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Dihydropyrimidine dehydrogenase circadian rhythm in mouse liver: comparison between enzyme activity and gene expression

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Abstract

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme of 5-fluorouracil (FU) catabolism. The relevance of the measurement of DPD activity for identifying DPD-deficient patients is lessened by circadian variability in DPD activity. Our purpose was to determine whether or not DPD mRNA is sustained by a circadian rhythm. Synchronised mice (male B6D2F1) were sacrificed at 3, 7, 11, 15, 19 or 23 Hours After Light Onset (HALO; eight mice per time-point). Liver DPD activity was determined by a radio-enzymatic assay and liver DPD expression by a reverse transcriptase-polymerase chain reaction (RT-PCR) enzyme-linked immunosorbent assay (ELISA) method. Mice synchronisation was controlled by leucocyte and neutrophil counts. Individual DPD activity ranged from 555 to 1575 pmol/min/mg prot; mean DPD activity was highest at 3 HALO (mean±standard error of the mean (S.E.M.); 1105±70) and lowest at 15 HALO (889±71). Individual liver DPD expression varied from 761 to 3481 units (DPD/β actin ratio); the mean was lowest at 3 HALO (1406±112) and highest at 15 HALO (2067±214). Cosinor analysis indicated that respective double amplitudes of DPD activity and expression were 21 and 30% of the 24-h mean. The acrophases for activity and expression were 6:40 and 14:10 HALO, respectively, meaning that maximum activity occurred 16 h after the maximum observed expression. These results, revealing the existence of a circadian rhythm in DPD expression, should stimulate further studies to enhance our understanding of the molecular mechanisms involved in the circadian regulation of the DPD enzyme.

Keywords: Circadian rhythm; Chronopharmacology; Drug metabolism; Dihydropyrimidine dehydrogenase

1. Introduction

5-Fluorouracil (FU) and recently developed FU oral prodrugs like capecitabine are currently major anticancer drugs. In eukaryotic cells, the first step of pyrimidine catabolism, including FU, is a reduction mediated by the ubiquitous dihydropyrimidine reductase or dihydropyrimidine dehydrogenase enzyme (EC 1.3.1.2, DPD) [1]. During the last decade, numerous experimental and clinical studies have focused on dihydropyrimidine dehydrogenase (DPD) [2–7]. DPD activity strongly influences FU plasma pharmacokinetics [1,2]. Relative DPD deficiencies measured in lymphocytes of cancer patients were reported [6] and, even though FU catabolism mainly occurs in the liver, patients with DPD deficiencies measured in lymphocytes exhibit impaired FU systemic clearance [2] and are exposed to severe FU-related toxicities [3]. However, the relevance of a single determination of DPD activity in lymphocytes as a means of identifying patients at risk for developing FU-related toxicities is lessened by circadian variability in DPD activity. Such a circadian rhythm was demonstrated in rats [8], in cancer patients [9] and in healthy human beings [10]. Of importance are the data of Grem and colleagues [10] which not only confirmed the significant variation of lymphocytic DPD activity throughout the day, with an individual peak/trough ratio between 2.1 and 3.2, but also demonstrated that

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the time of day when the peak occurred was randomly distributed over the 24-h period. As a consequence, sampling recommendations based upon the time of the day are of limited efficacy for reducing intra- and inter-subject variability of DPD activity. Thus, reliability and clinical usefulness of a single-point DPD determination appear to be limited.

The existence of such a circadian rhythm suggests a transcriptional and/or postranscriptional (translation level or protein allosteric modulation) control of DPD by the circadian clock. So far, the cellular mechanisms responsible for circadian variability in DPD activity are unknown. Of practical and clinical interest would be the observation that DPD expression remains invariant over the 24-h time scale, thus providing a strong alternative for the identification of DPD-deficient patients. However, no studies providing data on the possible rhythmicity of DPD mRNA have so far been published. Only a few genes are known to display a circadian rhythm at the mRNA level [11]. Recent studies have compared single-point determination of DPD activity with DPD expression, and conflicting results were found, depending on the tissue explored [12,13]. For instance, Uetake and colleagues [13] reported a significant relationship between DPD activity and DPD mRNA measured in human colorectal tumours whereas no relationship was demonstrated in normal mucosa. However, such comparative studies do not answer the question of circadian rhythm at the level of DPD mRNA. Finding an answer to this question was the main objective of the present experimental study conducted in synchronised mice. A sensitive and quantitative reverse transcription-polymerase chain reaction (RT-PCR) enzyme-linked immunosorbent assay (ELISA) method was specifically developed for that purpose in order to precisely measure DPD expression in mouse liver along with DPD activity over the 24-h scale.

