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Original Paper

Autoregulation of 5-Fluorouracil Metabolism

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5-Fluorouracil (5-FU) is a commonly used anticancer agent for the treatment of gastrointestinal, head and neck, and breast tumours. This study determined the influence of 5-FU on dihydropyrimidine dehydrogenase (DPD) activity, the enzyme responsible for its *in vivo* degradation. DPD activity was measured in mononuclear cells obtained prior to and after the administration of 5-FU in 20 patients with colorectal cancer. Following the results from the human studies, DPD activity was measured in Sprague–Dawley rat liver up to 72 h after administration of 5-FU 200 mg/kg as a single injection. Total liver P450 content and the production of testosterone metabolites (indicative of CYP3A activity) were also analysed to determine the specificity of 5-FU-associated alteration in rat liver metabolism. Human mononuclear cell DPD activity decreased by a median of 38.7% following the administration of 5-FU ($P = 0.001$). 5-FU-induced alterations in rat liver DPD were also observed, with the lowest activity occurring 48 h after injection (50% of control activity; $P = 0.009$). Rat liver DPD activity returned to near control values by 72 h postinjection. Rat liver total P450 content and CYP3A activity were not significantly different in 5-FU treated or control tissues. Thus, 5-FU demonstrates autoregulation of its metabolism through inhibition of DPD activity. Although this inhibition appears to be specific for DPD, the mechanism for enzyme inhibition is not clear. These findings may aid in the design of 5-FU treatment regimens and provide the basis for further studies into the regulation of DPD.

Key words: dihydropyrimidine dehydrogenase, enzyme inhibition, 5-fluorouracil, metabolism
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INTRODUCTION

THE PYRIMIDINE analogue 5-fluorouracil (5-FU) is an essential component of the treatment of gastrointestinal, head and neck, and breast tumours. 5-FU itself is not cytotoxic, but requires bioactivation by multi-step pathways, eventually leading to inhibition of thymidylate synthase or incorporation into RNA. The majority of an administered 5-FU dose (> 80%) is degraded by a three-step pathway which is catalysed by the initial and rate limiting enzyme, dihydropyrimidine dehydrogenase (DPD; E.C. 1.3.1.2) [1, 2]. Although 5-FU has a rapid clearance and a short half-life in humans, there is a large variation in 5-FU elimination amongst patients with cancer (5- to 10-fold) [3, 4]. The variability in 5-FU elimination may have clinical relevance, as measures of systemic exposure (AUC, Css) have been correlated with both toxicity and survival following 5-FU treatment [5–7]. In addition, severe toxicity or death has been observed after standard doses of 5-FU in patients with DPD deficiency [8–10]. DPD activity is highly variable in mononuclear cells, liver, and tumour tissue [10, 13]. The ratio of tumour to normal tissue DPD activity in patients with head and neck cancer was higher in those who did not respond to 5-FU-based chemotherapy than those achieving a complete response [12]. This suggests a role for DPD in drug resistance; whereby intratumoral DPD inactivates 5-FU, preventing it from forming cytotoxic nucleotides. These findings have fuelled our interest in understanding DPD-mediated regulation of 5-FU metabolism.

Little is currently known about the regulation of DPD in humans. Several studies in both human and rat have identified circadian variation in DPD activity with corresponding alteration in 5-FU plasma concentrations [14, 15]. This circadian variation is not correlated with plasma cortisol concentrations [16]. This variation may at least in part be responsible for the differences in effects observed with chronomodulation of administration of 5-FU [17]. The

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mechanism by which these changes in DPD occur has not been elucidated. The administration of hydrocortisone to 5-day-old rats led to an increase in liver DPD activity, but these findings could not be replicated in adult rats [18, 19]. Depletion of dietary protein or vitamin B2 has also been shown to decrease DPD activity in rat liver, but the relevance of this finding to humans has not been evaluated [20, 21]. Autoregulation of 5-FU activity has been previously identified (e.g. induction of the cellular target thymidylate synthase) [22], leading us to assess whether 5-FU influences its own catabolism (or inactivation) in humans with colorectal cancer.

