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CIRCADIAN RHYTHM OF OROTATE PHOSPHORIBOSYLTRANSFERASE, PYRIMIDINE NUCLEOSIDE PHOSPHORYLASES AND DIHYDROURACIL DEHYDROGENASE IN MOUSE LIVER

POSSIBLE RELEVANCE TO CHEMOTHERAPY WITH 5-FLUOROPYRIMIDINES

FARDOS N. M. NAGUIB,* SENG-JAW SOONG and MAHMOUD H. EL KOUNI
Department of Pharmacology and Comprehensive Cancer Center,
The University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

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Abstract—In female mice (30–35 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.*, there was a 24-hr cycle change ($P < 0.0001$, Cosinor analysis) in the activity of hepatic orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10), uridine phosphorylase (UrdPase; EC 2.4.2.3), and dihydrouracil dehydrogenase (DHUDase; 1.3.1.2) but not thymidine phosphorylase (EC 2.4.2.4). The peaks of both OPRTase and UrdPase activities occurred in the activity span at around 18 and 15 hours after light onset (HALO) and the trough at 6 and 3 HALO, respectively, when samples were taken every 4 hr. Conversely, the peak of DHUDase occurred in the rest span at around 3 HALO and the trough at 15 HALO. The maximal enzyme activities (3146 ± 172, 561 ± 25, and 6.7 ± 0.7 pmol/min/mg protein) was 210, 400 and 560% higher than the minimal activities (1507 ± 172, 139 ± 25, and 1.2 ± 0.7 pmol/min/mg protein), for OPRTase UrdPase, and DHUDase, respectively. A circadian rhythm 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 UrdPase and DHUDase activities with a period of 1 hr difference in the time of maximum and minimum enzyme activities. OPRTase, on the other hand, showed little change after 4 weeks of adaptation under the reverse light cycle. The circadian rhythm of these key enzymes of pyrimidine metabolism, the interrelationship of their activities, and their role in the regulation of uridine bioavailability could be of particular significance in modulating the therapeutic regimens with 5-fluorinated pyrimidines.

For over three decades, 5-fluorouracil (FUra)† and its 2′-deoxyriboside, 5-fluoro-2′-deoxyuridine (FdUrd), despite their clinical toxicity, have remained among the few drugs effective against solid tumors in humans. FUra is used mainly for the treatment of colorectal, ovarian, and breast carcinomas. FdUrd is used for the chemotherapy of hepatic metastasis of gastrointestinal adenocarcinoma and renal cell carcinoma.

It has been reported recently that the bioavailability, efficacy as well as host-toxicity of FUra [1–4] and FdUrd [5–10] follow a circadian rhythm in rodents and cancer patients. Thus, a fixed dose may have a therapeutic or toxic effect at one time point along a 24-hr time interval, but no effect at another. However, the mechanism underlying these 24-hr cyclic variations has not been fully understood. FUra and FdUrd must first be anabolized to the nucleotide level before their anti-cancer activity can be realized. However, these drugs are also subjected to catabolism and inactivation. Therefore, assessment of the activities of enzymes involved in the metabolism of FUra and FdUrd over a 24-hr period may elucidate the mechanism underlying the time-dependent variations in the bioavailability, host-toxicity and efficacy of these drugs.

The liver is considered to be the main organ implicated in the regulation of pyrimidine metabolism [11–16], and the balance between activity of hepatic enzymes involved in pyrimidine anabolism and catabolism determines the availability and hence the response to chemotherapy with fluorinated pyrimidines. Hepatic orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10), uridine phosphorylase (UrdPase, EC 2.4.2.3), thymidine phosphorylase (dThdPase, EC 2.4.2.4), and dihydrouracil dehydrogenase (DHUDase, EC 1.3.1.2) are crucial enzymes in the regulation of the catabolism or anabolism of FUra and FdUrd [for a review see Ref. 17]. Thymidine kinase (EC 2.7.1.21), which phosphorylates FdUrd in rapidly growing tissues, is essentially absent from the liver, and uridine-cytidine kinase (EC 2.7.1.48) is specific for pyrimidine ribosides and does not phosphorylate FdUrd.