2. Materials and methods

2.1. Animals

Forty-eight 5–6-week-old, male B6D2F1 mice were purchased from IFFA-Credo (L'Arbresle, France). They were stratified according to their body weight on arrival, then housed two per cage with water and food *ad libitum*. Mice were randomly allocated to one of four consecutive experiments, each comprising six groups. One to 3 days separated each of the four experiments. Each group corresponded to a different circadian time for the sacrifice of mice (3, 7, 11, 15, 19 or 23 Hours After Light Onset (HALO)), and two mice were taken per sampling time, i.e. giving a total of eight mice per time-point. For this purpose, a different shelf of an autonomous chronobiological animal facility (ESI-Flufrance, Arcueil, France) was used for each group. This facility has six soundproof, temperature-controlled compartments, each with its own programmable lighting regimen and constant supply of filtered air delivered at an adjustable rate. All mice were synchronised with an alternation of 12 h of light (L) and 12 h of darkness (D) (LD 12:12) for 3 weeks prior to the experiment. Adequate synchronisation of mice by means of these lighting regimens was assessed by the occurrence of a normal circadian variation in body temperature and counts of circulating leucocytes and neutrophils. Blood (200 μl) was sampled via a needle puncture of the right retro-orbital sinus and collected on ethylene diamine tetra acetic acid (EDTA)-K3 for immediate determination of total leucocyte and neutrophil counts (Cell-Dyn 3500, Abbott Diagnostic, Rungis, France). Mice were subsequently sacrificed and approximately 200 mg of liver tissue per mouse was obtained, separated into two aliquots (one for DPD activity measurement, the other for RNA extraction), immediately frozen and stored in liquid nitrogen until assayed.

2.2. DPD activity measurement

2.2.1. Chemicals

Dihydro 5-fluorouracil (FUH2), α-fluoro β-ureidopropionic acid (FUPA), and α-fluoro β-alanine (FBAL) were kindly provided by Roche Laboratories (Neuilly, France). ^{14}C-FU labelled at position 6 (55 Ci/mol) was obtained from Amersham (Buckinghamshire, UK). All other chemicals were obtained from the Sigma Chemical Co. (Sigma Diagnostics, St. Louis, MO, USA) and were of the highest purity available.

2.2.2. Enzyme activity

A frozen piece from each liver sample (mean weight: 38 mg; range: 30–50 mg) was homogenised in 1 ml buffer containing 10 mM Tris–HCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 10 mM sodium molybdate (pH 7.4) on an ice bed using a Polytron (Polylabo, Paris, France). The homogenate was centrifuged for 30 min at 105 000*g* (+2 °C). The resulting cytosolic fractions were immediately assayed for DPD activity. Cytosolic proteins were quantified using the Bradford assay (Bio-Rad SA, Munich, Germany) and bovine serum albumin (BSA) as a standard. DPD activity was measured according to the method described by Harris and colleagues [9]. The assay consisted of incubating 50 μl cytosol with ^{14}C-FU (50 μM final), β-nicotinamide adenine dinucleotide phosphate (250 μM final), and magnesium chloride (2.5 mM final). Total volume was 125 μl (in 35 mM sodium phosphate buffer, pH 7.5, containing sodium azide). The duration of incubation was 15 min at +37 °C. The reaction was stopped by the addition of 125 μl ice-cold ethanol followed by 30-min

storage at −20 °C. The samples were centrifuged (5 min, 400*g*) to remove proteins, and the supernatant was analysed for the presence of ^{14}C-FUH2, ^{14}C-FBAL and ^{14}C-FUPA using a high-pressure liquid chromatographic (HPLC) method. Detection was performed using a radioactive flow monitor (LD 506; Berthold, Widbad, Germany). DPD activity was calculated by taking into account the sum of FUH2, FBAL, and FUPA peaks. DPD activity was expressed as pmol ^{14}C-FU catabolised per min and per mg protein. Each sample was assayed in duplicate. The sensitivity limit was 5 pmol/min/mg protein. The inter-assay coefficient of variation of pooled cytosols was less than 10%, which also indicates the rather good stability of DPD activity during storage.

