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ORIGINAL ARTICLE

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Inter- and intraindividual variation in dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells

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Abstract *Purpose*: The activity of dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme in fluorouracil catabolism, has been reported to vary according to time of day. We wished to determine whether peak and trough DPD activities occurred at uniform times in six subjects, whether individual patterns fit a discernible profile, and whether such patterns were consistent and reproducible over time. *Methods*: Mononuclear cells were isolated from peripheral blood at 3-h intervals over a 24-h period on three different dates over a 6-month period. DPD activity was determined by incubating cellular lysates with [^{3}H]FUra and measuring [^{3}H]dihydrofluorouracil formation over time. *Results*: When the data were averaged by study date for each subject, the median value for the average DPD activity (11.0 pmol/min per 10^6 cells) was significantly different from both the median peak (21.1 pmol/min per 10^6 cells, $P = 0.004$) and median trough activities (4.0 pmol/min per 10^6 cells, $P = 0.002$). Within the six subjects, the average DPD activity for the three study dates differed by a median of 2.4-fold (range 1.2- to 4.8-fold). The time at which peak and trough DPD activities occurred varied between subjects: 8 of the 17 peaks (47%) occurred between 10:00 p.m. and 6:00 a.m., 6 (35%) occurred between 8:00 a.m. and 3:00 p.m., and 3 (18%) occurred between 5:00 p.m. and 8:15 p.m. Thus, the time of day when the peak occurred was essentially randomly distributed over the 24-h period of observation ($P = 0.68$). Ten (59%) of the trough DPD activities occurred between 7:00 a.m. and 3:00 p.m. The median interval between the peak and trough was 6.5 h. The data were also expressed as percent of the mean for each individual's 24-h sampling period, and reordered as time from peak rather than as the actual time of day. When the combined data for all cycles was considered, the trough occurred 6–9 h after the peak, and the DPD levels subsequent to the peak did not display merely random variation ($P = 0.0055$). *Conclusions*: DPD activity levels and the times at which peak and trough DPD activities occurred varied both between and within subjects. If fluctuations in DPD activity influence the tolerability of fixed-rate infusions of FUra, these data suggest that a single variable-rate infusion regimen may not be suitable for all patients nor for a given individual treated over several months.

Key words Circadian pharmacology · Dihydropyrimidine dehydrogenase · Fluorouracil

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Introduction

Fluorouracil (FUra) is an important component of the therapeutic armamentarium for various epithelial malignancies. Preclinical studies suggest that the duration of FUra exposure is a crucial determinant of cytotoxicity [1]. Because of its very short half-life, a variety of continuous infusion schedules have been developed. Some, but not all, randomized clinical studies have demonstrated that continuous infusion schedules are superior to bolus FUra in terms of response rates in advanced colorectal and head and neck cancers [2–7].

In several preclinical models, the time of administration of FUra and 5-fluoro-2′-deoxyuridine (FUDR) influences host toxicity [8–13]. Hematologic toxicity

is greater in mice treated with four weekly doses of 60 mg/kg FUra at 6:30 p.m. (during the ''lights off'' or active phase) compared to mice treated with FUra at 8:30 a.m. (during the ''lights on'' or resting/sleep phase) [10]. FUDR (1200 mg/kg i.p. bolus) is not lethal when administered to rats at 12:00 p.m. (midpoint of the resting phase), but kills 40% of rats when given at 4:00 a.m. (late active phase) [13]. A comparison of the toxicity of FUDR given by either constant rate or different sine-wave continuous infusion schedules in rats bearing SC-implanted adenocarcinoma FN-12762 has shown that the pattern which gives maximum drug flow during the late activity/early rest phase is less toxic and provides the most effective tumor control [11].

These observations may in part be explained by circadian-dependent changes in the rates of DNA and RNA synthesis. Peak DNA synthetic activity in normal tissues of the mouse occurs during the mid-dark (active) phase, which may account for the greater hematologic toxicity of fluoropyrimidines administered during the active phase [14]. Diurnal variation in the activity of enzymes involved with both FUra anabolism and catabolism may also partially explain these findings [13, 15, 16]. Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in FUra catabolism. When measured simultaneously, an inverse circadian relationship can be seen between the activities of DPD and thymidine kinase, the enzyme that converts FUDR to fluorodeoxyuridine monophosphate, in several tissues of rats [13]. Peak DPD activity occurs around 12:00 p.m. to 1:00 p.m. (mid-resting phase) in bone marrow and liver, while trough activity is seen between 11:00 p.m. and 1:00 a.m. (mid-active phase); the ratio of peak to trough values is about 2 [13].

