Int. J. Cancer: **88,** 479–485 (2000)
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Publication of the International Union Against Cancer

CIRCADIAN ORGANIZATION OF THYMIDYLATE SYNTHASE ACTIVITY IN NORMAL TISSUES: A POSSIBLE BASIS FOR 5-FLUOROURACIL CHRONOTHERAPEUTIC ADVANTAGE

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Fluoropyrimidines induce cytotoxicity, in part, by inhibiting the proliferation-coordinated enzyme thymidylate synthase (TS), which is essential for DNA synthesis. Tumor TS levels are clinically predictive of post-surgical tumor recurrence and of response to fluoropyrimidine chemotherapy. Fluoropyrimidine drug toxicity and efficacy each vary reproducibly in humans and animals, depending upon their circadian timing. *In vivo*, normal tissues and some tumor tissues exhibit circadian coordination of cellular proliferation. We therefore asked whether TS activity is coordinated rhythmically throughout the day in the normal proliferative tissues most damaged by fluoropyrimidine drugs. To assess tissue and time of day TS activity differences, we harvested normal tissues from female mice living on a 12:12 hr light:dark schedule at each of 6 different equispaced times throughout a 24 hr cycle and measured TS catalytic activity. We observed up to 10-fold differences *in vivo* in TS activity among different normal tissue types, roughly paralleling their proliferative state and relative fluoropyrimidine sensitivity. In normal tissues most damaged by fluoropyrimidines (bone marrow, small intestinal mucosa and oral mucosa/tongue), TS activity varies up to 2-fold throughout each day. In bone marrow, the circadian pattern of TS activity parallels the circadian rhythm in proliferation in this tissue. This circadian organization of TS, one of the primary fluoropyrimidine targets in normal tissues, probably contributes *in vivo* to the time of day differences in the toxic-therapeutic ratio of circadian-timed fluoropyrimidine drug therapy. *Int. J. Cancer* 88:479–485, 2000.
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5-fluorouracil (5-FU) most prominently causes cellular toxicity by inhibiting TS (thymidylate synthase; methylenetetrahydrofolate:deoxyuridine-5′-monophosphate C-methyltransferase; EC 2.1.1.45), although effects upon RNA also occur (Pinedo and Peters, 1988). TS is essential for *de novo* thymidylate production and, thereby, DNA synthesis. TS activity increases with cell proliferation (Jehn *et al.,* 1985; Maley and Maley, 1960; Navalgrund *et al.,* 1980; Pestalozzi *et al.,* 1995). TS expression in some types of human cancer (protein and mRNA levels) correlates inversely with *in vivo* fluoropyrimidine responsiveness and with the likelihood of cure following surgical resection (Johnston *et al.,* 1994, 1995; Leichman *et al.,* 1997; Lenz *et al.,* 1995, 1998).

Ubiquitous circadian organization has meaningful biomedical implications (Hrushesky, 1994). Both toxicity and antitumor efficacy of 5-fluorouracil (5-FU), one of the most commonly used anticancer drugs worldwide, depend reproducibly upon the time of day at which this drug is given to experimental animals and human beings (Wood and Hrushesky, 1996). In randomized, controlled clinical trials, circadian-optimized fluorodeoxyuridine (FdUrd) treatment of patients with metastatic kidney cancer and circadian-optimized 5-FU/leucovorin/oxaliplatin chemotherapy of patients with metastatic colon cancer diminishes gastrointestinal toxicity by 4- to 8-fold and marrow toxicity by 2- to 4-fold (Hrushesky *et al.,* 1990; Lévi *et al.,* 1997; von Roemeling and Hrushesky, 1990). Circadian-optimized fluoropyrimidine-based cancer therapy reproducibly doubles the objective tumor-response frequency for patients with metastatic colon cancer (Lévi *et al.,* 1997). Studies of normal human and rodent proliferative tissues, which are prominent targets of fluoropyrimidine toxicity, demonstrate that larger proportions of cells are undergoing DNA synthesis and mitosis at specific times within each day (Bjarnason *et al.,* 1999; Buchi *et al.,* 1989; Scheving *et al.,* 1983; Smaaland *et al.,* 1991; Wood *et al.,* 1998). Cytokinetic coordination throughout the day has also been demonstrated for a limited number of human cancers (Hrushesky *et al.,* 1998; Klevecz *et al.,* 1987; Smaaland *et al.,* 1993).

