Impact of Two Weekly Schedules of Oral Eniluracil Given with Fluorouracil and Leucovorin on the Duration of Dihydropyrimidine Dehydrogenase Inhibition

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ABSTRACT

Purpose: This study determined the effect of different weekly dosing schedules of 5-fluorouracil (5-FU)/leucovorin (LV)/eniluracil on dihydropyrimidine dehydrogenase (DPD) activity and plasma uracil levels.

Methods: Plasma and mononuclear cells were isolated from peripheral blood samples obtained before, during, and at various times after 5-FU/LV/eniluracil therapy. Two schedules were studied: 20 mg of eniluracil p.o. plus 30 mg of LV p.o. on days 1–3 with a single dose of 5-FU given day 2, or 30 mg of LV p.o. on days 1–2 with a single dose of eniluracil and 5-FU on day 2. DPD activity was determined with a radiotisotopic enzyme assay; the reaction products were separated by high-performance liquid chromatography. Plasma uracil levels were determined by gas chromatography-mass spectroscopy.

Results: During oral therapy, DPD activity was profoundly depressed, and uracil levels were strikingly elevated with both schedules. With the daily-for-3-days schedule, DPD activity was similar to baseline values by 3 weeks after the earlier eniluracil dose, whereas it appeared to recover earlier in patients receiving the single-dose schedule, reaching baseline values by 2 weeks. Although baseline uracil values did not predict DPD activity accurately, plasma uracil levels >0.95 μM were associated with significantly lower DPD activity (median, 18.4 versus 287.6 pmol/min/mg).

Conclusions: When eniluracil is given with 5-FU/LV, DPD inhibition appears to be influenced by schedule, and the time to recovery is much longer than has been observed with eniluracil given alone.

INTRODUCTION

The rate-limiting step of 5-FU2 catabolism is mediated by DPD, which converts 5-FU to dihydrofluorouracil (1). DPD is widely distributed throughout the body, and uracil and thymine are its natural substrates (2). Traditionally, 5-FU has not been given by the p.o. route because of incomplete and variable bioavailability. Several strategies have been developed to allow p.o. dosing, including the use of 5-FU prodrugs or inhibitors of DPD. Binding of the uracil analogue EU to DPD results in the formation of a covalent bond and, consequently, DPD inactivation, (3–5). Preclinical and clinical studies have shown that EU allows complete bioavailability of orally administered 5-FU and increases the 5-FU plasma half-life to 4.5–5 h (6–10).

The effect of EU monotherapy in humans on DPD activity in PBMCs has been examined in patients given a single dose of EU daily for 7 consecutive days at one of three dose levels: 0.74, 3.7, or 18.5 mg/m² (7). DPD activity was not detected 1 h after any of the three EU doses; DPD activity 24 h after dosing was 7, 2, and 2.5% of baseline, respectively. Two weeks after the seventh daily EU dose, DPD activity had returned to 60, 70, and 123% of baseline after 0.74, 3.7, and 18.5 mg/m², respectively. These results suggest that EU as low as 0.74 mg/m² daily provide near-complete DPD inactivation for at least 24 h after dosing, and that enzyme activity has largely recovered 14 days after the last dose.

We reported previously the results of a Phase I trial of oral EU/5-FU/LV in which patients received 20 mg of EU and 15 mg of LV twice daily on days 1–3, with a starting dose of either 10 or 15 mg/m² 5-FU given twice daily on day 2 repeated weekly for 3 of 4 weeks (10). DPD in PBMCs was inactivated completely on day 3 (12 h after the earlier EU dose) in 11 of 12 patients. DPD activity remained depressed at 5, 12, and 19 days after the last EU dose (mean) at 6, 21, and 59 pmol/min/mg, respectively. Baseline plasma uracil levels averaged 0.17 ± 0.05 μM and were markedly elevated during EU therapy (39 μM on day 3), which indirectly reflects DPD inactivation. The uracil levels remained elevated 5 and 12 days after EU therapy at 8.5 and 3.5 μM, respectively.