DNA synthesis in human bone marrow and intestinal mucosa has also been reported to follow a circadian pattern [17, 18]. When the proportion of cells in S phase was determined in bone marrow samples at 4-h intervals in 16 healthy subjects, the average time of the highest DNA synthetic rate was 12:14 p.m. ± 3:50 (mean ± SD), and 90% of the highest values occurred during the waking span (8:00 a.m. to 8:00 p.m.). The lowest levels tended to occur during the resting span (midnight to 4:00 a.m.), and were on average about twofold lower than the high values [17]. Buchi et al. measured cellular proliferation (by [^{3}H]thymidine uptake) in human rectal mucosa obtained by biopsy at 2–3-h intervals over a 24-h period in 24 healthy men. The highest rate of DNA synthesis occurred at 5:06 a.m. ± 3:48, and was on average 8.2-fold greater than the lowest rate [18]. Although the combined data for all study dates could be fitted to a cosinor model, there appeared to be considerable variability in the times of peak activity among the subjects. Further, these two studies suggest that the times of peak DNA synthesis may vary from tissue to tissue.

FUra plasma levels during continuous infusion have been reported to vary according to time of day [19, 20]. Petit et al. reported a 2.2-fold difference in FUra steady-state plasma levels (C*pss*) obtained from seven patients during a 5-day infusion of 1000 mg/m^2 per day (with IV cisplatin on day 1). Cosinor analysis of the entire sample set indicated a peak at 1:00 a.m., while the minimum value occurred at 1:00 p.m. [19]. With protracted infusion of 300 mg/m^2 per day FUra, however, Harris et al. found a fivefold difference in FUra C*pss* during a single cycle, and the maximum occurred between 9:00 a.m. and 2:30 p.m. in six of seven patients, while the peak was at 4:00 a.m. in one patient. When a cosinor analysis was applied to the combined data for all seven patients, the peak was at about 11:00 a.m., and the trough was at 11:00 p.m. [20]. Because the time of peak DPD activity and peak FUra plasma levels varied between subjects, the data were also expressed as time from peak. With this reordering, DPD activity was inversely related to FUra plasma levels [20].

These studies suggest that circadian patterns of sensitivity to fluoropyrimidines and DPD activity appear to be reproducible in laboratory rodents subjected to strictly controlled environmental conditions (12-h each of light and dark per day). The discrepancy between the times of day at which peak and trough FUra levels occurred in two clinical studies suggests that other factors, perhaps geographical, seasonal, individual sleep/wake habits, and/or administration of other drugs may influence FUra clearance. DPD is widely distributed in tissues throughout the body [1]. Several groups have reported that DPD activity in peripheral blood mononuclear cells reflects total body DPD activity [21–23]. We therefore sought to determine whether peak and trough DPD activity would occur at uniform times in six subjects, whether individual patterns fit a discernible profile, and whether such patterns were consistent and reproducible over a 6-month period. To this end, we measured DPD activity in peripheral blood mononuclear cells obtained at 3-h intervals over a 24-h period on three occasions over a 6-month period in six volunteers who were not restricted in terms of their usual routine.

Material and methods

Materials

Phosphate-buffered isotonic saline (PBS, pH 7.4) without calcium and magnesium was obtained from Biofluids (Gaithersburg, Md.). Gelman Acrodisc filters (LC13 PVDF, 0.45 μm) were obtained from PGC Scientifics (Gaithersburg, Md.). [^{3}H]FUra (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). FUra, dihydrofluorouracil, and other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

Study design

Six healthy laboratory workers participated voluntarily in this study. Subjects were studied on two or three separate occasions over a period of 6 months. On each study day, an indwelling peripheral venous catheter was placed with a heparin lock. Around 8:00 a.m., 12–16 ml of blood was collected in a heparinized tube, and repeat samples were obtained at 3-h intervals through 5:00 a.m. the following morning. The subjects were expected to maintain their usual work schedule and mealtimes during the week prior to the study dates. Five of the six subjects (A–E) normally awoke

between 6:00 a.m. and 8:00 a.m., and went to sleep between 10:00 p.m. and 2:00 a.m. One individual (subject F), in contrast, tended to wake up around noon, stay awake through the night, then retire between 6:00 a.m. and 7:00 a.m. The first and second study dates were 3 October and 13 December for all subjects; the third study date was 6 March in one subject, 28 March in three subjects, and 15 April in the other two subjects.

Isolation of peripheral blood mononuclear cells

To minimize variability, one experienced individual processed all the blood samples on each study date. An equal volume of PBS was mixed with each blood sample, and gently layered over Lymphocyte Separation Medium (LSM, Organon Teknica, Durham, N.C.) as recommended by the manufacturer [23, 24]. The samples were then centrifuged at room temperature for 30 min at 800 *g*. The cell layer at the plasma/LSM interface was gently collected and washed with 50 ml PBS, and the cells were collected by centrifugation. The purified mononuclear cells were suspended in 1 ml iced PBS. An aliquot was subjected to red blood cell lysis with one drop of potassium cyanide, and counted by hemacytometer. Viability of the mononuclear cells was tested by trypan blue exclusion and was routinely over 95%. The cells were spun at 200 *g* for 15 min at 4 °C, and the PBS was gently aspirated. The intact cell pellet was rapidly frozen and kept at −70 °C until the time of analysis. Because of the number of samples, the time needed to purify the mononuclear cells, and the relatively short interval between samples, it was not feasible to perform the DPD assay on fresh samples. Although DPD activity in intact cells decreases by a factor of ~2 as a result of the freezing process, it remains stable thereafter [22]. Since all samples were handled in a similar fashion, the impact of the freezing process should have been similar. The interval between the date the sample was acquired and the date the assay was run averaged 47 ± 9 days, and was not significantly different for the three study dates (data not shown).

