Relationship between Circadian-dependent Toxicity of 5-Fluorodeoxyuridine and Circadian Rhythms of Pyrimidine Enzymes: Possible Relevance to Fluoropyrimidine Chemotherapy

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ABSTRACT

Previous studies in experimental animals and patients have suggested a circadian variation in host toxicity following administration of 5-fluorodeoxyuridine (FdUrd) although the biochemical mechanisms are not fully understood. Thymidine kinase (TK; EC 2.7.1.21), the initial enzyme in the thymidine-phosphorylation pathway, is the first enzyme in the anabolism of FdUrd. Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2), is the rate-limiting enzyme in the pyrimidine catabolic pathway and has been shown to be the key enzyme in FdUrd catabolism. The present study examined the relationship between the suggested circadian variation in FdUrd toxicity and potential circadian variations in the activity of these enzymes. Initial studies in Sprague-Dawley rats confirmed that the time of FdUrd administration affected death rate and other drug-related toxicities including loss of body weight, diarrhea, and bone marrow suppression, with the least toxicity and highest survival rate being observed in rats receiving FdUrd at 12:00 noon and 4:00 p.m. and the greatest toxicity and lowest survival rate at 12:00 midnight and 4:00 a.m. Statistical analysis revealed a circadian pattern in FdUrd toxicity (Cosinor analysis, \( P < 0.001 \)). In subsequent studies with the same species, we simultaneously measured TK and DPD activities in several tissues at various times over 24 h. Under standardized light conditions (lights on, 6:00 a.m. to 6:00 p.m.; lights off, 6:00 p.m. to 6:00 a.m.), with sampling at 4-h intervals (4:00 and 8:00 a.m.; 12:00 noon; 4:00 and 8:00 p.m., and 12:00 midnight), a circadian variation in TK activity was observed (\( P < 0.001 \)), Cosinor analysis) in bone marrow, intestinal mucosa, liver, and spleen. In the same group of animals, a circadian pattern of DPD activity in liver and bone marrow was also observed (Cosinor analysis, \( P < 0.001 \)), that was inverse compared to the circadian variation in TK activity (Pearson correlation analysis, \( P < 0.05 \)). Further statistical analysis indicated that the observed circadian variation in FdUrd toxicity was correlated with the circadian variation of TK activity and inversely correlated with DPD activity (Pearson correlation analysis, \( P < 0.05 \)). Based on the above data, we conclude that the circadian pattern of TK and DPD activity may explain the observed circadian variation in toxicity as the time of FdUrd administration is varied. These results may be useful in the design of improved chemotherapeutic regimens using time-modified administration of FdUrd.

INTRODUCTION

FdUrd\(^1\) is a pyrimidine nucleoside analogue used primarily in the management of colorectal cancer metastatic to the liver (1–3) and has recently been shown to be potentially useful in the treatment of renal cell carcinoma (4). Because FdUrd has a very short half-life, continuous infusion may markedly increase the exposure of tumor cells to the drug and hence improve the tumor response (2). Unfortunately, dose-limiting toxicities occur frequently in patients receiving FdUrd by “flat” continuous infusion (i.e., no dose change during infusion over time), often requiring significant reduction of the administered dose or cessation of chemotherapy (5, 6). Even when FdUrd is given at low doses, severe enteritis often occurs (7). Recently, several studies in rodents (8, 9) and cancer patients (4, 10–12) suggested that the time of FdUrd administration may affect the extent of FdUrd toxicity in normal tissues. The biochemical basis for the suggested circadian-dependent host toxicity is not fully understood.

Studies have shown that the cytotoxic activity of FdUrd is mediated by its anabolism within the cell (2, 3). It is essential that FdUrd be phosphorylated to FdUMP for its anticancer effects or cytotoxicity. TK (EC 2.7.1.21), the first enzyme in the thymidine-phosphorylation pathway, is also the first enzyme in FdUrd anabolism. The extent of anabolism is determined by the activity of anabolic enzymes. The availability of FdUrd for anabolism is further regulated by its catabolism since more than 80% of administered fluoropyrimidines drugs are metabolized by the catabolic pathway (2, 13). Studies from our laboratory have demonstrated that the activity of DPD (EC 1.3.1.2), the rate-limiting enzyme in fluoropyrimidine catabolism, has a circadian variation in experimental animals (14, 15) and cancer patients (16). Studies in cancer patients receiving FURA have shown an inverse relationship between the activity of DPD in peripheral blood mononuclear cells and plasma concentration of FURA (16), demonstrating the importance of DPD in regulating FURA metabolism. Our laboratory has shown that DPD is also the key enzyme in FdUrd catabolism (17, 18), suggesting that a circadian rhythm of DPD activity may have a role in regulating the availability of FdUrd.

