Circadian clock coordinates cancer cell cycle progression, thymidylate synthase, and 5-fluorouracil therapeutic index

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Abstract
Dysregulated cellular proliferation is a characteristic property of cancer. We show that, despite this fact, cancers maintain high amplitude, circadian rhythms in their growth, DNA synthesis, and mitosis. These patterns are accompanied by the daily traverse of BMAL-1 protein between the cytoplasm, where it is produced, and nucleus, where it influences timing of cancer cell proliferation. This core clock gene product gates cancer cell proliferation by coordinating clock-controlled proteins, thymidylate synthase [thymidylate synthase activity (TSA) cell DNA replication], WEE-1 (cell mitosis), and vascular endothelial growth factor (growth). 5-Fluorouracil (5-FU)–induced host bone marrow and gut toxicity and tumor shrinkage following administration at six equi-spaced times of day allowed determination of circadian relationships among tumor growth, relevant clock, and clock-controlled proteins and dependence of 5-FU target availability (TSA) in normal and cancer tissues and resultant 5-FU toxic-therapeutic index. The time of day (hours after lights on) of low TSA in each tissue and tumor is respectively associated with greatest toxicity to that tissue and greatest tumor shrinkage. 5-FU treatment near daily awakening results in least damage to bone marrow and gut, greatest antitumor effect, and best survival. This time of day is associated with maximum tumor nuclear BMAL-1 and total cell WEE-1 protein. The described chain of events, for the first time, links cancer cell clock proteins, cancer cell DNA synthesis, proliferation, TSA, and 5-FU toxic-therapeutic index, explaining the dependence of cancer outcome on circadian timing of 5-FU. [Mol Cancer Ther 2006;5(8):2023 – 33]

Introduction
Cellular proliferation is organized within proliferating tissues throughout each day (1–4). In the usually quiescent liver, regeneration following partial hepatectomy results in tight circadian gating of cell division at specific times of day (5). The central circadian clock, in the suprachiasmatic nuclei of the hypothalamus, interacts with the environment through the pineal, retina, and hypothalamic-adrenal axis to generate and maintain daily rhythms. Core circadian clock proteins are shuttled from the cytoplasm to the nucleus as heterodimers at particular times of day, resulting in autoregulatory feedback loops with positive (BMAL-1 and CLOCK) and negative (PER1, PER2, PER3, CRY1, and CRY2) components (6, 7). The sequential processes of clock protein dimerization, nuclear transport, induction of transcriptional activation or repression through E boxes or other DNA-binding elements, phosphorylation, and degradation comprise the essential molecular clock works (8, 9).

Peripheral tissues also keep circadian time by coordinately expressing these same clock genes. This circadian temporal order of clock gene expression can be reproducibly induced in vitro (10). Up to 10% of the genome of peripheral tissues is transcriptionally expressed in a circadian-coordinated manner. These tissue-specific circadian clock-controlled genes temporally organize unique rate-limiting functions of that tissue. In bone marrow and gut epithelium, these functions are largely proliferative. In liver, these functions are primarily energetic and metabolic (11).

Cell cycle progression is tied to the circadian clock through clock-controlled WEE-1 protein availability. WEE-1 modulates cyclin-dependent kinases, inhibiting cyclin-dependent kinase, causing cell cycle arrest and subsequent circadian stage-dependent gating of cells at G2-M interface of the cell cycle (12, 13). People with cancer maintain meaningful, albeit sometimes disturbed, circadian organization until late in the disease (14–16). Spontaneous human cancers coordinate their proliferation within each day (17, 18). In tumor-bearing mice, tumor growth rates, tumor mitotic index, and the G1-S phase–regulated cyclin E protein are each circadian organized (14). The enzyme thymidylate synthase catalyzes the synthesis of thymidine, which is essential for DNA replication. The gene for thymidylate synthase is tied to the circadian clock. The
primary 5-fluorouracil (5-FU) drug target, thymidylate synthase activity (TSA), varies throughout the day in normal human and mouse tissues in parallel with tissue S-phase fraction (19, 20).

We herein show that circadian clock BMAL-1 nuclear protein translocation and WEE-1 protein content of tumor cells vary rhythmically throughout the day in *synchronization* with *in vivo* tumor growth, growth factor levels, mitotic index, S-phase progression, 5-FU drug target (TSA), and the toxic-therapeutic index of 5-FU. Knowing when in the day tumors reproducibly express proliferative targets relevant to cancer cell DNA synthesis or cancer cell division is useful for the optimal timing of successful cancer therapy.

**Materials and Methods**

**Animals**

Age-matched female CD2F1 mice, 10 to 14 weeks (Charles River, Portage, MI), were maintained in a 12-hour light (sleep phase) alternating with 12-hour dark (activity phase) with food and water *ad libitum*. All mice were synchronized to the lighting regime for 3 weeks. Time is expressed as hours after lights on (HALO).

