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CIRCADIAN RHYTHM OF HEPATIC URIDINE PHOSPHORYLASE ACTIVITY AND PLASMA CONCENTRATION OF URIDINE IN MICE

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Abstract—The activity of hepatic uridine phosphorylase (EC 2.4.2.3) in male mice (24–29 g) maintained in standardized conditions of 12 hr light (0600–1800 hr) alternating with 12 hr darkness (1800–0600 hr), food and water *ad lib.*, exhibited a circadian rhythm ($P < 0.0001$, Cosinor analysis). The peak of enzyme activity (559 ± 25 pmol/min/mg protein) occurred at 15 hr after light onset (HALO) with the nadir (139 ± 25 pmol/min/mg protein) at 3 HALO when samples were taken every 4 hr. Female mice showed essentially the same pattern. A circadian rhythm ($P < 0.0001$, Cosinor analysis) was also observed when the light–dark cycle was shifted (reverse cycle) so that the lights went on at 2200 hr and off at 1000 hr. Under the reverse cycle condition, there was a corresponding shift in the enzyme activity with a lag period of 3.5 hr in the time of maximum and minimum enzyme activities (i.e. the peak at 11 HALO and the nadir at 23 HALO) after a 2-week adaptation period. The lag period was reduced to 1 hr after 4 weeks of adaptation, and no further change was observed after 6 weeks of adaptation. The plasma concentration of uridine also exhibited a circadian rhythm ($P < 0.0001$, Cosinor analysis) with peak concentration (10 μM) occurring at 2 HALO and a nadir (5 μM) at 14 HALO. The circadian rhythm observed in the plasma concentration of uridine is the inverse of that for uridine phosphorylase activity. These results demonstrate that hepatic uridine phosphorylase plays an important role in the regulation of the uridine level in the blood which, in turn, may be involved in the humoral control of sleep by uridine. This may also be of clinical significance in enhancing the antitumor efficacy of the 5-fluorinated pyrimidines by modulating the time of their administration.

Hepatic uridine phosphorylase (EC 2.4.2.3) plays an important role in the metabolism of pyrimidines. It modulates the salvage pathway by anabolizing or catabolizing various pyrimidine nucleosides and their analogues [1–3]. It is also involved in the regulation of the levels of uridine in the plasma [4–6]. Furthermore, uridine phosphorylase is responsible for the degradation of several chemotherapeutic pyrimidine nucleoside analogues, in particular the 5-fluorinated pyrimidines, thus reducing their effectiveness [1, 3, 7–9]. Inhibitors of uridine phosphorylase have been shown to (a) increase the concentration and duration of uridine in plasma [4, 5, 10, 11] and heart perfusate [12], (b) increase the salvage of uridine by various tissues [5, 6, 10, 11], (c) increase the level of 5-fluoro-2′-deoxyuridine (FdUrd‖) in liver perfusates [13] and enhance its efficacy against human tumors [9, 14, 15], and (d) protect against 5-fluorouracil (FUra) [5, 6, 10] and FdUrd [16] toxicities.

Because of the importance of uridine phosphorylase in the regulation of pyrimidine metabolism and its role in cancer chemotherapy with pyrimidine analogues, we are interested in studying the physiological as well as the physical and kinetic properties of this enzyme. In the course of our attempts to purify the enzyme from mouse liver, we noticed that the extent of enzyme activity was dependent on the time the animals were killed. Therefore, it was of interest to determine the pattern of uridine phosphorylase activity in mice over a period of 24 hr and how this would affect the concentration of uridine in the plasma. We found that hepatic uridine phosphorylase activity, and plasma concentration of uridine, followed a circadian rhythm. This circadian rhythm may be involved in the humoral control of sleep by uridine and may also be of particular significance in modulating the therapeutic regimens with fluorinated pyrimidines. Preliminary reports have been presented [17, 18].

MATERIALS AND METHODS

Chemicals. [2-^{14}C]Uridine (56 Ci/mol) was obtained from Moravek Biochemicals Inc. (Brea, CA); Omnifluor scintillant from the New England Corp. (Boston, MA); silica gel G/UV$_{254}$ polygram thin-layer chromatography (TLC) plates from Brinkmann (Westbury, NY); and the protein assay kit from Bio-Rad Laboratories (Richmond, CA). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animals. Female and male CD-1 mice (Charles River Laboratories, Wilmington, MA) weighing 24–29 g were used in all experiments.

