Cancer Biology & Therapy 12:7, 557-568; October 1, 2011; © 2011 Landes Bioscience

Preliminary observations indicate variable patterns of plasma 5-fluorouracil (5-FU) levels during dose optimization of infusional 5-FU in colorectal cancer patients

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Keywords: colorectal cancer, chemotherapy, FOLFOX, 5-FU AUC, pharmacokinetic monitoring, OnDose

Efforts to improve efficacy and minimize toxicity have led to pharmacokinetic monitoring of plasma 5-fluorouracil (5-FU) levels in colorectal cancer patients undergoing chemotherapy. We observed variation in basal 5-FU levels in 21 patients and significant variation during subsequent dose optimization. Tumor KRAS, BRAF mutations and TS mRNA levels were determined. Regimens included FOLFOX6+Avastin (n = 8), FOLFOX6 (n = 11), FOLFIRI (n = 1) and FOLFOX4 (n = 1). Mutations identified in tumors included G12V KRAS (n = 2), G12A KRAS (n = 1), and V600E BRAF (n = 3). Six-of-11 patients with normalized tumor TS mRNA levels < 4.0 had a 5-FU AUC of 20 mg.h/L or greater, and 80% of patients (four of five) with TS levels > 4.0 had a plasma 5-FU AUC of less than or equal to 20 mg.h/L. Approximately 2/3 of patients achieved therapeutic 5-FU AUC levels with 0–2 dose adjustments while a sub-group of ~1/3 of patients slowly achieved therapeutic levels (> 3–4 dose increases leading to supra-therapeutic 5-FU and subsequent reductions to lesser than original doses). Liver metastases and tumor TS levels did not fully account for variable 5-FU AUC optimization patterns. The 5-FU level during continuous infusion was half-therapeutic in one patient who received FOLFOX4. The observed heterogeneous patterns at baseline and during dose optimization of 5-FU levels suggest variations in 5-FU metabolism among treated patients. Physiological and/or genetic differences underlying heterogeneity in 5-FU levels during dose optimization require further study of patient demographics, single nucleotide polymorphisms in Dihydropyrimidine Dehydrogenase (DPD), TS, or other genes that impact 5-FU metabolism and gene expression changes in liver after 5-FU therapy.

Introduction

5-Fluorouracil (5-FU) has been the cornerstone of colorectal cancer chemotherapy regimens for over five decades. However more than 80% of patients undergoing treatment will experience adverse side-effects from the chemotherapy.[1] The efficacy and side effects of chemotherapy are influenced by the dose calculated for each patient. The dose of 5-FU is conventionally calculated using the body surface area (BSA) method.

It has been shown that the efficacy of 5-FU is not optimized by BSA-based dosing. On the other hand, the use of AUC (defined as the area-under-the-curve when plasma 5-FU levels are plotted against a defined period of time) has been demonstrated to improve outcomes.[2-7] Although the data vary, most studies indicate that 5-FU may lack its desired therapeutic effect below an AUC of 20 mg.h/L^{-1}, whereas it may cause excessive toxicity above an AUC of 24 mg.h/L^{-1}.[3,4] Therefore, the optimum range for 5-FU AUC is considered to lie between an AUC of 20 and 24 mg.h/L to derive the greatest therapeutic benefit with minimal toxicity. Studies indicate that when using the conventional BSA-based method of 5-FU dose calculation, about 70–80% of patients do not achieve the desired 5-FU AUC range of 20–24 mg.h/L.[5]

The impact of keeping 5-FU plasma levels at therapeutic concentrations can be gleaned from observations that the overall survival with a 5-FU/leucovorin (FU/LV) regimen with pharmacokinetically-guided monitoring of 5-FU levels, is nearly equivalent to that observed with combination regimens involving FU/LV and oxaliplatin, and FU/LV and the topoisomerase inhibitor irinotecan. Thus, 5-FU (in the presence of leucovorin) may be as effective as these more toxic combinations, as long as it is present at the optimum levels in the blood.[2] There is also limited evidence suggesting that 5-FU pharmacokinetic monitoring may further improve the efficacy of a modified FOLFOX regimen.[5,6]

Given previous results that therapeutic benefit can be gained by achieving optimum plasma 5-FU levels, we have adopted