**Methylcholanthrene A-Induced Sarcoma and Tissue Procurements**

Ascitic tumor cell suspension of methylcholanthrene A-induced sarcoma was harvested from BALB/c female mice, and 5 × 10⁵ cells were inoculated s.c. on the backs of 90 CD2F1 mice during the activity cycle (14 HALO). Fifteen animals were sacrificed at mean tumor volume (length × width × height) of 500 mm³ at one of six times of day (2, 6, 10, 14, 18, and 22 HALO). Tumors were dissected away from skin and half was frozen in liquid nitrogen and the other half was fixed in 10% buffered formalin and embedded in paraffin blocks. The intestinal mucosa from the proximal 8 cm of the small intestine and bone marrow cells were prepared and stored at −80°C as described (19).

**Thymidylate Synthase Activity**

Frozen tissues were homogenized mechanically with a glass dounce homogenizer in 200 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaF, and 20 mmol/L β-mercaptoethanol. The homogenate was centrifuged and supernatant fluid was harvested and protein concentration was determined. Thymidylate synthase catalytic activity was determined using a tritiated water release assay following the method of Armstrong with changes as described (19).

**Western Blotting**

Tumor supernatant fractions were electrophoresed through 12% polyacrylamide gels and proteins transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were incubated with rabbit anti-rat thymidylate synthase antibody (1:1,000; Frank Maley, New York State Department of Health, Albany, NY) overnight at 4°C and then incubated with horseradish peroxidase–conjugated goat anti-rabbit antibody (1:20,000). Immunoreactive bands were visualized by chemiluminescence (SuperSignal Substrate, Pierce, Rockford IL) and quantitated using scanning densitometry. Equivalency of protein loading was assessed by actin content (rabbit polyclonal anti-actin antibody, Sigma, St. Louis, MO).

**Table 1. Circadian variation in tumor histopathology**

<table>
<thead>
<tr>
<th>Time of day (HALO) of sacrifice (mean ± SE)</th>
<th>ANOVA</th>
<th>Cosinor analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fc</td>
<td>P</td>
</tr>
<tr>
<td>2 (n = 7-12)</td>
<td>4.42 ± 0.9</td>
<td>5.08 ± 0.5</td>
</tr>
<tr>
<td>6 (n = 11-12)</td>
<td>4.62 ± 0.9</td>
<td>5.16 ± 0.8</td>
</tr>
<tr>
<td>10 (n = 10-11)</td>
<td>4.82 ± 0.9</td>
<td>5.46 ± 0.5</td>
</tr>
<tr>
<td>14 (n = 8-9)</td>
<td>4.92 ± 0.9</td>
<td>5.56 ± 0.5</td>
</tr>
<tr>
<td>18 (n = 7-11)</td>
<td>4.92 ± 0.9</td>
<td>5.56 ± 0.5</td>
</tr>
<tr>
<td>22 (n = 9-15)</td>
<td>5.6 ± 0.7</td>
<td>4.6 ± 0.5</td>
</tr>
</tbody>
</table>

Note: n = number of animals.
Abbreviation: NS, not significant.

*Absorbance per high-power field. HALO on 12-hour light/12-hour dark lighting schedule.
Tumor complete remission was complete disappearance of a palpable tumor mass and cure was survival for 4 months with no tumor on necropsy. Body weight and WBC counts were expressed as absolute value of area under the curve (AUC) by subtracting the value at drug injection from each daily observation for each mouse. A qualitative assessment of gastrointestinal toxicity was recorded as percentage of animals with perianal swelling at anytime during the treatment course.

**5-FU Toxic-Therapeutic Index**

5-FU toxicity was evaluated using absolute AUC WBC counts, absolute AUC body weight, and presence or absence of perianal swelling, whereas antitumor therapeutic effects was evaluated by tumor remission, decrease in tumor size, and survival. Mice were ranked (numerically

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**Figure 1.** A, highest nuclear BMAL-1 staining was seen in tumors resected at 14 HALO. B, tumors resected at 18 HALO show the minimum nuclear BMAL-1 staining. WEE-1 staining is mainly found in tumor cell cytoplasm. The highest intensity WEE-1 was seen in tumors resected at 14 HALO (C) and the lowest WEE-1 staining in tumors resected at 18 HALO (D). VEGF staining of tumors was minimum when resected at 14 HALO (E) and maximal at 18 HALO (F). Magnification, ×40.