2.3. DPD expression measurement (RT-PCR ELISA)

2.3.1. RNA extraction and RT

DPD expression was assayed on an aliquot of a liver fragment stored in liquid nitrogen. Each liver fragment corresponded to a given mouse sacrificed at a given experimental time in experiment 1, 2, 3 or 4. Total RNA was isolated using the QIAGEN mini RNA/DNA kit (QIAGEN, Courtaboeuf, France) based on RNA purification on an anion exchange resin column. Purified RNA was stored at −80 °C until RT-PCR. The day of the assay, RNA quality was checked by agarose gel electrophoresis and quantification was performed by densitometric analysis at 260 nm. Two μg of total RNA was preincubated for 5 min at 65 °C in a 40 μl final volume of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphate and 2 μM of random hexamers (Roche Diagnostics, Meylan, France). One hundred units of Expand Reverse Transcriptase (Roche Diagnostics, Meylan, France) and 40 units of human placenta ribonuclease inhibitor (Amersham Pharmacia Biotech, les Ulis, France) were then added and the mixture was incubated for 30 min at 42 °C followed by 5 min at 94 °C. The cDNAs obtained were stored at −20 °C.

2.3.2. Primers

Mouse *DPYD* gene has not yet been cloned. Thus, sequences were selected from homologous sequences obtained by alignment of cDNAs from the different species that have been cloned so far (human, *rattus norvegicus*, *bos taurus*, *sus scrofa*).

The oligonucleotides used for DPD amplification were:

DPD sense-strand: AAT AGG TTT GCC AGA ACC CA
nucleotide (nt.) 904-923 on *rattus norvegicus* mRNA.
DPD antisense-strand: ACA TCA CCA CCT GCA AAT AC
nt. 1484-1503 on *rattus norvegicus* mRNA which yields a 600 bp product.

Those used for amplification of the reference gene (*beta-actin*) were: beta-actin sense-strand:

AGG CTG TGC CCC TGT AT
nt. 332-351 on mouse mRNA.
beta-actin antisense-strand: TAG CTC TTC TCC AGG GAG GA
nt. 622-641 on mouse mRNA which yields a 310 bp product.

Two specific capture probes, 5′biotinylated and purified by HPLC (EUROBIO, les Ulis, France) and corresponding to each amplification product, were used for ELISA detection:

DPD capture probe: TCC CCA CGG AAG GTT ATA GT
nt. 1232-1251 on *rattus norvegicus* mRNA.
beta-actin capture probe: ACC TCA TGA AGA TCC TCA CC
nt. 485-504 on mouse mRNA.

2.3.3. PCR conditions

Briefly, 150 ng RNA equivalents were subjected to PCR amplification in a 100 μl final volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP), 190 μM of dTTP, 10 μM of dUTP labelled with digoxigenin, 5 units of Taq polymerase and 125 nM for the primer pair for actin and 500 nM for the primer pair for DPD. The multiplex amplification consisted of an initial 5 min incubation at 94 °C followed by 23 amplification cycles (94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min).

2.3.4. PCR ELISA

DPD and β actin amplifications were performed using the PCR-ELISA DIG labeling and the PCR-ELISA DIG detection kits (Roche Diagnostics, Meylan, France) as previously described by us in Ref. [14]. The digoxigenin labeling reaction of the PCR products was carried out during co-amplification of DPD and β actin for an optimal number of cycles, in the presence of digoxigenin-labeled dUTP. These labeled products were analysed with the two specific biotinylated capture probes, which allowed immobilisation of the hybrid to a streptavidin-coated microplate surface. The bound hybrid was detected by an anti-digoxigenin antibody–peroxidase conjugate. Peroxidase activity was evaluated by addition of the colorimetric substrate ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) and the absorbance was read at 405 nm. Results were arbitrarily expressed as 1000-fold the absorbance ratio (relative abundance, DPD/β actin).