* Corresponding author: Dr. Fardos N. M. Naguib, Department of Pharmacology, The University of Alabama at Birmingham, Birmingham, AL 35294. Tel. (205) 934-1131 or 934-4578; FAX (205) 934-8240.

† Abbreviations: DHUDase, dihydrouracil dehydrogenase; dThdPase, thymidine phosphorylase; DTT, dithiothreitol; FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; HALO, hours after light onset; OPRTase, orotate phosphoribosyltransferase; and UrdPase, uridine phosphorylase.

OPRTase is involved in the anabolism of FUra to its nucleotide FUMP [18]. DHUDase, on the other hand, is responsible for the rapid catabolism and inactivation of FUra [19]. UrdPase and dThdPase are active in the degradation of FdUrd to FUra, thus reducing its effectiveness [20–23]. Inhibitors of UrdPase and DHUDase were shown to increase the level of fluoropyrimidines in liver perfusates [24, 25] and plasma [3, 26–29], and to enhance their efficacy against human and murine tumors ([3, 23, 26–33]; unpublished results). Inhibitors of UrdPase have also been shown to reduce uridine catabolism [31–35] and to protect against FUra [31–33] and FdUrd [36] host-toxicities. This indicates an active role for UrdPase and increased uridine bioavailability in the mechanism of protection against the host-toxicities of 5-fluoropyrimidines. Therefore, in the present investigation the activities of hepatic OPRTase, DHUDase, UrdPase and dThdPase were assessed concomitantly in livers from the same mice over a period of 24 hr to ascertain whether or not their activities follow a circadian rhythm. We have shown previously that UrdPase activity from mouse liver exhibits a circadian rhythm [37]. Nevertheless, because enzyme activities as well as their circadian pattern can change with season, sex and age, it was important for the purpose of proper comparisons to study the activities of all four enzymes from the same tissue at the same time. We report here that OPRTase, UrdPase and DHUDase but not dThdPase follow a circadian rhythm. The interrelationship of the circadian rhythm of OPRTase, UrdPase and DHUDase and their role in the metabolism of FdUrd and FUra as well as uridine may explain the time dependency of host-toxicity and the response to chemotherapy with these fluoropyrimidines. A preliminary report has been presented [38].

MATERIALS AND METHODS

Chemicals. [2-^{14}C]Uridine (56 Ci/mol), [2-^{14}C]-thymidine (56 Ci/mol), and [6-^{14}C]uracil (56 Ci/mol) were obtained from Moravek Biochemicals Inc., Brea, CA; [6-^{14}C]orotate (46.9 Ci/mol) was from NEN Research Products, the Du Pont Co., Boston, MA; and silica gel G/UV_{254} polygram, polyethyleneimine cellulose 300 PEI/UV_{254} and cellulose CEL 300 UV_{254} polygram TLC plates were from Brinkmann, Westbury, NJ. The protein assay kit was from Bio-Rad Laboratories, Richmond, CA. All other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Animals. Female CD-1 mice (Charles River Laboratories, Wilmington, MA) weighing 30–35 g were used in all experiments.

Light cycles. Animals were housed for 4 weeks 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 were anesthetized by inhalation of Metofane. Livers were removed, weighed, minced and homogenized in 3 vol. of 20 mM potassium phosphate buffer (pH 8) containing 1 mM dithiothreitol (DTT) and 1 mM EDTA using a Polytron homogenizer (Brinkmann). The homogenates were stored in a refrigerator (4°) until all samples were collected; thereafter the activities of orotate phosphoribosyltransferase, uridine phosphorylase, thymidine phosphorylase and dihydrouracil dehydrogenase were determined concomitantly in each homogenate.

Enzyme assays. All assays were run at 37° under conditions where activity was linear with time and enzyme concentration. Reactions were started by the addition of extract and stopped by boiling in a water bath for 2 min followed by freezing. Precipitated proteins were removed by centrifugation. Substrates were separated from products in the supernatant by TLC and the radioactivity in the spots was determined on a percentage basis using a Berthold LB-2821 Automatic TLC-Linear Analyzer.

