Cell Cycle-Dependent Chronotoxicity of Irinotecan Hydrochloride in Mice

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ABSTRACT

The mechanisms underlying the circadian rhythm of the toxicity induced by irinotecan hydrochloride (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) were investigated from the viewpoint of the sensitivity of living organisms and the pharmacokinetics of the drug. ICR male mice were housed under standardized light-dark cycle conditions (lights on at 0700, off at 1900) with food and water ad libitum. The loss of body weight after an intraperitoneal injection of CPT-11 (100 mg/kg) was more serious in the late dark and the early light and milder in the late light. The lower toxicity of CPT-11 was observed when the level of DNA synthesis and type I DNA topoisomerase activity in bone marrow cells decreased and the higher toxicity was observed when these activities began to increase. There were circadian stage-dependent changes in the concentrations of CPT-11 and its major metabolite (SN-38; 7-ethyl-10-hydroxycamptothecin) in plasma. The higher concentrations of CPT-11 and SN-38 in plasma were observed when the level of CPT-11-induced toxicity increased. The present study suggests that the toxicity of CPT-11 is influenced by circadian rhythm-dependent processes.

The maximization of the antitumor effects and the minimization of the toxicity of antitumor drugs to normal tissues are important in cancer chemotherapy, because antitumor drugs can kill normal cells as well as tumor cells. The characteristic features of fatal intoxication with antitumor drugs including irinotecan are progressive weight loss, leukopenia, anorexia, bloody diarrhea, depression and coma. The dose and duration of treatment have been severely limited by the drug toxicities. One approach to increase the efficiency of cancer treatment is the administration of highly toxic drugs at times at which they are best tolerated. Use of a chronopharmacological strategy can improve tumor response to treatment, and overall survival rates and reduce drug toxicities in rodents and humans (Levi et al., 1987; Boughattas et al., 1989; Song et al., 1993; Labat et al., 1987; English et al., 1982; Bjarnason and Hrushesky, 1994). However, the exact mechanisms involved have not been yet clarified.

CPT-11 shows significant antitumor activity against a variety of solid tumors, including lung, colorectal and cervical cancers, and malignant lymphoma (Kojima et al., 1993). However, the dose and duration of treatment have been severely limited by serious side effects such as granulocytopenia and diarrhea. CPT and its analogs inhibit Topo I through the formation of stable Topo I-DNA-cleavable complexes (Hsiang et al., 1985; Hsiang and Liu, 1988; Hertzberg et al., 1989). The antitumor activity of CPT analogs correlates with the drug-induced accumulation of Topo I-DNA-cleavable complexes (Hsiang et al., 1989b) and with the degree of inhibition of DNA relaxation by Topo I (Jaxel et al., 1989). Topo I-DNA-cleavable complexes stabilized by CPT analogs appear to be responsible for DNA single-strand breaks (Hsiang and Liu, 1988; Mattern et al., 1987; Covey et al., 1989), and the production of these breaks in the S-phase interferes with or arrests the progress of the replication fork, which results in cell death (Hsiang et al., 1989a; Holm et al., 1989). These mechanisms offer plausible reasons for such phenomena as the inhibition by CPT analogs of nucleic acid synthesis in a time-dependent and S-phase-sensitive manner (Kessel, 1971; Horwitz et al., 1971; Kessel et al., 1972; Li et al., 1972; Drewinko et al., 1974) and induction of the degrada-

ABBREVIATIONS: CPT, camptothecin; CPT-11, irinotecan hydrochloride, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; Topo I, type I DNA topoisomerase; HPLC, high pressure liquid chromatography; AUC, area under the plasma-time concentration curve; MRT, mean residence time; VRT, variance of residence time; EDTA, ethylenediaminetetraacetic acid; I.D., internal diameter.
dation of DNA in an alkaline sucrose gradient (Horwitz and Horwitz, 1971; Spataro and Kessel, 1972, Abeelson and Penman, 1973). However, the influence of dosing time on CPT-11-induced toxicity and the relationship between the circadian rhythm of DNA synthesis and CPT-11-induced toxicity have not yet been investigated.

This study was designed to clarify the existence of CPT-11-induced chronotoxicity in mice. The mechanisms underlying the circadian rhythm of CPT-11-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 CPT-11-induced toxicity is associated with that of DNA synthesis was investigated.

**Methods**

**Animals.** ICR male mice (5 weeks old) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were housed 10 per cage under standardized light-dark cycle conditions (lights on at 7:00 A.M., off at 7:00 P.M.) at a temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water available ad libitum.