These results suggest that the duration of DPD inactivation as measured directly in PBMCs or by elevations in plasma uracil in patients receiving EU in combination with 5-FU and LV was much longer than observed in patients receiving EU alone. An important consideration was that life-threatening and fatal toxicities in patients have been reported in other studies when standard doses of fluoropyrimidines were given up to 31 days

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2 The abbreviations used are: 5-FU, fluorouracil; DPD, dihydropyrimidine dehydrogenase; LV, calcium leucovorin; EU, eniluracil; PBMC, peripheral blood mononuclear cell; CV, coefficient of variation.
after the last dose of 5-FU/EU (11), suggesting that DPD activity may have remained abnormally low in these individuals.

We therefore evaluated two attenuated weekly schedules of EU, either a single daily dose of 20 mg given with LV on days 1–3 and 5-FU on day 2, and subsequently, a single dose of EU given 1–12 h before 5-FU. DPD activity and plasma uracil were monitored before, during, and after completion of EU therapy. The pharmacodynamic effect of these two different dose schedules of EU is reported herein.

PATIENTS AND METHODS

Eligibility. Patients were accrued into this amended protocol between October 1998 and July 2000. Patients with solid tumors for whom a 5-FU/LV-based regimen represented a reasonable therapeutic approach or for whom no effective standard therapy was available were eligible provided they had adequate hematological, hepatic, and renal function as reported previously for the twice-daily schedule of EU on days 1–3 (10). This study had the approval of the local Institutional Review Boards and the Cancer Therapy Evaluation Program, National Cancer Institute. All patients gave written informed consent.

Treatment Plan. Tablets of EU (10 mg) and 5-FU (5 and 25 mg) administered p.o. were formulated by Glaxo Wellcome (Research Triangle, NC) and supplied by the Cancer Therapy Evaluation Program. Commercial sources were used for the i.v. 5-FU and p.o. LV. During the initial period, 15 mg of LV p.o. was given twice daily on days 1–3. On day 2, 2300 mg/m² 5-FU was given by continuous infusion over a 24-h period. The patient returned 2 weeks later to commence therapy with 20 mg of EU and 30 mg of LV p.o. daily on days 1–3. 5-FU was given p.o. on day 2. The starting dose of 5-FU was 15 mg/m², with the treatment repeated weekly for 3 or 4 weeks. Dose escalation was planned in cohorts of 3 or 6 patients with 25% increments until dose-limiting toxicity was seen in at least 2 patients at a given level. Sixteen patients were enrolled in this portion of the trial (patients 13 to 28) at one of four 5-FU dose levels: 15, 18.8, 23.4, and 29 mg/m². Ten patients were enrolled subsequently and received 30 mg of LV p.o. on days 1 and 2, with a single dose of 20 mg of EU given at either 12 h (patients 29–33) or 1 h (patients 34–38) before 29 mg/m² of 5-FU p.o. on day 2.

Pharmacodynamic Sampling Scheme. Peripheral blood was collected in heparinized tubes at the following times: before therapy; on days 2 and 3 of the first cycle of EU/LV/5-FU (before the daily drug administration); on day 8 (week 2, cycle 1); at the start of cycle 2; and when feasible, every 1–2 weeks after protocol treatment was stopped. The tubes drawn for measurement of plasma uracil levels were placed immediately on ice and centrifuged at 800 × g for 15 min at 4°C; the plasma was then frozen at −70°C until analysis. For patients 23–38, additional samples were obtained on the second day of period 1 before i.v. 5-FU and before period 2, 2 weeks later, to allow assessment of the potential variability of pre-EU DPD and plasma uracil levels.