OPRTase. OPRTase activity was measured by following orotidine 5′-monophosphate (OMP), orotidine, UMP, and uridine and uracil formation from [6-^{14}C]orotate [39]. The standard assay mixture contained 20 mM potassium phosphate buffer (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 2.5 mM 5-phosphoribosyl 1-pyrophosphate (PRPP), 0.05 mM [6-^{14}C]orotate (56.2 Ci/mol) and 25 μL of enzyme extract, in a final volume of 50 μL. The incubation was terminated after 30 min. After removal of precipitated proteins, 10 μL of the supernatant fluid was spotted on prewashed PEI TLC plates. The plates were developed first in distilled water to a front of 10 cm. They were then dried and redeveloped in 0.2 M lithium chloride. R_f values were OMP, 0.16; UMP, 0.51; orotate, 0.62; orotidine, 0.77; and uridine, 0.95. OPRTase activity was measured as the sum of OMP, orotidine, UMP, and uridine and uracil combined.

DHUDase. DHUDase activity was measured by following the formation of dihydrouracil, carbamyl-β-alanine and β-alanine from [6-^{14}C]uracil, as previously described [19]. The reaction mixture contained 20 mM potassium phosphate (pH 8), 1.0 mM EDTA, 2 mM DTT, 5 mM $MgCl_2$, 25 μM [6-^{14}C]uracil (56 Ci/mol), 100 μM NADPH and 25 μL of homogenate in a final volume of 50 μL. The incubations were terminated after 15 min. Uracil, dihydrouracil, carbamyl-β-alanine and β-alanine were separated on cellulose TLC plates developed in the top phase of a mixture of *n*-buta-nol: water: ammonia (90:45:15, by vol.). R_f values were: dihydrouracil, 0.46; uracil, 0.23 and carbamyl-β-alanine plus β-alanine, 0.09, respectively. DHUDase activity was determined as the sum of the products dihydrouracil, carbamyl-β-alanine and β-alanine.

Pyrimidine nucleoside phosphorylases. Nucleoside cleavage was measured isotopically by following the formation of nucleobases from their respective nucleosides as previously described [40]. The reaction mixture contained 20 mM potassium phosphate (pH 8), 1 mM EDTA, 1 mM DTT, 1 mM uridine or thymidine (6 Ci/mol) and 25 μL of enzyme in a final volume of 50 μL. The incubation was terminated

