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ORIGINAL ARTICLE

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Dihydropyrimidine dehydrogenase (DPD) rapidly regenerates after inactivation by eniluracil (GW776C85) in primary and metastatic colorectal cancer

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Abstract *Purpose*: Catabolism of 5-fluorouracil (5-FU) is primarily regulated by DPD. Inactivation of DPD using eniluracil is advantageous in that it renders 5-FU orally bioavailable with more predictable pharmacokinetics and blocks one of the major potential mechanisms of 5-FU chemoresistance. The purpose of this study was to initially document inactivation of DPD by eniluracil in primary and metastatic colorectal cancer (CRC) and then to assess the time-course of the regeneration of DPD activity in peripheral blood (and where possible, additional tissues). *Methods*: Of 28 patients entered, 23 were randomized to preoperative oral eniluracil (20 mg orally twice daily) or placebo prior to definitive resection of primary or metastatic CRC. Three patients were replaced, two because they had no residual tumor on pathologic evaluation and one for not taking the study drug. Patients received eniluracil 48, 36, 24 and approximately 12 h prior to surgical resection. In a second part of the study to document tissue regeneration of DPD, the additional five patients received eniluracil 144, 132, 120 and 108 h prior to surgical resection. DPD activity was measured in normal tissues, tumors and peripheral blood mononuclear cells (PBMC). Serum eniluracil and plasma uracil concentrations were determined before and through 28 days after eniluracil dosing. Data are presented as means ± SEM, and significance defined as $P < 0.05$. *Results*: Eniluracil inactivated DPD below the level of detection in primary and metastatic CRC as well as in normal tissues (0.0 pmol/min per mg protein) compared to primary tumor, metastatic tumor, PBMC, normal mucosa, and normal liver of patients receiving placebo (57 ± 12, 119 ± 19, 157 ± 22, 77 ± 12, 243 ± 24 pmol/min/mg protein, respectively; $P < 0.05$). At the time of surgery, serum eniluracil and uracil concentrations were 207 ± 36 ng/ml and 2700 ± 170 ng/ml in drug-treated patients. Within 6 days following treatment with eniluracil, serum eniluracil and uracil concentrations were undetectable, while DPD activity in PBMC had returned to baseline. The second group of patients ($n = 5$) were given eniluracil 8 and 7 days prior to surgery to evaluate DPD regeneration in normal tissues and primary CRC tissue. In samples of these tissues, collected 6 days after the last eniluracil dose, DPD activity approached baseline in normal mucosa, normal liver and primary tumor (28 ± 12, 94 ± 23 and 20 ± 8 pmol/min per mg protein, respectively). *Conclusions*: These results demonstrate that oral administration of eniluracil inactivated DPD below the level of detection in normal tissues as well as in primary and metastatic CRC. After discontinuation of eniluracil, DPD rapidly returned toward baseline within 6 days in PBMC, normal intestinal mucosa and normal liver.

Keywords Dihydropyrimidine dehydrogenase · Eniluracil · Colorectal cancer

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Introduction

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in pyrimidine catabolism [10]. DPD has

also been shown to be critical in the pharmacology of 5-fluorouracil (5-FU) [3]. Of particular interest are recent studies demonstrating that tumor DPD activity is often markedly elevated in some tumors, particularly those that are relatively resistant to 5-FU [5, 7]. We have recently shown the possibility of measuring DPD, in addition to other critical enzymes such as thymidylate synthase (TS), in needle biopsies by quantitative PCR [8]. Tumors with elevated DPD and/or TS have been shown to be less responsive to 5-FU and leucovorin with a decreased median survival of patients [11].