Determination of DPD activity

The cells were disrupted by sonication in 1.0 ml 135 m*M* sodium phosphate, pH 7.5; the supernatant was isolated after centrifugation (12 000 *g* for 30 min). The cellular lysate was prepared from an average of 8.1 ± 0.4 million mononuclear cells (mean ± SE). An aliquot (0.8 ml) of the supernatant was mixed with 0.1 ml 200 μ*M* [6-^{3}H]5-FUra (final specific activity 0.025 Ci/mmol) and 110 nmol nicotinamide adenine dinucleotide phosphate, reduced form (final volume 1.0 ml), and then incubated at 37 °C [25, 26]. At various times, aliquots were removed and extracted with an equal volume of ice-cold methanol. Following a 15-min incubation on ice, the samples were centrifuged at 12 000 *g* for 5 min. The supernatant was filtered through a 0.45 μm Gelman acrodisc filter, then frozen at −70 °C until analysis by reverse-phase HPLC. Buffer A was 0.5 m*M* tetrabutyl ammonium hydrogen sulfate (TBAHS) and 2.5 m*M* potassium phosphate (KH_2PO_4), pH 9.0; buffer B was 2.5 m*M* TBAHS and 2.5 m*M* KH_2PO_4, pH 9.0. A radially compressed C18 column (8 mm × 100 mm, 10 μm I.D.; Waters, Milford, Mass.) was first developed for 30 min with 100% buffer B, followed by 100% buffer A for 15 min. The run began with an isocratic gradient of 100% buffer A at 0.8 ml/min for 20 min, followed by a 5-min wash with 100% buffer B at 2 ml/min. The column was equilibrated with initial conditions for 15 min prior to the next run. Retention times (min) for cold standards were as follows: DHU, 6 min; FUra, 16 min. The distribution of radiolabelled metabolites was determined with an in-line liquid scintillation detector (Packard Instrument Co., Downers Grove, Ill.). The amount of catabolite formed was determined from the linear portion of the curve, and DPD activity was expressed as picomoles DHFU formed per minute per million nucleated cells. The lower limit of detection was 0.25 pmol/min per million cells.

To minimize variation, one individual with extensive experience performed all the DPD assays, and all eight samples from a subject's given study date were assayed on the same day. The reproduciblity of the assay in a given subject was very high: the variability in duplicate samples averaged 3.0 ± 4.8% (mean ± SD, median 8%, 25th percentile 1%, 75th percentile 3.3%, $n = 18$). The variability in DPD activity calculated from replicate samples at two or three discrete incubation times for this study averaged 17.5 ± 21.0% (mean ± SD, median 10.5%, 25th percentile 4.2%, 75th percentile 21.2%, $n = 54$).

Statistical analysis

When data for each study date were combined, the time of day at which the samples were drawn was rounded to the nearest hour (e.g. 2:00 a.m., 5:00 a.m., 8:00 a.m., 11:00 a.m., 2:00 p.m., 5:00 p.m., 8:00 p.m., 11:00 p.m.). The Mann-Whitney-Wilcoxon test was used for pairwise comparisons of groups of DPD levels. By expressing the DPD activity as a percentage of the mean DPD level by study date, any serial correlation was removed. Therefore, the Kruskal-Wallis test was used for comparisons across the eight times of day at which samples were obtained. In any test that was applied individually to the six subjects, however, only *P*-values < 0.0083 $(0.05 \div 6)$ were considered significant at the $P < 0.05$ level (Kruskal-Wallis test with Bonferroni correction for subset analysis). Chi-squared tests were derived from the exact multinomial distribution of the chi-squared statistic. The test of the negative Spearman rank correlation was performed by exact methods. Cosinor analysis was applied using a model that assumed that the peak DPD activity for all study dates for all subjects occurred at the same time of day. Because of unequal variances for different observations (i.e. the larger observed values had greater standard deviations), one of the assumptions of the cosinor model fitting procedure was violated. Therefore, the actual DPD activity levels were logarithmically transformed to substantially reduce that effect, and then the mean value for the study period was subtracted from each DPD value according to the actual time of day the samples were drawn. The values were then rescaled to have the same standard deviation on each study date. One level that was below the limits of detection was arbitrarily assigned the value 0.1 before transformation (since the smallest positive level detected was 0.27 pmol/min per million cells). The cosinor analysis was performed using the method of Tong, with the mean fixed at zero [27]. The distribution of the residuals was found to be consistent with normality. The SAS (Version 6.10, SAS Institute, Cary, N.C.) and StatXact-3 (Version 3.0, Cytel Software Corp., Cambridge, Mass.) statistical software packages were employed for all computations.

Results

Subject characteristics

Six healthy laboratory workers participated in this study, comprising five males and one female with a median age of 35 years (range 30 to 38 years). On the actual study dates, subjects A–E remained awake from the initial sample time through the seventh sampling time (about 2:00 a.m.), slept for the next 3 h, then arose for the final blood sample. Subject F remained awake for the entire 24-h sampling period on each of the three study dates. The times that blood samples were obtained from each subject are shown in Table 1.