The extent of toxicity of S-phase, cell cycle-specific, cytotoxic drug often depends upon the size of the proliferative fraction within the target tissue as well as the capacity of the tissue or host to anabolize and catabolize the drug. The activities of anabolic enzymes for fluoropyrimidine drugs are organized throughout each day in normal rodent tissues including uridine phosphorylase (UrdPase, EC 2.4.2.3) (el Kouni *et al.,* 1990), orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) (Naguib *et al.,* 1993) and thymidine kinase (TK, EC 2.7.1.21) (Zhang *et al.,* 1993). The activity of the rate-limiting enzyme for fluoropyrimidine catabolism, dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) in normal tissues (Daher *et al.,* 1991; Naguib *et al.,* 1993) and the clearance of 5-FU (Harris *et al.,* 1989, 1990) are circadian time-dependent in rodents and humans. The cancer patient to cancer patient consistency of the circadian time structure of 5-FU catabolism is unclear at this time (Harris *et al.,* 1990; Takimoto *et al.,* 1999). If this circadian organization is variable, it cannot easily explain the 2- to 8-fold time of day-dependent differences in 5-FU toxicity found in randomized clinical trials. Therefore, we hypothesized that the circadian organization of TS availability may also be relevant. We investigated whether TS activity varies rhythmically throughout each day in normal tissues that limit fluoropyrimidine use, including bone marrow, gastrointestinal mucosa and oral mucosa/tongue. We concurrently determined the relationship between TS activity and circadian proliferation patterns in the bone marrow.

MATERIAL AND METHODS

Animal study design

Female CD_2F_1 mice (11–15 weeks) were maintained on a 12 hr light (sleep phase) and 12 hr dark (activity phase) cycle with food and water freely available. Twenty-four-hour time is referred to as hours after light onset (HALO). Circadian-timed tissue samples were obtained from individual mice at one of 6 equispaced times throughout a 24 hr cycle by using six parallel groups of mice in different rooms, each with a 12:12 hr light-dark cycle staggered by the time of light onset. In one study, normal tissues from individual mice (n = 2–23) were procured at a single time of day (early activity, 14 HALO) to examine tissue differences in TS activity. Two circadian studies were performed (study 1, n = 8 mice/time of day; study 2, n = 15 mice/time of day) with bone marrow and small intestinal mucosa, and in one circadian study tongue samples, procured at each of 6 equispaced times throughout a 24 hr cycle at 2, 6, 10, 14, 18 and 22 HALO.

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Received 3 April 2000; Revised 5 June 2000; Accepted 13 June 2000

Fertility cycle determinations

To determine the fertility cycle phase of each mouse at sacrifice, serial daily vaginal smears on the 4 days prior to sacrifice and immediately prior to sacrifice were obtained, fixed and stained with hematoxylin and thiazine (Diff Quick, Baxter, Deerfield, IL). Serial daily readings of cellular ratios and abundance of cornified epithelial cells, polymorphonuclear cells and noncornified epithelial cells on each slide were used to classify smears into 1 of 4 estrous cycle stages (proestrus, estrus, metestrus or diestrus) (Allen, 1922).

Tissue handling

The proximal 8 cm of the small intestine, the cecal pouch of the colon and the stomach were removed and flushed with 20 ml of ice-cold Hank's balanced salt solution (HBSS), cut longitudinally and spread luminal side up on a glass microscope slide. Mucosal preparations of these organs were obtained by scraping the inverted segments with another glass slide. Bone marrow cells were obtained from both femurs of each mouse by removing the femoral ends, flushing the cavities with ice-cold HBSS and pooling the cells from 2 femurs. Whole organs such as liver, tongue, uterus and ovary (trimmed of fat) were recovered. Tissues were stored −80°C. Frozen tissues were thawed and homogenized in fixed volumes of ice-cold 200 mM TRIS-HCl, pH 7.4 with 100 mM NaF, 20 mM beta-mercaptoethanol and a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) by mechanical homogenization using 30 complete strokes with a glass dounce homogenizer. The homogenates were centrifuged at 4°C at 12,000× g for 10–20 min, the supernatant fluid recovered, and protein content determined by the dye binding method of Bradford (Bradford, 1976).

TS catalytic assay

TS catalytic activity was determined using a tritium release assay by the method of Armstrong (Armstrong and Diasio, 1982) with modifications. TS activity was determined on freshly prepared 12,000× g tissue supernatants in a final volume of 50 μl with 35 μl 200 mM TRIS-HCl, pH 7.4, 100 mM NaF, 20 mM beta-mercaptoethanol, protease inhibitors, 5 μl of cofactor solution (6.5 mM tetrahydrofolic acid, 65 mM $NaHCO_3$, 65 mM formalin, 0.25 M beta-mercaptoethanol and 40 mM sodium ascorbate, pH 7.0) and 10 μl of 50 μM {5-^{3}H}-2′-deoxyuridine 5′-monophosphate (20 Ci/mmol; Moravek Biochemical, Brea, CA) and 25 mM CMP. Samples were incubated in triplicate at 37°C. At 2 time points, the radioactivity in the acid-soluble fraction, after acid charcoal treatment, was determined. Background counts were consistently less then 5% of total input counts and standard deviations of triplicate determinations were less then 15%.