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‖ Abbreviations: FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; and HALO, hours after light onset.

Light cycles. Animals were housed in group cages with food and water *ad lib.* under the "normal light cycle" (light, 0600–1800 hr; dark, 1800–0600 hr). In the "reverse light cycle," the light–dark cycle was changed (light, 2200–1000 hr; dark, 1000–2200 hr).

Preparation of samples. Every 4 hr (at 1000, 1400, 1800, 2200, 0200 and 0600 hr) a group of five mice was anesthetized by inhalation of Metofane. For plasma uridine analysis, whole blood was collected by cardiac puncture with a 1-cc syringe and a 20-gauge needle. The blood thus obtained was mixed with 0.1 vol. of 3.8% sodium citrate and allowed to stand for 20 min, after which it was centrifuged for 5 min at 3000 rpm. The acid-soluble material in the serum obtained was extracted with 1 vol. of 1 N trioctylamine in 1,1,2-trichlorotrifluoroethene (Freon) and stored at −20° until analysis by HPLC. For determination of uridine phosphorylase activity, livers were removed, weighed, minced and homogenized in 3 vol. of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 1 mM EDTA using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenates were stored at −80° until all samples were collected, after which uridine phosphorylase activity was determined.

Analysis of uridine concentration in the plasma. Uridine was assayed by an HPLC method previously reported [5], utilizing a Rainin "Rabbit" dual pump system equipped with a Gilson model 112 fixed wavelength (254 nm) UV detector. The column was a Rainin Microsorb C-18 (25 cm × 4.6 mm) maintained at 8° with a mobile phase of 10 mM phosphoric acid containing 30 μM heptane sulfonic acid and adjusted to pH 3.1 with NaOH. Plasma extracts (200 μL) were injected with a Gilson model 231 automated sample processor, and the column was eluted at 1 mL/min. The retention time of uridine was 24.5 min.

Uridine phosphorylase assay. All assays were run at 37° under conditions where activity was linear with time and enzyme concentration. Nucleoside cleavage was measured isotopically by following the formation of uracil from uridine as previously described [2]. The reaction mixture contained 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 1 mM uridine (6 Ci/mol) and 25 μL of enzyme in a final volume of 50 μL. Reactions were started by the addition of extract, incubated for 30 min, and stopped by boiling in a water bath for 2 min followed by freezing. Precipitated proteins were removed by centrifugation. A 5-μL aliquot of the supernatant fluid was spotted on a silica gel TLC plate prespotted with 5 μL from a 10 mM solution of unlabeled carriers. Uridine was separated from uracil after the plates were developed with chloroform : methanol : acetic acid (90 : 5 : 5, by vol.). The R_f values were: uridine, 0.07; and uracil, 0.43. The amounts of radioactivity in uridine and uracil spots were determined on percentage bases using a Berthold LB-2821 Automatic TLC-Linear Analyzer.

Protein determination. Protein concentrations were determined spectrophotometrically by the method of Bradford [19] using bovine γ-globulin as a standard.

Statistical analysis. The data were analyzed by the Cosinor method [20] where the data were fitted to a cosine wave by the least-squares method. Four parameters were calculated: the mesor (rhythm-adjusted mean), the amplitude (maximum or minimum value from the mean), the acrophase (time of maximum or minimum value), and the period (length of one complete cycle).

RESULTS

In male mice maintained in standardized conditions of 12 hr light (0600–1800 hr) alternating with 12 hr darkness (1800–0600 hr), there was a 24-hr circadian cycle of uridine phosphorylase activity in the liver homogenates when samples were taken every 4 hr. Table 1 shows the Cosinor analysis. The peak of enzyme activity occurred at approximately 15 hr after light onset (HALO) and the nadir at 3 HALO. The maximal enzyme activity (559 ± 25 pmol/min/mg protein) was 4-fold higher than the minimal enzyme activity (139 ± 25 pmol/min/mg protein) (Table 1). Female mice showed essentially the same pattern (Table 1). Shifting the light–dark cycle by 16 hr, so that the lights went on at 2200 hr and off at 1000 hr resulted in a corresponding shift in the enzyme activity (Table 2 and Fig. 1). The peak activity occurred at approximately 11 HALO and the nadir at 23 HALO after a 2-week adaptation period (Table 2). However, there was a lag period of 3.5 hr in the time at which the maximum and minimum activities were observed. Extension of the adaptation period to 4 weeks reduced this lag period to 1 hr, i.e. maximum activity occurred around 14 HALO and minimum around 2 HALO (Table 2). Further adaptation up to 6 weeks did not reduce or abolish this 1-hr lag period (Table 2 and Fig. 1). In general, there was no significant difference in maximum and minimum enzyme activities between normal and reverse cycle after 2-, 4- or 6-week adaptation periods (Table 2).