Analysis of times of peaks and troughs in DPD activities

Data from a total of 17 study dates were evaluable for analysis (three study dates in five subjects, two study

Table 1 Time of blood samples during each 24-h sampling period. The values shown are the means ± SD or, for subject B, ± ½ range (*a* a.m., *p* p.m.)

	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth
A	7:50a ± 1:02	10:50a ± 0:17	1:53p ± 0:38	5:03p ± 0:29	7:50p ± 0:44	10:48p ± 0:34	1:25a ± 0:17	5:33a ± 0:38
B	7:35a ± 0:24	10:37a ± 0:23	1:35p ± 0:25	4:47p ± 0:17	7:30p ± 0:30	10:35p ± 0:25	1:20a ± 0:15	5:03a ± 0:03
C	7:53a ± 0:21	11:02a ± 0:05	1:56p ± 0:25	4:56p ± 0:15	7:55p ± 0:24	10:53p ± 0:21	1:45a ± 0:13	5:05a ± 0:09
D	7:45a ± 0:40	10:50a ± 0:31	1:50p ± 0:46	4:55p ± 0:23	7:48p ± 0:34	11:05p ± 0:34	1:41a ± 0:20	5:01a ± 0:03
E	8:25a ± 0:43	11:16a ± 0:15	2:06p ± 0:12	5:13p ± 0:23	8:15p ± 0:15	11:03p ± 0:15	2:05a ± 0:09	5:00a ± 0:00
F	8:49a ± 0:25	11:08a ± 0:10	2:03p ± 0:03	5:05p ± 0:05	8:04p ± 0:04	11:03p ± 0:03	1:53a ± 0:12	5:08a ± 0:14

dates in one subject). We first considered the observed peaks and troughs over the eight sample times on each study date. The average DPD activities for the combined study dates in each of the six subjects according to actual time of day are presented in Table 2. Mean, trough, and peak DPD activities (pmol/min per million cells) ranged from 8.2 to16.4 (2-fold), 4.0 to 11.1 (2.8-fold), and 12.2 to 24.5 (2-fold), respectively. The time of peak and trough DPD activities differed among the six subjects. The peak occurred between 11:00 p.m. and 2:00 a.m. in three subjects, between 5:00 a.m. and 8:00 a.m. in two subjects, but was in the late afternoon (5:00 p.m.) in one subject. The discrepancy between subject D's time of peak could not be explained by the subject's usual sleep/wake cycle. The trough DPD activity occurred around 2:00 a.m. in one subject, between 8:00 a.m. and 11:00 a.m. in three subjects, and between 5:00 p.m. and 8:00 p.m. in two subjects. The interval from peak to trough DPD activity was close to 12-h in only two subjects (E and F).

The time of day at which the peak and trough values occurred for the 17 study dates was then considered. Since eight samples were obtained over the 24-h period, the data were grouped into three intervals (Table 3). Eight of the 17 peaks occurred during the overnight interval, while ten of the trough values occurred during the mid-day interval. The median interval between the peak and trough was 6.5 h (mean 8.6 h). If the 17 peak and trough values were randomly distributed across these three intervals, the expected distribution might be six, six and five. The observed distribution indicated that the time of day when the peak occurred was essentially randomly distributed over the 24-h period. Although the trough values showed a less even distribution among the three intervals, the pattern was not significantly different from a uniform distribution ($P = 0.22$). Since subject F had an altered sleep/wake schedule compared to the other five subjects, the analysis was repeated with subject F's peak shifted by about 6 h. Even with this adjustment, however, the time when the peaks ($P = 0.42$) and troughs ($P = 0.22$) occurred appeared to be random.

Finally, a cosinor model was employed to select the time of peak and trough activities. Cosinor analysis assumes a particular symmetrical distribution of deviations from the mean, and stipulates that the fitted peak has to be 12-h before the trough. We found that the computer model did not produce an adequate fit of the data (data not shown), attributable to the wide variation in the time of peak and trough values and the intervals between peak and trough. Therefore, a model-free analysis was more useful in selecting the times of the peak and trough.

Analysis of DPD activity according to actual time of day

The variation in DPD activity between study dates was next examined (Table 4). Within the six subjects, the average DPD activity for the three study dates varied by a median of 2.4-fold (range 1.2- to 4.8-fold). With the 17

Table 2 Variation in DPD activities in peripheral blood mononuclear cells. (*a* a.m., *p* p.m.) Peripheral blood mononuclear cells were isolated from six volunteers at 3-h intervals for a 24-h period beginning at 8:00 a.m. on two or three separate occasions over a 6-month period. DPD activity in cellular lysates was determined by measuring the formation of [^{3}H]dihydrofluorouracil by HPLC as described in Methods. The data are presented as mean ± SD DPD activity (pmol/min per million cells) according to the time of day for each subject ($n = 3$), except for subject B (mean ± ½ range, $n = 2$). Since a cosinor model did not accurately describe the data, the times of the peak and trough DPD activity reflect the high and low values, and are indicated by *[P]* and *[T]*, respectively