Given the importance of anabolic and catabolic enzymes in determining FdUrd cytotoxic activity and therapeutic effects, the present study examined: (a) if the major anabolic enzyme (TK) follows a circadian pattern in host tissues where FdUrd toxicity primarily occurs; (b) if there is a relationship between the patterns of activity of anabolic and catabolic enzymes; and (c) if the suggested circadian-dependent FdUrd host toxicity is related to the potential circadian variations of these anabolic and catabolic enzymes.

MATERIALS AND METHODS

Chemicals and Radiochemicals

FdUrd, FURA, thymidine, ATP, MgCl\(_2\), NaF, dithiothreitol, phosphocreatine, creatine kinase, and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). \(^{2}\)H\(_2\)FUra (56 mCi/mmol) was obtained from Amersham Life Science (Brea, CA). \(^{3}\)H\(_2\)FUra (25 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The purity of FdUrd and unlabeled and labeled thymidine, as well as that of FURA, was determined by HPLC (14, 19–21) to be greater than 99%. Solvents were HPLC grade and all other chemicals used in the present study were the highest grade available.

Animals and Treatment Protocol

Study 1: Circadian-dependent FdUrd Toxicity

Male Sprague-Dawley rats weighing 40–50 g (Harlan Laboratories, Indianapolis, IN) were obtained and housed one to a cage with free access to food and water in a light- and dark-controlled room (lights on from 6:00 a.m. to 6:00 p.m. to 6:00 a.m.)
p.m. and lights-off from 6:00 p.m. to 6:00 a.m. (local time) with environmental temperature maintained at 25 ± 1°C and relative humidity at approximately 50%. All animals were given 4 weeks to adjust to their light/dark cycle. For the actual experiment, animals were randomly divided into 7 groups (10 rats in each group). Six groups received an equal dose of FdUrd (i.p. bolus, 1200 mg/kg body weight) at various times, i.e., 4:00 a.m., 8:00 a.m., 12:00 noon, 4:00 p.m., 8:00 p.m., and 12:00 midnight; one group as control received sterilized water. Each animal was weighed immediately before the injection of FdUrd to calculate the dose to be administered. The following toxic indices were used in the present study.

**Body Weight and General Condition.** Each animal was weighed every 3 days before injection of drug (or water as control) and every day after injection. Each animal was examined for toxicities including diarrhea and death by observing at 6-h intervals around the clock for 30 days. At the end of the experiments, the remaining rats were killed by pentobarbital sodium overdose in accordance with an institutional approval protocol.

**Hematological Parameters.** Hematological variables were measured 3 days before injection and 5, 12, and 19 days after injection. Measurements included WBC count, RBC count, and concentrations of hemoglobin and hematocrit (packed cell volume).

**Study 2: Determination of TK and DPD Activity**

Male Sprague-Dawley rats weighing 170–190 g (Harlan Laboratories, Indianapolis, IN) were obtained and housed 3 to a cage under the above light conditions for 4 weeks before study. To determine the variation of TK and DPD activity over 24 h, animals were sacrificed at various times of the day, i.e., 4:00 a.m., 8:00 a.m., 12:00 noon, 4:00 p.m., 8:00 p.m., and 12:00 midnight, by cervical dislocation in accordance with an institutional approval protocol.

**Preparation of Rat Spleen, Liver, and Kidney Homogenate.** These tissues were excised and washed with ice-cold physiological saline (0.9% NaCl). The cytosolic fraction was prepared by the method previously described (19, 22). In brief, each of the removed tissues was weighed, minced, and homogenized in 6 volumes of the homogenizing buffer (buffer A), i.e., 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM dithiothreitol, 50 mM thiourea, and 10% glycerol. The resulting homogenate was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant was removed and used in the subsequent enzyme assays.

**Intestinal Mucosa.** Following removal, the small intestinal contents were emptied and the intestine was washed with sterile ice-cold physiological saline. The small intestine was then opened and the intestinal mucosa was scraped off with a stainless-steel spatula. The removed intestinal mucosa was weighed and homogenized in 6 volumes of buffer A. After the homogenate was centrifuged at 100,000 × g for 60 min at 4°C, the supernatant was removed and used in the subsequent enzyme assays.