2.3.5. Validation

Reliability of RT-PCR quantification was tested by mixing a sample exhibiting a very low DPD expression

with a sample expressing a high DPD level. The five tested ratios of low/high expression mixture were the following: 0/100, 25/75, 50/50, 75/25 and 100/0. This approach was applied to the cDNA and RNA samples. Very strong correlations were observed between the expected and measured DPD/β actin ratio, with $r=0.975$ for cDNA mixtures and $r=0.991$ for RNA mixtures. Intra- and interassay reproducibility was further evaluated on an RNA pool extracted from a rat liver. The coefficient of variation was 7.33% for intra-assay reproducibility ($N=6$) and 19.41% for interassay reproducibility ($N=6$).

2.4. Statistics

Mean and standard error of mean were computed for each variable, for each experiment and for each sampling time point. Differences related to sampling time were tested with a 2-way analysis of variance (experiment and sampling time). Data were subsequently analysed both as raw values and, following transformation, as percentages of the 24 h-mean value of each experiment. The statistical significance of a cyclic variation was further documented by Cosinor analysis (considered as significant if $P<0.05$). Periods (τ) of 24 and 12 h were tested. The fitted cosine function best approximating all data allows parameters to be estimated using a linear least squares method. The parameters estimated from Cosinor analysis were the mesor (M, rhythm-adjusted mean), the double amplitude (2A, difference between minimum and maximum of fitted cosine function), and the acrophase (Ø, time of maximum in fitted cosine function, with light onset as Ø reference, so that time units are HALO); A and Ø could be approximated if $0.05<P<0.10$. Statistics were drawn up on the Statistical Package for the Social Sciences (SPSS) (Chicago, IL, USA).

3. Results

3.1. Control of mice synchronisation

Mean leucocyte and neutrophil counts were 6598 ± 251 (mean $\pm$S.E.M.) and 621 ± 30 cells/mm^3, respectively. Mean leucocytes ranged from 4404 ± 457 at 15 HALO to 9131 ± 671 cells/mm^3 at 7 HALO (Fig. 1). Mean neutrophil count ranged from 429 ± 54 at 15 HALO to 846 ± 128 cells/mm^3 at 3 HALO. Both circadian changes were statistically validated with ANOVA. Cosinor analysis further indicated that both cellular counts followed a circadian rhythm with acrophases at 5:50 HALO for leucocytes and 4:55 HALO for neutrophils. The double amplitude of these rhythms approximated 70% of the mesor (Fig. 1, Table 1). This circadian rhythm in leucocytes and neutrophils validates the fact that the animals were synchronised.

3.2. Temporal variability in DPD activity

Individual liver DPD activity ranged from 555 to 1575 pmol/min/mg protein. Mean liver DPD activity was highest at 3 HALO (1105 ± 70) and lowest values were observed at 15 and 23 HALO (889 ± 71 and

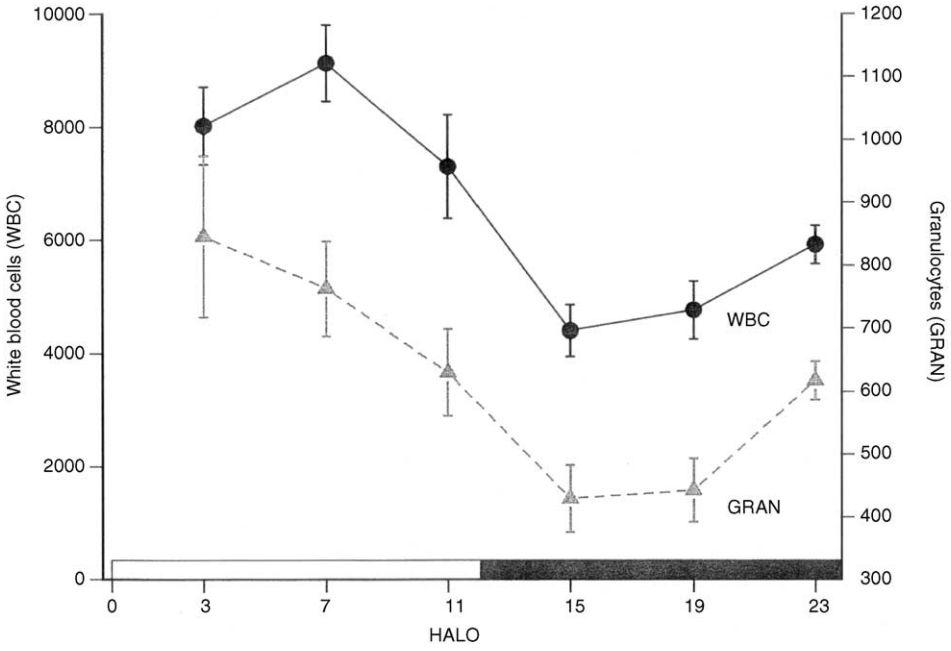

Fig. 1. Mean $\pm$ standard error of the mean (S.E.M.) of leucocyte and neutrophil counts according to sampling time (see Table 1 for statistical validation).