Time (± *SD*)	A	B	C	D	E	F
1:42a ± 0:20	5.8 ± 7.9 *[T]*	11.5 ± 1.5	21.9 ± 13.1 *[P]*	10.6 ± 6.2	17.6 ± 9.8	13.2 ± 3.4
5:08a ± 0:19	8.1 ± 5.5	6.5 ± 2.5	15.4 ± 4.0	12.8 ± 8.0	24.5 ± 12.3 *[P]*	15.1 ± 4.4
8:04a ± 0:42	17.5 ± 18.8	4.0 ± 0.1 *[T]*	9.7 ± 3.3 *[T]*	10.3 ± 5.9	13.2 ± 10.9	15.9 ± 8.8 *[P]*
10:58a ± 0:21	14.0 ± 4.2	6.8 ± 1.8	18.4 ± 10.0	6.9 ± 5.8 *[T]*	15.4 ± 19.3	9.6 ± 13.0
1:55p ± 0:27	8.4 ± 3.3	5.7 ± 0.4	18.7 ± 6.1	7.4 ± 2.2	11.9 ± 7.9	14.9 ± 13.8
5:00p ± 0:19	11.6 ± 2.1	11.5 ± 0.9	13.9 ± 6.9	20.0 ± 21.7 *[P]*	11.1 ± 11.4 *[T]*	14.3 ± 2.7
7:55p ± 0:28	12.4 ± 5.1	7.3 ± 2.7	14.2 ± 4.0	13.8 ± 8.7	21.8 ± 14.8	7.7 ± 7.5 *[T]*
10:58p ± 0:26	19.1 ± 6.0 *[P]*	12.2 ± 0.6 *[P]*	16.8 ± 9.6	8.2 ± 4.5	15.5 ± 6.1	12.1 ± 6.1
Mean (24 h period)	12.1 ± 4.6	8.2 ± 3.1	16.1 ± 3.7	11.2 ± 4.3	16.4 ± 4.7	12.8 ± 2.9
Peak/trough ratio	3.2	3.0	2.3	2.9	2.2	2.1

Table 3 Distribution of times of peak and trough DPD values. The data represent the times of peak and trough DPD values from all 17 study dates. For the statistical analysis, a *P*-value < 0.05 would indicate that the distribution is significantly different from a uniform or random distribution

	Interval			
	Overnight 11 p.m., 2 a.m., 5 a.m.	Mid-day 8 a.m., 11 a.m., 2 p.m.	Late-day 5 p.m., 8 p.m.	Random distribution? (Chi-squared test)
Peak	8 (47%)	6 (35%)	3 (18%)	Yes ($P = 0.68$)
Trough	4 (24%)	10 (59%)	3 (18%)	Yes ($P = 0.22$)

study dates combined, the median value for the average DPD activity (11.0 pmol/min per million cells) was significantly different from both the median peak (21.1 pmol/min per million cells, $P = 0.004$) and median trough activities (4.0 pmol/min per million cells, $P = 0.002$, Mann-Whitney-Wilcoxon test). The highly significant difference in the median values for the peak and trough groups ($P < 0.0001$) indicates that the range of the peak activities did not greatly overlap the range of the trough activities.

To properly interpret the apparent variation in the data according to actual time of day, we wished to detect whether there was any one time or any set of times at which the DPD activity was consistently higher or lower than at other times, over all study dates for a subject. Since there were appreciable differences between absolute DPD values on different study dates, the data were expressed as percentages of the mean for each study date. The statistical test was then applied to the combined data for two (subject B) or three study dates for each subject, and the following *P*-values reflect either 16 values (subject B, 2×8 time-points) or 24 values (3×8 time-points): A, $P = 0.14$; B, $P = 0.019$; C, $P = 0.21$; D, $P = 0.22$; E, $P = 0.037$; and F, $P = 0.66$. The uncorrected *P*-values suggested that the variations in the percentage mean DPD activity appeared to be different in only two of the six subjects (B, E). After correction for subset analysis, however, the variations in the percentage mean DPD values were not significantly different in any of the subjects. When the data were combined across all the subjects, the test had a *P*-value of 0.20.

To test whether the variation in DPD activities displayed a symmetrical distribution, we determined whether the values at 12-h intervals were significantly different. Each study date for each subject would thus contain four pairs 12-h apart (e.g. 2:00 a.m. and 2:00 p.m., 5:00 a.m. and 5:00 p.m., etc.). When the data were expressed as the percentage mean DPD activity on each study date, the differences in activity at 12-h intervals were significant in only one of six subjects: subject E ($P = 0.0025$, negative Spearman rank correlation), while the other subjects all had *P*-values > 0.40. Therefore, the variations in DPD activity did not conform to a symmetrical distribution in five of six subjects.

Because of the variability in DPD activities across study dates, it was possible that a small but consistent circadian pattern might be missed. When the data were expressed as the percentage mean DPD activity on each study date, the distribution appeared to be skewed, and thus did not meet the assumptions needed for cosinor analysis. In order to remove the effect of the nonsymmetrical distribution, the actual DPD activity levels

Table 4 Variation in DPD activities (expressed as pmol/min per million cells) across study dates (*b.d.* below detection, no formation of [^{3}H]dihydrofluorouracil was detected during a 2-h incubation of cellular lysate prepared from 6.9 million mononuclear cells, *a* a.m., *p* p.m.)