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**Figure 2.** Daily variation in tumor clock protein (BMAL-1, positive nuclear/total nuclear area; A), clock-controlled protein (WEE-1, absorbance per high-power field; B), and tumor mitotic index (C) from tumors resected at one of six times of day (HALO) during the 12 h of light (sleep) and 12 h of dark (activity). Points, mean with 12-h plus 24-h cosine fitted curves; bars, SE. Dashed lines and arrows, time of day – dependent interrelationships. Note that there is break in the vertical axis values.
ordered) independently for each variable: toxicity factors in descending order (higher numerical rank indicates low toxicity), whereas tumor therapeutic effect factors in ascending order (higher numerical rank indicates greater antitumor therapeutic effect). Toxicity and therapeutic scores are the average rank of (AUCWBC + AUCBW + perianal swelling) and [tumor size (treated versus control) + tumor remission + survival], respectively. The 5-FU toxic-therapeutic index is then computed as the sum of toxicity and therapeutic scores for each individual mouse. Higher toxic-therapeutic index values indicate simultaneous occurrence of low host drug toxicity with high tumor therapeutic effects. Low index values indicate occurrence of high host drug toxicity with low tumor therapeutic effects.

**Tumor Mitotic Index**

One H&E-stained section was prepared from each tumor and evaluated under Axioskop microscope (Axioskop, Carl Zeiss, Germany). Areas containing the most viable tumor cells in five consecutive images (300–400 tumor cells per image) were randomly taken from each section using ×40 objective lens and the AxioCam Digital camera and quantitated as described (14). Values are mean number of mitotic figures per field.

**Tumor Tissue Array**

A tissue array instrument (Beecher Instrument, Inc., Sun Prairie, WI) was used to construct tumor tissue array block (14). Multiple 5-μm sections were cut from the array block and mounted on the positively charged glass slides (Surgipath, Richmond, IL) for histopathologic and immunohistochemical examination.

**Protein Immunohistochemistry**

The tissue array sections were digested using pepsin (4 mg/mL in 0.01 N HCl) for 40 minutes and endogenous peroxidase activity was blocked by 3% H2O2 in PBS for 15 minutes. Primary antibody [anti BMAL-1 and WEE-1 rabbit polyclonal antibodies and anti–vascular endothelial growth factor (VEGF)-1 mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA] was applied at 1:250, 1:1,000, and 1:400, respectively, and incubated overnight at 4°C. For BMAL-1 and WEE-1 protein stain, the secondary antibody (goat anti-rabbit IgG) and AB complex (Vectastain avidin-biotin complex method kit, Vector Laboratories, Inc., Burlingame, CA) were applied. Color was developed by 3,3′-diaminobenzidine tetrahydrochloride (Peroxidase substrate kit 3,3′-diaminobenzidine, Vector Laboratories; ref. 14). Sections were counterstained with Harris’ hematoxylin (Sigma). For VEGF-1, the secondary biotinylated rabbit anti-mouse antibody and ExtrAvidin peroxidase (B-6398 and E-8386, Sigma) were applied. The color was developed by aminoethylcarbazole substrate (AEC-101 kit, Sigma). Sections were counterstained with 1% methyl green solution (Sigma). Immunohistochemical stains without primary antibody served as negative controls.

**Quantitation of Total Cellular BMAL-1, VEGF-1, and WEE-1 Protein Immunostain**

Areas, which represented average levels of the BMAL-1, VEGF-1, and WEE-1 protein stain, consisting of only viable tumor cells, were selected from each tissue core. Digital images were taken from these areas using AxioVision camera (Carl Zeiss) and analyzed using Vision Lite (Clemex Technologies, Longueuil, Quebec, Canada). The target objectives in these images were defined and selected by a preset intensity. The average intensity of the objectives was measured as described (14).

**Quantitation of Nuclear BMAL-1 Protein Immunostain**

Two patterns of BMAL-1 protein were observed, nuclear and cytoplasmic. Immunostained images were quantified using the Vision Lite. The nuclear BMAL-1 stain within tumor cells was highlighted by a preset intensity and the area of tumor nuclear positive stain was measured. Total nuclear area of the tumor cells was estimated at the
coordinated regions of an adjacent tumor array section, which was used for negative control, and the tumor cell nuclei were counterstained with hematoxylin. The total nuclear area was assayed with a similar procedure. The ratio \( \frac{R_{\text{BMAL-nuclear}}}{A_{\text{total}}} \) of tumor nuclear positive-stained area \( A_{\text{BMAL}} \) to the total tumor nuclear area \( A_{\text{total}} \) was calculated as: \( R_{\text{BMAL-nuclear}} = \frac{A_{\text{BMAL}}}{A_{\text{total}}} \).

**Statistical Analysis**

For each numerical value, mean and SEs were calculated and graphed. Comparison of means across six circadian times of day (HALO) was assessed by one-way ANOVA (SAS Inc., Cary, NC). Circadian rhythm pattern was assessed by Cosinor analysis (Chronolab version 4.5, Bioengineering & Chronobiology Laboratory, Pontevedra, Spain). Average values based on the six circadian times were graphed as a double plot over 48 hours with SEs.