The DPD inactivator, eniluracil, potentially offers several distinct advantages over intravenous 5-FU chemotherapy including: (1) eniluracil makes 5-FU orally bioavailable; (2) eniluracil decreases the variable pharmacokinetics of 5-FU which is primarily due to variable amounts of DPD among patients, therefore resulting in more rational dosing of 5-FU; (3) eniluracil, by inducing DPD deficiency, combined with a fixed dose of 5-FU avoids the problems of partial or complete DPD deficiency; (4) eniluracil, by inactivating DPD, avoids the known circadian variation of DPD that can be responsible for varying levels of 5-FU in patients receiving 5-FU protracted infusion; and (5) eniluracil, by inactivating tumor DPD, may make the tumor that has become resistant due to increased DPD activity more sensitive to 5-FU. One prior report has documented the effects of eniluracil on DPD activity in primary colorectal cancer (CRC) [1], but little information is available on the effects of eniluracil on DPD activity in primary and metastatic CRC as well as the time-course of DPD regeneration in peripheral blood mononuclear cells (PBMC) and tissues. The time-course of DPD regeneration in PBMC only has been evaluated in one prior study of patients being treated with 5-FU and eniluracil concurrently [6]. That study suggested that PBMC regenerate DPD after a number of weeks. The time-course of the regeneration of DPD activity in PBMC and tissues is of interest, since the presence of DPD could have toxic consequences if another fluoropyrimidine drug were administered too early.

The clinical efficacy of eniluracil has been recently evaluated in a large multicenter trial of CRC patients [12]. No improvement in the overall survival compared with 5-FU and leucovorin was demonstrated. Since all tumors do not overexpress DPD, the proposed benefits of eniluracil may not have been completely realized. Further clinical investigation may be warranted in the subset of patients who clearly show overexpression of DPD.

The objectives of this study were to (1) evaluate DPD inactivation by eniluracil in primary and metastatic CRC and accessible adjacent normal tissues; (2) examine the time-course of DPD regeneration in PBMC, together with an assessment of changes in uracil and eniluracil concentrations in peripheral blood; and (3) obtain an initial insight into the extent of tissue regeneration of DPD activity in primary CRC 1 week after eniluracil administration.

Materials and methods

Patients and clinical protocol

From 28 October 1998 through 21 October 1999, 28 patients were entered on study. Consent was obtained from all patients on a protocol approved by the University of Alabama at Birmingham (UAB) Institutional Review Board in accordance with the Helsinki guidelines. Ultimately, the data from 25 patients were available for analysis with 3 patients replaced, two due to no residual tumor on final pathologic evaluation and one for not taking the study drug. The patients were divided into two groups. Group I comprised 20 patients with resectable primary or metastatic CRC randomized to eniluracil 20 mg orally twice daily or placebo, who after operation underwent serial blood sampling in order to evaluate DPD activity in normal and tumor tissues as well as in PBMC. Blood was analyzed for DPD activity, and eniluracil and uracil concentrations at baseline, on the day of operation and then on postoperative days 5–7, 14, 21 and 28 (see Fig. 1a). Drug or placebo was given 48, 36, 24 and 12 h prior to operation, with the last dose taken as close as possible to midnight the night before hospital admission. All patients were contacted to make sure drug or placebo were taken, and a calendar was used to record the time of dosing. Group II comprised five patients with resectable primary CRC all of whom received eniluracil 20 mg 144, 132, 120 and 108 h prior to operation to provide additional insight into recovery of DPD in normal and tumor tissues in comparison with the primary CRC patients in the control arm of group I. Blood was sampled as above (see Fig. 2). Table 1 shows the clinicopathologic characteristics of the patients.

Tissue peparation

Tissues were collected in the operating room, immediately snap-frozen and placed in liquid nitrogen. Normal colorectal mucosa, normal liver and primary and metastatic CRC (stored at −80°C) were washed with ice-cold physiological saline (0.9% NaCl), weighed, minced and homogenized in four volumes of buffer A (35 m*M* potassium phosphate, pH 7.4, 2.5 m*M* magnesium chloride and 10 m*M* 2-mercaptoethanol). The resulting homogenate was centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant was removed and used in subsequent DPD enzyme assays. The amount of protein in each sample was determined according to the method of Bradford [2] prior to the DPD enzyme assay.

Measurement of plasma eniluracil and uracil

Plasma eniluracil and uracil concentrations were assayed using gas chromatography-mass spectrometry by Triangle Laboratories (Durham, N.C.). The plasma concentration range for the eniluracil calibration curve was 1.0 to 1000.0 ng/ml with a coefficient of variation of less than 8.7%. The accuracy (bias as percent of nominal) was 94.5 to 104.8%. The plasma concentration range for the uracil calibration curve for was 100.0 to 6000.0 ng/ml with a coefficient of variation of less than 4.2%. The accuracy (bias as percent of nominal) was 89.7 to 106.2%.