MATERIALS AND METHODS

Patients

The analysis of the influence of 5-FU on DPD activity was performed in 20 patients with colorectal cancer receiving a modified 'de Gramont regimen', consisting of folinic acid 200 mg/m^2 intravenously (i.v.) over 2 h then 5-FU 600 mg/m^2 i.v. over 30 min followed by 600 mg/m^2 i.v. infused over 22 h [4]. This bolus/infusion was administered on days 1 and 2 with cycles repeated every 14 days. All patients had normal haematological, renal, and hepatic function tests during this study period and did not require anti-emetic therapy. Blood samples (20 ml) were obtained in heparin vacutainer tubes prior to the start of chemotherapy and 48 h later following the cessation of chemotherapy. Blood samples were obtained between 0930 h and 1100 h and at the end of infusion samples were taken within 20 min of the time the presample was obtained, to avoid significant influence of circadian variation. Mononuclear cells were purified using a Ficoll-hypaque gradient and the cells were washed in phosphate buffered saline (PBS) prior to storage in 35 mM $NaPO_4$ buffer with 10% glycerol.

Rodent studies

Thirty male Sprague–Dawley rats (320–350 g) were randomly assigned to two treatment groups. One group was administered 5-FU (200 mg/kg) prepared in 0.9% NaCl as a single intraperitoneal (i.p.) injection. The other group received an i.p. injection of 0.9% NaCl. At 24, 48 and 72 h after treatment, five rats from each treatment group were killed by exposure to carbon dioxide, cervical dislocation, and the livers were then removed. The livers were washed in PBS and snap frozen in liquid nitrogen, before being stored at − 80°C. The rats were maintained on a 12 h, light/dark cycle (lights on at 0700 h) with free access to standard laboratory chow and water for 2 weeks prior to the studies. The livers were excised between 0900 h and 1100 h to minimise the influence of circadian variation on DPD activity.

DPD activity analysis

Mononuclear cell DPD activity was measured as previously described [11]. In brief, cells were lysed by a three cycle freeze/thaw procedure using dry ice and centrifuged for 20 min at 12000 ***g*** at 4°C. A reaction mixture (125 µl) consisting of 125 µM NADPH, 20 µM (^{14}C)-5-FU, buffer A (35 mM KPO_4 (pH 7.4), 2.5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol) and 50 µl cytosol (0.05–0.1 mg protein) was incubated for 90 min at 37°C in a shaking water bath. The reaction was stopped by the addition of an equal volume of ice cold ethanol and the supernatant was assayed for 5-FU catabolites using high performance liquid chromatography (HPLC) with on-line radioactivity detection. Separation was achieved using a hypersil ODS (25×0.46) and sperisorb ODS (25×0.46) column in tandem with a mobile phase of 1.5 mM KPO_4 buffer pH 8 with 5 mM tetrabutylammonium hydrogen sulphate at a flow rate of 1 ml/min. To assess DPD activity in rat liver, tissues were thawed, weighed and homogenised in 4 ml of buffer A with 0.25 M sucrose, 1 mM aminoethylisothiouronium bromide, 1 mM benzamidine and 5 mM Na_2EDTA. The resulting homogenate was centrifuged at 10 000 ***g*** for 60 min at 4°C. The cytosolic fraction was then incubated with the co-factors described above for 45 min at 37°C in a shaking water bath. The reaction was terminated by the addition of ice cold ethanol and the supernatant assayed for 5-FU catabolites using HPLC. Each sample was assayed in triplicate and expressed as pmol of product formed per minute per mg of protein (pmol/min/mg protein).