Table 1
Results from statistical analyses of circadian changes in study variables

Variables		ANOVA	Cosinor			
		P value	*P* value	Mesor ±S.E.M.	Double amplitude ±S.D.	Acrophase (HALO) (h:min±min)
Leucocytes	cells/mm^3	<0.0001	<0.0001	6598±251	4774±1798	5:55±90
	% 24-h mean	<0.0001	<0.0001	100	72±23	5:50±70
Neutrophils	cells/mm^3	0.001	<0.0001	621±30	426±216	4:50±120
	% 24-h mean	0.003	<0.0001	100	67±34	4:55±120
DPD activity	pmol/min/mg	0.007	0.05	986±30	196	6:10
	% 24-h mean	0.003	0.0009	100	21±13	6:40±160
DPD expression	Ratio DPD/actin	0.04	0.05	1835±78	543	14:30
	% 24-h mean	0.02	0.0009	100	30±19	14:10±160

S.E.M., standard error of the mean; S.D., standard deviation; DPD, dihydropyrimidine dehydrogenase; HALO, Hours After Light Onset.

898±63, respectively) (Fig. 2). A circadian variation was statistically validated with both ANOVA, and Cosinor analysis for $\tau = 24$ h (Table 1). A 12-h rhythm was not validated. A better definition of the circadian parameters was obtained by Cosinor analysis of data expressed as % of each experiment's 24-h mean (Table 1). Thus, the double amplitude of the rhythm represented approximately 20% of the mesor, and the acrophase was located at 6:40 HALO.

3.3. Temporal variability in DPD expression

Individual liver DPD expression ranged from 761 to 3481 units (DPD/β actin ratio) throughout the 24-h period. The average DPD expression was lowest at 3 HALO (1406±112) and highest at 11 and at 15 HALO (2055±198 and 2067±214, respectively) (Fig. 3). Both ANOVA and Cosinor analysis validated significant changes throughout the 24-h time scale (Table 1); the 12-h rhythm was not validated. Cosinor analysis of data transformed as% of each experiment's 24-h mean indicated that the double amplitude of the rhythm was 30% of the mesor, and its acrophase occurred at 14:10 HALO (Table 1).

3.4. Relationship between DPD activity and expression rhythms

Fig. 4 illustrates the 24-h period cosine curves of DPD activity and DPD expression with data expressed as % of each experiment's 24-h mean. The maximum DPD activity occurred at approximately 6 HALO, that is nearly 16 h after the maximum observed for DPD expression (14 HALO).

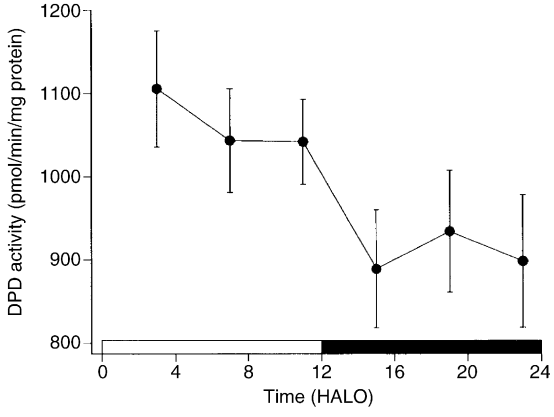

Fig. 2. Mean±standard error of the mean (S.E.M.) of liver dihydropyrimidine dehydrogenase (DPD) activity as a function of sampling time (eight mice per time point, see Table 1 for statistical validation).