Measurement of DPD activity in PBMC

PBMC were obtained from blood samples (50 ml) drawn from a peripheral vein into evacuated collection tubes containing heparin. After centrifugation at 500 *g* for 30 min at 25°C over

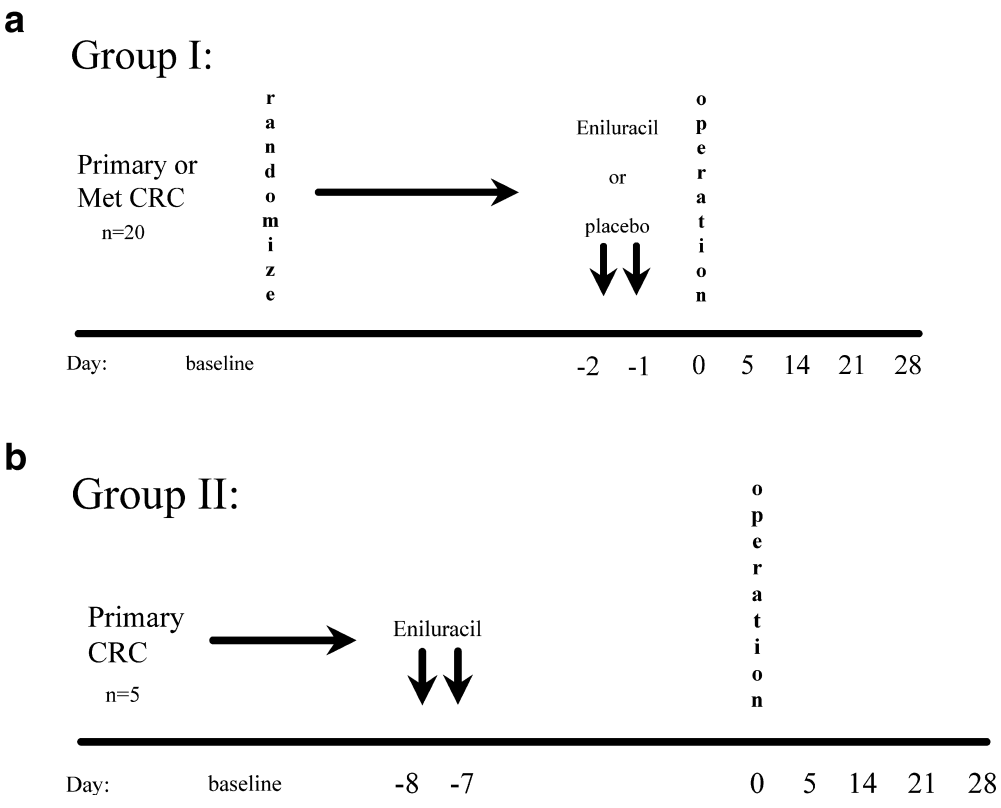

Fig. 1 **a** Study design for group I. **b** Study design for group II

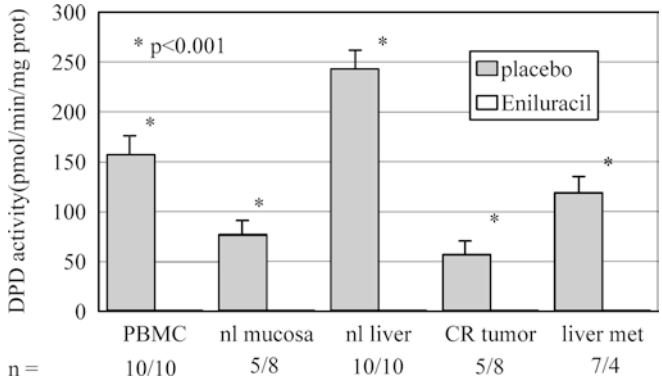

Fig. 2 Effect of 48 h of eniluracil pretreatment on DPD activity in PBMC, normal tissue, and primary and metastatic colorectal cancer (*CR* colorectal, *nl* normal, *met* metastatic)

