTX toxicity is
associated with that of DNA synthesis was investigated. The treatment of animals was based on the Guidelines for Animal Experiments at the Kyushu University.

MATERIALS AND METHODS

Animals
ICR male mice (5-weeks-old) were purchased from Charles River Japan, Inc. (Yokohama). Mice were housed 10 per cage in a standardized light-dark cycle (lights on at 0700, off at 1900) at a room temperature of 24±1°C and with food and water available ad libitum.

Preparation of dosing solutions
MTX (Methotrexate Injection; Lederle Japan Co., Tokyo) was used at a dose of 400 mg/kg for the intraperitoneal (i.p.) route. The dosage of MTX was chosen based on the preliminary experiment as indices of body weight loss and leukopenia. The drug was dissolved in sterilized distilled water to yield an appropriate concentration. 3',5',9-3H-MTX (10-20 Ci/mmol), ACS (aqueous counting scintillant) and NCS (nuclear-chicago corporation solubilizer) were obtained from Amersham (Tokyo). Norit I (charcoal activated, neutralized powder) was obtained from Nacalai Tesque, Inc. (Kyoto). Silicone oil (SH 550) was obtained from Toray Dow Corning Silicone (Tokyo). Other reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka), Kanto Chemical Co. (Tokyo) and Sigma Chemical Co. (St. Louis, MO, USA).

Influence of MTX dosing time on body weight loss
Groups of 5 mice were intraperitoneally injected with 400 mg/kg MTX at 0900, 1300, 1700, 2100, 0100 or 0500. The mice were weighed daily and checked throughout the experiment. Body weight loss was calculated as percent change for each mouse from the initial treatment day (day 0).

Influence of MTX dosing time on leukocyte number
Groups of 5 mice were intraperitoneally injected with 400 mg/kg MTX at each of the six times as described above. Blood samples (20 μl each) were drawn by orbital sinus collection using micropipettes (Drummond Scientific, Broomall, PA, USA) on day 3 after MTX injection. Leukocyte number was measured by Sysmex F-300 (Towa Denshi, Kobe). Because of the physiological circadian rhythm in leukocyte number, the leukocyte number decreasing effect of MTX was also expressed as percent change of the corresponding time-qualified controls.

Circadian rhythm of cell cycle in bone marrow cells
The circadian rhythm of the cell cycle in bone marrow cells was determined by the method of Sletvold and Laerum (11). Groups of 10 mice were killed at each of the six times as described above, and their femurs were removed. Thereafter, femurs were flushed with 5 ml of 0.9% NaCl solution (2.5 ml from each end of the bone). The cell suspension from both femurs was pooled and centrifuged at 800 rpm for 10 min at 4°C. The pellets were washed twice with 10 ml of ice-cold 0.14 M NaCl and 0.01 M sodium phosphate (pH 7.4) and then resuspended in 2 ml of the same buffer. Afterwards, the cells were fixed dropwise in ice-cold 96% ethanol and stored at 4°C overnight. Ethanol-fixed bone marrow cells were washed twice in 10 ml of ice-cold 0.14 M NaCl−0.01 M sodium phosphate (pH 7.4). Thereafter, 1 ml of ribonuclease A (1 mg/ml in 0.14 M NaCl−0.01 M sodium phosphate (pH 7.4)) per 1×10^6 cells was added, and incubated for 60 min at 37°C. For specific staining of DNA, 1 ml of propidium iodide (0.05 mg/ml in 0.1% sodium citrate solution) per 3×10^6 cells was added. The proportion of cells in the G0+G1 phase, the S phase and the G2+M phase of the cell cycle was determined by the EPICS Elite flow cytometer (488 nm; Coulter Co., Hialeah, FL, USA). The total number of cells analyzed from each sample was 10,000. Propidium iodide binds stoichiometrically to DNA. When excited by the proper wavelength, it fluoresces proportionally to the amount of DNA in the cell. Thus, a cell in G0+G1 phase having 2N amount of DNA gives off half as much fluorescence as a cell in G2+M phase having 4N DNA. A cell in the S phase has a fluorescence in between cells in G0+G1 and G2+M. Thus a flow cytometer allows quantitation of the fluorescence of a rapidly flowing stream of appropriately stained cells.