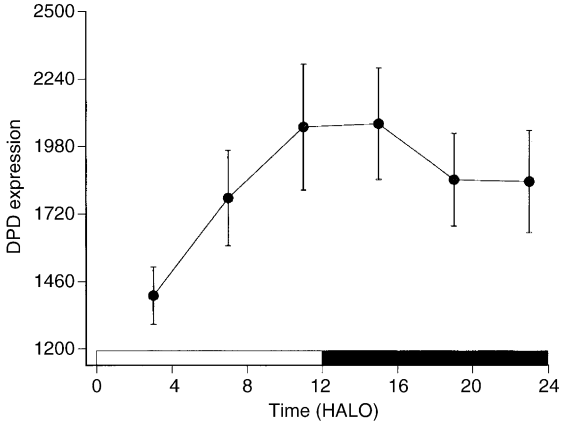

Fig. 3. Mean±standard error of the mean (S.E.M.) of liver DPD expression (ratio dihydropyrimidine dehydrogenase (DPD)/β actin) as a function of sampling time (eight mice per time point, see Table 1 for statistical validation).

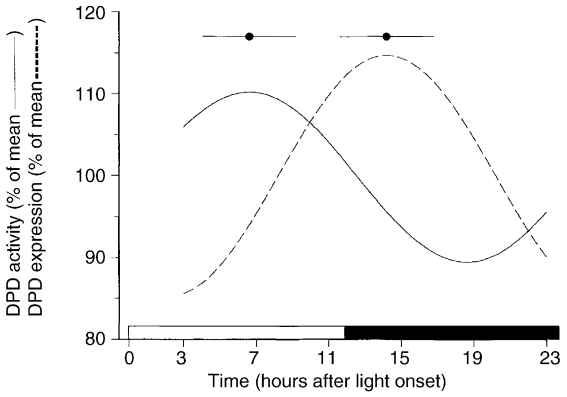

Fig. 4. Relationship between 24-h cosine curves of dihydropyrimidine dehydrogenase (DPD) activity (—) and DPD expression (- - - -), with data converted as % of each experiment's 24-h mean. The respective acrophases (and 95% confidence limits) of both rhythms are shown at the top of the figure.

4. Discussion

Importantly, the circadian rhythm demonstrated in human beings for DPD activity measured in blood lymphocytes [9,10] significantly influences FU pharmacokinetics. In fact, Petit and colleagues [15] reported that a constant-rate FU infusion resulted in a circadian rhythm in FU plasma concentration, with amplitude and maximum depending upon infusion duration, daily dose and patient characteristics. Accordingly, the circadian variability in DPD activity also results in a coincident variation in FU tolerability, both in laboratory rodents and in cancer patients : the time of highest DPD activity corresponds to that of best FU tolerability [16,17]. Such observations have led to the development of chronomodulated delivery schedules, that have demonstrated better tolerability and greater antitumour activity than constant rate infusion in patients with metastatic colorectal cancer patients [18–20].

Regarding the usefulness of DPD activity measurement for predicting FU-related severe toxicities, the circadian rhythm in DPD activity results in unpredictable intra- and intersubject variability, even if tissues are obtained at the same clock hour in all subjects. This impairs the reliability of a single-point DPD activity determination for identifying at-risk subjects. It is thus important to know whether this circadian variability affects DPD measurement at the transcriptional level. Such information would make it possible, or not, to use DPD expression as a more faithful indicator than DPD activity for identifying patients with DPD deficiency.

Previous studies have firmly established the existence of a circadian rhythm for DPD activity measured in rat liver and bone marrow [8], as well as in mouse liver [21]. In the present study, the validation of artificial synchronisation of mice (12 h of light and 12 h of dark) is provided by the demonstrated leucocyte and neutrophil circadian rhythms, with characteristics similar to those reported earlier by us and others [22,23]. The present study confirms the existence of a circadian rhythm for DPD activity measured in the liver of healthy mice, with a maximum occurring at 3 HALO, i.e. during the rest span of the mice (light span). This time is very close to that reported earlier in female CD1 mice by Naguib and colleagues [21] (4 HALO). In our study, however, DPD activity remained near maximum throughout the light span. It must be stressed that the circadian double-amplitude presently found was much lower than that reported by Naguib and colleagues [21]. These differences in circadian pattern and amplitude may reflect differences related to mouse strain, sex or age, as well as housing conditions.

The present study demonstrates for the first time that DPD expression displays a significant circadian rhythm, with a trough at 3 HALO, in the early rest span (early light), and a maximum at 15 HALO, a few hours after the onset of nocturnal activity (darkness). Thus, the trough in DPD expression precisely coincided with the peak in DPD activity. Using cosinor analysis, the time lag between the acrophase of DPD expression and activity was 16 h. DPD is a complex protein with two identical subunits [24]. Although *in vitro* translation studies have shown that large full-length proteins can be quickly synthesised and assembled, circadian changes in protein conformation have been reported and may be relevant for DPD. In addition, while protein synthesis results from transcription, enzymatic activity can be regulated by many factors [25].