PBMCs were isolated by Ficoll-Hypaque density centrifugation, and erythrocytes were lysed with a brief hypotonic lysis step. Intact cell pellets were stored at −70°C until analysis. The frozen cell pellet was suspended in 300 μl of DPD assay buffer, and the cellular lysate was isolated as described previously (10, 12). Lysate containing 200–300 μg of protein (determined by the Bio-Rad protein assay kit; Bio-Rad, Hercules, CA) was incubated in a final volume of 1 ml of DPD assay buffer containing 20 μM [14C]5-FU (final specific activity, 0.009 μCi/nmol; Moravek Biochemicals, Brea, CA) and 250 μM NADPH-reduced form in a 37°C shaking water bath. At 15-min intervals, a 250-μl aliquot was removed and transferred to a 1.5-ml microcentrifuge tube containing an equal volume of 100% ice-cold methanol. After incubation on ice for 15 min, the sample was centrifuged, and the methanol-soluble supernatant was filtered through a 0.2 μm Gelman Acrodisc filter. Donor Buffy coats were used as a positive control with each set of reactions. A sample that contained all assay ingredients except a source of protein served as the negative control and permitted correction for any possible degradation of the radiolabeled 5-FU used in the enzymatic reaction (which averaged 1.19 ± 0.46%).

Analytic Methods. 5-FU was separated from its catabolites by an ion-pairing high-performance liquid chromatography method using a Waters (Milford, MA) high-performance liquid chromatography system with an in-line Radiomatic 500TR series Flow Scintillation Analyzer (Packard Instruments, Meriden, CT) as described previously (10, 12). DPD activity was defined as the pmol catabolites formed per min per mg protein using the average of all four time points. The amount of protein used in the assays averaged 270 ± 60 μg. The DPD reaction was linear for 60 min; linear regression of the amount of DHFU formed versus time (15–60 min) produced an average r² value of 0.996 ± 0.005 for 58 peripheral blood samples obtained before EU dosing.

Uracil was determined in 0.2-ml aliquots of plasma by a validated gas chromatography-mass spectrometry method as described previously using [15N₂]uracil as the internal standard (13). Graphical analysis was performed with SigmaPlot 2001 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Patient Characteristics. Twenty-six patients were treated on the amended clinical protocol. Seventy-three % had colorectal cancer, 11.5% had pancreatic cancer, 7.6% had cholangiocarcinoma, and the remainder had other histologies. The median age was 61.5 years (range, 32–80 years), and two-thirds were male. The percentage of patients receiving 5-FU or irinotecan previously was 88 and 62%, respectively.

Inter- and Intraindividual Variation in Pretreatment DPD Activity. PBMCs were obtained the morning before receiving period 1 5-FU therapy in 24 patients (samples taken at 9:12 a.m. ± 58 min). DPD activity averaged 309.7 ± 125.3 pmol/min/mg, with a 5.6-fold range among patients (median, 295.0 pmol/min/mg; range, 114–641 pmol/min/mg). The degree of interindividual variation is expected (14, 15). Serial blood samples were also obtained in a subset of 15 patients on a median of 3 separate days over a 2-week period to assess the variation in DPD activity before receiving EU therapy. Considerable intraindividual variation in the baseline samples was evident (Fig. 1), with an average (±SD) CV of 31.4 ± 15.2%. To provide a positive control and a measure of day to day variability in the assay, aliquots of Buffy coats isolated from six pooled donor samples were sequentially analyzed on 4–10
Intrasubject variation in DPD activity. PBMCs were isolated from 15 patients on a median of three separate occasions (range, 2–5) over a 2-week period before receiving EU therapy. DPD activity is shown as the means, except in patients 23 and 24, for whom the mean ± ½ range is shown (n = 2); bars, SD.

The baseline uracil level obtained before the start of the 24-h 5-FU infusion during period 1 averaged 0.30 ± 0.11 μM, and the range was 5-fold (0.09–0.48 μM). Ten patients had several baseline samples obtained on different days; the CV averaged 13.5 ± 4.2% (data not shown).