Subject	Test period	Mean DPD activity	DPD peak		DPD Trough		Interval peak to trough (h)
			Activity	Time	Activity	Time	
A	1	17.0 ± 8.5	37.5	9:00a	11.7	2:20p	5.3
	2	10.2 ± 6.2	17.8	8:10p	0.4	1:35a	5.4
	3	9.2 ± 8.3	25.8	10:10p	0.3	7:00a	8.8
B	1	9.0 ± 3.1	12.8	11:00p	3.8	8:00a	9.0
	3	7.4 ± 3.7	13.0	1:05a	4.0	5:00a	3.9
C	1	18.8 ± 4.9	25.2	11:00a	11.1	5:00p	6.0
	2	8.6 ± 2.1	11.9	2:20p	5.8	11:10p	8.8
	3	20.9 ± 7.3	33.4	2:00a	8.6	7:30a	5.5
D	1	18.2 ± 11.5	44.8	5:00p	9.4	2:00p	21.0
	2	4.5 ± 1.0	6.0	8:15p	3.5	11:15a	15.0
	3	11.4 ± 5.5	21.1	5:00a	3.6	10:15a	5.2
E	1	27.4 ± 6.8	37.3	11:00a	20.0	2:00p	3.0
	2	16.1 ± 9.0	32.2	5:00a	4.1	5:40p	12.7
	3	5.7 ± 3.1	10.4	5:00a	1.1	11:30a	6.5
F	1	19.5 ± 5.8	29.9	2:00p	12.6	2:00a	12.0
	2	8.0 ± 4.6	16.6	5:25a	2.8	11:05a	5.7
	3	11.0 ± 7.0	19.0	8:42a	b.d.	8:05p	11.4
Mean ± SD		13.1 ± 6.3	23.2 ± 11.2		6.2 ± 5.4		8.6 ± 4.6
Median		11.0	21.1		4.0		6.5

required rescaling and transformation. The result was not significant for the best fitting model with a peak at 11:00 p.m. ($P = 0.17$, data not shown). The cosinor analysis was repeated after shifting subject F's peak back 6 h to adjust for the altered sleep/wake cycle. The fit improved after this adjustment ($P = 0.035$, data not shown). Thus, there did seem to be a weak circadian pattern after much of the variation had been removed. The amount of standardization needed to detect this pattern, however, suggests that the apparent statistical significance of the result was much greater than its clinical importance.

Analysis according to time from peak DPD activity

To account for the possibility that different cycles might have peaks at disparate times of day for an individual subject, the percentage mean DPD activity was shifted so that the peaks occurred at hour zero (Fig. 1). There was a tendency for the trough to fall 6–9 h after the peak. Using the time from peak DPD activity, we tested whether the percentage mean DPD values were significantly different at any time over the 24-h sampling period. The resulting P-values were smaller than before for five of the six subjects: A, 0.017; B, 0.3; C, 0.032; D, 0.13; E, 0.002; and F, 0.048 (Kruskal-Wallis test). This analysis suggested that the DPD activities were indeed different throughout the 24-h period in four of the six subjects. The apparent significance, however, may simply reflect the fact that the DPD levels at the time of the peak were by definition higher than the rest of the values. To ensure that the significance did not result merely from clustering the highest values at the same point, the data for individual subjects were also analyzed with the hour zero (peak) data omitted. The secondary analysis provided a P-value below 0.05 only for subject E: $P = 0.026$. After applying the correction for subset analysis, however, this P-value was no longer significant, indicating that the null hypothesis was not rejected for any of the six subjects.

Analysis of the combined data for each study date across all the subjects according to time from peak (Fig. 2) again suggested a tendency for the trough to fall 6–9 h after the peak. When the combined data for all cycles for all subjects was considered (Fig. 2), the percentage DPD activities over the 24-h period were significantly different ($P < 0.0001$). When the analysis was repeated with the peak omitted, there was still a significant difference in the remaining seven times ($P = 0.0055$) owing to the consistency of this trough. By