**Preparation of dosing solutions.** CPT-11, SN-38 and CPT were kindly supplied by Yakult Honsha Co., Ltd. (Tokyo, Japan). CPT-11 was used at an intraperitoneal (i.p.) dose with 100 mg/kg of CPT-11. The drug was dissolved in sterilized boiled water (80°C) to yield an appropriate concentration of 100 mg/10 ml. CPT-11 was administered by injection with a 23-gauge needle connected to a 0.5-ml syringe. The volume of drug solutions administered was 10 ml/kg. Propidium iodide and ribonuclease A were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents, purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) were of analytical grade and used without further purification.

**Influence of CPT-11 dosing time on loss of body weight.** Groups of 10 mice were intraperitoneally injected with 100 mg/kg of CPT-11 at 0900, 1300, 1700, 2100, 0100 or 0500 hr. The mice were weighed daily and monitored throughout the experiment. Body weight loss was calculated as the percentage change for each mouse from the initial treatment day (day 0).

**Influence of CPT-11 dosing time on leukocyte counts.** Groups of 10 mice were intraperitoneally injected with 100 mg/kg of CPT-11 at each of the six times outlined above. Twenty-microliter blood samples were drawn by orbital sinus collection with micropipettes (Drummond Scientific, Broomall, PA) on day 3 after CPT-11 injection. Leukocyte counts were determined as the percentage change for each mouse from the initial treatment day (day 0).

**Circadian rhythm of cell cycle in bone marrow cells.** The circadian rhythm of cell cycle in bone marrow cells was determined by the method of Sletvold and Laerum (1988). Groups of 10 mice were sacrificed at each of the six times outlined 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 4 min at 4°C. The pellets were washed once with 10 ml of the above-mentioned buffer and resuspended at a density of 5 × 10⁶ cells/ml. The cell suspension was centrifuged at 5000 rpm for 4 min at 4°C. The pellets were resuspended in 50 μl of phosphate buffer/0.35% Triton X-100 and centrifuged at 5000 rpm for 4 min at 4°C. The pellets were resuspended in 100 μl of extraction buffer [1 M Tris-HCl (pH 8.0) (20 μl)/5 M NaCl (70 μl)/14 M 2-mercaptopethanol (10 μl)/10 mg/ml bovine serum albumin (5 μl)/H₂O (895 μl)]. After 30 min, the cell suspension was centrifuged at 7000 rpm for 15 min at 4°C. The supernatant was stored at −80°C. Topo I activity was measured by the relaxation of supercoiled plasmid DNA using a Topo I assay kit (TopoGEN, Inc, Columbus, OH). The 20 μl assay mixture contained 16 μl H₂O, 2 μl of 10× assay buffer [100 mM Tris-HCl (pH 7.5), 1 mM EDTA] and 1 μl of test sample. After 30 min at 37°C the reactions were terminated by the addition of 5 μl of stop buffer/loading dye (5% sarkosyl, 0.125% bromophenol blue, 25% glycerol). The reaction product was digested with 1.25 mg/ml proteinase K (1 μl) at 37°C for 60 min. The relaxed plasmid substrate DNA and the reaction product with the supercoiled DNA in buffer without any enzyme fraction were used as markers. The samples were loaded onto a 0.8% agarose gel. The dimensions of the agarose gel were 10 × 11 cm (width by length) and the gel tank was 12.9 × 24.7 cm (width by length). The running buffer was 1× TAE (50 × contains 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA) and the gel was run at 50 V at room temperature for 4 hr in a PFCO-2 system (Taitai, Saitama, Japan). The gel was then stained with ethidium bromide (1 μg/ml) for 30 min, destained in distilled water and photographed under UV light (302 nm). The amount of DNA was quantified using a NIH image analysis program on a Macintosh. Topo I activity was calculated from the ratios of relaxed DNA to total DNA (relaxed DNA + supercoiled DNA).

**Influence of CPT-11 dosing time on pharmacokinetics of CPT-11 and SN-38.** Groups of six mice were intraperitoneally injected with 100 mg/kg of CPT-11 at 1700 or 0500 hr. Blood samples (approximately 50 μl for each sample) were drawn by orbital sinus collection by micropipettes at 0.25, 0.5, 1, 2, 4, 6 and 24 hr after CPT-11 injection. Blood samples were immediately centrifuged at 3000 rpm for 1 min at 4°C. The CPT-11 and SN-38 concentrations in plasma were determined by a method described previously (Kaneda and Yokokura, 1990). A mixture of plasma sample (10 μl), 0.1 mM disopropyl fluorophosphate, internal standard (CPT, 0.125 or 1.25 μg/ml, 25 μl) and methanol (375 μl) was mixed with an automatic mixer (S-100, Taitai, Saitama, Japan) for 30 s and centrifuged at 3000 rpm for 10 min to deproteinize the samples. The supernatant was evaporated on a Speed Vac Plus SC110A (Savant Instruments, Inc., Farmingdale, NY) for 30 min. The residue was dissolved in 200 μl of a solution containing tetrahydrofuran/50 mM KH₂PO₄ and 5 mM heptanesulfonate (25:75, v/v), pH 2.0. The insoluble substance was removed by centrifugation at 10,000 rpm for 3 min. Twenty or 50 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 Detector, Shimadzu), a chromatopac (C-R1B, Shimadzu), a guard column (TSK-
GEL ODS-80TS, 5 μm, 3.5 mm I.D. × 15 mm, Toyo soda, Tokyo, Japan) and an analytical column (TSK-GEL ODS-80TS, 5 μm, 4.6 mm I.D. × 150 mm, Toyo soda). The mobile phases consisted of tetrahydrofuran/50 mM KH2PO4, 5 mM heptanesulfonate (25:75 v/v, pH 4.0) and tetrahydrofuran/50 mM KH2PO4, 5 mM heptanesulfonate (32:68 v/v, pH 4.0) for CPT-11 and SN-38, respectively. The flow rate was 0.8 ml/min. The fluorospectromonitor was set at an excitation wavelength of 370 nm and an emission wavelength of 430 nm for CPT-11 and at 380 nm and 550 nm for SN-38. The peak areas were integrated by a data processor.