**Bone Marrow.** Bone marrow cells were harvested by flushing the femurs with sterile ice-cold physiological saline. In brief, after removing the distal and proximal ends of femur, each end of the bone was punctured with an 18-gauge needle fitted with a 10-ml syringe containing 5 ml of sterile ice-cold physiological saline. The bone was then held with a forceps over a test tube and the needle was inserted into the hole in the proximal end. The sterile ice-cold physiological saline (5 ml) was flushed through the bone into the tube by applying gentle, steady pressure to the plunger. The syringe was then filled with 5 ml of air which was gently forced through the bone to remove the remaining saline and bone marrow in the shaft. The second flush was conducted from the distal end to the proximal end with saline (5 ml) followed by air (5 ml). The resulting suspension of bone marrow cells was centrifuged at 800 × g for 5 min at 4°C. The resulting cell pellet was resuspended in 1 ml of the buffer A. The cells were lysed by sonication 5 times for periods of 10 s. Following centrifugation at 20,000 × g for 30 min, the supernatant was removed and diluted for use in the subsequent enzyme assays.

**Enzyme Assay: TK Activity.** Cytosolic TK activity was assayed using the method described previously by Cheng (22). In brief, the reaction mixture (2 ml, final volume) contained 1.5 ml of the stock assay solution (20 μM [2-14C]thymidine-2.5 mM ATP-2.5 mM MgCl2-12.5 mM NaF-2 mM dithiothreitol-4.5 mM phosphocreatine-6 units/ml creatine kinase-1% bovine serum albumin in 0.2 M Tris-HCl, pH 7.5) and 0.5 ml of enzyme solution. This mixture was incubated at 37°C. The enzyme reactions were stopped at the designated times by spotting 50 μl of the reaction mixture on a Whatman DE-81 paper disc; the disc was then dropped immediately into 95% alcohol (10 ml/disc) and washed twice with 95% alcohol for 5 min. The disc was then dried and placed in a 7.5-ml vial containing 6.5 ml of scintillation liquid (Econo-Safe; RPI, Mt. Prospect, IL). The radioactivity in each vial was quantitated by liquid scintillation spectrometry (LS 5801; Beckman, Irvine, CA). The blank value was obtained by conducting the assay with denatured enzyme solution which had been heated at 100°C for 3 min. This reaction was linear for at least 4 h, providing at least 10% thymidine is still present in the reaction mixture. TK activity was expressed as the amount of product (TMP) formed/h/mg protein.

**Enzyme Assay: DPD Activity.** Enzyme activity was determined using an HPLC method we have previously described (14, 20). The removed liver tissue was weighed, minced, and homogenized in 4 volumes of the homogenizing buffer (buffer B) containing 35 mM potassium phosphate (pH 7.4), 2.5 mM MgCl2, and 10 mM 2-mercaptoethanol, in the presence of 0.25 mM sucrose, 1 mM benzamidine, 1 mM aminoethylisothiourea bromide, and 5 mM EDTA. The resulting homogenate was centrifuged at 100,000 × g for 60 min at 4°C. The cytotoxic fraction was removed and used in the subsequent assays. The bone marrow sample for DPD assay was prepared using the method described above. For the DPD assay, the reaction mixture (2 ml) contained buffer B (pH 7.4), 200 μM NADPH, 20 μM [14H]FdUra, and 100 μl of the above cytosolic fraction. The mixture was incubated at 37°C, and 350 μl of the reaction sample were taken out at various times and added into the same volume of ice-cold ethanol to stop the reaction. The sample was then kept in a freezer (−20°C) for 30 min and then filtered through a 0.2-μm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis. Separation of FdUra and its catabolites was performed by reversed-phase HPLC using a Hewlett-Packard 1050 HPLC system equipped with a filter spectrophotometric detector and chromatographic terminal (HP 3396 Series II Integrator). Two Hypersil 5-μm columns (Jones Chromatography, Littleton, CO) were used in tandem as the stationary phase. The columns were eluted at a flow rate of 1.0 ml/min with the mobile phase containing 1.5 mM potassium phosphate, pH 8.0, with 5 mM tetrabutylammonium hydrogen sulfate. Fractions (1-ml) were collected into 7-ml scintillation vials, using a Redifrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ) and were mixed with 5.5-ml scintillation solvent. The radioactivity in each fraction was quantitated by liquid scintillation spectrometry described above. Under these conditions, typical retention times for dihydrofluorouracil and FdUra were 9 and 21 min, respectively. The DPD activity was expressed as the amount of product (FdUra catabolites) formed/min/mg protein.