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Submitted: 09/12/11; Accepted: 09/12/11
DOI:10.4161/cbt.12.7.18059

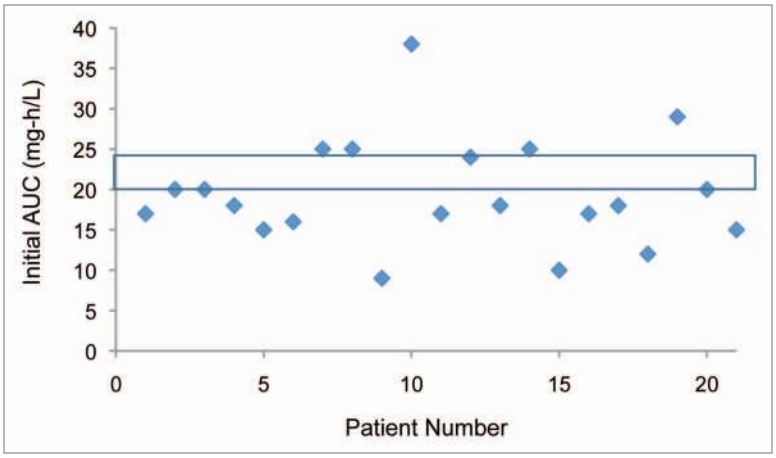


Figure 1. A scatter plot of initial AUC of individual patients observed in the study. The box outlines data points that are within the therapeutic range of 5-FU (20–24 mg.h/L). Four data points lie within this area.

routine pharmacokinetic monitoring of 5-FU in colorectal cancer patients receiving 5-FU-based chemotherapy regimens in our clinical practice. Our study has identified novel and heterogeneous patterns in 5-FU levels, at baseline and during dose optimization, suggesting variations in 5-FU metabolism among patients.

Results

Patient demographics and baseline 5-FU AUC following initial body surface area (BSA) dosing. Patient characteristics are summarized in **Table 1**. The median age of the patients was 59 y. Both genders were equally represented in the sample (11 male and ten female). Stages III and IV were also equally represented (12 Stage III and 9 Stage IV). The patients have been assigned to groups based on the pattern of the variability of their 5-FU AUC levels. These patterns are further described below. Of 21 patients, only four patients achieved an AUC within the target range without any dose titration (**Fig. 1**). These patients are referred to as Group A. In another eight patients (Group B), the target 5-FU AUC was achieved after adjusting the administered dose once or twice (**Fig. 2**).

Identification of a sub-group of colorectal cancer patients requiring multiple dose adjustments to achieve a 5-FU AUC in the therapeutic range. In a subset of our patients (n = 6) the observed changes in actual AUC did not correlate well with the adjustments made on the administered dose. In two patients (assigned to Group C), sub-therapeutic 5-FU plasma levels did not increase up to the target range despite an incremental (recommended) dose increase in the first two consecutive treatments but were seen to exceed the upper limit of the target range after the third dose adjustment. In one patient, an increase of 120 mg/m^2 in the first two consecutive doses of 5-FU failed to increase the AUC levels but caused an increase after the third adjustment from an AUC level of 15–28 mg.h/L (**Fig. 3A**). A similar observation can be made from the results of another patient (**Fig. 3B**).

Our observations demonstrate the importance of monitoring plasma 5-FU levels even after the therapeutic range has been achieved. In two patients (Group D), 5-FU AUC levels showed an upward trend exceeding past the target range of 20–24 mg.h/L after a continued use of the fixed therapeutic dose in the absence of any dose adjustments. This phenomenon was also observed when a therapeutic dose was reduced leading to an increase in the AUC levels beyond the upper limit of the target range. In the first patient, the therapeutic range was reached after one adjustment. Yet, even after using the same dose in the next cycle, the AUC levels exceeded the therapeutic range. (**Fig. 4A**). In the second patient, the therapeutic range was also reached after one adjustment. Yet, even when the administered dose was decreased, the AUC levels increased (**Fig. 4B**).

Lower plasma 5-FU AUC level in a patient treated with FOLFOX4 as compared with patients treated with FOLFOX6. FOLFOX 6 was administered to the majority of our patients in this study. However, we report the case of a patient who received FOLFOX4 (**Fig. 5**). The plasma levels in this patient were found to be half the lower limit of the therapeutic range. This patient was assigned to Group E.