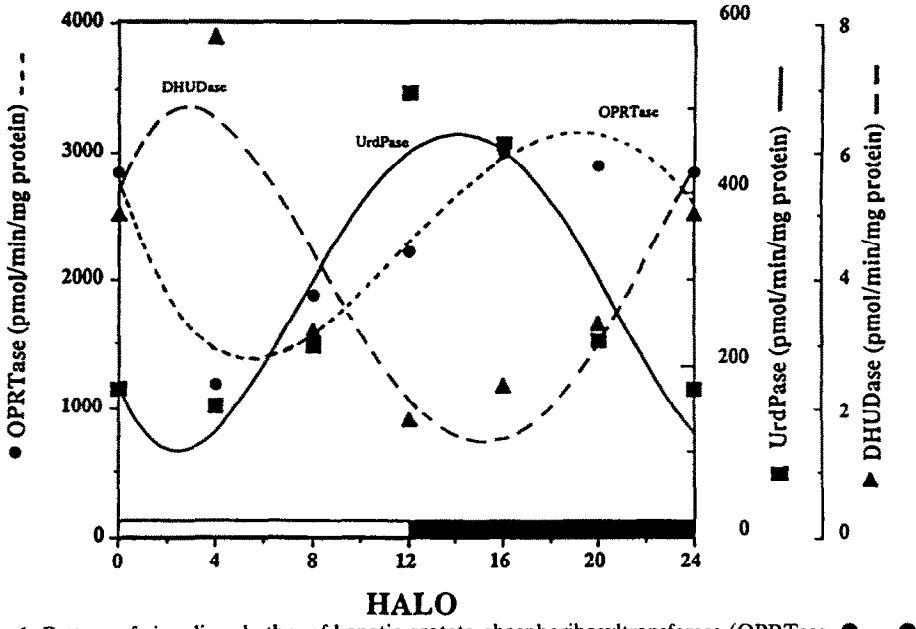

Fig. 1. Pattern of circadian rhythm of hepatic orotate phosphoribosyltransferase (OPRTase, ●···●), uridine phosphorylase (UrdPase, ■——■) and dihydrouracil dehydrogenase (DHUDase, ▲---▲) in mice kept under a "normal" cycle of 12 hr light (0600–1800 hr) alternating with 12 hr darkness (1800–0600 hr) with food and water *ad lib*. Each data point represents the average from three determinations from at least five mice. The curves are best fit computer-generated cosine curves as determined by the Cosinor method [42].

after 30 min. Protein precipitates were removed by centrifugation. A 10-μL aliquot of the supernatant fluid was spotted on silica gel TLC plate. Uridine and thymidine were separated from their respective nucleobases after the plates were developed with chloroform : methanol : acetic acid (90:5:5, by vol.). The R_f values were: uridine, 0.07; thymidine, 0.14; uracil, 0.43; and thymine, 0.62.

Protein determination. Protein concentrations were determined spectrophotometrically by the method of Bradford [41] using bovine γ-globulin as a standard.

Statistical analysis. The data were analyzed by the Cosinor method [42] whereby 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 female 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 cycle change in the activity of hepatic OPRTase, DHUDase and UrdPase, but not dThdPase, when samples were taken every 4 hr (Fig. 1 and Table 1). OPRTase achieved peak activity after UrdPase activity reached its peak and began its decline. During this period of maximum activities of OPRTase and UrdPase, DHUDase activity was at its nadir (Fig. 1). Table 1 shows the Cosinor analysis. The peaks of OPRTase and UrdPase occurred at 18 and 15 hours after light onset (HALO) and the trough at 6 and 3 HALO, respectively. Conversely, the peak of DHUDase occurred at 3 HALO and the trough at 15 HALO. The maximal enzyme activity (3146 ± 172, 561 ± 25 and 6.7 ± 0.7 pmol/min/mg protein) was 210, 400 and 560% higher than the minimal enzyme activity (1507 ± 172, 139 ± 25 and 1.2 ± 0.7 pmol/min/mg protein) for OPRTase, UrdPase and DHUDase, respectively (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 for UrdPase and DHUDase. The peak activities occurred at approximately 14 and 4 HALO and the nadir at 2 and 16 HALO for UrdPase and DHUDase, respectively, after the 4-week adaptation period (Table 1). The time of maximum or minimum activity of OPRTase, on the other hand, did not shift under the reverse light cycle after the 4-week adaptation period. The peak activity occurred between 2400 and 0100 hr and the nadir between 1200 and 1300 hr under both normal and reverse cycles. In general, there was no significant difference in maximum and minimum enzyme activities between normal and reverse cycle after the 4-week adaptation period (Table 1).

DISCUSSION

The present study clearly demonstrates that there is a circadian rhythm for OPRTase, DHUDase and

Table 1. Rhythmometric values of single Cosinor analysis of the activities (pmol/min/mg protein) of orotate phosphoribosyltransferase (OPRTase), uridine phosphorylase (UrdPase), thymidine phosphorylase (dThdPase) and dihydrouracil dehydrogenase (DHUDase) in mouse liver under normal (light, 0600–1800 hr; dark, 1800–0600 hr) and reverse (light, 2200–1000 hr; dark, 1000–2200 hr) cycles of light and darkness after 4 weeks of adaptation

