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CPT-11 Alters the Circadian Rhythm of Dihydropyrimidine Dehydrogenase mRNA in Mouse Liver

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Combination chemotherapy consisting of 5-fluorouracil (5-FU) and 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carboxycamptothecin (CPT-11) is a promising regimen for gastrointestinal cancer. The circadian-dependent efficacy and toxicity of 5-FU are related to the circadian variation in the activity of dihydropyrimidine dehydrogenase (DPD), which is a rate-limiting enzyme in the pyrimidine catabolic pathway. To optimize the schedule of the CPT-11 plus 5-FU combination, we investigated the effect of CPT-11 on the circadian rhythm of DPD *in vivo*. In control mice, the *DPD* mRNA level in the liver was significantly higher at 14:00 than that at 02:00. After intravenous administration of CPT-11 (30 mg/kg) at 20:00, the circadian rhythm of the *DPD* mRNA level in the liver was no longer observed 18 h later (14:00), but it was unaffected 6 and 18 h later (at 14:00 and 02:00) when CPT-11 was given at 08:00. In addition, a dose-dependent lengthening of the period of the circadian rhythm of *DPD* was observed for 42 h after intravenous injection of CPT-11 at 20:00. The levels of DPD protein and activity at 21 h after administration of CPT-11 (at 17:00) were significantly higher than at 9 h (at 05:00). These results suggest that CPT-11 may influence the circadian rhythm of *DPD* at the transcriptional level. Modulation of the circadian rhythm of DPD by CPT-11 may be a factor in optimizing the combination of 5-FU and CPT-11.

Key words: CPT-11 — Dihydropyrimidine dehydrogenase — Circadian rhythm

7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carboxycamptothecin (CPT-11) is one of the most potent therapeutic agents for the treatment of solid tumors, including lung, colorectal, and cervical cancer, and malignant lymphoma,[1, 2] and combination regimens of CPT-11 and 5-fluorouracil (5-FU) have recently been evaluated in metastatic colorectal cancer patients.[3, 4] Combination chemotherapy consisting of CPT-11, 5-FU, and leucovorin has been reported to be well tolerated and to have greater efficacy than 5-FU alone for first-line metastatic colorectal cancer.[5]

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in 5-FU catabolism, and it has been found to exhibit circadian variation in experimental animals[6, 7] and cancer patients.[8] A study in cancer patients treated with 5-FU showed an inverse relationship between DPD activity in peripheral blood mononuclear cells and the plasma concentration of 5-FU,[8] and the circadian variation in 5-FU toxicity in rats has been reported to be inversely correlated with the circadian variation in DPD activity.[9] The relevance of chronotherapy using 5-FU, folinic acid, and oxaliplatin was investigated in phase II trials in metastatic colorectal cancer patients.[10]

The mechanisms regulating circadian rhythms in a variety of species have recently been clarified. In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus has been shown to be a circadian pacemaker,[11] and the human and mouse homologues of the mammalian *period* gene, which plays a central role in circadian rhythms, have been isolated.[12, 13] 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits the synthesis of heterogeneous nuclear RNA and mRNA at the transcription initiation level by interfering with RNA polymerase II function.[14, 15] DRB was found to shift the phase and lengthen the period of the circadian rhythm of isolated eyes of *Aplysia*.[16] Camptothecin, a transcription inhibitor whose action is mediated by inhibition of DNA topoisomerase I (topo I),[17] has been observed to have similar effects on circadian rhythms. Based on these findings, it can be speculated that transcriptional regulation through topo I inhibition might alter the circadian rhythm. We therefore hypothesized that CPT-11, a clinically useful derivative of camptothecin, shifts the circadian rhythm of DPD and affects the efficacy and toxicity in combination therapy of CPT-11 and 5-FU. Accordingly, the purpose of this study was to investigate the effect of CPT-11 on the circadian rhythm of DPD *in vivo*.

MATERIALS AND METHODS

Materials CPT-11 was obtained from Yakult Co., Ltd. (Tokyo) and dissolved in saline for intravenous injection into mice. Anti-DPD polyclonal antibody was provided by

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Animals C57BL male mice (8 weeks old; 19–22 g) were obtained from Charles River Japan Inc. (Yokohama) and exposed to a 12-h light/12-h dark cycle (lights on at 08:00 and off at 20:00) for at least 1 week before the day of the experiment.