**Statistical analysis.** The percentage of cells in each cell cycle phase (G0+S+G2+M) were calculated according to Multicycle, a cell cycle analytical software package (Coulter Co., Hialeah, FL). Statistical moment analysis was used to calculate the pharmacokinetic parameters such as area under the plasma-time concentration curve (AUC), mean residence time (MRT) and variance of residence time (VRT). The statistical significance of differences between groups was validated by analysis of variance, the Bonferroni method and Student’s t test. A probability level of < .05 was considered to be significant.

**Results**

**Influence of CPT-11 dosing time on loss of body weight.** The time course of body weight change after CPT-11 (100 mg/kg i.p.) injection showed a significant dosing time-dependent difference (P < .01, fig. 1). Mean maximum body weight loss was observed between days 3 and 4 after CPT-11 injection. The minimum mean body weight loss was observed after CPT-11 injection at 1700 hr. Moreover, the maximum mean body weight loss was observed after CPT-11 injection at 0500 or 0900 hr. Recovery from subsequent body weight loss was faster in mice injected with the drug in the late light and the early dark than in mice injected with the drug in the late dark and the early light.

**Influence of CPT-11 dosing time on leukocyte counts.** The leukocyte counts of mice given saline showed a significant circadian rhythm dependence with higher values in the light and lower values in the dark (P < .01, fig. 2). The higher values were observed at 1300 and 1700 hr and the lowest values at 2100 hr. The leukocyte counts of mice on day 3 after CPT-11 (100 mg/kg i.p.) injection also showed a significant circadian rhythm dependence with higher values in the light and lower values in the dark (P < .01, fig. 2). The higher values were observed at 1300 and 1700 hr and the lowest values at 2100 hr. The leukocyte counts of mice on day 3 after CPT-11 (100 mg/kg i.p.) injection were significantly larger in mice injected with the drug at 0500 than at 1700 hr (P < .05, respectively, table 1).

**Circadian rhythm dependence of cell cycle in bone marrow cells.** A significant circadian rhythm dependence was demonstrated for G0+G1, S and G2+M phases (P < .01, respectively, fig. 3). The proportion of cells in the G0+G1 phase showed a peak at 0500 hr and a trough at 1300 hr. The proportion of cells in the S phase showed higher levels at 0900 and 1300 hr and lower levels at 1700 and 2100 hr. The proportion of cells in the G2+M phase showed a peak at 1700 hr and a trough at 0900 hr. These results were interrelated in that the cells in the G0+G1 phase enter the S phase and later the G2+M phase.

**Time-dependent change in Topo I activity in bone marrow cells.** Topo I activity in bone marrow cells was significantly higher in cells prepared at 0700 hr than in cells prepared at 1900 hr (P < .05, fig. 4).

**Influence of CPT-11 dosing time on pharmacokinetics of CPT-11.** Plasma CPT-11 concentrations at 0.25 and 0.5 hr after CPT-11 (100 mg/kg i.p.) injection were significantly higher in mice injected with the drug at 0500 than at 1700 hr (P < .05, respectively, table 2). The VRT was significantly larger in mice injected with the drug at 0500 than at 1700 hr (P < .05, respectively, fig. 4).