Relationships between tumor thymidylate synthase (TS) expression and baseline 5-FU AUC levels. To begin to understand the possible mechanisms that contribute to the variability of 5-FU levels observed in the study, the thymidylate synthase (TS) expression levels of the tumors of the patients were plotted against the initial patient AUC's (**Fig. 6A**). A TS level below 4 is considered low, according to the manufacturer that routinely analyzes this parameter. Patients that had high TS levels (i.e., above 4) tended to have AUC's lower than the minimum therapeutic range of 20 mg.h/L. However, the difference in the distribution of the AUC levels between the low and high TS level patient groups was not statistically significant by the Wilcoxon rank sum test ($p > 0.05$). The initial AUC levels of the patients in different groups were plotted (**Fig. 6B**). Although the number of patients in Groups C and D were small, preliminary observations indicate that patients with low AUC's needed more than 3–4 dose adjustments before therapeutic levels of 5-FU can be achieved.

Discussion

Although the advantages of administering 5-FU doses based on actual 5-FU plasma levels of patients have been reported in the literature,[2,8] its use in the clinic has been slow. We have presented in our preliminary case series evidence to show that actual 5-FU plasma levels of patients cannot be taken for granted for its therapeutic efficacy using BSA methodology alone. There is a marked individual variation in the bioavailability of plasma 5-FU levels that has been well documented in the literature and reaffirmed in our series of 21 patients. In addition, we have reported data that indicate that 5-FU metabolism in patients changes as they undergo cycles of chemotherapy. We, thus, propose that the practice of treating patients with doses that are based on their height and weight (BSA methodology), should be

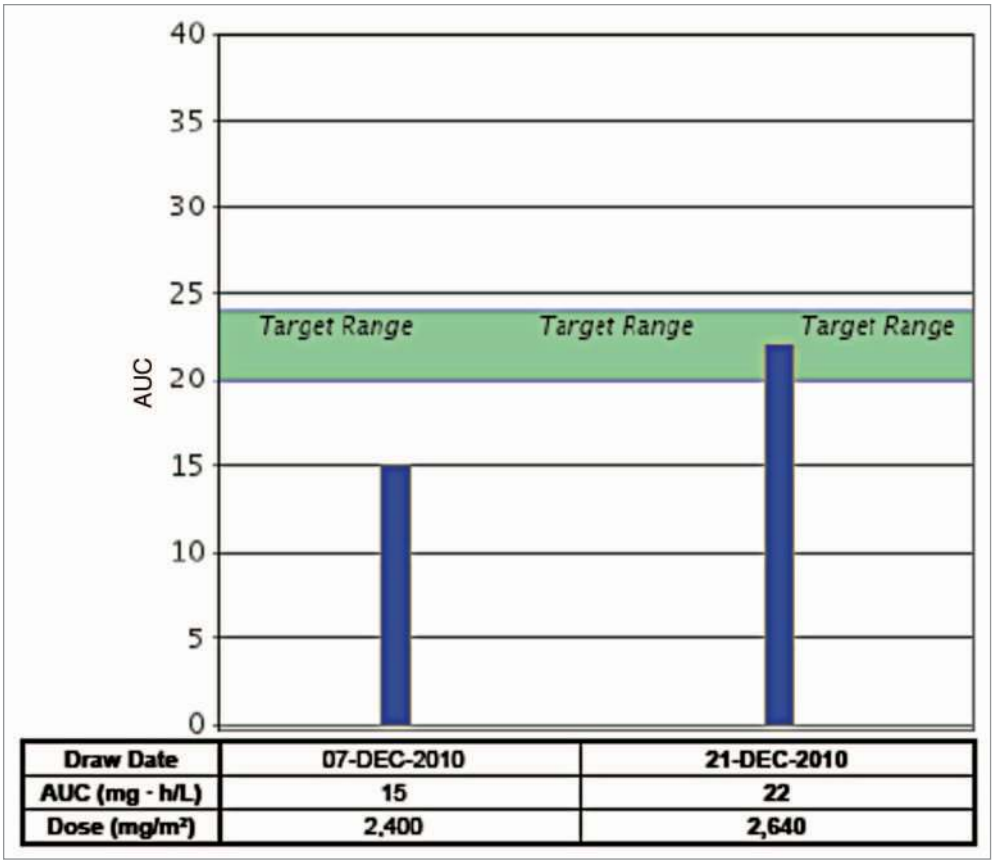
Table 1. Patient Characteristics.