Treatment schedule

Experiment 1: Influence of CPT-11 administration time on DPD mRNA levels. Mice (one mouse in each group) were intravenously injected with CPT-11 (30 mg/kg) at 08:00 or 20:00, and control mice were injected with physiological saline. Mice were sacrificed at 6 and 18 h after the injection, and their brain and liver were removed and stored at −80°C until used.

Experiment 2: Dose-dependent effects of CPT-11 on hepatic levels of DPD mRNA. Mice (one mouse in each group) in 4 groups were intravenously injected with different doses of CPT-11 (10, 30, 60, and 100 mg/kg) at 20:00, and control mice were injected with physiological saline. The liver of some of the animals was quickly removed at 6, 18, 30, or 42 h after the injection.

Experiment 3: Influence of CPT-11 on DPD activity and protein levels. Mice in separate groups were intravenously injected with one of two doses of CPT-11, 30 or 60 mg/kg, at 20:00, and control mice were injected with physiological saline. Mice (3 mice in each group) were sacrificed at 9 h (at 05:00) or 21 h (at 17:00) after the injection, and liver tissue was collected and stored.

Northern blot analysis Total RNA was isolated from liver tissue with Isogen (a guanidine HCl/phenol procedure; Nippon Gene, Tokyo). Total RNA (20 μg) was denatured with formaldehyde, electrophoresed through 1% agarose formaldehyde gel, transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK), and immobilized by UV cross-linking. The *DPD* cDNA probe was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml, Daiichi Pure Chemicals, Tokyo) by using a random prime labeling system (Rediprime, Amersham) and purified with a Chroma Spin-10 column (Clontech, Palo Alto, CA). The membrane was prehybridized for 2 h at 42°C in hybridization solution [50% (v/v) formamide, 0.65 *M* NaCl, 0.1 *M* sodium-pipes (pH 6.8), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 5 m*M* ethylenediamine-*N,N,N′N′*-tetraacetic acid, disodium salt, dihydrate (EDTA), 100 μg/ml salmon sperm DNA], and hybridized overnight with the probe at 42°C. The membrane was then washed with 2× saline-sodium citrate solution (SSC) and 0.1% SDS for 10 min at 42°C, with 1× SSC and 0.1% SDS for 20 min at 65°C, followed by washing with 0.1× SSC and 0.1% SDS for 20 min at 65°C under the same conditions. The *GAPDH* cDNA probe was hybridized as described above and used as an internal control for RNA integrity and normalization of RNA loading. Bio Max film (Kodak, Rochester, NY) was exposed to the radioactivity on the membrane under an intensifying screen at −80°C for autoradiography, and the radioactivity was quantitated densitometrically with a scanner (CanonScan 600, Tokyo) and Photoshop 5.0 (Adobe, San Jose, CA).

Preparation of liver cytosol The liver was washed with ice-cold saline, weighed, minced, and homogenized in 3 volumes of homogenization buffer [35 m*M* potassium phosphate buffer (pH 7.4) containing 0.25 *M* sucrose, 1 m*M* EDTA, 1 m*M* mercaptoethanol and protease inhibitor (Complete, Boehringer Mannheim GmbH, Mannheim, Germany)]. Each homogenate was centrifuged at 600*g* for 10 min at 4°C, and the supernatant was centrifuged at 8000*g* for 10 min at 4°C. This supernatant was centrifuged at 105 000*g* for 1 h at 4°C, and the cytosol fraction (supernatant) was collected as the enzyme source. The protein concentration was determined with BCA Protein Assay Reagent (PIERCE, Rockford, IL).

Western blot analysis Cytosol samples (75 μg each) were mixed with sample buffer (pH 6.8, 80 m*M* Tris-HCl, 0.1 *M* dithiothreitol, 15% glycerol, 2% SDS, and 0.6% bromophenol/ethanol) in a final volume of 10 μl, and heated for 5 min at 95°C. Proteins were separated on 7.5% SDS-polyacrylamide gel and transferred onto an Immobilon polyvinylidene difluoride transfer membrane (Millipore, Bedford, MA). The blot was blocked by 5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% Tween-20 overnight at 4°C. The membrane was incubated with rabbit anti-DPD polyclonal antibody (1:1000 dilution) in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature, and washed four times with PBST. Next, the DPD bands on the membrane were visualized with biotinylated anti-rabbit IgG (1:400 dilution; Vector Laboratories, Peterborough, UK) followed by an avidin/biotin-horseradish peroxidase ABC kit (Vector Laboratories, Burlingame, CA) and POD immunostaining kit (Wako Pure Chemical Co., Osaka). The DPD proteins on the membrane were quantified densitometrically with a scanner and Photoshop 5.0.