**Protein Determination**

The amount of protein in the sample was determined by the method of Lowry et al. (23).

**Statistical Analysis**

In the toxicity study, 10 animals were used in each group. The differences among the treatment groups and the control were analyzed by analysis of variance or χ2 test as appropriate. The effect of time as a factor on FdUrd toxicity was analyzed by the Cosinor method (24) described below. In the study of TK and DPD activity, three animals were utilized for each time point. Data from each animal were the means of 5 determinations. The time series data were analyzed by the Cosinor method (24). A cosine wave was fitted to the data by the method of least squares. Four parameters were calculated in the analysis, including the mesor (the rhythm-adjusted mean), amplitude (maximum or minimum value from the adjusted mean), acrophase (time of maximum or minimum value), and period (the length of one complete cycle). In determining the correlation between TK and DPD activity and between toxic parameters and enzyme activities, Pearson correlation coefficients were calculated; the significance level was set at P < 0.05.

**RESULTS**

Circadian-dependent Toxicity of FdUrd

Lethal toxicity secondary to FdUrd differed significantly among the Sprague-Dawley rats receiving FdUrd by i.p. bolus at the dose of 1200 mg/kg body weight at various times, with the highest survival rate (100%) observed in rats receiving the drug at 12:00 noon (6 HALO) and the lowest survival rate (60%) at 4:00 a.m. (22 HALO) (χ2 test,
Further statistical analysis revealed that the survival rate follows a circadian pattern (Cosinor analysis, \(P < 0.001\); Fig. 1). Autopsy showed that the most obvious causes of death were secondary to toxic effects on bone marrow and intestine.

Decreased WBC, RBC, hemoglobin, and packed cell volume were observed in rats treated with FdUrd. Statistical analysis revealed that FdUrd-related hematomal toxicity differed significantly among treatment groups, depending on the time of FdUrd administration (Cosinor analysis, \(P < 0.001\); data not shown). Loss of body weight was observed in all rats receiving FdUrd compared to control with the maximum loss of body weight observed in rats receiving FdUrd at 4:00 a.m. (22 HALO) (analysis of variance, \(P < 0.05\)). A circadian variation in the loss of body weight was shown by Cosinor analysis (\(P < 0.001\), data not shown). Diarrhea was one of the major toxic signs observed in the rats receiving FdUrd. All rats treated with FdUrd had decreased food and water consumption in the first week after administration of FdUrd. Toxic effects in the eyes were also observed in rats treated with FdUrd.

**Circadian Rhythms of TK and DPD Activity**

Since we confirmed the suggested circadian variation in FdUrd host toxicity in Sprague-Dawley rats, we further utilized the same species (housed under the same light conditions) to simultaneously measure the activity of two major enzymes (TK and DPD) in FdUrd metabolism in order to investigate the biochemical basis for circadian-dependent FdUrd toxicity.

**Circadian Pattern of TK Activity.** Statistical analysis indicated that there was a significant circadian variation of TK activity in bone marrow, intestinal mucosa, spleen, and liver (Cosinor analysis, \(P < 0.0001\); Fig. 2, Table 1). Under standard light conditions, i.e., lights on from 6:00 a.m. to 6:00 p.m. and lights off from 6:00 p.m. to 6:00 a.m., with samples taken every 4 h, peak TK activity in bone marrow and intestinal mucosa was at approximately 4:00 a.m. (22 HALO) and trough activity at approximately 4:00 p.m. (10 HALO), demonstrating an approximately 2–10-fold difference in enzyme activity between maximum and minimum points, respectively (Fig. 2, A and B; Table 2).

A circadian pattern of liver cytoplasmic TK activity was also observed (Cosinor analysis, \(P < 0.0001\); Fig. 2C; Table 1), with peak TK activity at approximately 2:00 a.m. (8 HALO) and trough activity at approximately 2:00 p.m. (8 HALO), demonstrating an approximately 2-fold difference in enzyme activity between maximum and minimum points (Table 2). The TK activity in kidney was very low with no significant difference among rats examined at various times (data not shown).

A circadian pattern of spleen cytoplasmic TK activity was also observed and consistent with an earlier study from our laboratory using the same species (19). In the present study, peak TK activity in
Correlation Between TK and DPD Activity. As illustrated in Table 4, correlation analysis demonstrated a similar circadian pattern of TK activity in bone marrow, intestinal mucosa, liver, and spleen (P < 0.05–0.001). There was also a correlation between DPD activities in the liver and bone marrow (P < 0.01). Further correlation analysis demonstrated an inverse correlation between DPD activity and TK activity in the above tissues (Table 4).