Patient	Age	Sex	Diagnosis	KRAS/ BRAF	TS	Stage	Regimen	Initial AUC	Group	Liver mets	5-FU dose reduction
1	75	M	Colon cancer	WT/WT	1.87	IV	FOLFOX6 + Avastin	17	B/C	Yes	Yes based on AUC of 32
2	67	M	Colon cancer	G12A/WT	1.34	III	FOLFOX6	20	A	No	No (yes for oxaliplatin)
3	50	M	Colon cancer	WT/WT	6.41	III	FOLFOX6	20	A	No	No (yes for oxaliplatin)
4	53	F	Rectal cancer	NA	NA	III	FOLFIRI	18	B	No	No
5	40	M	Rectal cancer	WT/WT	6.99	IV	FOLFOX6 + Avastin	15	B	Yes	No (yes for oxaliplatin)
6	51	F	Colon cancer	WT/WT	NA	III	FOLFOX6	16	C/D	No	No (yes for oxaliplatin)
7	60	F	Colon cancer	WT/V600E	1.75	IV	FOLFOX6 + Avastin	25	D	Yes	Yes based on AUC of 25
8	76	F	Colon cancer	G12V/WT	2.71	III	FOLFOX6	25	B	No	Yes based on AUC of 25
9	34	M	Colon cancer	NA	NA	III	FOLFOX4	9	E	No	No (yes for oxaliplatin)
10	62	F	Colon cancer	WT/WT	5.72	III	FOLFOX6	38	B	No	Yes based on AUC of 38
11	55	M	Colon cancer	WT/WT	5.56	III	FOLFOX6	17	C*	No	No (yes for oxaliplatin)
12	43	F	Colon cancer	WT/WT	3.5	IV	FOLFOX6 + Avastin	24	A	No	Yes based on later toxicity (yes for oxaliplatin)
13	47	F	Colon cancer	G12V/WT	1.64	IV NED	FOLFOX6	18	B	No	No
14	61	F	Rectal cancer	NA	NA	III	FOLFOX6	25	C	No	Yes based on AUC of 25 (yes for oxaliplatin)
15	57	M	Colon cancer	WT/WT	NA	III	FOLFOX6	10		No	No due to diarrhea
16	61	M	Colon cancer	WT/WT	1.99	III	FOLFOX6	17	B	No	Yes based on later AUC of 25 (yes for oxaliplatin)
17	63	M	Colon cancer	WT/WT	1.03	IV	FOLFOX6 + Avastin	18	C	No	Yes based on AUC of 27, 29
18	43	M	Colon cancer	WT/V600E	1.9	IV	FOLFOX6 + Avastin	12	C**	Yes	Yes based on later AUC of 30 (yes for oxaliplatin)
19	63	M	Rectal cancer	WT/WT	3.58	III	FOLFOX6	29	B	No	Yes based on later AUC of 30
20	68	F	Rectal cancer	WT/WT	3.68	IV	FOLFOX6 + Avastin	20	A	No	No (therapy in progress)
21	59	F	Colon cancer	WT/V600E	6.76	IV	FOLFOX6 + Avastin	15		No	No (therapy in progress)

The patients were assigned to an AUC optimization group based on the pattern of variability of their 5-FU levels. The groups were designated as follows: A) patients that achieved an AUC within the target range without any dose titration; B) patients where the target 5-FU was achieved by adjusting the administered dose once or twice; C) patients that did not respond to two dose adjustments but at the third adjustment, experienced a spike in 5-FU level, exceeding the target range; D) patients that demonstrated increases in 5-FU levels (past the target range) despite receiving previously established therapeutic dose or even less than the therapeutic dose and E) patient that received FOLFOX4.

re-evaluated especially in the era of readily available commercial testing for 5-FU.