Immunohistochemical staining for DPD The paraffin sections of liver were dried, deparaffinized, and incubated in 3% hydrogen peroxide and methanol to inhibit endogenous peroxidase activity. Nonspecific binding sites were blocked with PBS, 3% bovine serum albumin (BSA), and 10% fetal bovine serum (FBS) for 1.5 h. The sections were incubated with rabbit anti-DPD polyclonal antibody diluted with blocking buffer (1:250 dilution) for 1 h, and after having been washed with PBS, they were treated with biotinylated secondary goat anti-rabbit IgG (1:200 dilution; Vector Laboratories) for 30 min. The sections were then incubated with the ABC kit for 30 min, and DPD was visualized with 3,3-diaminobenzidine (0.05%) as the substrate. The sections were counterstained with veronal acetate-buffered 1% methyl green solution (pH

4.0). Liver tissue staining was graded as 1+ (weak), 2+ (moderate), or 3+ (strong). Negative control slides incubated without primary antibodies did not show any specific signal.

Determination of DPD activity in the liver cytosol DPD activity was estimated on the basis of the rate of production of 5,6-dihydrothymine from thymine in liver cytosol. Linear relationships between the rates of formation of 5,6-dihydrothymine from thymine and both the concentration of cytosolic protein (equivalent to 0–75 μg protein) and incubation time (0–12 min) were observed in our preliminary experiments. Based on these results, the cytosol incubation mixture, which contained 0.4 ml of 0.5 *M* potassium phosphate buffer (pH 7.4), 0.2 ml of 0.5 m*M* EDTA disodium, 0.2 ml of cytosol (equivalent to 50 μg of protein), and 100 μ*M* thymine was preincubated at 37°C for 5 min. The enzyme reaction was initiated by adding 0.2 ml of an α-nicotinamide adenine dinucleotide phosphate (NADP) generating system containing 8 m*M* NADP, 80 m*M* glucose 6-phosphate, 60 m*M* magnesium chloride, and 12.5 U/ml glucose 6-phosphate dehydrogenase. After an incubation of the mixture at 37°C for 10 min, the enzyme reaction was terminated by adding 6 ml of ethyl acetate. To this reaction solution, 1.0 ml of 0.1 *M* ammonium acetate buffer (pH 3.0) and 0.1 ml of 5-bromouracil (1.0 μg/ml) as an internal standard were added, and it was shaken for 10 min at 200 cycle/min. After centrifugation at 1000*g* for 10 min, 4 ml of the organic layer was evaporated at 40°C. The residue was dissolved in 100 μl of the HPLC mobile phase and a 50 μl aliquot of this solution was injected into the PU-980 HPLC system (JASCO, Tokyo). An L-column ODS (4.6 ø×250 mm, Chemicals Evaluation and Research Institute, Tokyo) with a guard filter (Waters Associates, Milford, MA) was used to determine 5,6-dihydrothymine in the cytosolic samples. The mobile phase was 0.05 *M* sodium phosphate buffer (pH 3.0). The flow rate was 1.0 ml/min, and the eluate was monitored at 205 nm.

Statistical analysis DPD protein levels and activities at the different doses of CPT-11 were statistically analyzed for homogeneity by using Bartlett's test. Multiple comparisons of their mean values at the different doses of CPT-11 were performed by ANOVA followed by Scheffe's multiple comparison procedure. All statistical analyses were performed with the SPSS 7.5J program (SPSS Inc., Chicago, IL). A P value of <0.05 was considered statistically significant throughout the study.

RESULTS

Effect of CPT-11 administration time on the *DPD* mRNA level in mouse tissue To determine whether CPT-11 treatment affects the circadian rhythm of *DPD* in mice, the *DPD* mRNA level in liver and brain was examined by northern blot analysis 6 and 18 h after injecting mice with CPT-11 at 08:00 or 20:00 (Fig. 1).