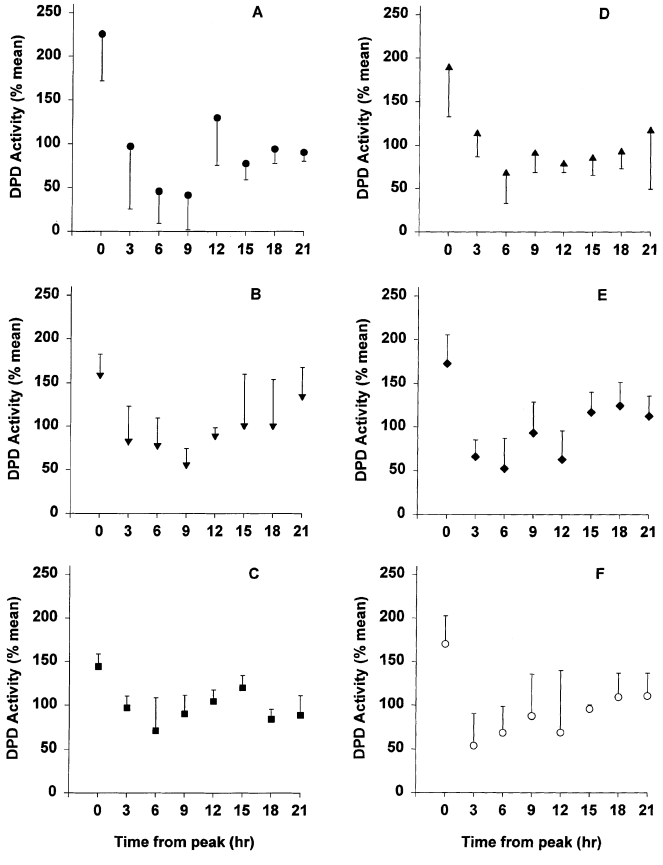

Fig. 1A–F Variation In DPD activity for each subject (**A–F**). The percentage of the mean DPD activities for each time-point (rounded to the nearest hour) for each individual's study dates were shifted to express the time at which the peak DPD level occurred as time zero hours. The data for all study cycles for each individual are then shown as the mean ± SD (subjects A, C–F) or the mean ± ½ range (subject B) DPD level as a function of the time from peak DPD activity

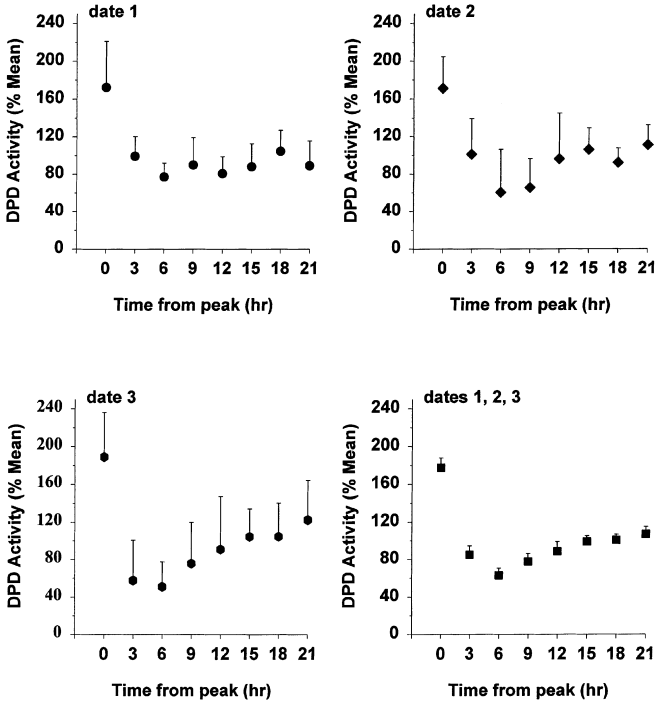

Fig. 2 Variation In DPD activity across study dates. The data are presented as the percentage of the mean DPD activity for each cycle according to time from peak. Cycles 1 and 3 are the means ± SD for all six subjects, while Cycle 2 is the mean ± SD for five subjects. The bottom right panel shows the combined data for all 17 cycles (mean ± SE)

reordering the data as time from peak rather than actual time of day, therefore, the DPD levels subsequent to the peak did not have merely random variation.

Discussion

Variable-rate infusion schedules of FUra and FUDR using programmable pumps are being tested in the clinical setting in an effort to minimize host toxicity [28–33]. Von Roemeling and Hrushesky have examined the clinical toxicity of FUDR administered as either a fixed-rate infusion (0.15 mg/kg per day for 14 days) or the same dosage as a variable-rate infusion (15% of the total dose from 9:00 a.m. to 3:00 p.m.; 68% from 3:00 p.m. to 9:00 p.m.; 15% from 9:00 p.m. to 3:00 a.m.; 2% from 3:00 a.m. to 9:00 p.m.) [28]. In both a six-patient crossover pilot study and a 30-patient randomized trial, the incidence and severity of diarrhea was significantly lower with the variable-rate infusion program [28]. In a subsequent dose-escalation study, patients tolerated an average of 1.5-fold more FUDR with minimal toxicity with the variable-rate infusion [29].

Levi and colleagues have tested a variable-rate infusion regimen of FUra, calcium leucovorin and oxaliplatin given daily for 5 days every 3 weeks. An 11.5-h infusion of 120 mg/m^2 oxaliplatin was given from 10:15 a.m. to 9:45 p.m. with a sinusoidal profile and the peak at 4:00 p.m., while FUra/leucovorin were given concurrently from 10:15 p.m. to 9:45 a.m. with peak delivery at 4:00 a.m. [31, 32]. A randomized trial comparing this chronomodulated regimen versus fixed-rate infusion of all three drugs over 24 h in patients with metastatic colorectal cancer indicated that severe mucositis occurred more frequently in patients receiving fixed-rate infusion (89% vs 18%) [32]. The median FUra dose (500 vs 700 mg/m^2 per day) and the response rate (32% vs 53%) were lower in patients receiving fixed-rate infusions. It is possible, however, that the difference in toxicity and clinical outcome may have been due to concurrent 24-h infusion of all three drugs rather than a 12-h daily exposure to each drug (with a 12-h drug-free period) as opposed to chronomodulation per se.