15 ml Histopaque, the PBMC fraction was removed carefully, washed three times with phosphate-buffered saline, added to 300 µl buffer A containing 35 mmol/l potassium phosphate, pH 7.4, 2.5 mmol/l magnesium chloride, and 10 mmol/l 2-mercaptoethanol, then frozen at −70°C until assay. At the time of assay, the PBMC were slowly thawed. The sample was placed on ice and sonicated three times for 10 s with a 30-s interval between sonications. After centrifugation at 16,000 *g* for 30 min at 4°C, the supernatant was removed and used in the subsequent enzyme assay. Protein was measured using the method of Bradford [2].

DPD enzyme activity was determined as previously reported [9]. Final DPD activities are expressed as picomoles per minute per milligram protein.

Statistics

The Biostatistics Unit of the UAB Comprehensive Cancer Center provided a computer-generated randomization scheme and treatment allocation schedule. Randomization was accomplished according to a blocked randomization schedule. Computer-generated codes and randomization were implemented through a closed envelope system. All data are presented as means ± SEM. Data were compared by paired or unpaired *t*-tests as appropriate. Significance was defined as $P < 0.05$.

Results

The clinicopathologic data of the patients in group I and group II are presented in Table 1. Two patients in group I presented with synchronous primary and metastatic disease, and therefore data were acquired from both primary and metastatic tumors for analysis.

Group I

Effect of 48 h of eniluracil on DPD activity in PBMC and tissues

Figure 2 shows the DPD activity in PBMC, normal colorectal mucosa, normal liver, primary colorectal tumors and colorectal liver metastases in group I receiving eniluracil and placebo. In patients receiving placebo, DPD activity was highest in normal liver and lowest in primary CRCs. Although not statistically significant, it is interesting to note that DPD activity in colorectal liver

Table 1 Clinicopathologic features of the patients

	Group I		Group II
	Placebo	Eniluracil	
Patients			
No. analyzed	10	10	5
Age (years)	61 ± 3	58 ± 3	69 ± 4
Gender (F/M)	4/6	6/4	0/5
Race (black/white/other)	2/8/0	1/9/0	0/5/0
Tumors (primary/metastatic)	6/4	4/6	5/0
Tumor location			
Colon/rectum	3	6	5
Liver	5	4	0
Both	2	0	0

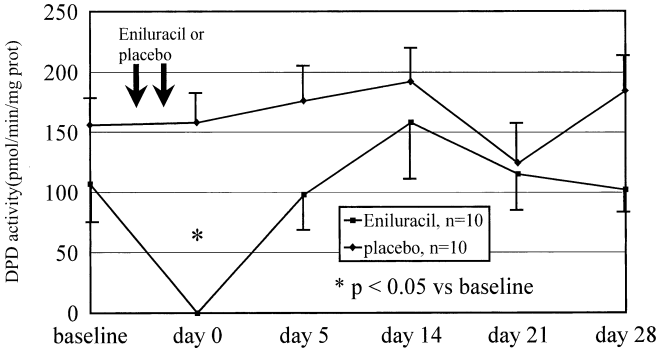

Fig. 3 Pharmacokinetics of DPD activity (regeneration) in PBMC after 48 h eniluracil pretreatment

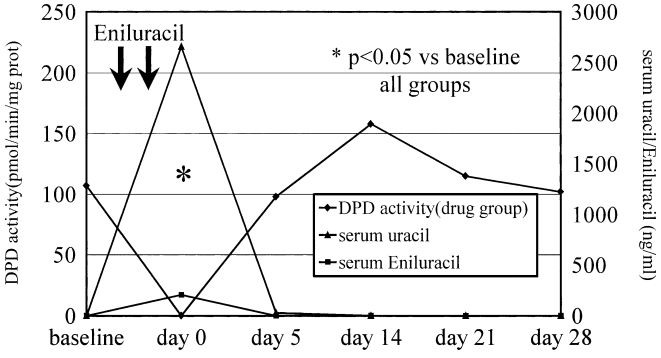

Fig. 4 Pharmacokinetics of DPD activity (regeneration) in PBMC, and serum eniluracil and serum uracil concentrations after 48 h of eniluracil pretreatment

metastases was higher than in primary CRCs. Patients receiving eniluracil had inactivation of DPD below the level of detection in all normal tissues, primary CRCs, colorectal liver metastases and PBMC ($P < 0.001$ vs placebo).