In the control mice, the *DPD* mRNA level in the liver (relative ratio of *DPD*/*GAPDH*; 0.65) was markedly higher at 02:00 than that at 14:00 (*DPD*/*GAPDH*; 0.41). By contrast, no expression of *DPD* was detected in the brain at 6 or 18 h after administration of CPT-11 at 08:00 or 20:00. When CPT-11 was administered at 08:00, the *DPD* mRNA level (*DPD*/*GAPDH*) in the liver was 0.50 and 0.27 at 6 and 18 h later (at 14:00 and 02:00), respectively. These variations in *DPD* levels at 14:00 and 02:00 were similar to those in the controls. When CPT-11 was administered at 20:00, on the other hand, the *DPD* mRNA level at 14:00 (*DPD*/*GAPDH*; 0.35) was almost the same as at 02:00 (*DPD*/*GAPDH*; 0.29), suggesting that CPT-11 affects the *DPD* mRNA level when administered at 20:00.

Dose-dependent effect of CPT-11 on hepatic level of *DPD* mRNA One of four doses of CPT-11 (10, 30, 60, and 100 mg/kg) was administered to mice in different groups at 20:00, and the *DPD* mRNA level was measured by northern blot analysis (Fig. 2). At 6 h (at 02:00) after administration, no difference in *DPD* mRNA expression was observed between the four different doses of CPT-11. *DPD* mRNA levels, on the other hand, decreased dose-dependently at 18 h (at 14:00) after administration of CPT-11.

The diurnal variation of *DPD* was determined to clarify whether CPT-11 decreased the expression of *DPD* mRNA or altered the phase and period of the circadian rhythm of *DPD*. The circadian rhythm of *DPD* in the control mice exhibited the 24-h cycle determined from the oscillating pattern in Fig. 3, whereas at the 100 mg/kg dose, it extended dose-dependently to a 48-h cycle (Fig. 3).

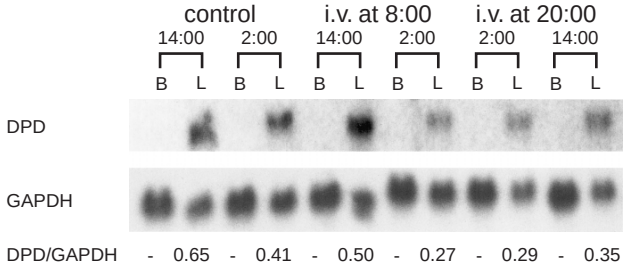

Fig. 1. Influence of administration time of CPT-11 on the *DPD* mRNA level. Total RNA was extracted from mouse brain and liver tissue at 6 and 18 h after intravenous injection of CPT-11 (30 mg/kg) at 08:00 or 20:00 in mice. Total RNA (20 μg/lane) was subjected to northern blot analysis with a ^{32}P-labeled *DPD* cDNA probe (first panel, which resulted in a 4.7 kb transcript). The northern blots were then stripped and rehybridized with a *GAPDH* cDNA probe (second panel, which yielded a 0.9 kb transcript). The *DPD*/*GAPDH* ratio represents the ratio of the integrated optical densities. B, brain; L, liver.

These results suggest that CPT-11 dose-dependently shifts the phase of the circadian rhythm of *DPD*.

Effect of CPT-11 on the DPD protein level in liver cytosol The liver DPD protein level in the CPT-11-treated mice was examined by western blotting. At 6 and 18 h after administration of CPT-11 (at 02:00 and 14:00), no differences in DPD protein level were observed between the three doses of CPT-11 (data not shown). In view of the lag-time in the translation process, the protein levels at 9

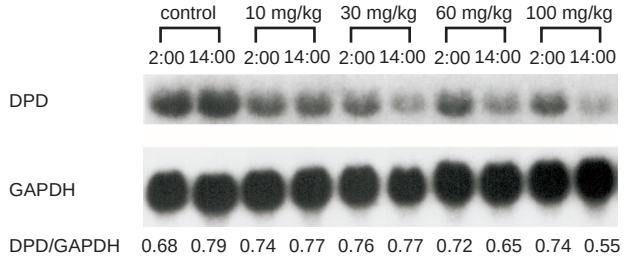

Fig. 2. Dose-dependent effects of CPT-11 on hepatic levels of *DPD* mRNA after intravenous injection of CPT-11 at 20:00. Mice were intravenously injected with CPT-11 (10, 30, 60, and 100 mg/kg) or saline (control) at 20:00, and the liver was removed at 6 or 18 h later (02:00, 14:00). Total RNA (20 μg/lane) extracted from the liver was subjected to northern blot analysis with a ^{32}P-labeled *DPD* cDNA probe (first panel) and a *GAPDH* cDNA probe as a loading and transfer control (second panel). The *DPD*/*GAPDH* ratio represents the ratio of the integrated optical densities.