Time-dependent change of DHFR activity in bone marrow cells
The DHFR activity in bone marrow cells was determined by radioligand (3H-MTX) binding assay (12) since MTX shows a 1:1 molar stoichiometric reaction with DHFR. In the light period (0900–1300) or the dark period (2100–0100), groups of 5 mice were killed and their femurs were removed. Cell pellets were prepared by the method described above and resuspended at a density of 1×10^6 cells/ml. The suspensions were sonicated at 0°C, using three 10-sec bursts at maximum miniprobe power and 20 sec of chilling between bursts. The suspension was centrifuged at 9000 rpm for 10 min at 4°C. Aliquots (0–150 μl) of cytosolic extract were incubated for 10 min at 20°C with 800 μl of 100 μM NADPH and 50 μl of 0.15 μM 3H-MTX in a total volume of 1.0 ml in 0.01 M potassium phosphate buffer, pH 6.0 containing 0.15 M potassium chloride. Non-bound radioligand was removed by addition of 500 μl of a 5% (W/V) aqueous suspension of Norit I containing 1% dextran. After 1 min, the mix-
ture was filtered through a 0.45-μm filter (Kanto Chemical Co., Tokyo) using a disposable 2.5-ml syringe. Aliquots of 500 μl of filtrate were added to 10 ml ACS and counted in a liquid scintillation counter (Aloka LSC-3500; Aloka, Tokyo). Protein concentrations of cytosolic extract were determined by DC protein assay (Bio-Rad, Tokyo). Radioligand binding, expressed in picomoles, was plotted as a function of volume of cytosolic extract. Least squares analysis indicated a linear relationship. DHFR level (pmol/ml) in the cell extract was calculated from the slope of this curve. From these values, the original densities of the cell suspension (10^7 cells/ml) and the protein content (mg/10^7 cells), the intracellular DHFR level was converted into pmol/10^7 cells and pmol/mg protein.

**Circadian rhythm of folate and its metabolites in plasma**

Groups of 5 mice were killed at each of the six times as described above. Blood samples were drawn by heart puncture and centrifuged at 3000 rpm for 5 min. The concentrations of folate and its metabolites in plasma were determined by high pressure liquid chromatography (HPLC) as described by Shin et al. (13). The mixture of plasma (200 μl), internal standard (2-sulfamoylacetyl-phenol, 20 μg/ml, 50 μl) and methanol (300 μl) was mixed for 30 sec and centrifuged for 5 min. The supernatant was filtered with a 0.45-μm microfilter (Millipore, Tokyo) and evaporated by Speed Vac Plus SC110A (Savant Instruments, Inc., New York, NY, USA) for 50 min. The residue (100 μl) was injected into the HPLC system. The HPLC system had the following setup: a pump (655A-11 Liquid Chromatograph; Hitachi, Tokyo), a detector (655A Variable Wavelength UV Monitor, Hitachi), a chromatopac (C-RIB; Shimadzu, Kyoto), a guard column (5 μm, RP-8, 4 mm x 4 mm I.D.; Merck, Dermstadt, Germany) and an analytical the column (C8 column, RP-8 (5 μm), 250 mm x 4 mm I.D.; Merck). The UV wavelength for detection was 288 nm. A constant temperature of 40°C by the column oven (Model CTO-2A, Shimadzu) and a flow rate of 1 ml/min were maintained. The mobile phase was a mixture of 50 mM KH2PO4 (pH 5.0)-acetonitrile-methanol (92 : 1 : 5, v/v). All folate-related compounds were dissolved in 0.2% sodium ascorbate solution and evaporated by Speed Vac Plus SC110A (Savant) and then reconstituted at 37°C. The reaction was terminated by centrifugation at 13,000 rpm for 7 sec at 4°C. MTX concentrations in plasma were determined by fluorescence polarization immunoassay (TDX; Abbott Laboratories, North Chicago, IL, USA).