Effect of eniluracil on time-course of DPD regeneration

Figure 3 shows the DPD activity in PBMC from patients receiving eniluracil or placebo at baseline, on the day of operation and then on postoperative days 5–7, 14, 21 and 28. There was a statistically significant reduction in DPD activity (below detection) in PBMC at the time of operation and then recovery to baseline within 5–7 days after operation. There were no significant differences compared to baseline in DPD activity on postoperative days 14, 21 and 28. There were no significant differences in DPD activity between baseline and any of the time-points in PBMC from patients receiving placebo.

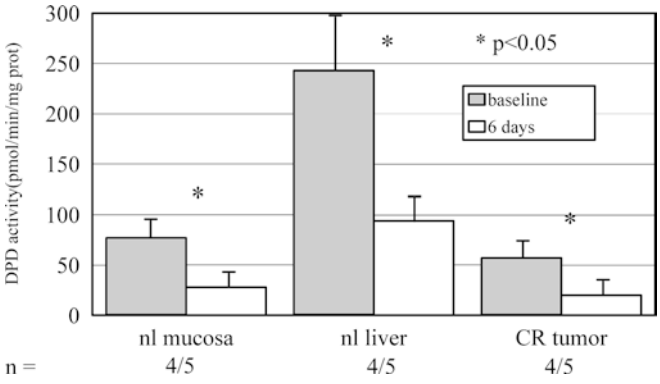

Fig. 5 Tissue DPD activity after oral eniluracil treatment on preoperative days 8 and 7 compared with baseline DPD activity in the placebo group for the same tissues (*CR* colorectal, *nl* normal)

Effect of 48 h of eniluracil on plasma eniluracil and uracil concentrations

Figure 4 shows the plasma concentrations of eniluracil and uracil at baseline, on the day of operation and then on postoperative days 5–7, 14, 21 and 28. This is compared with the DPD activity in PBMC for reference. There was a statistically significant increase in plasma eniluracil and uracil concentrations corresponding to inactivation of DPD activity in PBMC. Eniluracil and uracil concentrations were essentially undetectable by postoperative days 5–7 and this corresponded to recovery of DPD to baseline in PBMC.

Group II

Effect of eniluracil given 1 week prior to operation on tissue DPD activity compared to baseline DPD activity in the placebo arm of group I

Figure 5 shows that the DPD activities in normal mucosa, normal liver and primary CRC were returning to approximate baseline values, although still significantly different by the unpaired *t*-test.

Discussion

This study demonstrated that administration of oral eniluracil within 48 h of surgery inactivated DPD below the level of detection in PBMC, normal mucosa, normal

liver, primary CRC and metastatic CRC to the liver in human subjects. Inactivation of DPD activity in PBMC correlated with peaks of plasma eniluracil and uracil. Similarly, rapid return of DPD activity in PBMC was associated with undetectable concentrations of plasma eniluracil and uracil by approximately postoperative days 5–7. Lastly, tissue regeneration of DPD activity approached baseline control values by 6 days after administration of drug, but appeared to lag behind activity regeneration in PBMC.

There have been several attempts to develop an oral fluoropyrimidine chemotherapy through various manipulations of biochemical pathways (capecitabine, tegafur, etc.) [4]. At present, capecitabine (Xeloda) is the only agent with FDA approval. Eniluracil was developed to inactivate DPD which normally metabolizes up to 85% of 5-FU. By blocking this enzyme, 5-FU becomes orally bioavailable, circadian variations that are normally present are reduced, and importantly one mechanism for tumor chemoresistance may be eliminated. One previous study limited to primary CRC also demonstrated inactivation of DPD in normal colorectal mucosa and tumor [1]. These authors demonstrated no change in DPD mRNA expression and confirmed that the effect of eniluracil occurs at the enzyme level and that eniluracil does not affect gene expression in the short term. The present study demonstrated inactivation to below the level of detection in normal mucosa, normal liver, primary CRC and metastatic CRC to the liver. This is an important finding, since the primary role of adjuvant and therapeutic chemotherapy is to affect microscopic or macroscopic metastatic tumor cells, respectively.