Table 3 shows that the plasma concentrations of uridine also followed a circadian rhythm ($P < 0.0001$, Cosinor analysis). The time of maximum concentration of uridine (10 μM) occurred at 2 HALO, while the lowest concentration (5 μM) occurred at 14 HALO (Table 3). The highest concentration of uridine exceeded the minimum by approximately 2-fold (Table 3). Figure 2 shows the circadian pattern of plasma uridine concentrations and its relationship to that of hepatic uridine phosphorylase activities measured from the same mice. The circadian rhythm of plasma uridine concentrations was the inverse of the circadian rhythm of hepatic uridine phosphorylase activity. The maximum plasma uridine concentration coincided with the nadir of uridine phosphorylase activity at approximately 0800 (2 HALO), while the lowest concentration occurred at 2000 hr (14 HALO) around the time when uridine phosphorylase activity was highest.

DISCUSSION

The present study clearly demonstrates that there is a circadian rhythm for uridine phosphorylase activity in mouse liver with peak enzyme activity at approximately 14–15 HALO and the nadir at 2–3

Table 1. Rhythmometric values of single Cosinor analysis of hepatic uridine phosphorylase in male and female mice under normal cycles of light and darkness (light, 0600–1800 hr; dark, 1800–0600)*

Sex	Mesor† ± SE	Amplitude‡ ± SE	Maximum activity§ ± SE	Time of maximum activity‖ ± SE	Minimum activity¶ ± SE	Time of minimum activity‖ ± SE	r^2	P
Males	350 ± 15	210 ± 21	560 ± 25	15.0 ± 0.4	140 ± 25	3.0 ± 0.4	0.80	< 0.0001
Females	396 ± 33	313 ± 47	709 ± 58	14.4 ± 0.6	83 ± 58	2.4 ± 0.6	0.74	< 0.0001

* Activities (pmol/min/mg protein) were determined in thirty animals in each group.
† Rhythm-adjusted mean from thirty animals in each group.
‡ Maximum or minimum value from the mean.
§ Calculated by adding the amplitude to the mesor (rhythm adjusted mean).
‖ Calculated from the acrophase parameter of the Cosinor analysis and expressed as HALO (hours after light onset).
¶ Calculated by subtracting the amplitude from the mesor (rhythm adjusted mean).

Table 2. Rhythmometric values of single Cosinor analysis of uridine phosphorylase activities in mouse liver under normal (light, 0600–1800 hr; dark, 1800–0600) and reverse (light, 2200–1000 hr; dark, 1000–2200 hr) cycles of light and darkness after different periods of adaptation*

Adaptation period	Cycle	Mesor† ± SE	Amplitude‡ ± SE	Maximum activity§ ± SE	Time of maximum activity‖ ± SE	Minimum activity¶ ± SE	Time of minimum activity‖ ± SE	r^2	P
Two weeks	Normal	430 ± 24	388 ± 34	818 ± 41	14.3 ± 0.3	42 ± 41	2.3 ± 0.3	0.83	<0.0001
	Reverse	416 ± 38	309 ± 54	725 ± 66	10.9 ± 0.7	107 ± 66	22.9 ± 0.7	0.55	<0.0001
Four weeks	Normal	386 ± 22	276 ± 31	662 ± 38	14.3 ± 0.4	110 ± 38	2.3 ± 0.4	0.74	<0.0001
	Reverse	431 ± 28	369 ± 40	800 ± 49	13.5 ± 0.4	62 ± 49	1.5 ± 0.4	0.76	<0.0001
Six weeks	Normal	440 ± 27	384 ± 38	824 ± 47	14.4 ± 0.6	56 ± 47	2.4 ± 0.4	0.79	<0.0001
	Reverse	449 ± 26	354 ± 37	803 ± 45	12.6 ± 0.4	95 ± 45	0.7 ± 0.4	0.77	<0.0001