Enzyme	Cycle	Mesor* ± SE	Amplitude† ± SE	Maximum activity‡ ± SE	Time of maximum activity§ ± SE	Minimum activity‖ ± SE	Time of minimum activity§ ± SE	r^2	P
OPRTase	Normal	2327 ± 100	820 ± 141	3146 ± 172	18.4 ± 0.7	1507 ± 172	6.4 ± 0.7	0.56	< 0.0001
	Reverse	2304 ± 157	715 ± 221	3019 ± 271	2.8 ± 1.2	1589 ± 271	14.8 ± 1.2	0.28	0.012
UrdPase	Normal	350 ± 14.8	211 ± 20.5	561 ± 25.3	14.9 ± 0.5	139 ± 25.3	2.9 ± 0.5	0.80	< 0.0001
	Reverse	365 ± 14.3	194 ± 19.1	559 ± 24.1	13.7 ± 0.8	171 ± 24.1	1.7 ± 0.8	0.79	< 0.0001
dThdPase	Normal	190 ± 10.4	24.6 ± 14.7	215 ± 18.1	9.8 ± 2.3	166 ± 18.1	21.9 ± 2.3	0.05	0.26
	Reverse	207 ± 10.7	19.7 ± 21.6	227 ± 18.2	14.6 ± 2.8	187 ± 18.2	2.7 ± 2.8	0.07	0.41
DHUDase	Normal	4.0 ± 0.6	2.8 ± 0.6	6.7 ± 0.7	2.8 ± 0.8	1.2 ± 0.7	14.9 ± 0.8	0.59	< 0.0001
	Reverse	3.6 ± 0.6	0.6 ± 0.9	4.2 ± 0.2	4.2 ± 1.2	3.0 ± 0.2	16.2 ± 1.2	0.27	< 0.014

* Rhythm-adjusted mean (pmol/min/mg protein) from 30 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).

UrdPase but not dThdPase activity in mouse liver. The peak UrdPase and OPRTase activities occurred at 15 and 18 HALO, with their nadir occurring at 3 and 6 HALO, respectively, and UrdPase peak and nadir activities preceding those of OPRTase by 3 hr (Table 1). In contrast, DHUDase peak activity took place at 3 HALO at the time when UrdPase was at its nadir. The nadir of DHUDase occurred at 15 HALO around the time when UrdPase was at its maximum activity. Reversing the light–dark cycle resulted in a corresponding shift in the enzyme activities of UrdPase and DHUDase but not that of OPRTase. A 4-week acclimatization period to new light–dark cycle conditions seems adequate to achieve near complete reversal of the circadian pattern of UrdPase and DHUDase but not OPRTase (Table 1). This suggests that an extended time of acclimatization beyond the 4-week period may be required to completely reverse OPRTase activity.

Our studies [Ref. 38 and Table 1] are the first reports on a circadian rhythm for OPRTase and the absence of rhythmical variations for dThdPase. The lack of circadian variation for dThdPase has been confirmed in rat liver [43]. The time-dependent variation of UrdPase (Table 1 and Fig. 1) confirms our previous reports [37, 38] and that of DHUDase is also in agreement with results on the rhythmical variation of DHUDase from human blood mononuclear cells [4] and rat liver [44]. This is also the first report on the interrelationship among the circadian rhythms of OPRTase, UrdPase, dThdPase and DHUDase.

The time-dependent bioavailability, host-toxicity and efficacy of FUra and FdUrd observed in experimental and clinical studies [1–10] may be explained by the present results. FdUrd is cleaved to FUra by dThdPase and UrdPase [20–23]. Since UrdPase but not dThdPase showed circadian rhythmical variations (Table 1 and Fig. 1), it is not unreasonable to assume that an increase of UrdPase activity would be accompanied by an increase in conversion of FdUrd to FUra. The FUra thus generated would then be subjected to anabolism to FUMP by OPRTase or catabolism to dihydroFUra by DHUDase. The present results show that the peak activities of UrdPase and OPRTase occurred sequentially (15, 18 HALO) with the peak activity of UrdPase preceding that of OPRTase by approximately 3 hr. At approximately the same time (15 HALO) DHUDase activity was at its nadir. It is noteworthy that DHUDase is not only saturable but also strongly inhibited by its substrates [19]. It follows that increased UrdPase adding to steady dThdPase activities would flood an already diminished DHUDase with substrates (FUra, uracil and thymine), triggering substrate inhibition, and hence leaving FUra intact for anabolism by OPRTase. Under physiological conditions neither thymine nor uracil is a substrate for OPRTase [18]. This suggests that, at least in mice, the fate and consequently the chemotherapeutic efficacy of FdUrd and FUra at a given time may be determined by the balance between anabolic and catabolic activities of these enzymes of pyrimidine metabolism. This contention is supported by the fact that the circadian variation of the catabolic DHUDase is reported to be the

inverse of the plasma concentration of FUra in patients [4] and rats [3] receiving infusion of FUra at a constant rate, i.e. high DHUDase activity corresponds to low FUra concentration.