Western blot analysis

Cyclin E levels were determined by Western blot analysis of the 12,000× g supernatants of bone marrow samples (Chu *et al.,* 1991). Equal of amounts of supernatant protein were separated by PAGE and transferred to PVDF membranes (Laemmli, 1970; Towbin *et al.,* 1979). The remaining protein binding sites were blocked (5% dry milk, 10 mm Tris, pH 7.4) and washed with TBS-0.25% Tween. Cyclin E was detected using 1:500 dilution of rabbit polyclonal anticyclin E antibody (Santa Cruz, Santa Cruz, CA). Following binding of a 1:20,000 dilution of a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL), the signal was developed by chemiluminescent detection (Pierce Super Signal kit). Quantitation was done by densitometric scanning. Samples are expressed as a ratio to a common standard sample run on all gels. Equivalency of protein loading was assessed by actin content (rabbit polyclonal anti-actin antibody; Sigma) using the method of multiple antigen detection (Krajewski *et al.,* 1996). Pellets from these bone marrow homogenates contained little, if any, cyclin E staining material.

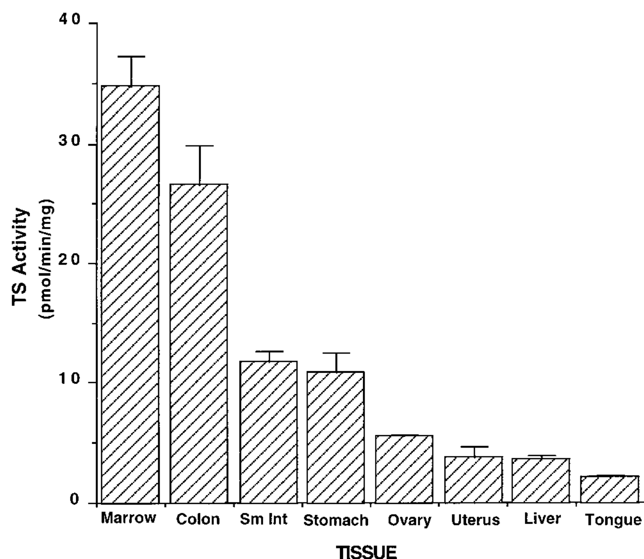


FIGURE 1 – Thymidylate synthase (TS) activity varies nearly 10-fold in different tissues of female CD_2F_1 mice measured at a single time of day. Values are mean TS catalytic activity ± standard error in 12,000× g supernatants of tissues (n = 2–23 mice) harvested during the early activity phase (14 hr after light onset, HALO). Stomach, small intestine and cecal colonic samples are mucosal preparations, while those of the liver, tongue, uterus, ovary and bone marrow are whole-organ preparations.

Statistics

Variance among mean values across more than 2 groups (e.g., 6 times of day) were contrasted using one- or two-way analysis of variance (ANOVA). Circadian time patterns were also analyzed for the presence and shape of a significant 12 hr, 24 hr and simultaneous 12 plus 24 hr rhythm using cosinor analysis (Halberg *et al.,* 1972). This analysis uses the least squares method to fit one or more cosine functions to the raw data. The rhythm weighted average value (mesor), double amplitude of the rhythm (predicted peak to trough difference), acrophase (times of day predicted when the maximum daily values are most likely to occur) are derived from this nonparametric analysis. Correlations among variables was tested by linear regression analysis (R = Pearson correlation coefficient, *p*-values). Depicted values are the mean ± one standard error for grouped data from individual tissues derived from individual mice (n = 8–17 mice/circadian time group). Results are considered statistically significant if $p < 0.05$.

RESULTS

Reliable measurement of TS activity in small tissue samples

We adapted a TS catalytic assay for small sample size and investigated the variation in tissue TS activity with overall known differences in tissue proliferation by surveying several tissues in female CD_2F_1 mice at one time of day. TS activity in murine tissues is reproducibly measured with mean values (95% confidence intervals) for bone marrow of 34.8 (29.5, 40.2), small intestinal mucosa 11.8 (9.7, 13.8) and tongue 2.2 (2.0, 2.5) pmol/min/mg protein when measured at a single time of day (early activity, 14 HALO). TS activity among different tissue types varies up to 10-fold (Fig. 1).