**Results**

**Daily Rhythms in Tumor Nuclear BMAL-1 Protein Translocation, Tumor Clock-Controlled-Protein (WEE-1), and Tumor Size**

The total tumor cell optical intensity of BMAL-1 remained unchanged throughout the day (Table 1). However, BMAL-1 nuclear protein concentration varied significantly throughout the day, with strong nuclear BMAL-1 staining at 2, 6, 10, and 14 HALO (Fig. 1A), whereas at 18 and 22 HALO, tumors showed weakly BMAL-1 nuclear positivity or were negative (Fig. 1B). The circadian pattern of nuclear tumor BMAL-1 (12-hour plus 24-hour fit; \( P < 0.001 \); Table 1) showed two peaks at 1:30 and 12:30 HALO (Fig. 2A), with a major trough between 18 to 22 HALO. Tumor WEE-1 clock-controlled protein showed mainly a cytoplasmic pattern. Circadian WEE-1 expression showed a circadian rhythm (24-hour fit, \( P = 0.047 \); 12-hour plus 24-hour fit, \( P = 0.060 \); Table 1), with the highest tumor staining at 12:30 HALO and lowest staining at 20:00 HALO (Figs. 1C and D and 2B), which parallels the second daily peak and trough in nuclear BMAL-1. The peak in WEE-1 lags behind the peak in BMAL-1 and then WEE-1 gradually accumulates in the cytoplasm during early sleep phase. Tumor mitotic index, which is regulated by WEE-1, showed a 2-fold difference (12-hour plus 24-hour fit; \( P = 0.012 \); Table 1), with a major daily peak at 9 HALO and a smaller peak at 21 HALO (Fig. 2C). Therefore, daily peaks in tumor WEE-1 protein are followed by a decline in tumor mitotic index. Later in the day, the daily trough in tumor WEE-1 protein is followed by a surge in tumor cell mitotic index (Fig. 2).

### Table 2. Circadian variation in tumor size and thymidylate synthase in tumor and normal tissues

<table>
<thead>
<tr>
<th>Time of day (HALO) of sacrifice (mean ± SE)</th>
<th>ANOVA Rhythm fitted (h)</th>
<th>Cosinor analysis Circadian peaks (HALO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (mm³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (n = 14-16)</td>
<td>988 ± 96</td>
<td>5.22</td>
</tr>
<tr>
<td>6 (n = 16-19)</td>
<td>753 ± 71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 (n = 14-19)</td>
<td>1,073 ± 82</td>
<td>12</td>
</tr>
<tr>
<td>14 (n = 16-17)</td>
<td>783 ± 87</td>
<td>0.080</td>
</tr>
<tr>
<td>18 (n = 16-19)</td>
<td>901 ± 67</td>
<td>&lt;0.001 10:30, 22:30</td>
</tr>
<tr>
<td>22 (n = 16-17)</td>
<td>1,292 ± 119</td>
<td>24</td>
</tr>
<tr>
<td>TSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor*</td>
<td>17.8 ± 1.8</td>
<td>6.26</td>
</tr>
<tr>
<td>Small intestine*</td>
<td>6.7 ± 1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bone marrow*</td>
<td>85.8 ± 8.6</td>
<td>12</td>
</tr>
<tr>
<td>Tumor thymidylate synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymidylate synthase protein†</td>
<td>1.26 ± 0.32</td>
<td>12</td>
</tr>
<tr>
<td>Thymidylate synthase mRNA†</td>
<td>0.24 ± 0.03</td>
<td>&lt;0.001 1:00</td>
</tr>
</tbody>
</table>

Note: \( n \) = number of animals.

†Absorbance (sample/standard).

‡TS/18S (% phosphorimage units).
The circadian rhythm in tumor size \((P < 0.001; \text{Fig. 3C; Table 2})\) parallels the rhythm in tumor mitotic index, coupling circadian coordination of tumor clock protein (BMAL-1) and tumor clock-controlled regulator of mitosis (WEE-1), tumor cell division (mitotic index), and cancer growth.