Measurement of testosterone metabolites (6β-hydroxy and 2β-hydroxy

To assess the specificity of 5-FU-associated alterations in liver metabolism, total P450 content and the production of testosterone metabolites were analysed. Alterations in total P450 content would indicate a generalised effect of 5-FU. The 6β-hydroxy and 2β-hydroxy metabolites of testosterone are indicative of CYP3A activity, representing one of the major P450 isozymes in hepatic tissue. Rat liver microsomes were prepared from the same liver samples as used for DPD measurement by differential centrifugation using standard methodology. After preparation, protein [23] and total cytochrome P450 concentrations [24] were determined and microsomal suspensions were stored at − 80°C until required for use. The testosterone metabolite assay was a modification of a previously described method [25]. Reaction mixtures contained a total volume of 1 ml, consisting of 1 mg microsomal protein, 0.1 M Tris–HCl buffer (pH 7.4), testosterone (25 µl of a 10 mM solution in methanol) and a 1 mM NADPH generating system (1 mM NADP, 5 mM isocitric acid, 5 mM $MgCl_2$, 0.1 M Tris–HCl buffer (pH 7.4) and 1 unit isocitrate dehydrogenase). Reactions were carried out at 37°C, pre-incubated with testosterone for 5 min and initiated by the addition of the NADPH generating system.

After 5 min (the formation of both metabolites is linear up to 30 min), the reactions were terminated by the addition of 6 ml ice cold dichloromethane. An internal standard (100 µl; 10 mg/ml solution of 11β-hydroxytestosterone in methanol) was added and the extraction was performed by mixing on a rotary mixer for 30 min followed by centrifugation for 3 min (1500 ***g***). The organic phase was evaporated to dryness under nitrogen. Residues were reconstituted in 300 µl of mobile phase A and 100 µl injected on to the HPLC column. The quantification of the testosterone metabolites was based on a modification of the method of Funae and Imaoka [26]. The HPLC system consisted of a Pharmacia LKB gradient pump, a UV detector set at 254 nm and a heated (50°C) Waters C_{18} Novapak column. The metabolites were separated using a gradient mobile phase delivered at a flow rate of 1 ml/min. Mobile phase A consisted of methanol (25% v/v) and distilled water (75% v/v), whilst mobile phase B was a mix of methanol (63.5% v/v), distilled water (35% v/v) and acetonitrile (1.5% v/v). The gradient shifted from 25% buffer B to 100% buffer B over 30 min, followed by 25% buffer B for 10 min. Standard curves were constructed in the concentration range 0–3 µg for 6β-hydroxytestosterone and 2β-hydroxytestosterone. Unknown concentrations were determined by

comparison of metabolite: internal standard peak-height ratio with those of the calibration curve.

Statistical analysis

The influence of 5-FU on patient mononuclear cell DPD activity was assessed using the Wilcoxon test. The differences in DPD activity, total P450 content, 6β-hydroxytestosterone metabolite, and 2β-hydroxytestosterone metabolite in rat liver from either 5-FU-treated or control animals were assessed using the Kruskal–Wallis and two-sample *t*-tests.

RESULTS

Mononuclear cell DPD activity was measurable in all 20 patients with colorectal cancer. Median pretherapy activity was 258.6 pmol/min/mg protein (range 115.4–506.8), while median activity after therapy was 159.8 pmol/min/mg protein (range 68.6–315). DPD activity was a median 100.8 pmol/min/mg protein lower in the post-therapy sample when compared with pretherapy DPD activity ($P = 0.001$). A median decrease of 38.7% (range 67.8% decrease to 45% increase) in activity was seen with the administration of 5-FU (Figure 1). There was no significant difference in leucocyte counts between the pre- or post-therapy periods.

The influence of 5-FU pre-exposure on *in vivo* DPD activity was confirmed in rat liver (Figure 2). Evidence for a decrease in DPD activity was seen 24 h after injection and activity was 50% of control values 48 h after injection ($P = 0.009$). DPD activity then returned to near control values 72 h after the injection. There was no statistically significant difference in DPD activity in the control livers at the 24, 48 or 72 h time points. Rat liver DPD activity ranged from 345.5 to 491 pmol/min/mg protein in the control liver samples. Total P450 content ranged from 0.99 to 1.52 nmol/mg protein, 6β-hydroxy activity ranged from 0.47 to 2.56 nmol/min/mg protein, and 2β-hydroxy activity ranged from 0.146 to 0.393 nmol/min/mg protein in the control liver samples. Although P450 content and CYP3A activity were highly variable in the various treatment groups, there was no statistically significant difference in samples from 5-FU-treated or control animals (Table 1).