Effect of EU on DPD Activity. PBMCs were isolated pretreatment, on days 2 and 3 of the first week of oral therapy before the daily drug dosing, and at various intervals after the previous dose of EU. As shown in Fig. 2, DPD activity in PBMCs was diminished profoundly on days 2 and 3 of the initial week of oral EU/5-FU/LV therapy with both the daily-for-3-days and single-dose schedules. A difference in the extent of DPD recovery (pmol/min/mg) was apparent in the samples taken on day 8, week 2 (median = 21.4 and 137.0 pmol/min/mg, 5 and 6.5–7 days after the last EU dose given at week 1, respectively) and at the start of cycle 2 (median = 51.6 and 143.3 pmol/min/mg, 12 and 13–13.5 days after the last EU dose given at week 3, respectively). These results indicate that a single dose of 20 mg of EU given within 24 h before oral 5-FU provides a profound degree of DPD inactivation. DPD activity appears to recover more quickly (~2 weeks) after a single weekly EU dose given with oral 5-FU/LV compared with the daily-for-3-days schedule (~3 weeks).

Comparable increases in plasma uracil were seen during days 1–3 of the first week of oral EU/5-FU/LV therapy on both schedules (Fig. 3). With the daily-for-3-days schedule, the uracil levels in the plasma samples obtained on day 3 (24 h after the oral 5-FU dose, 24 h after two daily doses of EU) were consistently higher (2.0 ± 0.5-fold) than that measured before p.o. 5-FU dosing. Although the uracil levels declined thereafter, the plasma levels remained elevated by 16.9 ± 11.2-fold and 5.9 ± 3.6-fold compared with the pretherapy sample 5 and 12 days after the last EU dose, respectively. Uracil levels were similar to baseline values by 19 days after EU.

A similar increase in uracil was evident in the samples obtained 12 and 36 h after the single EU dose (12 h before and 24 h after the oral 5-FU dose). The plasma uracil levels remained elevated over baseline by 13.3 ± 14.2-fold and 3.6 ± 3.2-fold 6–6.5 and 13 days after EU, respectively. Because there were few data beyond day 13 after EU, it is not possible to precisely estimate when the plasma uracil levels returned to baseline on this single weekly-dose schedule.

Although pretreatment uracil levels did not accurately predict for DPD activity in PBMCs (data not shown), when all paired samples for uracil and DPD activity were analyzed, patients with uracil values lower than the median of 0.95 μM had significantly higher DPD activity than patients whose uracil values were above the median (Fig. 4).

DISCUSSION

Studies evaluating the impact of EU monotherapy on DPD activity have demonstrated that the enzyme is inactivated rapidly and remains so for at least 24 h after dosing, and that enzyme recovery occurs by 7–14 days after therapy (7, 16). Because EU does not have single agent antitumor activity, its clinical development has involved its use as a modulator of 5-FU catabolism (17). Only a few studies have evaluated the impact of EU on DPD activity and uracil levels when given in combination with 5-FU/LV (10, 18). A 20-mg dose of EU given twice daily for 7 days in combination with oral 5-FU at doses ranging from 2.5 to 5 mg/m² and radiation therapy in patients with head and neck cancer led to complete or near-complete DPD inactivation during therapy, but the time to enzyme recovery was not assessed (18). In our initial study that evaluated weekly 20-mg EU p.o. given with 15 mg of LV twice daily on days 1–3 and 5-FU given twice daily on day 2, we found that DPD activity in PBMCs remained inhibited to an average of 24% of the baseline value 19 days after the prior EU dose (10).
say (limit of quantitation, 0.025 μM) allowed us to measure accurately the baseline samples in all patients. In several patients in whom serial plasma samples were available after EU therapy, uracil values were projected to require 4–5 weeks to reach baseline levels, which was consistent with the delay in DPD recovery in PBMCs.

These unexpected results led us to evaluate in turn two attenuated schedules of weekly EU given as a single daily dose for either 3 days or 1 day along with LV (once on days 1–3 or days 1–2) and 5-FU (once on day 2) and to determine the effect on DPD inhibition. We found that DPD inactivation during the first 3 days of oral therapy was comparable with both schedules, but recovery of DPD activity in PBMCs appeared to occur more quickly with the single weekly dose schedule compared with the three-consecutive-days schedule (~2 weeks versus 3 weeks).