**Daily Rhythms in Tumor Clock-Controlled Protein (VEGF), Tumor S-phase Marker (TSA), and Tumor Size**

Tumor VEGF (clock-controlled gene) protein shows a prominent bimodal circadian variation (12-hour plus 24-hour fit; \(P = 0.004; \text{Table 1}\)), with a major peak at 19:00 HALO and a minor peak at 7:00 HALO (Fig. 1E and F). Tumor TSA shows two major peaks at 7:30 and 19:30 HALO (12-hour plus 24-hour fit; \(P < 0.001; \text{Fig. 3A}\)). Daily peaks and troughs in tumor VEGF paralleled the daily peaks and troughs in tumor TSA (Fig. 3). Each of these peaks is followed 4 hours later by two daily peaks in tumor size, thereby concurrent with putative tumor growth rate because growth necessarily precedes any size change. The 2-fold circadian variation in tumor TSA is paralleled by a 6- to 7-fold variation in thymidylate synthase protein (Table 1). Tumor thymidylate synthase RNA levels vary minimally throughout the day, consistent with our findings in human oral mucosa (20).

**Circadian Dependence of TSA and 5-FU Antitumor Efficacy and Host Toxicity**

The 5-FU-induced tumor response (treated/control tumor size), on the day of maximal tumor regression, shows a large time of day dependence \((P = 0.048; \text{Fig. 4B; Table 3})\), with maximum cancer response (lowest values, greatest inhibition) at two times of day of 5-FU administration, 6 HALO (34.9 ± 7.2%) and 14 HALO (31.2 ± 5.7%). Tumor complete remissions were seen when 5-FU was given at 2 or 14 HALO, with one tumor cure at 14 HALO (Table 3). The average life span of tumor-bearing mice, following circadian-timed 5-FU, is circadian dependent with one major peak (longest life span) when 5-FU is given at 14 HALO (36.5 ± 3.3 days), a second minor peak at 6 HALO. The shortest life span followed 5-FU given at 10 HALO (21.3 ± 23 days; \(P = 0.028; \text{Fig. 4C}\)) supported by survival analysis \((P < 0.001; \text{Fig. 5})\).

5-FU-induced myelotoxicity, the fall and recovery of circulating WBC concentration, was bimodally circadian time dependent (12-hour plus 24-hour fit; \(P < 0.001; \text{Table 3}\)). 5-FU was most toxic to bone marrow (largest absolute WBC AUC) when 5-FU is administered at 10 HALO with a second minor peak at 22 HALO \((P < 0.001; \text{Table 3})\). 5-FU-induced decline in body weight was circadian time dependent, with the most severe decline (largest absolute body weight AUC) when 5-FU is given at 9:30 HALO and at 21:30 HALO \((P = 0.001; \text{Fig. 6C})\). This toxicity pattern is similar to 5-FU-induced myelotoxicity (Fig. 6). Gastrointestinal toxicity, measured by frequency of 5-FU-induced perianal swelling, was circadian time dependent with highest toxicity at 6 to 10 HALO and at 18 HALO \((P = 0.012; \text{Table 3})\). Bone marrow, small intestinal mucosa, and tumor show time of day–dependent differences in TSA with rhythms best described by two daily peaks \((P < 0.001; \text{Figs. 3A, 4A, and 6A; Table 2})\) occurring at tissue-specific times of day. Tumor TSA correlates inversely with degree of 5-FU antitumor effect and therapeutic life span prolongation when 5-FU is given during the daily activity span (12–24 HALO; shaded area).
body weight decline (Fig. 6). The frequency of perianal swelling likewise varies inversely with the TSA in the small intestine for the majority of the circadian cycle (6–22 HALO; Fig. 7).

**5-FU Toxic-Therapeutic Index**

Numerical rank of absolute AUC WBC counts, absolute AUC body weight, and presence or absence of perianal swelling were combined to measure 5-FU toxicity, whereas antitumor therapeutic effects was evaluated by tumor remission, tumor size, and survival. The 5-FU toxic-therapeutic index is then obtained as the sum of resulting drug toxicity and therapeutic scores. Higher toxic-therapeutic index values indicate simultaneous occurrence of low host 5-FU toxicity with high tumor therapeutic effects. Results showed that 5-FU toxic-therapeutic index is circadian rhythmic with two major peaks ($P < 0.001$; Fig. 8; Table 3). The most optimal time of day for both 5-FU toxicity and antitumor efficacy was at 14 HALO (117.23 ± 8.2) followed by a second optimal time at 6 HALO (108.23 ± 4.4), whereas the worst time of day for both 5-FU toxicity and antitumor efficacy is at 10 HALO (72.9 ± 5.4).