Circadian coordination of TS activity in normal proliferative tissues

In 2 studies, TS activity was measured throughout a full 24 hr circadian cycle in each of 3 normal tissues that usually limit the dose of fluoropyrimidine therapy, e.g., the bone marrow, small intestinal mucosa and the (oral mucosa) tongue. Small intestinal

mucosa TS activity varies 2-fold throughout the day from minimum values of 6.5 ± 1.5 and 9.5 ± 0.9 pmol/min/mg to maximum values of 12.3 ± 1.4 and 20.2 ± 2.9 pmol/min/mg. Time of day differences in small intestinal TS are significant by analysis of variance with a common time of day for maximum values in mid-daily activity phase (Table I). Cosinor analysis, concurrently fitting 12/24 hr periods, supports the existence of a small 12 hr component and a more robust 24 hr component with peak values predicted at 18:45 HALO (mid-activity phase) and a second minor peak at 7:00 HALO (mid-sleep phase; Table 1, Fig. 2).

From a single study (study two), TS activity was also quantitated in another alimentary tissue, the tongue, from the same mice whose TS rhythms in bone marrow and small intestinal mucosa had been defined. Tongue TS activity varies nearly 2-fold throughout the day from a minimum of 2.2 ± 0.1 to a maximum of 4.1 ± 0.5 pmol/min/mg in a single study. Time of day differences in tongue TS are significant by analysis of variance and cosinor analysis with both robust 12 hr and 24 hr components, which are best modeled by a simultaneous 12/24 hr period fit (Table I, Fig. 2). Maximum daily values occur both in late sleep (9:00 HALO) and again in late activity (21:00 HALO).

Bone marrow TS activity varies nearly 2-fold throughout the day from a minimum of 31.6 ± 5.9 and 24.5 ± 5.0 pmol/min/mg to a maximum of 42.6 ± 2.4 and 50.6 ± 4.9 pmol/min/mg. These time of day differences in bone marrow TS are significant by analysis of variance. Cosinor analysis supports the presence of a rhythm in both studies for a 12 hr period fit and for the simultaneous fit of 12/24 hr periods with maximum daily values in late sleep (9:30 HALO) and again in late activity (23:00 HALO) (Table I, Fig. 2). There are no differences throughout the day in tissue supernatant protein yield for each tissue, bone marrow cell number or organ wet weight.

From the results of the 2 studies combined, using simultaneous 12/24 hr period fits, the daily pattern of variation in TS activity is fairly similar in the bone marrow and oral mucosa with 2 peaks each day in late sleep and late activity (Fig. 2). The small intestinal mucosa shows a slightly different circadian pattern of TS activity with one more predominant peak each day in the late activity phase. All 3 tissues show near maximal levels of TS in the late activity phase of the circadian sleep-wake cycle. Other times of day demonstrate divergence of TS activity patterns among these tissue types.

Fertility cycle effects upon TS activity

Pooled results from the 2 studies of the bone marrow and small intestinal mucosa were assessed by two-way analysis of variance for both circadian effects (6 times of day) and estrous cycle stage (4 cycle stages) effects upon TS activity (24 cells with sample size range of 3–11 per group). The time of day effects upon TS activity were highly significant in each tissue ($p < 0.05$). The phase of the estrous cycle had no effect upon TS activity in bone marrow or in small intestine. There was no significant interaction between the time of day and stage of the estrous cycle.

Covariation of bone marrow TS activity and proliferation throughout the day

Cyclin E is known to be required and rate limiting for entry of cells into S phase of the cell cycle. The expression of cyclin E has been shown to peak at the G_1-S phase transition and decay rapidly during early S phase (Dulic *et al.,* 1992; Koff *et al.,* 1992). The circadian variation in cyclin E protein content, used as a marker of G_1/S phase, was quantitated by Western blot analysis in the same bone marrow preparations from study two. A 5-fold variation throughout the day in bone marrow cyclin E protein content was observed (Fig. 3, ANOVA $p = 0.034$). The rhythm in bone marrow cyclin E protein is best described by a simultaneous 12/24 hr period fit by cosinor analysis ($p = 0.016$) with peak values in the middle of the sleep phase (6:00 HALO) and again in the middle of the activity phase (17:00 HALO). These 2 peaks in bone marrow cyclin E content precede the 2 TS activity peaks by several