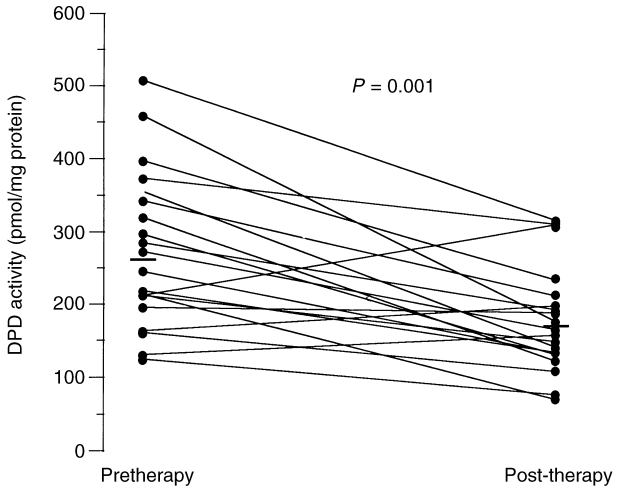

Figure 1. Change in mononuclear cell dihydropyrimidine dehydrogenase (DPD) activity after administration of intravenous 5-fluorouracil ($n = 20$). Each point represents 1 patient. Horizontal lines indicate median values.

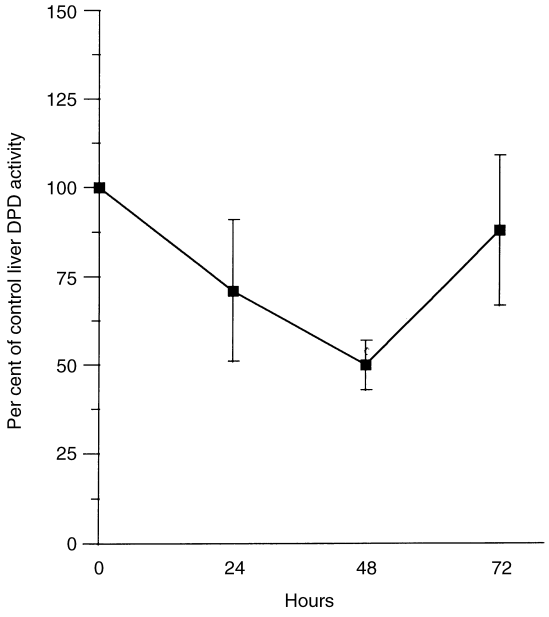

Figure 2. Alterations in rat liver dihydropyrimidine dehydrogenase (DPD) activity after 5-fluorouracil injection (time zero).

DISCUSSION

This study demonstrates for the first time autoregulation of 5-FU catabolism through inhibition of DPD. This inhibition was first observed in patients with colorectal cancer receiving 5-FU by bolus/infusion and was also demonstrated in an animal model analysing liver tissue, the primary site of *in vivo* 5-FU metabolism. This effect appears to be specific in nature as neither total P450 content nor CYP3A activity were altered in the livers of 5-FU-treated animals. Based on previous studies [27], the 38.7% decrease in mononuclear cell DPD activity could be associated with a 20–30% increase in 5-FU systemic exposure. However, 5-FU pharmacokinetics were not measured in this study. These findings may have important implications for both the use of 5-FU and the design of new treatment regimens. For instance, the results provide a rationale for protocols which require less 5-FU to be administered 48 h after initiation of therapy, due to the lower rate of degradation by DPD. This could have

Table 1. *Influence of* 5*-fluorouracil (*5*-FU) on rat liver* P450 *content,* 6β*-testosterone activity, and* 2β*-testosterone activity. Values expressed as mean ± standard deviation* (n = 9)

	24 h	48 h	72 h
P450 content (nmol/mg protein)			
Control	1.12 ± 0.09	1.09 ± 0.11	1.25 ± 0.22
5-FU treated	1.31 ± 0.10	1.27 ± 0.04	1.38 ± 0.20
	$P = 0.09$	$P = 0.11$	$P = 0.49$
6β-testosterone (nmol/min/mg protein)			
Control	1.06 ± 0.42	1.06 ± 0.63	1.33 ± 1.01
5-FU treated	1.44 ± 1.16	0.88 ± 0.10	1.85 ± 0.75
	$P = 0.73$	$P = 0.68$	$P = 0.53$
2β-testosterone (nmol/min/mg protein)			
Control	0.23 ± 0.05	0.29 ± 0.03	0.24 ± 0.10
5-FU treated	0.27 ± 0.14	0.31 ± 0.22	0.30 ± 0.08
	$P = 0.76$	$P = 0.89$	$P = 0.42$

important influences on tolerability and dose intensity. Although further investigations are required before initiation of such an approach, it is a feasible option, as it is similar to the protocols currently under investigation where chronomodulated 5-FU administration is taking place to account for the circadian variation in DPD activity [17].