Circadian Pattern of DPD Activity. In the same group of animals, DPD activity in liver was shown to follow a circadian variation (Fig. 3A; Table 3), with peak DPD activity at approximately 1:00 p.m. (7 HALO) and trough activity at approximately 1:00 a.m. (19 HALO), demonstrating an approximately 2-fold difference in enzyme activity between maximum and minimum points (Table 3). The DPD activity in bone marrow was also shown to follow a similar circadian pattern as DPD activity in liver (Fig. 3B; Table 3).

Table 2 Values and times of maximum and minimum TK activities in the intestinal mucosa, bone marrow, and spleen of rats under standard light-dark conditions.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TKmax ± SE</th>
<th>TKmin ± SE</th>
<th>TKmax/TKmin</th>
<th>Tmax ± SE</th>
<th>Tmin ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal mucosa</td>
<td>0.411 ± 0.034</td>
<td>0.040 ± 0.034</td>
<td>10.27</td>
<td>3.82 ± 0.32</td>
<td>15.89 ± 0.32</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>23.40 ± 2.996</td>
<td>15.110 ± 2.996</td>
<td>1.55</td>
<td>3.73 ± 1.63</td>
<td>15.81 ± 1.63</td>
</tr>
<tr>
<td>Liver</td>
<td>0.732 ± 0.070</td>
<td>0.423 ± 0.070</td>
<td>1.73</td>
<td>1.67 ± 0.22</td>
<td>13.75 ± 0.22</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.889 ± 0.362</td>
<td>1.535 ± 0.362</td>
<td>2.53</td>
<td>2.07 ± 0.90</td>
<td>14.15 ± 0.90</td>
</tr>
</tbody>
</table>

Table 3 Rhythmometric summary of single Cosinor analysis of DPD activity (nmol/min/mg protein) in liver and bone marrow Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Liver</th>
<th>Bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesor ± SE</td>
<td>2.39 ± 0.134</td>
<td>0.409 ± 0.019</td>
</tr>
<tr>
<td>Amplitude ± SE</td>
<td>0.716 ± 0.134</td>
<td>0.121 ± 0.026</td>
</tr>
<tr>
<td>DPDmax ± SE</td>
<td>2.955 ± 0.190</td>
<td>0.530 ± 0.032</td>
</tr>
<tr>
<td>DPDmin ± SE</td>
<td>1.523 ± 0.190</td>
<td>0.288 ± 0.026</td>
</tr>
<tr>
<td>DPDmax/DPDmin</td>
<td>1.94</td>
<td>1.84</td>
</tr>
<tr>
<td>Tmax ± SE</td>
<td>13.08 ± 0.51</td>
<td>11.53 ± 0.10</td>
</tr>
<tr>
<td>Tmin ± SE</td>
<td>1.16 ± 0.51</td>
<td>23.6 ± 0.10</td>
</tr>
<tr>
<td>R²</td>
<td>0.655</td>
<td>0.576</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Closer examination of the above data shows a correlation between circadian variation of FdUrd toxicity and circadian variation of TK activity in several tissues including bone marrow and intestinal mucosa that are the major sites of FdUrd toxicity. As illustrated in Table 5, there was inverse correlation between TK activity in several tissues and the survival rate of rats treated with FdUrd; the higher the TK activity, the lower the survival rate (higher toxicity). There was also a correlation between DPD activity in the liver and the survival rate; the higher the DPD activity, the higher the survival rate (lower toxicity).

DISCUSSION

The purpose of the present study was to investigate the biochemical mechanisms responsible for the suggested circadian variation in FdUrd host toxicity, particularly the effects of FdUrd metabolizing enzymes. Initial study in male Sprague-Dawley rats demonstrated that FdUrd host toxicity, including death, bone marrow suppression, and gastrointestinal toxic effects, differs among rats treated with FdUrd, depending on the time of FdUrd administration. In subsequent studies with the same species, we demonstrated that the activity of TK, the first enzyme in FdUrd anabolism, follows a circadian pattern in several tissues including bone marrow and intestinal mucosa. More interestingly, in the same group of animals, the activity of DPD, the key enzyme in FdUrd catabolism was also shown to follow a circadian pattern which was inverse compared to the circadian pattern of TK activity. Finally, statistical analysis demonstrated a correlation between the observed circadian-dependent FdUrd toxicity and the circadian variations in TK and DPD activity.