**Table 1**

<table>
<thead>
<tr>
<th>Time after Drug Injection</th>
<th>Time of Drug Injection</th>
<th>Statistical Significance</th>
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<tbody>
<tr>
<td></td>
<td>1700 hr</td>
<td>0500 hr</td>
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<tr>
<td>4 days - 26.39 ± 6.32</td>
<td>-45.15 ± 4.34</td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>5 days - 22.48 ± 3.40</td>
<td>-38.90 ± 3.20</td>
<td>P &lt; .05</td>
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Change in leukocyte count was calculated as the percentage change for each mouse from the initial treatment day (day 0). Each value is the mean with S.E. of 10 observations.
A significant circadian rhythm dependence was demonstrated for CPT-11-induced loss of body weight. This is consistent with the finding reported by Labat et al. (1987), who showed that MTX affecting DNA synthesis is more toxic in the late dark and early light. A significant circadian rhythm dependence was also shown for CPT-11-induced leukopenia which constitutes one of mechanisms of CPT-11-induced loss of body weight. The circadian rhythm dependence of drug susceptibility could be caused by that of the sensitivity of living organisms to drugs and/or the pharmacokinetics of drugs (Ohdo et al., 1988, 1995, 1996; Reinberg and Smolensky, 1982; Halberg and Halberg, 1984).

CPT and its analogs inhibit Topo I through the formation of stable Topo I-DNA cleavable complexes (Hsiang et al., 1987, 1995, 1996; Reinberg and Smolensky, 1982; Halberg and Halberg, 1984). Generally, CPT-11 is thought to specifically affect DNA synthesis and, therefore, is regarded as cell-cycle specific (Li et al., 1972; Tobey, 1972). Namely, DNA synthesis is irreversibly inhibited by CPT-11, and S-phase cells cannot progress into the G2 phase of the cell cycle. The DNA synthesis and Topo I activity decreased and the higher toxicity was observed when DNA synthesis and Topo I activity increases. However, the inhibition of the

### Discussion

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activity of dihydrofolate reductase by MTX is significantly greater when the activity of dihydrofolate reductase increases (Labat et al., 1987). The same might be true in CPT-11.

The main adverse effects of CPT-11 are gastrointestinal problems including diarrhea and vomiting in addition to myelosuppression (Negoro et al., 1991). Diarrhea comprises both early and delayed types. Vomiting and delayed diarrhea are induced by many other antitumor drugs, but early diarrhea which occurs immediately after dosing is a rare adverse effect. CPT-11 has an acetylcholine-like action (Takayanagi et al., 1989). The inhibition of acetylcholinesterase by CPT-11 relates to the occurrence of early defecation or diarrhea and vomiting (Kawato et al., 1993). Acetylcholinesterase activity begins to decrease in the late dark, whereas it begins to increase in the late light (Bhattacharya and von Mayersbach, 1981). The highest loss of body weight was observed when the acetylcholine level increased, and the lowest body weight loss was observed when it decreased. The inhibition of acetylcholinesterase by CPT-11 might vary depending on dosing time. In this study, the diarrhea occurring immediately after dosing was not observed in ICR mice, even after high-dose administration of CPT-11. BALB/c mice do not experience diarrhea, whereas athymic nude mice experience bloody diarrhea after CPT-11 administration (Araki et al., 1993). Thus, the sensitivities to mucosal injury vary among species of animals. Further studies are necessary to clarify the role of the cholinergic system in the toxicity induced by CPT-11.

Because SN-38, the active metabolite of CPT-11, possesses a much stronger growth inhibitory activity against tumor cells than does CPT-11 in vitro (Kojima et al., 1993), both CPT-11 and SN-38 concentrations in plasma were determined. A dosing time-dependent change was observed in plasma CPT-11 and SN-38 concentrations with higher levels in the late dark and lower levels in the late light. The higher plasma CPT-11 and SN-38 concentrations were observed when the CPT-11-induced toxicity increased. CPT-11 is distributed rapidly from the intraperitoneal site of injection (Kaneda and Yokokura, 1990) and is converted to SN-38 by carboxylesterase which occurs immediately after CPT-11 administration. SN-38 is then converted to SN-38 glucuronide and is deconjugated by intestinal microflora to SN-38. About 60% of the CPT-11 administered is excreted into the bile and urine without being metabolized. SN-38 is mainly excreted into the bile. There are significant circadian rhythms in enzyme activity (Halberg and Halberg, 1984), renal function (Cal et al., 1986), blood flow (Labrecque et al., 1988) and plasma protein (Vachon and Savoie, 1987). The highest levels of the enzyme activity are observed when plasma CPT-11 and SN-38 concentrations decrease. Therefore, the circadian rhythm of physiological functions can be considered to be the mechanism underlying the dosing time-dependent changes in plasma CPT-11 and SN-38 concentrations.

The present study indicates that the circadian rhythm of CPT-11-induced toxicity is related to the circadian rhythm of the sensitivity of living organisms to and the pharmacokinetics of the drug. Therefore, the choice of dosing time associated with the circadian rhythm of DNA synthesis and the chronopharmacokinetics of CPT-11 may help to achieve a rational chronotherapeutic strategy, reducing the toxic effects of CPT-11 and/or increasing its therapeutic effects.

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References


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