The circadian rhythm of UrdPase described in our studies (Fig. 1; [37, 38]) correlates with that reported for FdUrd host-toxicity in mice [5] and rats [8]. This may be explained by the role of UrdPase in modulating uridine metabolism and concentration. Inhibitors of UrdPase were shown to increase the concentration and salvage of uridine [14, 31–35] and to protect against host-toxicity of FdUrd [36], as well as FUra [31–33]. Therefore, the rhythmical surge observed in UrdPase activity (Fig. 1) could lead to a decrease in uridine concentration which in turn would result in increased host-toxicity and diminished therapeutic index for FdUrd. Indeed, we have established that plasma uridine concentration follows a circadian rhythm which is the inverse of that of UrdPase activity [37]. This effect of the circadian rhythm of UrdPase activity on uridine concentation could explain the relationship between the circadian rhythm of UrdPase activity and that for the therapeutic index of FdUrd and probably would also play a role in the mechanism underlying the circadian efficacy of FUra as well.

The correlation between the rhythmical variations for the activities of OPRTase and UrdPase may also underlie the balance between synthesis and catabolism of uridine in the liver. More than 90% of plasma uridine entering the liver by the portal vein is degraded in a single pass while constant amounts of uridine from *de novo* biosynthesis is released into the hepatic vein blood [11, 12]. Activity of hepatic UrdPase is the first step in elimination of plasma uridine delivered to the liver [14, 15, 32]. Our present (Table 1 and Fig. 1) and previous results [37, 38] show that hepatic OPRTase, UrdPase and plasma uridine concentration exhibit circadian rhythm. The increase in hepatic UrdPase activity results in a concomitant increase of uridine catabolism in the liver and lower uridine concentration in the plasma [37]. However, as seen from the present results, such an increase in UrdPase activity is followed by a corresponding rise in the activity of OPRTase, indicating an increase in *de novo* pyrimidine biosynthesis to compensate for the increased degradation of uridine. Indeed, the peak activity of hepatic OPRTase observed in the present study occurred at 18 HALO which concurs with the time reported for maximum incorporation of orotic acid into pyrimidine components of acid-soluble extract, RNA and DNA in rat liver [45].

Finally, the circadian rhythm of enzymes involved in the metabolism of FdUrd and FUra as well as that of uridine could be modulated to enhance the bioavailability and therapeutic indices of FUra and FdUrd by the use of enzyme inhibitors. It has been shown that the effects of the circadian variation of DHUDase in rodents can be abolished by administration of a DHUDase inhibitor (e.g. 3-cyano-2,6-dihydroxypyridine) resulting in enhanced bioavailability and efficacy of FUra [3]. Similarly, inhibitors of UrdPase (e.g. 5-benzylacyclouridines [20–22, 30, 40, 46]) could modify the effects of the circadian rhythm of UrdPase activity and enhance the efficacy of FdUrd administered by continuous infusion by reducing FdUrd degradation and maintaining a constant chemotherapeutic concentration of the drugs in the plasma especially in cases of regional chemotherapy. The benzylacyclouridines were shown to prevent FdUrd degradation [20–23], to improve the therapeutic indices of FdUrd and FUra [23, 31–33], to increase the concentration and salvage of uridine [14, 31–35], and to protect from FdUrd and FUra host-toxicities [31–34, 36]. Such modulation of the circadian rhythm of enzymes of pyrimidine metabolism by the use of inhibitors may open new avenues for designing more efficient regimens for cancer chemotherapy with these 5-fluoropyrimidines.

In conclusion, we have demonstrated that hepatic OPRTase, UrdPase and DHUDase follow circadian rhythms. These results along with those derived from circadian-dependent treatment protocols in experimental and clinical studies emphasize the potential clinical importance in cancer chemotherapy of the time of administration of 5-fluorinated pyrimidines that are activated or deactivated by OPRTase, UrdPase or DHUDase as well as drugs that inhibit these enzymes.

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