It has been shown that the time of drug administration may markedly affect host toxicity and possibly therapeutic effects (25–28) with a circadian variation in FdUrd toxicity having been suggested.
**CIRCADIAN RHYTHMS OF PYRIMIDINE ENZYMES AND FdUrd TOXICITY**

Table 4 Correlation analysis for relationship between TK activity and DPD activity in Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TK-IM</th>
<th>TK-BM</th>
<th>TK-S</th>
<th>TK-L</th>
<th>DPD-L</th>
<th>DPD-BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK-IM</td>
<td>1.00</td>
<td>0.862</td>
<td>0.600</td>
<td>0.616</td>
<td>-0.502</td>
<td>-0.166</td>
</tr>
<tr>
<td>TK-BM</td>
<td>1.00</td>
<td>0.683</td>
<td>0.566</td>
<td>0.562</td>
<td>-0.562</td>
<td>-0.264</td>
</tr>
<tr>
<td>TK-S</td>
<td>1.00</td>
<td>0.419</td>
<td>0.656</td>
<td>0.656</td>
<td>-0.588</td>
<td>-0.470</td>
</tr>
<tr>
<td>TK-L</td>
<td>1.00</td>
<td>0.012</td>
<td>0.662</td>
<td>0.662</td>
<td>-0.366</td>
<td></td>
</tr>
<tr>
<td>DPD-L</td>
<td>1.00</td>
<td>0.012</td>
<td>0.662</td>
<td>0.662</td>
<td>-0.366</td>
<td></td>
</tr>
<tr>
<td>DPD-BM</td>
<td>1.00</td>
<td>0.012</td>
<td>0.662</td>
<td>0.662</td>
<td>-0.366</td>
<td></td>
</tr>
</tbody>
</table>

* IM, intestinal mucosa; BM, bone marrow; S, spleen; L, liver.

* P < 0.01.

* P < 0.001.

* P < 0.0001.

* P < 0.05.

Table 5 Correlation analysis for relationship between FdUrd lethal toxicity and enzyme activities of TK and DPD in Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Correlation coefficient*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK-IM</td>
<td>-0.617</td>
<td>0.192</td>
</tr>
<tr>
<td>TK-BM</td>
<td>0.812</td>
<td>0.049</td>
</tr>
<tr>
<td>TK-S</td>
<td>-0.585</td>
<td>0.031</td>
</tr>
<tr>
<td>TK-L</td>
<td>-0.596</td>
<td>0.211</td>
</tr>
<tr>
<td>DPD-L</td>
<td>0.911</td>
<td>0.012</td>
</tr>
<tr>
<td>DPD-BM</td>
<td>0.497</td>
<td>0.316</td>
</tr>
</tbody>
</table>

* Pearson correlation coefficients were computer-calculated by linear correlation analysis between rat survival rate and enzyme activity.

* IM, intestinal mucosa; BM, bone marrow; S, spleen; L, liver.

(4, 8–12). However, it is difficult, using the previous data, to interpret the biochemical basis for the time-related toxicity and to correlate circadian variation in FdUrd toxicity with enzyme activity in the FdUrd metabolic pathway because of various factors including species and age of experimental animals, season, time, and interval of drug administration (and/or enzyme sampling). The experimental design in the present study has several advantages over previous investigations. These include the use of the same species in all toxicity and enzyme studies, simultaneous determination of activity of both TK (FdUrd anabolic enzyme) and DPD (FdUrd catabolic enzyme) in the same group of animals, and conduct of all experiments within 3 months. Confirmation of circadian-dependent toxicity of FdUrd in Sprague-Dawley rats enabled us to use the same species to further study the variation of FdUrd metabolic enzymes and interpret the correlation between the observed circadian-dependent FdUrd toxicity and potential variations in activities of these enzymes.

In the present study, we have demonstrated for the first time that TK activity in rat bone marrow and intestinal mucosa varies over 24 h. Under standard light conditions, a circadian rhythm in TK activity in intestinal mucosa was shown by Cosinor analysis to be highly significant (Table 1; P < 0.0001) with maximum activity at approximately 4:00 a.m. (22 HALO) and minimum activity at approximately 4:00 p.m. (10 HALO; Table 2). Maximum activity exceeded minimum activity by approximately 10-fold. A similar circadian rhythm in TK activity in bone marrow was also shown by Cosinor analysis to be highly significant (Table 1; P < 0.0001) with maximum activity at approximately 4:00 a.m. (22 HALO) and minimum activity at approximately 4:00 p.m. (10 HALO; Table 2). Maximum activity exceeded minimum activity by approximately 55%. Circadian variations of TK activity in intestinal mucosa and bone marrow may have an important role in FdUrd toxicity, since these tissues are the major sites of toxicity (2–4, 9, 10).