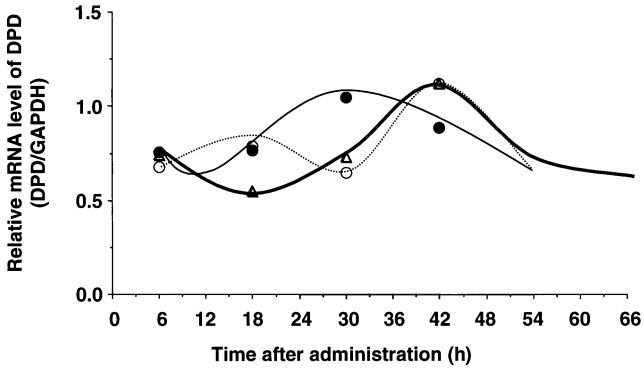

Fig. 3. Dose-dependent effects of CPT-11 on the circadian rhythm of hepatic levels of *DPD* mRNA after intravenous injection of CPT-11 at 20:00. Total RNA (20 μg/lane) extracted from liver at various times after administration of CPT-11 (30 mg/kg, ●; 60 mg/kg, △) or saline (control, ○) at 20:00 was subjected to northern blot analysis as described in "Materials and Methods." The relative mRNA level of *DPD* is expressed as the *DPD*/*GAPDH* ratio of integrated optical densities.

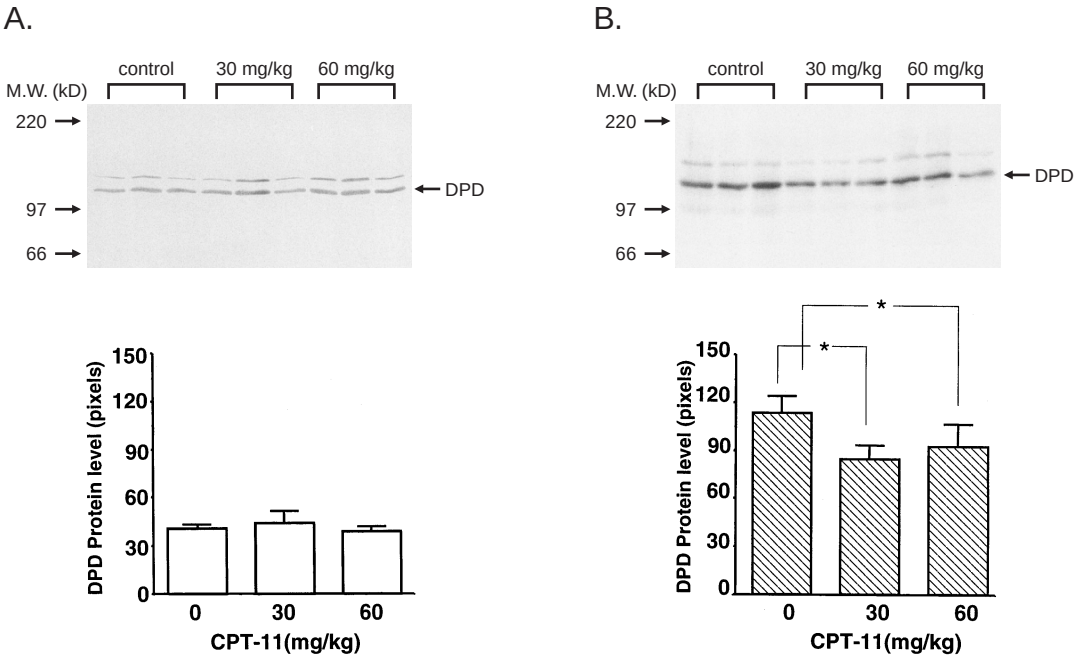

Fig. 4. DPD protein level in liver cytosol after intravenous injection of CPT-11 at 20:00. Cytosol fractions were obtained from the liver as described in "Materials and Methods." The proteins (75 μg protein/lane) at 9 h (at 05:00; A) or 21 h later (at 17:00; B) were stained with anti-DPD polyclonal antibody by immunoblotting. The arrow indicates 116 kDa. The band corresponding to DPD protein was measured densitometrically after immunoblotting. Each value represents the mean±SD of triplicate determinations. * $P<0.05$.