TABLE I – THYMIDYLATE SYNTHASE ACTIVITY IN NORMAL PROLIFERATIVE TISSUE THROUGHOUT THE DAY

Tissue	Study	Time of day (HALO) TS activity (pmol/min/mg)						Ratio of change	Analysis					Peak times (HALO)
		Sleep phase			Activity phase				ANOVA		Cosinor (*p*-values)			
		2	6	10	14	18	22		*p*	F	12 hr	24 hr	12/24 hr	
Small Intestine	1	9.6 ± 0.9	11.8 ± 1.3	6.5 ± 1.5	12.3 ± 1.4	10.1 ± 1.3	8.8 ± 1.1	1.9	0.030	2.8	0.040	0.650	0.120	
Small Intestine	2	10.5 ± 2.8	9.5 ± 0.9	11.7 ± 1.9	11.5 ± 1.4	20.2 ± 2.9	16.7 ± 2.3	2.1	0.004	3.8	0.260	0.002	0.002	
SI Combined	1,2	8.8 ± 1.2	10.3 ± 0.8	8.9 ± 1.0	11.8 ± 1.0	16.1 ± 2.1	13.9 ± 1.7	1.8	<0.001	4.5	0.190	<0.001	<0.001	7:00, 18:45
Tongue	2	2.7 ± 0.2	3.2 ± 0.2	4.1 ± 0.5	2.2 ± 0.1	3.2 ± 0.3	3.7 ± 0.1	1.9	<0.001	6.1	<0.001	0.560	<0.001	9:00, 21;00
Bone Marrow	1	32.0 ± 2.8	42.6 ± 2.4	41.9 ± 6.9	31.6 ± 5.9	39.9 ± 2.8	42.4 ± 4.7	1.4	0.28	1.3	0.020	0.670	0.073	
Bone Marrow	2	48.2 ± 4.0	40.4 ± 4.7	50.4 ± 4.8	36.5 ± 2.5	24.5 ± 5.0	50.6 ± 4.9	2.1	<0.001	5.0	0.002	0.031	<0.001	
BM Combined	1,2	42.6 ± 3.2	41.2 ± 3.1	47.4 ± 3.9	34.8 ± 2.6	30.7 ± 3.6	47.6 ± 3.6	1.6	0.002	4.0	0.003	0.045	<0.001	9:30, 23:00

Values are means from individual mouse tissue determinations (n = 8–17 mice/HALO) (pmol/min/mg) ± standard error. Time of day is expressed as hours after light onset (HALO). Peaks in mean value(s) throughout the day are underlined. Analyses are from analysis of variance (ANOVA) for time of day effect and cosinor analysis for the presence of a rhythm using a 12 hr, 24 hr or a simultaneous 12/24 hr period fit. Ratio of change is the highest mean value divided by the lowest mean, as a measure of the mean fold differences throughout the day. Peak times are times of day (in HALO) predicted for peak values of TS from the 12/24 hr period fit (see Fig. 2). SI = small intestine, BM = bone marrow.