A different regimen employed a 14-day variable-rate infusion of FUra (200 mg/m^2 per day) plus leucovorin (5 mg/m^2 per day) with 50% of the daily dose given between midnight and 6:00 a.m. with the peak between 3:00 and 4:00 a.m. [33]. In six patients who developed grade 2 or worse toxicity, the infusion schedule was modified such that the peak infusion rate occurred between 9:00 and 10:00 p.m. This change was accompanied by reduced toxicity in all six patients, and three patients were able to receive further FUra dose escalation.

It is not clear why there should be differences in the "optimal" time of administration of the highest infusion rate of FUra and FUDR in these various studies. In addition, there does not seem to be an adequate explanation for the 12-h discrepancy between the time of peak plasma levels of FUra during fixed rate infusion in two separate clinical studies [19, 20]. DPD activity can be influenced both directly and indirectly by several factors, including enzyme content, the presence of inhibitors, and the level of the NADPH cofactor. However, the biochemical or molecular basis for diurnal fluctuations in DPD activity has not yet been established, but is a subject of ongoing laboratory investigation.

Given the pivotal role that DPD plays with respect to both FUra and FUDR clearance, we sought to determine whether DPD activity differed at various times during a 24-h period in six volunteers, if so, whether the pattern was reproducible in individual subjects, and whether the pattern was similar between subjects. A potential source of variability is that during the 24-h study period, the subject will of necessity have a disturbed sleep pattern. However, this same issue pertains to studies in both laboratory animals and in patients receiving FUra infusions: the lights must be switched on and the subject disturbed to obtain the sample.

Using the actual DPD activities and the correct time of day the samples were obtained, we found that the time at which the peak and trough DPD activities occurred varied markedly between normal subjects. Further, this pattern of DPD activity within individuals also varied over time. In order to remove some of the possible sources of variation that might obscure any small but consistent pattern, the data were "standardized" in several ways. For example, using the combined, raw DPD activity from the 17 study points, significant differences were evident when the median values for the peaks, troughs, and means were compared. If the same comparison were to be made after transforming the data to percentages of the mean, the results would be much more significant because all the peaks would be above 100%, all the troughs below 100%, and all the means exactly 100%. Such standardization removed some of the cycle-to-cycle variation in the means, and thus provided sharper distinctions. The data conformed to a cosinor fit only when the data expressed as percentage mean DPD activity were subjected to log transformation, centered and rescaled by study date, and the time of peak for subject F shifted back 6 h to adjust for the altered sleep schedule.

When the data were reordered as time from peak DPD activity rather than the actual time of day, however, the combined data for all subjects and all study dates indicated that the DPD activity values differed significantly, and that the DPD levels after the peak did not have merely random variation. Although some regular changes in DPD activity occurred over the course of a day, the pattern did not conform to the anticipated profile, since the interval from peak to trough was 6–9 h rather than 12 h.

When the data were analyzed by individual or by cycle, the results generally became less significant, as a result of having less data in any one analysis and of having to correct for the multiple analyses. These individual results, however, helped to show the heterogeneity among the subjects, which is one of the sources of

variation mentioned above. Fleming et al. measured FUra plasma levels at 3-h intervals during the first two treatments in 28 patients receiving a weekly 24-h infusion of 1000 or 1800 mg/m^2 FUra with leucovorin (500 mg/m^2) [34]. Among 53 study dates, the actual peak occurred at either 3 a.m. or 6 a.m. on only 45% of occasions and the actual trough occurred at either 3 p.m. or 6 p.m. on only 32% of occasions. Among 25 patients in whom pharmacokinetic data were available on two consecutive occasions 1 week apart, the time of the peak and trough occurred within 3 h of each other in only 44% and 40% of patients, respectively. The considerable inter- and intrapatient variation in time of peak and trough FUra plasma levels on different study dates is thus in keeping with our observations.

Given human genetic diversity, heterogeneity of lifestyles with different sleepwake cycles, the geographical and seasonal changes which influence the hours of daily light exposure, and the possible influences of other drugs, hormones, feeding/fasting, and rate of cell proliferation on circadian rhythms, it may be overly simplistic to assume that potential diurnal variations in a single parameter such as DPD activity between individuals in a given patient population would be similar, or even that the activity versus time of day profile in an individual would be reproducible over time. If diurnal changes in DPD activity influence the toxicity and response to infusional fluoropyrimidine therapy, our data suggest that a single variable-rate infusion regimen might not be suitable for all patients, or even for an individual patient over a period of several months. To further address these questions in a clinical context, we are currently analyzing FUra plasma levels obtained at 3-h intervals over a 24-h period in patients receiving fixed-rate FUra infusions daily for 3 days every 3–4 weeks to determine whether the variation in FUra plasma levels fits a circadian profile, and whether an individual's pattern is reproducible from one cycle to the next.

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