* Activities (pmol/min/mg protein) were determined in thirty animals in each group.
† Rhythm-adjusted mean from thirty animals in each group.
‡ Maximum or minimum value from the mean.
§ Calculated by adding the amplitude to the mesor (rhythm adjusted mean).
‖ Calculated from the acrophase parameter of the Cosinor analysis and expressed as HALO (hours after light onset).
¶ Calculated by subtracting the amplitude from the mesor (rhythm adjusted mean).

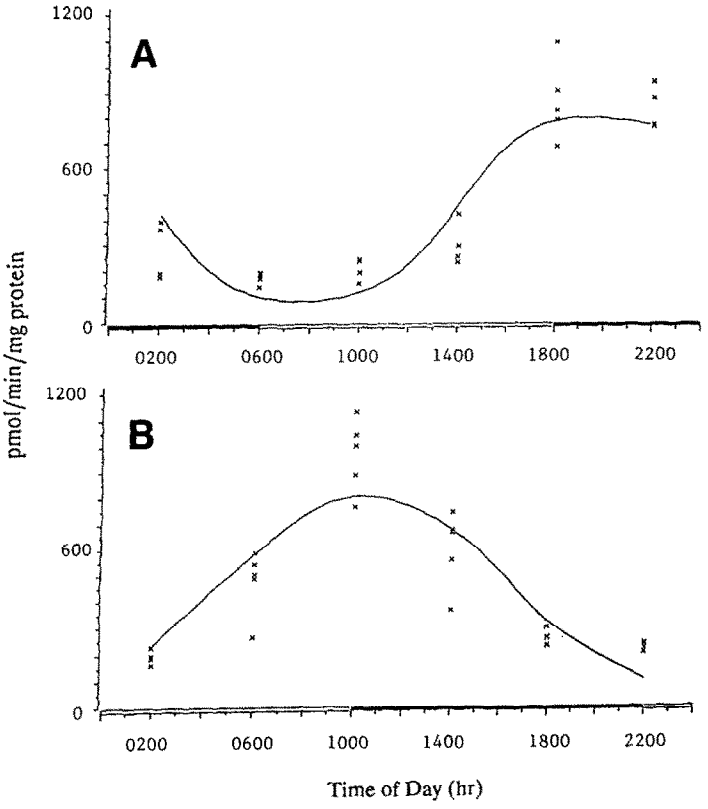

Fig. 1. Pattern of circadian rhythm of hepatic uridine phosphorylase from mice kept for 6 weeks under cycles of 12 hr light alternating with 12 hr darkness with food and water *ad lib.* (A) "normal" light cycle (light, 0600–1800 hr; dark 1800–0600 hr). (B) "reverse" light cycle (light, 2200–1000 hr; dark, 1000–2200 hr). Each data point represents three determinations of uridine phosphorylase activity in the liver homogenate of one mouse. The curve is a bestfit computer-generated cosine curve as determined by the Cosinor method [20].

HALO (Tables 1 and 2, and Fig. 1). Reversing the light–dark cycle resulted in a corresponding shift in the enzyme activity. Approximately a 4-week acclimatization period to new light–dark cycle conditions was required to achieve near complete reversal of the circadian pattern of uridine phosphorylase. The circadian rhythm of uridine phosphorylase described here is the first report on rhythmical variation of any of the nucleoside phosphorylase activities.

At the present time it is not known whether the circadian variation in uridine phosphorylase activity reflects changes in the total amount of enzyme, the presence of inhibitory material in the extract, or changes in the kinetic properties of the enzyme. Preliminary studies, however, suggest that the circadian rhythm of uridine phosphorylase may be hormonally regulated. Administration of hydrocortisone 6 hr prior to killing the mice resulted in increased uridine phosphorylase activity and a shift in the time of peak activity when compared to untreated controls (Niedzwicki JG and el Kouni MH, unpublished results).

The present results also establish that the plasma concentration of uridine exhibits a circadian rhythm and that the peak concentration of plasma uridine occurred around 2–3 HALO, the period when uridine phosphorylase activity was at a nadir, and the minimum concentration occurred at 14–15 HALO when the enzyme activity was highest (Tables 1–3 and Fig. 2).