EU pharmacokinetics do not appear to be affected by concomitant 5-FU at doses ranging from 1.0 to 1.8 mg/m² twice daily, and the duration of DPD inactivation clearly exceeds the projected plasma exposure to EU (9, 11). The addition of EU to 5-FU ± LV is intended to result in greater cytotoxicity by prolonging the plasma half-life of 5-FU and preventing its catabolism. DPD inhibition lasts much longer than can be accounted for by the prolonged half-life of 5-FU given with EU, but the increased plasma exposure to 5-FU leads to increased intracellular formation of active metabolites (19–22).

An analogous situation has been reported with the antiviral agent sorivudine, which has been evaluated as therapy for herpes infections (23–26). Sorivudine is metabolized by enteric bacteria to 5-bromovinyl-uracil, which is an irreversible, mechanism-based inhibitor of DPD. Nakamura et al. (23) reported that concurrent exposure to sorivudine and tegafur, an oral prodrug of 5-FU, was associated with a 5- and 7-fold increase in the 5-FU plasma half-life and area under the plasma concentration time curve. The effect of sorivudine monotherapy on DPD activity determined in 19 human subjects indicated that enzyme activity had returned to baseline in 18 subjects by 2 weeks, and by 3 weeks in all subjects (24). Within the first 2 months after the commercial release of sorivudine in Japan, however, several life-threatening toxicities and fatalities were reported in patients who received fluoropyrimidines several weeks after sorivudine therapy (25). This tragic experience supports our observation that the time to DPD recovery appears to be longer when an irreversible enzyme inhibitor is given with fluoropyrimidines.

It remains to be seen whether DPD recovery might occur more quickly in tissues with a high rate of proliferation. Elevation in plasma uracil levels most likely reflects hepatic DPD inactivation, because this organ has the greatest content of DPD in the body. In the absence of a pronounced stimulus, the liver and PBMCs are not generally considered to be actively proliferating tissues. Because of the enormous contribution of the liver to 5-FU clearance, however, the duration of DPD inactivation in this organ by any irreversible inhibitor of DPD including EU and sorivudine likely dictates the proper safe interval at which conventional doses of 5-FU or analogues can be introduced.

The wide interpatient variability in baseline DPD activity (14, 15, 27, 28), as well as the intrapatient variation in DPD activity in PBMCs, is consistent with earlier reports (29). In contrast, we found much less variation in the results obtained from sequential analysis of aliquots from given donor samples over a period of months and in plasma uracil levels determined over a 2-week period. A crucial question is what constitutes a “safe” DPD activity level that will allow full-dose administration of fluoropyrimidines. Some investigators have suggested a cut point of 100 pmol/min/mg (27, 28), but this cut point may vary depending on the patient population, gender, method used to isolate the mononuclear cells, and the laboratory. Our analysis of paired uracil plasma samples and DPD activity suggests
that patients with a plasma uracil level >0.9 μM have significantly lower DPD activity in PBMCs than subjects with lower uracil levels. Although this type of retrospective correlation is not sufficient to prospectively identify patients who should not receive standard doses of fluoropyrimidines, it provides a hypothesis that could be tested prospectively in future clinical trials. A confounding factor is the difference in the limits of quantitation in published uracil assays, which vary from 0.025 to 8.9 μM, depending on the methodology (13, 18, 30). Assays that use mass spectrometric detection have much greater sensitivity than detection by UV absorption, and the use of [15Ni2]uracil as the internal standard obviates potential interference from endogenous plasma uracil.

On the basis of the disappointing results of two randomized trials comparing the worth of the 28 of 35 days schedule of oral EU/5-FU (10 and 1 mg/m² twice daily) to a standard monthly schedule of bolus 5-FU modulated by LV in advanced colorectal cancer, the pharmaceutical sponsor has suspended evaluation of EU (31, 32). Our pharmacodynamic data suggest that the doses of EU used in these pivotal studies may greatly exceed the dose needed for enzyme inactivation. Other DPD-inhibitory strategies are being evaluated in the clinic currently, including the combination of uracil with florafur, S-1, and BOF-A2. Our findings suggest that greater attention should perhaps be paid to defining the proper modulatory dose of DPD inhibitors that are given with fluoropyrimidines early during clinical evaluation.

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