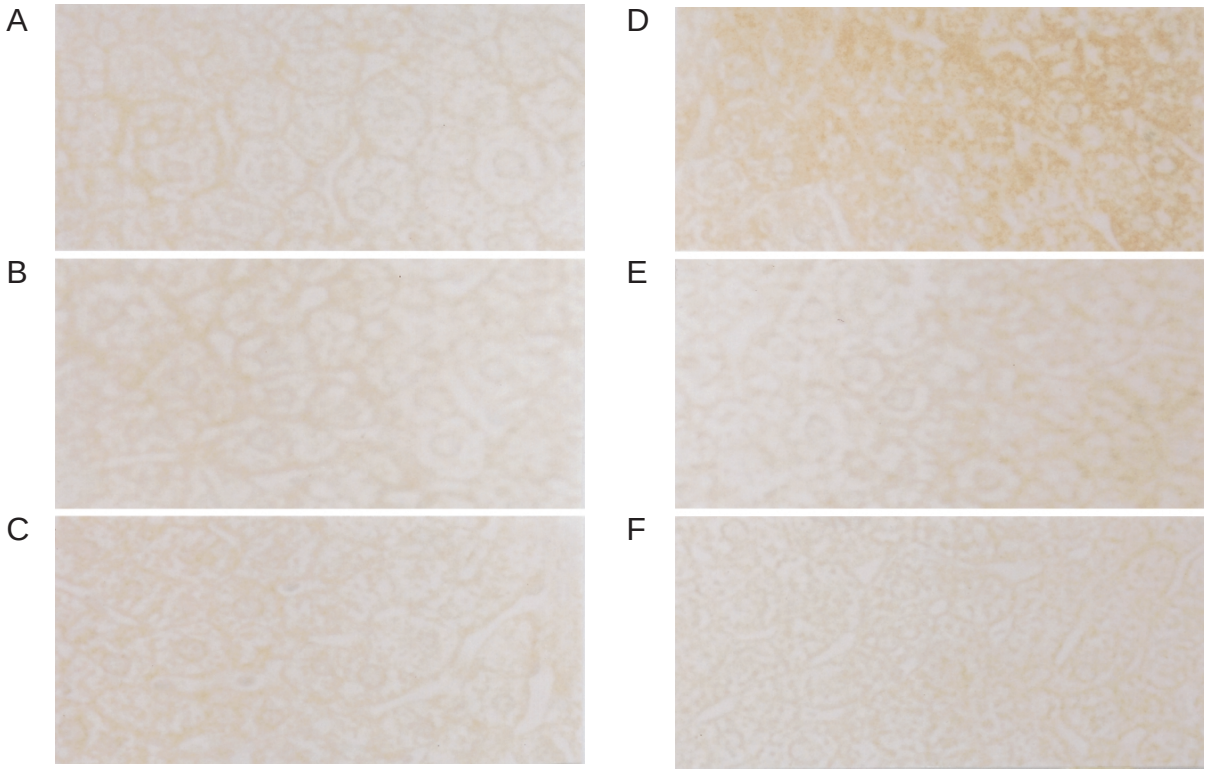

Fig. 5. Immunohistochemistry of DPD expression in mouse liver. CPT-11 was intravenously injected (0, 30, and 60 mg/kg) at 20:00, and liver tissue was removed 9 h later (at 05:00; A, B, C) and 21 h later (at 17:00; D, E, F). Paraffin sections of liver tissue were stained with anti-DPD antibody as described in "Materials and Methods." Original magnification ×40.

and 21 h after administration of CPT-11 (at 5:00 and 17:00) might reflect the mRNA levels at 6 and 18 h (at 02:00 and 14:00), respectively, after administration. The protein level in the liver sample at 05:00 was unaffected by any dose of CPT-11 (Fig. 4A). At 17:00, the DPD protein levels were significantly lower in the CPT-11 treated groups than in the control ($P<0.05$, Fig. 4B).