These findings have led us to postulate that the circadian rhythm of hepatic uridine phosphorylase and that of uridine concentration in the plasma may be involved in the humoral control of sleep. Uridine is an important metabolite for the normal function of the brain and much of the adult brain's requirement for uridine is known to be satisfied by uridine transported from the blood [21, 22]. Furthermore, it has been reported that uridine, but not uracil, is one of the active components of sleep-promoting substances [for a review see Ref. 23]. Thus, our findings suggest that the circadian rhythm of uridine phosphorylase may play an important role in regulating the levels of uridine in the brain as well as in the blood, and that this fluctuation of uridine levels in the brain, in turn, may be intimately connected with the humoral control of sleep. It is interesting to note that mice are nocturnal animals and rest or "sleep" in the light and that the peak of uridine concentration in the plasma was observed at 2 HALO, a short period after the onset of light (0600 hr) and around the time at which uridine phosphorylase activity was at its minimum. These results suggest that uridine phosphorylase inhibitors, e.g.

Table 3. Rhythmometric values of single Cosinor analysis of the plasma concentration of uridine in mice under normal (light, 0600–1800 hr; dark, 1800–0600) cycles of light and darkness*

Compound	Mesor† ± SE	Amplitude‡ ± SE	Maximum activity§ ± SE	Time of maximum activity‖ ± SE	Minimum activity¶ ± SE	Time of minimum activity‖ ± SE	r^2	P
Uridine	7.7 ± 0.3	2.5 ± 0.5	10.2 ± 0.6	2.2 ± 0.8	5.2 ± 0.6	14.2 ± 0.8	0.52	< 0.0001

* Activities (μM) were determined in thirty animals in each group.
† Rhythm-adjusted mean from thirty animals.
‡ Maximum or minimum value from the mean.
§ Calculated by adding the amplitude to the mesor (rhythm adjusted mean).
‖ Calculated from the acrophase parameter of the Cosinor analysis and expressed as HALO (hours after light onset).
¶ Calculated by subtracting the amplitude from the mesor (rhythm adjusted mean).

benzylacyclouridine [1–3, 14, 15, 24], may prove useful in regulating sleep and disorders of circadian phase such as "jet-lag" by controlling the concentration of plasma uridine.

Furthermore, modulation of the concentration of plasma uridine by the circadian rhythm of uridine phosphorylase could also reflect on cell functions that are regulated via the pyrimidine receptors which have been reviewed recently [25], and uridine phosphorylase inhibitors could help in studying and further understanding the role of these receptors.

The role of circadian rhythms in drug bioavailability and efficacy in cancer chemotherapy has attracted a great deal of attention in recent years [26, 27]. It has been shown that the efficacy, toxicity and bioavailability of several anticancer drugs, most notably the 5-fluorinated pyrimidines, vary drastically over a 24-hr cycle [28–32]. However, the mechanism underlying these 24-hr cyclic variations is not fully understood. These variations could be attributed to alteration in activities of enzymes involved in the inactivation or activation of these drugs such as uridine phosphorylase and/or to fluctuation in plasma uridine concentration which is known to affect the efficacy and prevent the toxicity of these drugs [5, 6, 11, 16]. It has been recognized that the liver is the main organ responsible for the catabolism of pyrimidines, and that hepatic uridine phosphorylase plays a major role in the catabolism of pyrimidine nucleosides and their analogues as well as the regulation of plasma uridine concentrations [4–6, 13, 33–37].