Autoregulation of drug metabolism has been observed with other medications. The anticonvulsant carbamazepine has been shown to rapidly induce its own metabolism through an increase in P450 expression [28]. This induction of enzyme activity has also been shown to alter the metabolism of co-administered medications. For example, metabolism of teniposide is much more rapid in patients treated with anticonvulsants than those receiving no anticonvulsant therapy [29]. A similar picture has been seen for cyclophosphamide, where auto-induction of metabolism was seen within 2 days of administration in a study of patients receiving bone marrow transplantation [30]. Not only did cyclophosphamide cause a 65% increase in its own clearance, but the metabolism of dexamethasone was also increased by a similar degree. While the kinetic and mechanistic features of enzyme induction by both carbamazepine and cyclophosphamide are incompletely understood, an increase in mRNA transcription leading to increased enzyme synthesis has been proposed based on the *in vivo* experiments, where there is a lag period after exposure to the inducing agent before evidence of induction can be detected.

However, in this study, it was inhibition of DPD activity, not enzyme induction, that was found after 5-FU treatment. Auto-inhibition of drug metabolism is a relatively rare occurrence with very few cases described in the literature. Although the specific mechanism behind the inhibition in this study has not been fully elucidated, it may involve alterations in mRNA translation and/or conformation as a result of incorporation of a fraudulent fluorinated uracil moiety [31]. This is supported by the time course in which 5-FU altered DPD activity, with a lag period of 48 h required before maximal inhibition occurred. 5-FU has a rapid systemic clearance and is not detectable in plasma after 2 h postinjection. The time course data point away from there being a direct drug–protein interaction between 5-FU and the DPD enzyme which alters protein conformation or reduction capacity, similar to that seen for cimetidine inhibition of P450s [32]. This is further supported by the fact that the production of 5-FU metabolites during *in vitro* incubation is linear in both liver and mononuclear cells. An alternative mechanism for the observed findings is feedback inhibition of DPD activity by F-β-alanine. DPD is the initial, and rate limiting step in the conversion of uracil and 5-FU to β-alanine and F-β-alanine, respectively [1]. The role of β-alanine as a putative neurotransmitter may necessitate a mechanism for regulating its production, as well as preventing production of false analogues such as F-β-alanine. However, little is known about the control of β-alanine production in mammalian systems.

The absence of any influence of 5-FU administration on total liver P450 content or CYP3A activity is consistent with the previous finding that 5-FU did not influence rat liver total P450 content, aminopyrine demethylase, cyclophosphamide 4-hydroxylase, aniline hydroxylase, androstenedione 6β- and 16α-hydroxylase and Δ^4-3-oxo-steroid 5α-oxidoreductase activity on day 1, 4, or 7 postinjection of a single i.p. injection of 5-FU (120 mg/kg) [33]. This is in contrast to a previous study where inhibition of rat liver para-nitro-anisol, analine and antipyrine metabolism was observed 7 days after administration of 5-FU (120 mg/kg) [34]. Enzyme activity was 36–55% of control values and was not associated with a change in total liver protein content.

Previous studies have shown an effect on drug metabolising capacity following the administration of 5-FU 24 mg/kg/day for 5 days [33]. A significant decrease in androstenedione 6β- and 16α-hydroxylase and cyclophosphamide 4-hydroxylase activity was observed 4 and 7 days after completion of therapy. The influence of chronic 5-FU administration on DPD activity in either humans or rodents was not evaluated in our study. Persistent alterations in mRNA and induction of the thymine-less state through the inhibition of thymidylate synthase may indeed result in more extensive and non-specific alterations in drug metabolism after repeated administration of 5-FU.

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