We have previously demonstrated a circadian rhythm in TK activity in spleen, with the same species, in association with the light-dark cycle (19). In the present study, we further confirmed the circadian pattern of TK in rat spleen (Table 1; P < 0.0001) with maximum activity at approximately 2:00 a.m. (20 HALO) and minimum activity at approximately 2:00 p.m. (10 HALO; Table 2). Maximum activity exceeded minimum activity by approximately 2-fold. These results were in agreement with the previous data (19), with a 1-h lag in the time of maximum (and minimum). This difference between the two experiments was not statistically significant.

The physiological mechanisms responsible for the circadian variation in TK activity have not been determined in the present study but may include the shift of activity (sleep/wake), feeding/fasting, and the patterns of cell proliferation. Many important functions have been demonstrated to be dependent on time of day. For example, DNA and RNA synthesis in several rodent tissues such as bone marrow and intestinal mucosa have a circadian pattern (29–36). Rhythmic changes in cytokinetics have been extensively documented in murine tissues and have been used to explain the observed rhythms in anticancer drug susceptibility (29). Circadian rhythms have been demonstrated with the mitotic index and DNA synthesis of rat stomach, duodenum, rectum, and bone marrow (29–32). TK is known to be important in pyrimidine nucleotide biosynthesis and DNA replication (37, 38). In the present study, a circadian pattern of TK activity in bone marrow and intestinal mucosa was demonstrated, suggesting a correlation between the circadian pattern of TK activity and DNA synthesis.

Previous studies from our laboratory utilizing Sprague-Dawley rats housed under similar experimental conditions have shown that DPD activity follows a circadian pattern (14, 15). However, the relationship between anabolic and catabolic enzymes has not been examined. In the present study, we simultaneously determined DPD and TK activities. Of particular interest is the inverse rhythmic (circadian) pattern of DPD compared to the circadian pattern of TK. At the time TK is at its maximum activity, DPD is approaching its minimum activity. Similarly, at the time TK is at its minimum activity, DPD is approaching its maximum activity. Statistical analysis revealed an inverse correlation between DPD and TK activities (Table 4). This suggests that regulation of the circadian variation of critical catabolic and anabolic enzymes may be important in maintaining metabolic homeostasis. Thus, it is probably advantageous to decrease catabolic activities in order to increase anabolic activities most efficiently. Although previous investigators have emphasized the importance of the balance between anabolism and catabolism in the nucleic acid pathways (39), the present study, to our knowledge, may be the first experimental evidence for the role of circadian variation of critical enzymes in anabolism and catabolism in orchestrating overall pyrimidine metabolism. However, the mechanisms responsible for the circadian variations of TK and DPD activity have not been investigated but may be related to new enzyme synthesis and/or regulation of preexisting enzymes. Further studies in gene expression and protein regulation are needed.

We hypothesize that a circadian pattern of TK activity in bone marrow and intestinal mucosa and inverse circadian pattern of DPD activity in liver may regulate the metabolism of pyrimidine nucleoside drugs and may explain the circadian variation of toxicities observed with time-modified FdUrd administration in humans and experimental animals. Data from the present study support this hypothesis (Tables
4 and 5). We have demonstrated that TK activity in different tissues follows a similar circadian pattern by Cosinor analysis (Fig. 2, A–D; Table 2) and correlation analysis (Table 4). This is also true for DPD activity in liver and bone marrow (Fig. 3, A and B; Tables 3 and 4). Correlation analysis revealed an inverse relationship between DPD and TK activity (Table 4). These results enabled us to use the liver DPD activity and bone marrow TK activity as representative of each enzyme to analyze the relationship between circadian pattern of FdUrd toxicity and circadian pattern of its major metabolizing enzymes (TK and DPD). As illustrated in Fig. 4, if FdUrd is given at the time of the lowest TK activity (and the highest DPD activity), the FdUrd toxicity would be the lowest; in contrast, if FdUrd is given at the time of the highest TK activity (and the lowest DPD activity), the FdUrd toxicity would be the highest.