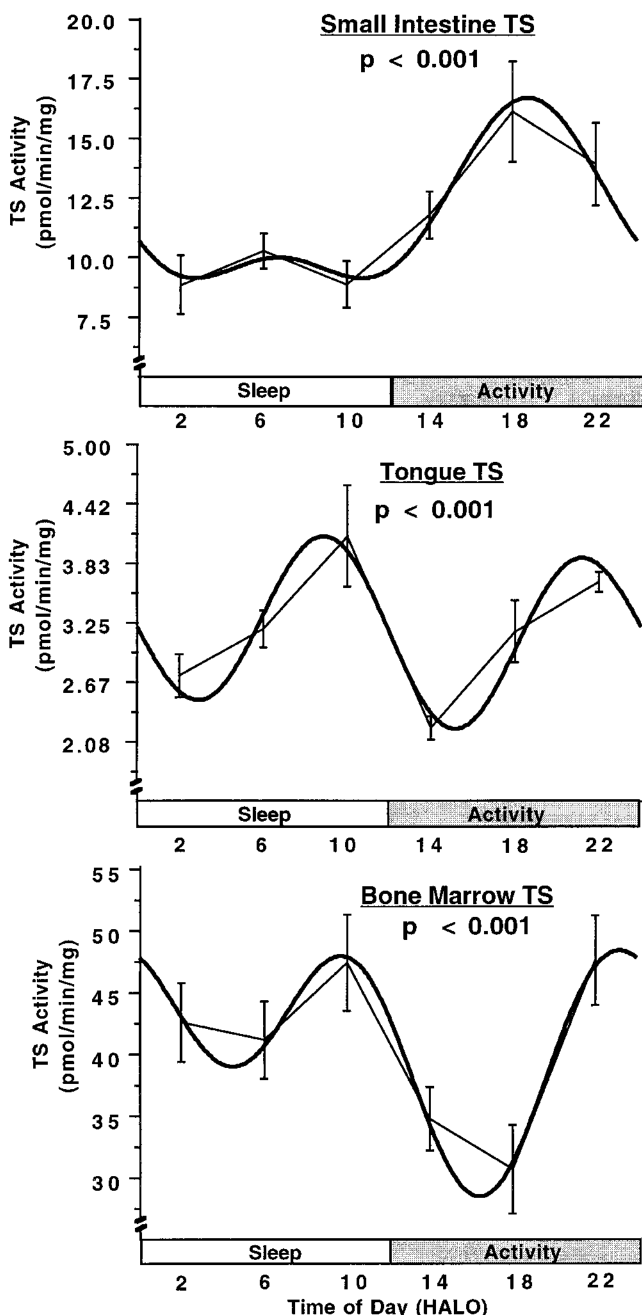


FIGURE 2 – TS activity throughout the day in normal proliferative tissues of CD_2F_1 mice. Mean values of TS activity ± standard errors) are shown at each of 6 different times of day in the small intestinal mucosa and bone marrow from 2 studies and tongue from 1 study. The predicted curves and *p*-values from cosinor analysis are shown for simultaneous 12/24 hr period fit. These normal tissues show up to 2-fold differences in TS activity throughout the day with 2 peaks observed, often unequal in magnitude, per 24 hr cycle.

hours. The percentage of bone marrow cells in S phase of the cell cycle, by flow gated DNA content in this same strain of female CD_2F_1 mice, varies significantly throughout the day with a lower amplitude of 1.2- to 1.3-fold differences. Peak values in bone marrow S phase fraction are seen in the late sleep phase (10:10 HALO) and again in the late activity phase (22:10 HALO, Fig. 3). The 2 daily peaks in S phase predominance of the bone marrow are preceded in time, by about 4 to 5 hr, by the two peaks in the G_1-S phase marker cyclin E protein, both of which support the circadian coordination of bone marrow proliferation throughout the day. The circadian rhythm in bone marrow TS, with 2 peaks in activity per day (9:30 and 23:00 HALO), co-varies with these rhythms in proliferation with the best correlation being with the percent S-phase fraction of the marrow (linear correlation of mean marrow TS activity and mean percent S phase at each circadian time, R = 0.960, $p = 0.0023$).