Immunohistochemistry of DPD expression in mouse liver To confirm the difference in DPD protein level in the liver between the CPT-11-treated and untreated mice, liver tissue obtained at 6 and 18 h after administration of CPT-11 (at 02:00 and 14:00) was immunohistochemically stained with anti-DPD antibody. 3,3-Diaminobenzidine staining was observed in the cytoplasm of the hepatocytes. No difference in staining was observed in the liver samples obtained at 05:00 after administration of CPT-11 (grade 1, Fig. 5, A, B and C), whereas the cytoplasm of CPT-11-untreated liver cells obtained at 17:00 was stained more diffusely and more deeply for DPD than at 05:00 (grade 3, Fig. 5D). Intensity was weak in the samples obtained from CPT-11-treated mice (60 mg/kg) (grade 1, Fig. 5F). These results were consistent with the results of western blotting.

Effect of CPT-11 on DPD activity in liver cytosol To determine whether CPT-11 affects DPD activity, we measured DPD activity in the cytosol fraction of hepatocytes at 9 and 21 h (at 05:00 and 17:00) after intravenous injection of CPT-11 at 20:00. The DPD activity of the samples obtained at 05:00 was similar at all three different doses of CPT-11 (181.4±4.8, 163.1±18.2, and 180.2±30.4 pmol/min/mg protein at 0, 30, and 60 mg/kg, respectively, Fig. 6A). On the other hand, at 17:00, DPD activity was decreased in the hepatocyte cytosol of mice treated with CPT-11, and the activity of the samples treated with 30 mg/kg (176.6±19.6 pmol/min/mg protein) and 60 mg/kg (172.3±41.4 pmol/min/mg protein) was significantly lower than that of the control (237.4±34.4 pmol/min/mg protein) ($P<0.05$, Fig. 6B). These results were consistent with the results of western blotting.

DISCUSSION

Many circadian rhythms in physiology and behavior have been described in a wide range of species, from bacteria to humans.[18] Because there are significant circadian rhythms in enzyme activity,[19, 20] renal function,[21, 22] blood

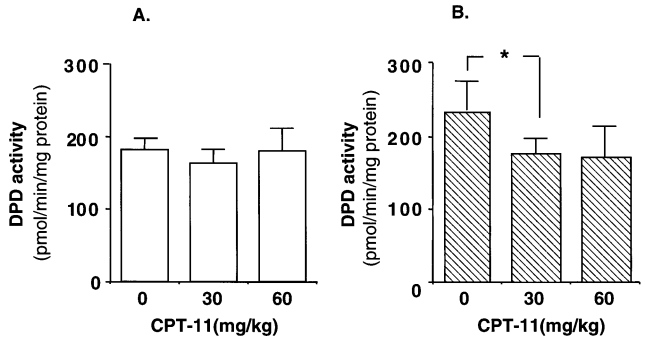

Fig. 6. DPD enzyme activity in liver cytosol after intravenous injection of CPT-11 at 20:00. Cytosol fractions were obtained from the liver at 9 h (at 05:00; A) and 21 h (at 17:00; B) after intravenous injection of CPT-11 (30 and 60 mg/kg) or saline (control) at 20:00. DPD enzyme activity in liver cytosol was determined by HPLC by measuring the rate of production of dihydrothymine from thymine (100 μM). Each value represents the mean±SD of triplicate determinations. $*$ $P<0.05$.