**Correlation of Host Tumor Cell Circadian Clock Protein Expression, Circadian TSA, and 5-FU Susceptibility**

The most optimal time of day for 5-FU (low host toxicity and high antitumor efficacy), at 14 HALO, is the time of day with highest tumor nuclear BMAL-1 and WEE-1 protein, which coincides with lowest daily values of untreated tumor size, minimum daily tumor VEGF, and TSA (Table 3). The worst time of day for 5-FU (high host toxicity and low antitumor activity), at 22 HALO, is

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**Table 3. Circadian-dependent 5-FU effects on host toxicity, antitumor efficacy, and toxic-therapeutic index**

<table>
<thead>
<tr>
<th>Time of day (HALO) of 5-FU treatment (5-FU)</th>
<th>Statistical analysis</th>
<th>Cosinor analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ($n = 14-15$)</td>
<td>6* ($n = 15$)</td>
<td>10† ($n = 15$)</td>
</tr>
<tr>
<td>14† ($n = 15$)</td>
<td>18 ($n = 15$)</td>
<td>22† ($n = 15$)</td>
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<tr>
<td>Test stat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhythm fitted (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circadian peak (HALO)</td>
<td></td>
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</tbody>
</table>

**Antitumor efficacy**

Tumor response (treated/control)

<table>
<thead>
<tr>
<th>Time to death (d)</th>
<th>24.9 ± 1.8</th>
<th>27.6 ± 2.4</th>
<th>21.3 ± 2.3</th>
<th>36.5 ± 3.3</th>
<th>27.6 ± 2.4</th>
<th>26.8 ± 2.9</th>
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<td>Survival</td>
<td>1 CR</td>
<td>2 CR, 1 C</td>
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<tr>
<td>Test stat</td>
<td>3.96</td>
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<tr>
<td>$P$</td>
<td>12</td>
<td>12</td>
<td></td>
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<tr>
<td>Rhythm fitted (h)</td>
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<tr>
<td>$P$</td>
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<td>0.002</td>
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<tr>
<td>Circadian peak (HALO)</td>
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<td>12</td>
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**Host toxicity**

WBC AUC (absolute, days 0-6, ×1,000)

<table>
<thead>
<tr>
<th>18.3 ± 1.5</th>
<th>24.6 ± 4.3</th>
<th>47.1 ± 6.1</th>
<th>26.1 ± 3.4</th>
<th>18.8 ± 1.9</th>
<th>22 ± 1.8</th>
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<tbody>
<tr>
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<td>$P$</td>
<td>12</td>
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<td>$P$</td>
<td>0.004</td>
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<tr>
<td>Circadian peak (HALO)</td>
<td>12</td>
<td>12</td>
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</tbody>
</table>

**Body weight AUC (absolute, days 0-6)**

<table>
<thead>
<tr>
<th>12 ± 1.5</th>
<th>12.4 ± 1.1</th>
<th>16.5 ± 1.6</th>
<th>10.0 ± 1.0</th>
<th>12.4 ± 1.8</th>
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<tbody>
<tr>
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<tr>
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<tr>
<td>Circadian peak (HALO)</td>
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**Perianal swelling (affected/total animals, %)**

<table>
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<td>$P$</td>
<td>12</td>
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<td>Rhythm fitted (h)</td>
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<tr>
<td>$P$</td>
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<tr>
<td>Circadian peak (HALO)</td>
<td>12</td>
<td>12</td>
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**Toxic-therapeutic index**

<table>
<thead>
<tr>
<th>103.6 ± 4.5</th>
<th>108.3 ± 4.4</th>
<th>72.9 ± 5.4</th>
<th>117.2 ± 2.3</th>
<th>103.5 ± 4.1</th>
<th>93.5 ± 6.2</th>
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<td>Rhythm fitted (h)</td>
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<tr>
<td>$P$</td>
<td>0.392</td>
<td>&lt;0.001</td>
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<tr>
<td>Circadian peak (HALO)</td>
<td>12</td>
<td>12</td>
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</tr>
</tbody>
</table>

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Note: $n$ = number of animals.

Abbreviation: CR, complete response; C, cure.

*With second optimal time of day at 6 HALO.

†With worst time of day for both 5-FU toxicity and antitumor efficacy was 10 and 22 HALO.

‡Most optimal time of day for both 5-FU toxicity and antitumor efficacy was 14 HALO.

§ANOVA unless noted otherwise.

## Sixth day after 5-FU injection when first death is observed [(tumor at day 6−day 0) / (control at day 6−day 0)] × 100.

*Based on a $\chi^2$ test of independence.

**Toxic-therapeutic index is the sum of overall toxicity and tumor therapeutic scores calculated for individual mice (see Materials and Methods).
associated with lowest daily tumor nuclear BMAL-1 and WEE-1 protein, which coincides with highest daily values in untreated tumor size, maximum daily tumor VEGF concentration, and TSA.

Discussion
Cancer cell DNA synthesis and division are crisply coordinated within each day in human and rodent tumors as they are in normal tissues (14, 17, 18, 21). In mice, tumor growth rate, blood flow, and VEGF protein content vary throughout each day (14, 22, 23). The circadian organization of Bmal-1 and other clock gene RNA levels is variably maintained in murine cancers and is associated with tumor circadian coordination of cell cycle progression throughout the day (14, 22). In regenerating liver, cell division is restricted to certain times of day by the circadian clock through clock-controlled gene products, such as WEE-1, a cyclin-dependent kinase inhibitor, which gates mitosis (5, 13).