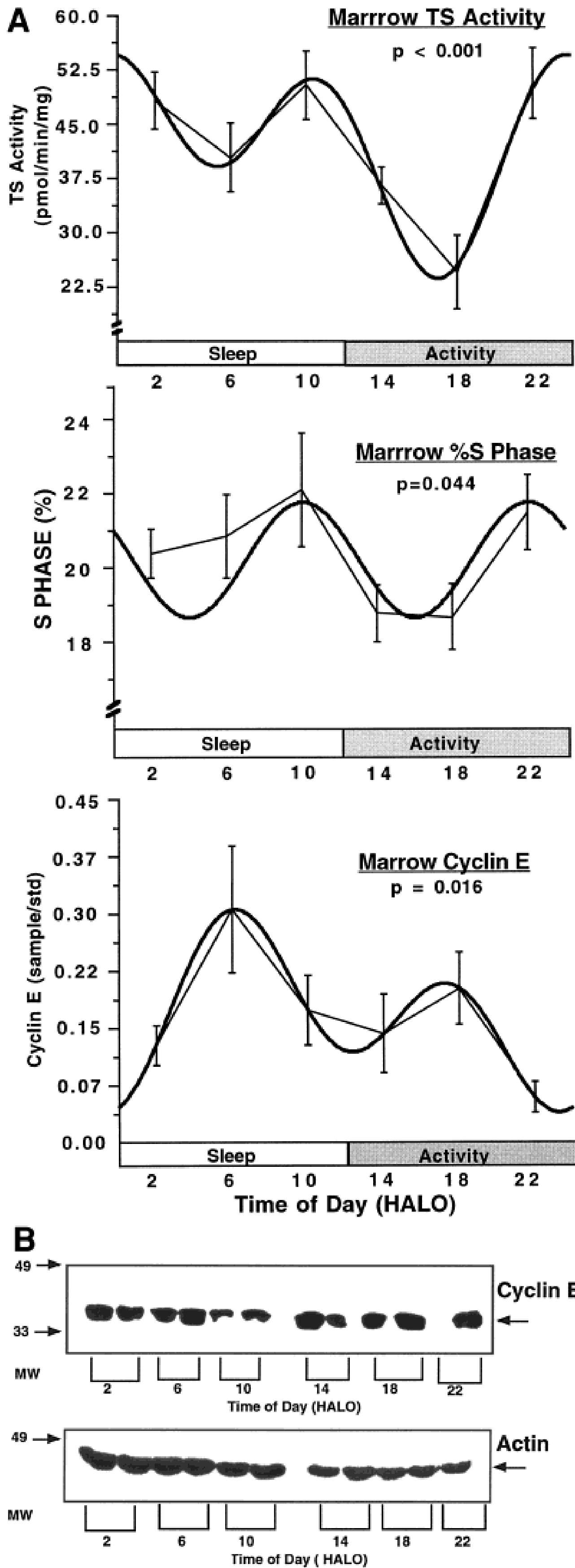
DISCUSSION

Ten-fold differences in TS activity characterize different normal tissues. Tissue TS activity roughly parallels the proliferative activity reported for these tissues with higher TS being associated with tissues with higher S-phase fraction (Burns *et al.,* 1983) and the relative sensitivity of that tissue to 5-FU damage. In each tissue that is sensitive to fluoropyrimidine drug effects, TS activity varies nearly 2-fold within each day. In the bone marrow *in vivo,* the circadian pattern in TS activity tightly parallels the circadian rhythm in the proportion of these cells in S phase of the cell cycle and is preceded in time by a similar rhythm in the G_1-S phase marker cyclin E protein content. The basis for the circadian variation in TS activity may therefore be explained, in part, by the circadian coordination of tissue proliferation. These data are the first to demonstrate the circadian coordination of a medically important cytotoxic drug target, TS activity, in each of the target tissues that limit the achievable 5-FU dose intensity. This circadian organization of the primary 5-FU molecular target is likely to explain, at least in part, why fluoropyrimidine timing, within the day, determines its toxic-therapeutic ratio (Lévi *et al.,* 1997).

Fertility cycle-stage effects on 5-FU toxicity have been reported (Hrushesky *et al.,* 1999; Wood and Hrushesky, 1997). We did not, however, see significant effects of estrous cycle stage on TS activity in the bone marrow or small intestinal mucosa. Our sample size and the distribution of estrous stages across all times of day, however, are not entirely balanced, therefore, further studies are warranted in order to confirm this lack of effect of fertility cycle stage upon TS activity in these 2 tissues.

In vitro differences in TS activity of 30% to 100% have been associated with 2- to 8-fold differences in IC_{50} concentrations of 5-FU or FdUrd (Johnston *et al.,* 1992; Ju *et al.,* 1998). Thereby, these 2-fold circadian TS differences seen *in vivo* may easily explain the 2- to 8-fold circadian differences in 5-FU toxicity seen in randomized clinical trials. Furthermore, in clinical studies comparing groups of cancer patients whose tumors respond to 5-FU with those who do not, mean differences in tumor TS values (FdUMP [5′-fluoro-2′deoxyuridine-5′-monophosphate] ligand binding, TS protein or TS mRNA) have most consistently been on the order of 2- to 4-fold (Johnston *et al.,* 1995; Leichman *et al.,* 1997; Lenz *et al.,* 1995). It remains to be determined whether the absolute tissue levels of TS activity and/or the extent of TS inhibition by 5-FU and/or the subsequent extent of TS protein induction following 5-FU will best predict and more fully explain the circadian differences in normal and tumor tissue fluoropyrimidine sensitivity.

Previous studies, both *in vitro* and *in vivo* systems, have demonstrated increasing fluoropyrimidine resistance with increased TS activity under spontaneous or nondrug pressured conditions. However, there does not appear to be a simple 1 to 1 relationship between relative differences in TS activity and differential sensitivity to fluoropyrimidine drugs in these settings nor with the *in vivo* circadian studies reported here (Chu and Allegra, 1996). Although the exact basis for this complex relationship has yet to be fully defined, possible explanations might include the secondary effects of translational and post-translational controls over TS activity levels and potential contributions of non-TS-based mechanisms (DNA, RNA effects) of fluoropyrimidine toxicity. *In vivo,* the relationship between the level of tissue drug target and that of the tissue drug effect may also be complex because of different cell types with differing characteristics within the same tissue.