The present original data have several potentially important implications. Firstly, they almost exclude the possibility of using DPD expression as a substitute for DPD activity measurement in order to circumvent the problem of circadian variability. However, such a conclusion is based on data obtained in healthy liver tissue, and extrapolation to tumour tissue will need to be validated. Secondly, the present data suggest that the circadian rhythm in DPD activity is mainly sustained by a circadian rhythm at the transcriptional level. In addition, the possibility of post-transcriptional regulation responsible for part of the circadian variation in DPD activity cannot be totally ruled out. However, a post-transcriptional regulation would exert only a minor influence since the circadian double-amplitude in DPD expression and in DPD activity were similar, i.e. 30 and 21% of the 24-h mean, respectively. The expression of several transcription factor genes is regulated during the 24-h time scale by a set of interacting circadian genes in mammals [26–28]. It has been shown that the rhythmic transcription of one or more clock genes produces rhythmic levels of clock RNA that, in turn, produce rhythmic levels of clock proteins [29]. Similar mechanisms may be involved in the circadian regulation of DPD expression and activity. Also of interest is the recent discovery of circadian clocks in peripheral organs like the liver, giving rise to the concept of peripheral

clocks [11,30]. These clocks are controlled by a set of clock genes which in turn regulate the expression of clock-controlled genes. Therefore, the DPD gene may also be a target of peripheral clocks. Cloning and functional characterisation of the *DPYD* gene promoter has recently been published in Ref. [31]. The present data may stimulate further investigations of the molecular mechanisms responsible for regulating DPD expression at the promoter level.