The utility of eniluracil as the mainstay of adjuvant fluoropyrimidine-based therapy is limited since no improvement in efficacy has been shown in recent phase III trials [12]. However, this compound may become clinically relevant if additional data become available suggesting that there is a population of CRC patients who have elevated DPD (one form of chemoresistance). It is interesting to note that DPD activity was higher in metastatic lesions than in primary tumors in those who did not receive eniluracil, although this did not reach statistical significance in the present study. Elevated DPD activity in tumors has been correlated with decreased responses to fluoropyrimidine chemotherapy [5, 11]. The present results, in conjunction with those of previous studies, suggest that metastatic lesions may be more resistant to the effects of 5-FU chemotherapy due to increased DPD activity and that eniluracil at standard doses can inactivate this activity in vivo. Studies to evaluate efficacy in this population should be considered.

Concern has been raised about the duration of DPD inactivation by eniluracil and the clinical significance in patients treated with 5-FU and eniluracil. Eniluracil inactivates DPD to below the level of detection and the only mechanism for regeneration of enzyme activity is through production of more enzyme [13]. In one prior study, the regeneration of DPD was evaluated in 12 patients with adenocarcinomas of the gastrointestinal tract being treated with 5-FU intravenously [6]. In this study, DPD activity was found to be approximately 25% of baseline 19 days after administration. This issue was addressed in the present study, and DPD activity in PBMC was demonstrated to be rapidly regenerated within 5–7 days after a single course of drug. DPD activity was also found not to be affected long-term after cessation of drug and there was no significant difference during the 4 weeks after administration. The differences between the studies may lie in the fact that the prior study included a relatively small number of patients who had previously undergone a median of two prior chemotherapy regimens. DPD activity regeneration in tissue appeared to lag behind that in PBMC.

The results suggest that PBMC recovered the ability to create DPD to near-normal activity in roughly 7 days (group I, treatment arm), but there was still a statistically significant reduction in tissue DPD activity at 1 week (group II). One mechanism for the difference may be that PBMC are manufactured at a more rapid rate than colorectal tissues and therefore, as well as current PBMC beginning to make DPD, new cells may also make DPD. This may be important clinically if eniluracil is combined with 5-FU and/or other myelosuppressive drugs since DPD may not regenerate as fast in PBMC in this situation. We believe that this may also be a reason for differences between the findings of previous studies suggesting that DPD activity does not regenerate as fast [6]. Additionally, it must be kept in mind that the group who received eniluracil 7 days prior to operation comprised only five patients and there was no randomized comparison with the patients who received eniluracil twice daily from 48 h before operation. Unfortunately, it is difficult to serially sample human tissues in the context of a biologic study but, despite this limitation, it is clear that tissues regenerated DPD in a relatively short time. In future studies, regeneration of DPD after repeated courses of 5-FU and eniluracil should be examined to confirm these preliminary observations.

Monitoring regeneration of DPD enzyme activity has been greatly simplified with a recent HPLC technique [9] developed in our laboratory, but not commercially available in kit form. Plasma uracil has been suggested to potentially serve as a surrogate since its concentrations are inversely related to DPD activity. Normally, plasma uracil concentrations are undetectable, but they can be elevated in patients who are completely DPD-deficient or who have had DPD inhibited pharmacologically [14]. The present study pharmacologically produced complete DPD deficiency with eniluracil and there was a corresponding rise in plasma uracil concentrations.

In summary, eniluracil inactivated DPD below the level of detection in normal tissues as well as in primary and metastatic CRC in human subjects. We demonstrated that enzyme activity rapidly regenerated in

PBMC within 5–7 days after cessation of the drug and appeared to regenerate somewhat more slowly in a small sample of tissues. Preliminary evaluation of metastatic disease in this study suggested that DPD activity may be elevated in some patients, and this could be one mechanism of chemoresistance. Although eniluracil did not show efficacy in all patients with CRC [12], the results suggest that eniluracil may provide certain advantages over 5-FU chemotherapy, especially in patients with high DPD levels.

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