flow,[23] and protein binding,[24] the pharmacokinetics and pharmacodynamics of many drugs are affected by circadian variations.[25, 26] DPD, a major catabolic enzyme of 5-FU, has been reported to exhibit a circadian rhythm, and the variations have been correlated with the efficacy and toxicity of 5-FU in experimental animals[6, 7] and cancer patients.[8] Zhang *et al.* have reported that DPD activity in rat liver exhibits a circadian variation, with peak DPD activity at 01:00–02:00 and trough activity at 13:00–14:00, and an approximately 2-fold difference in activity between the maximum and minimum points.[9] In our study, the *DPD* mRNA level in mouse liver at 02:00 was markedly higher than that at 14:00 (Fig. 1), and this finding was consistent with the above-described results. Dose-dependent changes in the period of the circadian rhythm of *DPD* mRNA levels in mice were observed in response to CPT-11 treatment at 20:00 (Fig. 2). We repeated the northern blotting experiment more than twice, and the same effect of CPT-11 on the circadian rhythm was confirmed by real-time RT-PCR (data not shown). These results suggest that CPT-11 regulates the circadian rhythm of *DPD* at the transcription level in a manner similar to that of DRB and camptothecin.[16] After the present study, the molecular mechanism of action of CPT-11 at the transcription level of *DPD* remains unclear. Recently, a 1.85 kb 5′ flanking region upstream of the translation start site in exon 1 of the human *DPD* gene (*DPYD* gene, GenBank Accession No. AF214571) was cloned.[27] *Cis*-acting elements, including TATA and inverted CCAAT motifs, were identified within this region. This flanking region was able to up-regulate transcription in cultured human pancreatic carcinoma Hs766T cells. The mRNA level of *DPD* has been found to be higher in the human liver than in the colon,[28] and DPD protein level and activity are higher in human liver and PBMCs than in other tissues.[29] Putative binding sites for liver-enriched transcription factors have been identified in the 1.85 kb 5′ flanking region of the human *DPD* gene,[27] and tissue-specific DPD expression may therefore be controlled by liver-enriched transcription factors, such as NHF-4 and C/EBP. For all of these reasons, we hypothesize that CPT-11 affects *cis*-acting elements in the 5′ flanking region of the *DPD* gene or its *trans*-acting factors. CPT-11 may also affect the stability of *DPD* mRNA.

CPT-11 inhibits gene transcription via inhibition of topo I,[17] and circadian rhythms of topo I activity and DNA synthesis have been found in mouse bone marrow cells.[30] Intraperitoneal injection of CPT-11 when topo I activity and DNA synthesis in mice bone marrow cells begin to increase (in the middle of the night and in the early morning) resulted in higher toxicity in mice, and higher plasma concentrations of CPT-11 and its active metabolite SN-38 have been observed in mice injected with CPT-11 at 05:00 than at 17:00.[30] Thus, it is likely that the pharmacokinetics and pharmacodynamics of CPT-11 itself are affected by the circadian rhythm.

Uetake *et al.* have reported a significant correlation between DPD activity and mRNA expression in the tumor tissue of patients with colorectal cancer, but not in normal mucosa.[31] Takenoue *et al.*, on the other hand, have reported that the immunohistochemical score was correlated with DPD protein expression and activity in human mononuclear cells, colorectal cancer cell lines and colorectal cancer specimens, but not with mRNA expression.[32] *DPD* mRNA levels have been reported to be significantly lower in colorectal tumor tissue than in normal tissue, but no difference was observed at the protein or enzyme activity level, suggesting transcriptional and translational regulation of DPD activity.[33] In our study, the activity of the samples obtained at 6 and 18 h after CPT-11 administration (at 02:00 and 14:00) did not reflect the *DPD* mRNA levels at the same times (data not shown). The protein levels and the immunohistochemistry scores at 05:00 and 17:00 appeared to reflect the mRNA levels at 02:00 and 14:00, respectively (Figs. 4–6), suggesting that a lag-time exists in the translation process.

We demonstrated that CPT-11 affects the circadian rhythm of DPD activity, suggesting that the anticancer effect of 5-FU may be affected by circadian modification of DPD by CPT-11. The chronopharmacology of CPT-11 and 5-FU will be helpful in reducing the toxic effects of 5-FU and CPT-11 and in increasing their therapeutic potential. There have been no consistent data on the optimal sequence of CPT-11 and 5-FU in previous studies. It has been reported that CPT-11 before 5-FU was the best sequence and yielded a significant increase in therapeutic

index over the other schedules tested in rats: CPT-11 simultaneously with 5-FU, and 5-FU 24 h before CPT-11.[34] On the other hand, discrepant results have been reported between *in vivo*[35] and *in vitro*.[36] CPT-11 followed by 5-FU yielded the highest toxicity in mice of the three schedules mentioned above, and higher antitumoral activity was noted when CPT-11 and 5-FU were administered sequentially.[35] Based on our findings, the sequence of 5-FU before CPT-11 might be the best schedule, because the pharmacokinetics and pharmacodynamics of 5-FU would be less affected by CPT-11-induced modification of the circadian rhythm of DPD. We plan to investigate the efficacy of a circadian-based combination schedule of CPT-11 and 5-FU in a colorectal tumor model.

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