**Time-dependent change of MTX uptake and efflux in bone marrow cells**

In the light period (0900–1300) or the dark period (2100–0100), groups of 5 mice were killed and their femurs were removed. Cell pellets were prepared by the method described above and resuspended at a density of 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with penicillin (50 U/ml), streptomycin (50 U/ml) and 10% fetal bovine serum. In MTX uptake experiments, the suspensions were preincubated for 30 min at 37°C and incubated at 37°C with 0.1 μM 3H-MTX. At various time intervals (3, 5, 15, 30, 45 min), aliquots (0.5 ml) of the reaction suspension were removed to microtubes containing 0.7 ml oil (silicone oil : liquid paraffin, 4 : 1). The reaction was terminated by centrifugation at 13,000 rpm for 7 sec at 4°C. The pellets were solubilized in 0.2 ml of NCS overnight at 37°C. The entire tube and its contents were transferred to vial containing 8 ml of ACS and counted in a liquid scintillation counter. In addition to MTX uptake experiments, the amount of MTX remaining within cells, as an index of MTX efflux, was investigated since the cellular retention of MTX reflects the DHFR-binding capacity and the inhibitory effect of DNA synthesis in the cell (6). Cell suspension (1 x 10^6 cells/ml) was preloaded with 0.1 μM 3H-MTX for 60 min at 37°C and centrifuged for 3000 rpm for 5 min at 4°C. The pellets were resuspended in MTX-free medium and then incubated at 37°C. At various time intervals (5, 15, 30, 45, 60 min), aliquots (1 ml) of the reaction suspension were removed to microtubes containing 0.7 ml oil. Thereafter, the samples were treated in the same manner as the uptake experiments.

**Statistical analyses**

The percentage of cells in each cell cycle phase (G0+G1, S, G2+M) was calculated according to Multicycle, cell cycle analytical software (Coulter Co.). Statistical moment analysis was employed to calculate the pharmacokinetic parameters such as area under the curve (AUC), mean residence time (MRT) and variance of residence time (VRT). Student's t-test was used to compare DHFR activities between the light period and dark period. Other data were statistically evaluated by analysis of variance (ANOVA). A probability level of <0.05 was considered to be significant.

**RESULTS**

**Influence of MTX dosing time on body weight loss**

The time course of body weight change after MTX (400 mg/kg, i.p.) injection showed a significant dosing-time-
dependent difference (P<0.01, Fig. 1). Mean maximal body weight loss was achieved between days 3 and 5 after MTX injection. The smallest mean body weight loss was observed after MTX injection at 2100. Moreover, the maximal mean body weight loss was observed after MTX injection at 0500. Recovery from subsequent body weight loss was faster in mice injected with the drug in the late light period and the early dark period than in mice injected with the drug in the late dark period and the early light period.

Influence of MTX dosing time on leukocyte number

The leukocyte number of the control group showed a significant circadian rhythm with higher value in the light period and lower value in the dark period (P < 0.01, Fig. 2). The peak value was obtained at 1300 and the lowest one at 2100. The leukocyte number of mice on day 3 after MTX (400 mg/kg, i.p.) injection showed no significant circadian rhythm. The percent change of leukocyte number, expressed as percent change of the corresponding time-qualified control, showed a significant difference as a function of dosing time (P < 0.01, Fig. 3). The decrease of leukocyte number was largest in mice injected with the drug at 1300 and the smallest in mice injected with the drug at 2100.

Circadian rhythm of cell cycle in bone marrow cells

A significant circadian rhythm was demonstrated for the G0 + G1, S and G2 + M phases (P < 0.01, respectively, Fig. 4). In the G0 + G1 phase, the peak was found at 0500 and the trough was found at 1300. In the S phase, higher levels were found at 0900 and 1300 and lower levels were found at 1700 and 2100. In the G2 + M phase, the peak
was found at 1700 and the trough was found at 0900.