The mechanisms responsible for the correlation of circadian variation of TK and DPD activity and FdUrd toxicity have not been investigated. As indicated above, it is essential that FdUrd be phosphorylated to FdUMP by TK for its anticancer effects or cytotoxicity. It may be predicted that the circadian variation of TK activity will result in the changes of intracellular FdUMP levels and further determine the amount of FdUrd-derived nucleotides and, subsequently, the FdUrd-induced cytotoxicity. More interestingly, the availability of FdUrd for anabolism is further regulated by its catabolism (2, 13) and DPD, the rate-limiting enzyme in fluoropyrimidine catabolism, which has been shown to have an inverse circadian pattern compared with TK. As shown in Fig. 4, at the time DPD is at its minimum activity, the availability of FdUrd for anabolism is predicted to be the highest, and TK is approaching its maximum activity. Therefore, much higher intracellular FdUMP levels would be expected and this may explain the highest toxicity observed. Similarly, at the time DPD is at its maximum activity, the availability of FdUrd for anabolism is predicted to be the lowest, and TK is approaching its minimum activity and so lower intracellular FdUMP levels would be expected and the toxicity should be lowest as was observed in the present study. Further studies to evaluate intracellular FdUMP levels are needed.

The relevance of the present study to clinical application of FdUrd deserves additional comments. Extrapolation of circadian rhythms from rodents to humans must consider differences in the life cycles. Rats are more active in the dark and less active in the light. Humans are just the opposite: more active in the light and less active in the dark. In addition, experimental animals were housed under a strictly controlled light-dark cycle and therefore are likely to have similar circadian patterns. In contrast, humans have varying life styles with different sleep-wake schedules and therefore the degree of circadian variation may be more variable than observed with experimental animals. In the present study, peak DPD activity and trough TK activity in the rat occurred at the middle to late stage of the resting span. This agrees with the circadian pattern of human DPD activity in peripheral blood mononuclear cells (15). Data on potential circadian variation in human TK activity are not currently available. However, several studies have shown that DNA synthesis in human bone marrow and intestinal mucosa follows a circadian pattern (40–43). For example, Smaaland et al. reported (40) that in human bone marrow the highest DNA synthesis occurred during waking span (8:00 a.m. to 8:00 p.m.) with the lowest DNA synthesis was observed during resting span (12:00 midnight to 4:00 a.m.). In another study, they demonstrated DNA synthesis in bone marrow follows a circadian pattern with peak activity at 1:00 p.m. (activity span) and trough activity at 1:00 a.m. (rest span) (42). In the present study, we demonstrate a circadian pattern of TK activity in rat bone marrow with peak activity at approximately 4:00 a.m. (activity span) and trough activity at approximately 4:00 p.m. (rest span). These results are in agreement with the DNA synthesis activity in human bone marrow. Further studies in humans should evaluate the relationship between pyrimidine metabolism, DNA synthesis, and the therapeutic effects and toxicity of fluoropyrimidine drugs.

It should also be noted that circadian based chemotherapy has yet to be shown to have widespread clinical utility and more preclinical and clinical studies are needed. One of the key therapeutic issues is whether the major enzymes in FdUrd anabolism and catabolism in tumor tissue also vary, particularly follow a circadian pattern, compared with normal tissues. If the enzyme activities in tumor tissue have no circadian (or inverse) pattern compared to normal tissues, the therapeutic advantage of drug administration is obvious when FdUrd is given at the time of the lowest TK activity (and the highest DPD activity) to diminish host toxicity. In contrast, if these enzymes follow the same circadian pattern in tumor tissues, administration of FdUrd at the time intended to avoid host toxicity might also lead to decreased activation of FdUrd resulting in decreased therapeutic effect. Further studies should compare the major enzyme activities in tumor tissues compared with normal tissues in a tumor-bearing animal model at various times of the day.

In summary, the present study demonstrates: (a) TK activity follows a circadian pattern in bone marrow and intestinal mucosa which are the major sites of FdUrd toxicity; (b) there is an inverse relationship between the major anabolic (TK) and catabolic (DPD) enzymes in FdUrd metabolism; and (c) the circadian variation in FdUrd toxicity is positively correlated with the circadian variation in TK activity but negatively correlated with the circadian variation in DPD activity. These results may be useful in the design of improved fluoropyrimidine chemotherapy regimens.

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Relationship between Circadian-dependent Toxicity of 5-Fluorodeoxyuridine and Circadian Rhythms of Pyrimidine Enzymes: Possible Relevance to Fluoropyrimidine Chemotherapy

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