Circadian coordination of S phase has been demonstrated in genetically diverse humans in tissues of the bone marrow, rectal mucosa and oral mucosa with persistence of the circadian proliferation rhythm even in the fasting state (Bjarnason *et al.,* 1999; Buchi *et al.,* 1991; Smaaland *et al.,* 1991). Cell cycle-specific protein markers, such as cyclin E, in oral mucosa of genetically diverse human beings (Bjarnason *et al.,* 1999), and as demonstrated here in mouse bone marrow, also vary throughout the day. Based upon these clinical findings, our current work and the proliferation-dependent coordination of TS gene expression, one would expect proliferative tissues in humans to demonstrate circadian coordination of TS activity. Preliminary studies in human oral mucosa demonstrate a circadian rhythm in TS activity that also parallels the circadian rhythms in G_1-S phase markers and support this conclusion (Bjarnason *et al.,* 1999, 2000). Whether the circadian TS patterns in normal tissues from tumor-bearing hosts are similar to or differ from the circadian patterns of TS in normal tissues from nontumor-bearing hosts is not known. It is not clear to what extent these findings are generalizable to tumor tissues in preclinical rodent models and spontaneous human cancers. More limited numbers of studies demonstrate that some murine and human cancers, in fact, do maintain circadian coordination of DNA synthesis and cellular division (Hrushesky *et al.,* 1998; Klevecz *et al.,* 1987; Smaaland *et al.,* 1993).

Seasonal rhythms in not only reproductive functions but also in nonreproductive tissue function have been described in rodents and humans. Those relevant to our current work include human seasonal susceptibility to the hematopoietic toxicity of chemotherapy (Hrushesky, 1982) and the seasonal effects upon the overall levels and the circadian pattern or phasing of circadian rhythms in parameters of tissue DNA synthesis in bone marrow and gastrointestinal tissue in rodents and humans (Haus *et al.,* 1984; Laerum *et al.,* 1988; Sothern *et al.,* 1995). Our 2 studies were not performed at the exact same time of the year, therefore it is possible that some of the variability between our studies might reflect seasonal effects upon TS circadian patterns. Seasonal effects on these circadian tissue TS activity patterns can only be evaluated by completing many more of these studies at equi-spaced times throughout each of several years.

Differences among tissues in their circadian patterns of TS activity, such as we observed here in the mouse, might be explained by the known differences among normal tissue circadian S-phase patterns. Thus different normal tissues, in the mouse and in human beings, can be expected to and do demonstrate different times of day for peak values in DNA synthesis. The control of TS gene expression is complex and occurs at multiple levels including transcriptional, post-transcriptional and translational mechanisms (Chu *et al.,* 1991; Johnson, 1994). It is not known at what level these circadian differences in TS activity are controlled. Much of the work describing the control of TS gene expression has been done in cell lines *in vitro*. Our data demonstrate that circadian

FIGURE 3 – (*a*) Bone marrow samples were analyzed for both TS activity and cyclin E protein content (Western blot analysis) in samples from study 2 and in an additional study in CD_2F_1 mice for S-phase fraction (DNA content by flow cytometry, modified from Wood *et al.,* 1998). Values are the means ± standard error. The predicted curves and *p*-values from cosinor analysis are shown for simultaneous 12/24 hr period fit. The 2-fold daily variation in bone marrow TS activity parallels a 20% to 30% difference in the daily rhythm in bone marrow S-phase fraction, as supported by linear regression of mean values of the 2 endpoints at each time of day ($R = 0.96$, $p = 0.0023$) and similar predicted times of day for peak values by cosinor analysis. Bone marrow cyclin E protein content varies 5-fold throughout the day with the times of day of peak values, predicted by cosinor analysis, just preceding in time those for bone marrow TS activity and S-phase fraction. (*b*) A Western blot of bone marrow samples (2 samples at each time of day except for 1 sample for 22 HALO) stained for cyclin E and then reprobed and stained for actin as a loading control.

dynamic control may be used to better understand the *in vivo* control of this critical gene.

As we begin to understand the molecular basis for the circadian schedule-dependent advantages in fluoropyrimidine therapeutic index, we will also be able to enhance the toxic-therapeutic ratios of new drugs that target TS. At least 6 new cytotoxic anticancer drugs directly targeting TS have recently entered our chemotherapeutic armamentarium (Brandt and Chu, 1997). As *"primum non nocere"* is the first tenet of medicine, these 2-fold circadian differences in the availability of the molecular target of these agents must be carefully considered in fully optimizing the benefits and minimizing the toxicities of each of these important new drugs as well as 5-FU.

ACKNOWLEDGEMENTS

This study was supported in part by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs (PAW, WJMH), Robison Family Foundation and National Institutes of Health grants RO1 CA31635 and RO1 CA50749 (WJMH). We thank Frank Maley (NY State Laboratories) for advice in establishing TS catalytic assays and Robert Clark for technical assistance. This work is dedicated to DWL, WC, AD and their survivors.

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