The circadian rhythm of uridine phosphorylase described in the present study is the inverse of what was reported for the efficacy of FdUrd in rats [31], i.e. high uridine phosphorylase activity corresponds to low FdUrd efficacy. This suggests that uridine phosphorylase is involved in FdUrd degradation and hence reduces its efficacy. This proposition is given further credence by our finding that the activity of thymidine phosphorylase, the other pyrimidine nucleoside phosphorylase involved in the cleavage of FdUrd, does not follow circadian variations [18]. Therefore, uridine phosphorylase inhibitors may enhance the efficacy of nucleoside analogues administered by continuous infusion by preventing their degradation and maintaining a constant chemotherapeutic concentration of the drugs in the plasma. This rationale is supported by our recent findings [18] and those of others [30, 38] showing that the activity of dihydrouracil dehydrogenase (EC 1.3.1.2), the enzyme responsible for the degradation of FUra, also exhibits a circadian variation. The circadian variation of dihydrouracil dehydrogenase [18, 30] is the inverse of that of plasma concentration of FUra in patients [28, 30] and rats [29] receiving continuous infusion of FUra at a constant rate. In rats, this circadian variation is abolished by administration of 3-cyano-2,6-dihydroxypyridine, a dihydrouracil dehydrogenase inhibitor, resulting in enhanced bioavailability and efficacy of FUra [29]. Therefore, similar modulation of the circadian rhythm of uridine phosphorylase by uridine phosphorylase inhibitors may open new avenues for designing more efficient regimens for cancer chemotherapy with the fluoropyrimidines. This also suggests that the time of administration of drugs that

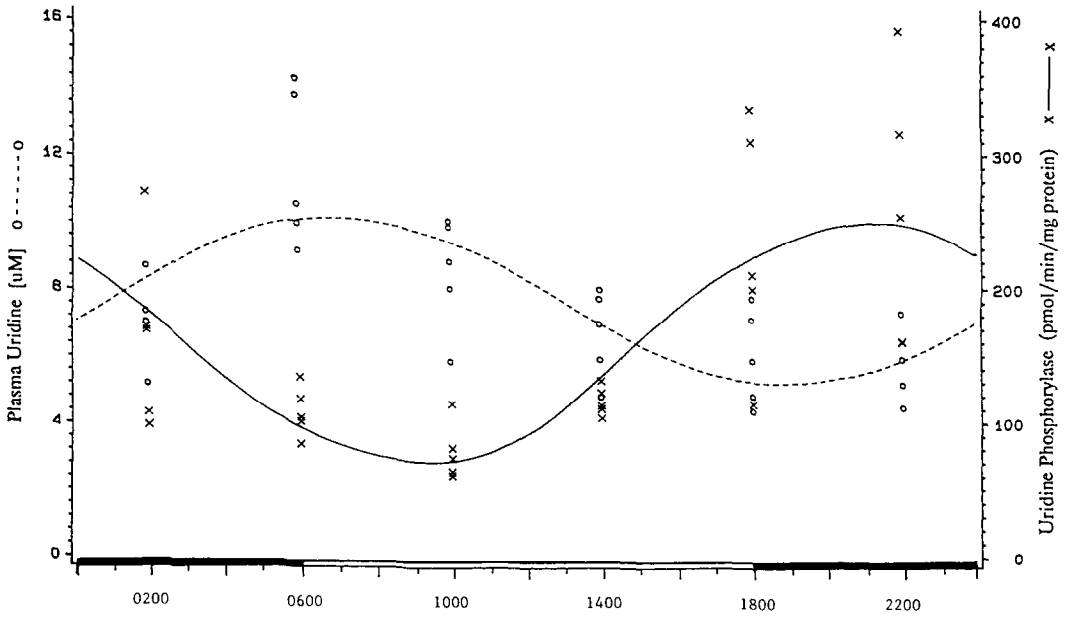

Fig. 2. Pattern of circadian rhythm of plasma uridine concentration (O--O) and hepatic uridine phosphorylase (×——×) in mice kept for 6 weeks under "normal" cycle of 12 hr light (0600–1800 hr) alternating with 12 hr darkness (1800–0600 hr) with food and water *ad lib*. Each data point represents three determinations from one mouse. The curve is a bestfit computer-generated cosine curve as determined by the Cosinor method [20].

are broken down (e.g. FdUrd [1–3, 7–9]) or activated (e.g. 5′-deoxy-5-fluorouridine [3, 39, 40]) by uridine phosphorylase, as well as drugs that inhibit this enzyme (e.g. benzylacyclouridines [1–3, 14, 15, 24]), should be considered an important factor in order to maximize their effectiveness.

In conclusion, we have demonstrated that uridine phosphorylase and plasma uridine concentrations follow a circadian rhythm. These observations may have far-reaching effects in the fields of sleep and cellular physiology as well as in cancer chemotherapy, and suggest that uridine phosphorylase inhibitors may have broad applications in these areas.

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