**Time-dependent change of DHFR activity in bone marrow cells**

DHFR activity in bone marrow cells was significantly higher in cells prepared in the light period than in cells prepared in the dark period ($P < 0.05$, Fig. 5).

**Circadian rhythm of folate and its metabolites in plasma**

Figure 6 shows that significant circadian rhythms were demonstrated for folate and its metabolites (FA: $P < 0.01$, 5-CH$_3$-THF: $P < 0.05$, 5-CHO-THF: $P < 0.01$, total folate: $P < 0.01$). FA levels were higher at 0500 and 0900 and lowest at 1700. 5-CHO-THF levels were highest at 0100 and lowest at 1300. 5-CH$_3$-THF levels were highest at 0900 and lowest at 1300.
Influence of MTX dosing time on pharmacokinetics of MTX

There was a significant dosing-time-dependent difference in the time course of plasma MTX concentrations after MTX (400 mg/kg, i.p.) injection (P < 0.01, Fig. 7). A significant dosing-time-dependent difference was demonstrated for plasma MTX concentrations at 24 hr after MTX injection (P < 0.01, Fig. 8). The higher MTX concentrations were found in mice injected with the drug at 0900 and 1300. The lower ones were found in mice injected with the drug at 1700 and 2100. There was a significant dosing-time-dependent difference in MTX pharmacokinetic parameters (Table 1). MRT and VRT were significantly larger in mice injected with the drug at 0900 or 1300 than at 1700 or 2100 (P < 0.05, respectively). However, no significant difference was found in AUC.

### Table 1. Influence of dosing time on MTX pharmacokinetic parameters after MTX (400 mg/kg, i.p.) injection at six different circadian stages

| Time of drug injection (clock hours) | AUC (mg·hr/liter) | MRT (hr) | VRT (hr²) |
|-------------------------------------|-------------------|----------|------------|
| 0900                                | 283 ± 21          | 1.94 ± 0.24 | 42.1 ± 13.3 |
| 1300                                | 294 ± 24          | 2.15 ± 0.37 | 63.7 ± 23.9 |
| 1700                                | 248 ± 14          | 1.28 ± 0.04 | 11.8 ± 1.2  |
| 2100                                | 256 ± 16          | 1.39 ± 0.07 | 12.7 ± 2.8  |
| 0100                                | 275 ± 17          | 1.90 ± 0.08 | 28.8 ± 3.0  |
| 0500                                | 274 ± 19          | 1.58 ± 0.13 | 21.1 ± 4.7  |

Each value is the mean of 8 observations with S.E.
**Time-dependent change of MTX uptake and efflux in bone marrow cells**

A significant time-dependent difference was observed in the time course of intracellular $^3$H-MTX concentrations (uptake) after an exposure of bone marrow cells prepared in the light period or the dark period to $^3$H-MTX (0.1 $\mu$M) ($P<0.05$, Fig. 9). The amount of MTX accumulated within cells was significantly higher in cells prepared in the light period than in the dark period. A significant time-dependent difference was also observed in the time course of intracellular $^3$H-MTX concentrations (efflux) after a resuspension of bone marrow cells prepared in the light period or the dark period in MTX-free medium ($P<0.01$, Fig. 10). Bone marrow cells prepared in the light period had a slower efflux rate than those prepared in the dark period. Namely, the amount of MTX remained within cells was significantly higher in cells prepared in the light period than in the dark period.

**DISCUSSION**

A significant circadian rhythm was demonstrated for MTX-induced body weight loss and leukopenia. It is consistent with the finding reported by Labat et al. (9) who showed that MTX is more toxic in the late dark period and the early light period. The circadian rhythm of drug susceptibility could be caused by that of the sensitivity of living organisms to drugs and/or the pharmacokinetics of drugs (14-18).