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References

1. Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* 1989, **16**, 215–237.
2. Fleming R, Milano G, Thyss A, *et al*. Correlation between dihydropyrimidine dehydrogenase activity in peripheral mononuclear cells and systemic clearance of fluorouracil in cancer patient. *Cancer Res* 1992, **52**, 2899–2902.
3. Fleming RA, Milano G, Gaspard MH, *et al*. Dihydropyrimidine deshydrogenase activity in cancer patients. *Eur J Cancer* 1993, **29**, 740–744.
4. Beck A, Etienne MC, Cheradame S, *et al*. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumor sensitivity to fluorouracil. *Eur J Cancer* 1994, **30**, 1517–1522.
5. Etienne MC, Cheradame S, Fischel JL, *et al*. Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. *J Clin Oncol* 1995, **13**, 1663–1670.
6. Milano G, Etienne MC, Pierrefite V, Barberi-Heyob M, Deporte-Fety R, Renée N. Dihydropyrimidine dehydrogenase deficiency and fluorouracil related toxicity. *Br J Cancer* 1999, **79**, 627–630.
7. Grem JL, Danenberg KD, Behan K, *et al*. Thymidine kinase, thymidylate synthase and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's Anticancer drug screen. *Clin Cancer Res* 2001, **7**, 999–1009.
8. Harris BE, Song R, He YJ, Soong SJ, Diasio RB. Circadian rhythm of rat liver dihydropyrimidine dehydrogenase, possible relevance to fluoropyrimidine chemotherapy. *Biochem Pharmacol* 1988, **37**, 4759–4762.
9. Harris BE, Song R, Soong SJ, Diasio RB. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. *Cancer Res* 1990, **50**, 197–201.
10. Grem JL, Yee LK, Venzon DJ, Takimoto CH, Allegra CJ. Inter- and intra-individual variation in dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells. *Cancer Chemother Pharmacol* 1997, **40**, 117–125.
11. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 1998, **93**, 929–937.
12. Tanaka-Nozaki M, Onda M, Tanaka N, Kato S. Variations in 5-fluorouracil concentrations of colorectal tissues as compared with dihydropyrimidine dehydrogenase (DPD) enzyme activities and DPD messenger RNA levels. *Clin Cancer Res* 2001, **7**, 2783–2787.
13. Uetake H, Ichikawa W, Takechi T, Fukushima M, Nihei Z, Sugihara K. Relationship between intratumoral dihydropyrimidine dehydrogenase activity and gene expression in human colorectal cancer. *Clin Cancer Res* 1999, **5**, 2836–2839.
14. Castillo L, Milano G, Santini J, Demard F, Pierrefite V. Analysis of retinoic acid receptor β expression in normal and malignant laryngeal mucosa by a sensitive and routine applicable reverse transcription-polymerase chain reaction-linked immunosorbent assay method. *Clin Cancer Res* 1997, **3**, 2137–2142.
15. Petit E, Milano G, Levi F, Thyss A, Bailleul F, Schneider M. Circadian rhythm varying plasma concentration of 5-fluorouracil during a five day continuous venous infusion at constant rate in cancer patients. *Cancer Res* 1988, **48**, 1976–1979.
16. Zhang R, Lu Z, Liu T, Soong SJ, Diasio RB. Relationship between circadian-dependent toxicity of 5-fluorodeoxyuridine and circadian rhythms of pyrimidine enzymes: possible relevance to fluoropyrimidine chemotherapy. *Cancer Res* 1993, **53**, 2816–2822.
17. Metzger G, Massari C, Etienne MC, *et al*. Spontaneous or imposed circadian changes in plasma concentrations of 5-fluorouracil coadministered with folinic acid and oxaliplatin relationship with mucosal toxicity in patients with cancer. *Clin Pharmacol Ther* 1994, **56**, 190–201.
18. Lévi F, Zidani R, Vannetzel JM, *et al*. Chronomodulated versus fixed infusion rate delivery of ambulatory chemotherapy with oxaliplatin, 5-fluorouracil and folinic acid in patients with colorectal cancer metastases. A randomized multiinstitutional trial. *J Natl Cancer Inst* 1994, **86**, 1608–1617.
19. Lévi F, Zidani R, Misset JL, for the International Organization for Cancer Chronotherapy. Randomized multicentre trial of chronotherapy with oxaliplatin, fluorouracil, and folinic acid in metastatic colorectal cancer. *Lancet* 1997, **350**, 681–686.
20. Curé H, Chevalier V, Adenis A, *et al*. Phase II trial of chronomodulated infusion of high-dose fluorouracil and l-folinic acid in previously untreated patients with metastatic colorectal cancer. *J Clin Oncol* 2002, **20**, 1175–1181.
21. Naguib FNM, Soong SJ, El Kouni MH. Circadian rhythm of orotate phosphoribosyltransferase, pyrimidine nucleoside phosphorylases and dihydrouracil dehydrogenase in mouse liver. Possible relevance to chemotherapy with 5-fluoropyrimidines. *Biochem Pharmacol* 1993, **45**, 667–673.
22. Haus E, Lakatua D, Swoyer J, Sackett-Lundeen L. Chronobiology in hematology and immunology. *Am J Anat* 1983, **168**, 467–517.
23. Tampellini M, Filipski E, Liu X-H, Li X-M, François E, Lévi F. Circadian rhythm in docetaxel tolerability and efficacy in mice. *Cancer Res* 1998, **58**, 3896–3904.
24. Shiotani T, Weber G. Purification and properties of dihydropyrimidine dehydrogenase from rat liver. *J Biol Chem* 1981, **256**, 219–224.
25. Queiroz-Claret C, Queiroz O. Multiple levels in the control of rhythms in enzyme synthesis and activity by circadian clocks: recent trends. *Chronobiol Int* 1990, **7**, 25–33.
26. Dunlap J. An end in the beginning. *Science* 1998, **280**, 1548–1549.
27. Gekakis N, Staknis D, Nguyen HB, *et al*. Role of clock protein in the mammalian circadian mechanism. *Science* 1998, **280**, 1564–1567.
28. Foulkes N, Borjigin J, Snyder SH, Sassone-Corsi P. Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Trends Neurosci* 1997, **20**, 487–492.
29. Lakin-Thomas PL. Circadian rhythms, new functions for old clock genes? *Trends Genet* 2000, **16**, 135–142.
30. Torra IP, Tsibulsky V, Delaunay F, *et al*. Circadian and glucocorticoid regulation of Rev-erbalpha expression in liver. *Endocrinology* 2000, **141**, 3799–3806.
31. Collie-Duguid ESR, Johnston SJ, Powrie RH, *et al*. Cloning and initial characterization of the human DPYD gene promoter. *Biochem Biophys Res Comm* 2000, **271**, 28–35.