We find that, in circadian-coordinated tumor-bearing hosts, tumors show a daily rhythm in nuclear BMAL-1 protein translocation, which parallels the daily rhythm in tumor WEE-1 protein content. As expected, tumor cell division (mitotic index) is gated throughout each day inversely with tumor WEE-1 with a predictable lag. Tumor size is greatest at times of day of high mitotic index and decreases as cell division declines. Daily peaks in tumor VEGF protein and thymidylate synthase protein and activity are each followed, in circadian time, by an increase in tumor size, potentially coupling tumor clock gene transcription and protein translation to clock-controlled gene expression and protein translation with tumor cell cycle progression and tumor growth throughout each day. Both thymidylate synthase overexpression and certain thymidylate synthase gene polymorphisms are associated with 

\textit{in vitro} and \textit{in vivo} 5-FU resistance (24, 25). Circadian coordination of tumor thymidylate synthase content and activity is associated with a daily rhythm in the toxic-therapeutic ratio of the proliferation-dependent thymidylate synthase–targeted drug, 5-FU. This circadian organization of 5-FU therapeutic index is explained by concurrent circadian gating of the 5-FU drug target, thymidylate synthase protein and enzyme activity in nucleated bone marrow cells, small intestinal mucosal cells, and cancer cells within the tumor-bearing mouse. The best time of day (early activity, 2 hours after daily arising) for 5-FU administration (low host toxicity and high tumor response) is associated with the maximum daily tumor nuclear BMAL-1 protein content and total cell WEE-1 protein concentration, which

![Figure 5. Kaplan-Meier survival curves of tumor-bearing mice following administration of 5-FU at one of six times of day (HALO). Survival is best at 14 HALO and worst at 10 HALO ($P = 0.004$; log-rank $\chi^2 = 17.1$).](image)

![Figure 6. Relationship between the circadian rhythm in bone marrow TSA in untreated mice (A), 5-FU-induced myelotoxicity (WBC absolute AUC over days 0–6; B), and body weight decline (absolute AUC over days 0–6) of mice following 5-FU treatment at one of six times of day (C). There is a meaningful relationship between marrow TSA, 5-FU marrow toxicity, and 5-FU-related body weight decline during the activity phase. (10–24 HALO; shaded area).](image)
is coincident with lowest daily average tumor size and VEGF content as well as the lowest daily tumor TSA. The worst time of day (late activity) for 5-FU (high host toxicity and low tumor response) is associated with the minimum daily tumor nuclear BMAL-1 and total cell WEE-1 protein content, which is coincident with the highest daily tumor size and VEGF content as well as the highest daily tumor cell TSA. Therefore, we conclude that tumor cell division seems to be coordinated throughout the day by the daily rhythm in clock BMAL-1 protein nuclear translocation, which gates clock-controlled WEE-1 protein availability. This relationship predicts, if not controls, the time of day of the highest and lowest TSA and the resultant 5-FU toxic-therapeutic index. Our results suggests this circadian coordination of tumor cell DNA synthesis and proliferation as a general mechanism by which the circadian clock within cells coordinates susceptibility to all drugs which target proliferation pathways.

Tumor growth factors and other receptors are also circadian organized in cancer cells (14, 22). We find tight covariation of VEGF levels and tumor size throughout each day, consistent with previous demonstration that higher tumor VEGF levels are associated with tumor growth (22). Our circadian tumor VEGF protein pattern is identical to that of Koyanagi et al. (22) with a peak at 2 to 6 HALO. We also find a second daily peak in tumor VEGF protein at 18 to 22 HALO. These two studies differ in the type of tumor, mouse strain, and sex. The circadian variation in tumor VEGF protein seems biologically meaningful because the in vivo response to several angiogenesis inhibitors also varies similarly with the circadian time of their administration.

Other circadian clock genes modulate cyclophosphamide toxicity in mice without tumors (26). The time of day when the activity of CLOCK/BMAL-1 transactivation complex peaks are the times of day with lowest cyclophosphamide toxicity to the host, 10 to 14 HALO. Circadian clock mutants (Cry1−/− and Cry2−/−), which constitutively express high levels of the CLOCK/BMAL-1 complex, show diminished sensitivity to cyclophosphamide toxicity at all times of day. Conversely, mutant mice with deficient CLOCK/BMAL-1 complex (Clock−/− or Bmal-1−/−) have greater susceptibility to host cyclophosphamide toxicity. No tumor susceptibility was evaluated in these studies.