MTX is generally thought to act specifically on the process of DNA synthesis and, therefore, is regarded as cell-cycle-specific (6, 7). MTX mediates its cytotoxic effects through inhibition of DHFR, which results in a depletion of intracellular tetrahydrofolate pools and leads to a decline of DNA synthesis (6, 7). The DNA synthesis and DHFR activity in bone marrow cells showed a prominent circadian-stage-dependent change. The circadian rhythm was associated with the circadian rhythm of MTX-induced toxicity. Namely, lower toxicity was observed when DNA synthesis and DHFR activity decreased, and higher toxicity was observed when they increased. A circadian rhythm was also demonstrated for folate and its metabolites in plasma. Folate levels were higher between 0500 and 0900 and lowest at 1700. The circadian rhythm of folate level in plasma corresponds to that of food intake in rodents, confined mostly to the dark phase (19). The circadian rhythm of folate level in plasma can be associated with that of sensitivity of living organisms to MTX since folate plays an important role in DNA and protein synthesis (20, 21). These results suggest that the circadian rhythm of MTX-induced toxicity is due to that of DNA synthesis and DHFR activity in bone marrow cells and plasma folate level from viewpoint of the sensitivity of living organisms to MTX.

There was a circadian rhythm of plasma MTX concentration with the highest level in the light period and the lowest one in the dark period. The higher plasma MTX concentration was observed when MTX-induced toxicity increased. MTX is absorbed rapidly and completely from the intraperitoneal site of injection (22). It is metabolized to only a minor extent and is excreted largely unchanged in urine, when a high dose of MTX is injected. Both renal blood flow and glomerular filtration rate have been found to follow circadian rhythms, with a maximum during the active period of animals (23, 24). The circadian rhythm of renal function seems to cause the circadian rhythm of plasma MTX concentration. The amount of MTX accumulated within bone marrow cells, as an index of MTX uptake, and the amount of MTX remaining within cells, as an index of MTX efflux, were significantly greater in cells prepared in the light period than in the dark period, although the same concentration of MTX was added to medium in both cases. MTX uptake and efflux in bone marrow cells after MTX injection in vivo was not investigated in the present study. However, the time-dependent difference in MTX uptake and efflux in vitro should increase in vivo, when considering the circadian rhythm of plasma MTX concentration in vivo. Although the transport of MTX by cells has several pathways (25), MTX uptake varies depending on the circadian change in the redox state of the cell membrane (26). It might be true in the case of present study. The cellular retention of MTX is considered to reflect the DHFR-binding capacity in cells (27, 28). Therefore, the circadian change in cellular DHFR activity shown in the present study seems to contribute to that in the efflux of MTX. These results suggest that the circadian rhythm of MTX-induced toxicity is due to that of plasma MTX concentration and MTX uptake and efflux in bone marrow cells.

Whether the mechanism underlying the circadian rhythm of MTX-induced toxicity is more closely related to the sensitivity of the living organisms to MTX or the pharmacokinetics of the drug is not obvious from present study. If the reduced toxicity observed in the dark period depends on lower plasma MTX concentration, presumably the tumor cells of an affected organism may also be exposed to lower levels of MTX. This might well impair the desired cytotoxic effect of the drug. If the reduced toxicity observed in the dark period depends on lower DNA synthesis in bone marrow cells, an important point may be to find a different phase in the circadian rhythm of DNA synthesis between normal cells and tumor cells. The DNA synthesis in tumor cells does undergo a significant circadian rhythm with a different phase from normal cells (4). The finding suggests that a chronotherapeutic administration of a drug with its main effect on the S phase.
during the time of higher DNA synthesis in tumor cells and lower DNA synthesis in normal cells could result in a maximized therapeutic index. These points should be clarified in an animal model with tumor.

The present study indicates that the circadian rhythm of MTX-induced toxicity was demonstrated in relation to that of the sensitivity of the living organisms to the drug and the pharmacokinetics of the drug. Therefore, the choice of dosing time associated with the circadian rhythm of DNA synthesis and the chronopharmacokinetics of MTX may help to achieve rational chronotherapeutics, reducing the toxic effects of MTX.

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