The molecular level of 5-FU target (thymidylate synthase) circadian control is of interest. We find minor time of day differences in thymidylate synthase mRNA, yet 6- to 8-fold differences in thymidylate synthase protein and 2-fold variations in thymidylate synthase enzyme activity throughout the day in cancer cells. For this important therapeutic target (thymidylate synthase), molecular mRNA profiling alone would be unrevealing of these circadian dynamics. Tumor thymidylate synthase protein content and TSA have been used to successfully predict 5-FU tumor response in experimental and human cancers (24, 25). Because protein and enzyme activity change throughout the day in cancer cells, the time of day of tissue collection of thymidylate synthase protein activity affects the accuracy of such assays for predicting thymidylate synthase–targeted drug toxicity and cancer response.

More generally, this work has clear clinical implications for the interpretation of all proliferation-based tumor prognostic indicators. Like thymidylate synthase (27) and VEGF (28) content, mitotic index, one of the most venerable...
and reliable predictors of cancer outcome, changes predictably more than 2-fold each day in our two tumor models (sarcoma and breast cancer; ref. 14). Tumors obtained early in daily activity (first morning case) might be judged as more highly malignant, whereas the same tumor resected in the early evening (last case) would be less proliferative and judged less malignant. This is equally true for spontaneous human cancers (17). Therefore, accurate evaluation of potentially all proliferation-based prognostic indicators demands that tumor samples obtained at the same time of day be compared to more accurately predict outcome.

These observations are relevant to the anticancer drug development process. Any agent targeting molecular processes tied to cancer cell proliferation will prove to have less or more anticancer efficacy depending on when in the day it is given. Our work uses three established therapeutic targets: mitosis (cell division), thymidylate synthase (DNA synthesis), and VEGF (angiogenesis and growth) as concrete examples. For TSA, we show how the circadian organization of this important target explains large predictable differences in 5-FU-induced bone marrow and gut damage as well as its therapeutic anticancer activity. Our data further show that the normal tissue-damaging effects of 5-FU can be dissociated from its anticancer effects by giving the drug at the time of day when thymidylate synthase target activity is abundant in normal tissues yet low in tumor tissue and thereby more easily suppressed within the cancer compared with gut and bone marrow. Our data show that this temporal organization is most probably controlled by cancer cell expression and nuclear translocation of circadian clock proteins, which in turn direct the expression of proliferation-specific circadian clock-controlled proteins, WEE-1, and thymidylate synthase, which gates cancer cell mitosis and DNA synthesis, respectively.

In summary, we find that the circadian clock, as represented by BMAL-1 nuclear translocation, beats twice every 24 hours in mouse tumors. Following these beats, three sets of clock-controlled protein increase and decrease: WEE-1 gates tumor cell mitosis; TSA gates 5-FU sensitivity and therapeutic index; and VEGF, along with other clock-controlled genes, coordinates cancer blood flow and growth (29, 30). This work shows for the first time the more or less complete chain of events responsible for repeated experimental and clinical findings that time of day proliferation-targeted anticancer drug, such as 5-FU, is given, determines its therapeutic index (31–33). It shows the temporal relationship among peripheral clock function (BMAL-1 nuclear translocation); clock-controlled protein (WEE-1) that control the availability of either mitosis or DNA synthesis or growth related drug targets (TSA and VEGF); the actual physiologic result of this coordination of these processes (tumor size and growth); target susceptibility of bone marrow, intestine, and tumor to a DNA replication–targeted agent (5-FU); and host toxicity and tumor shrinkage and survival of mice bearing 5-FU-treated cancers. The description of this chain of events does not prove causality but provides strong circumstantial evidence for it. If cancer cell proliferation remains under the control of circadian clocks and clock-controlled proteins, then creating drugs that mimic or control these molecules foreshadows the development of an entirely novel strategy to control cancer.

Acknowledgments

We thank Dr. Frank Maley for his advice with thymidylate synthase assays and the gift of anti-thymidylate synthase antibody, Dr. David W Lincoln III for thymidylate synthase measurements, and Denise Peace for technical assistance.

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Circadian clock and therapeutic index

In the article on the circadian clock and therapeutic index in the August 2006 issue (1), some entries in Table 3 on page 2029 appeared in the wrong columns. For the row “Tumor remission,” the entry “1CR” should have appeared under 2 HALO of “Time of day (HALO) of 5-FU treatment (5-FU)/(n = 14-15)” and the entry “2CR, 1C” should have appeared under 14 HALO of “Time of day (HALO) of 5-FU treatment (5-FU)/(n = 14-15).”

Reference
Molecular Cancer Therapeutics

Circadian clock coordinates cancer cell cycle progression, thymidylate synthase, and 5-fluorouracil therapeutic index


*Mol Cancer Ther* 2006;5:2023-2033.

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