In a subset of our patients, multiple dose adjustments were required to achieve the desired therapeutic range as determined by Gamelin et al.[4] Moreover, we observed that some patients despite repeated dose adjustments over 3–4 two-week cycles did not reach target AUC levels initially but suddenly exceeded the upper limit of the target range with the following dose adjustments.



Draw Date	07-DEC-2010	21-DEC-2010
AUC (mg · h/L)	15	22
Dose (mg/m²)	2,400	2,640

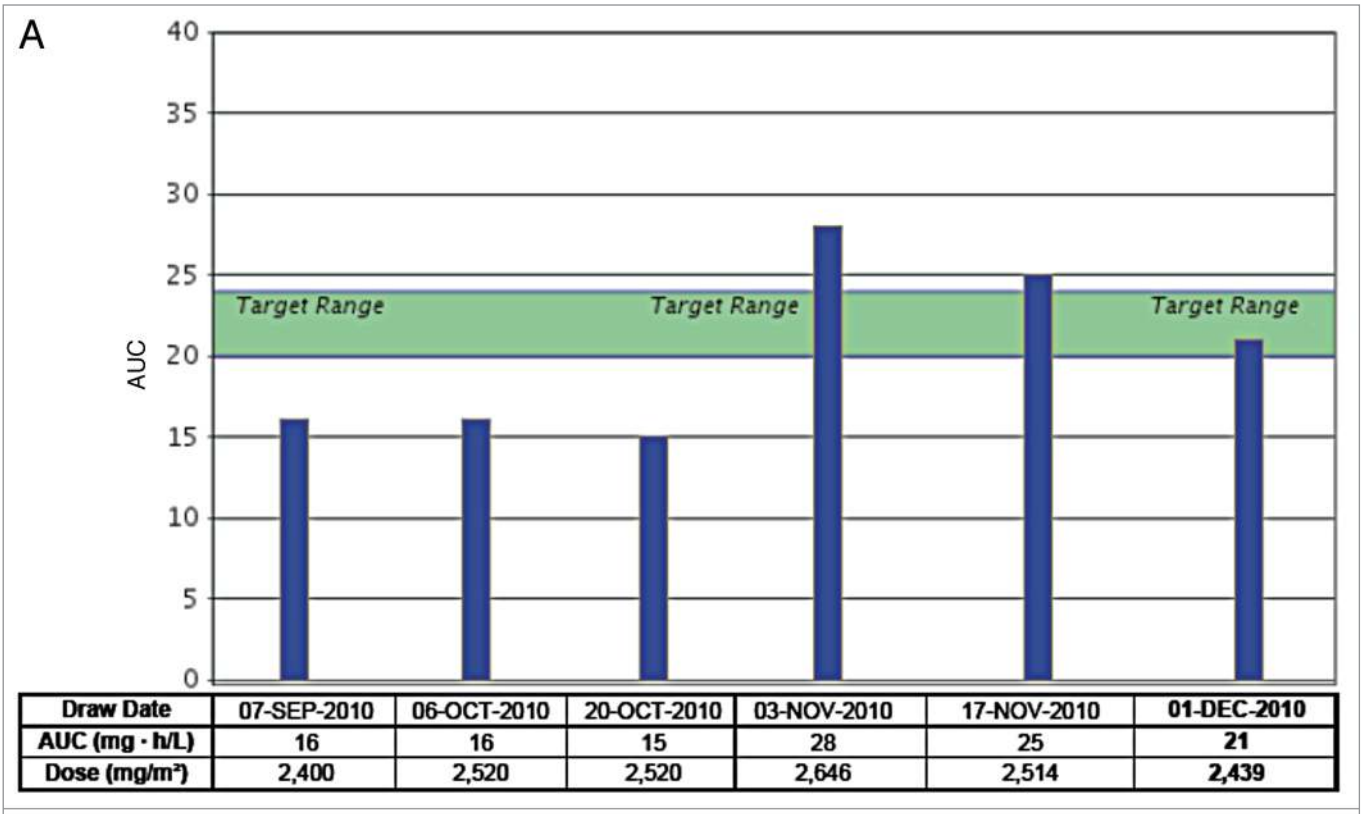
Figure 2. A representative Ondose test result on a patient that had therapeutic 5-FU level after one or two dose adjustments.

This necessitated a dose reduction to an amount less than their first dose that was initially deemed sub-therapeutic. The increase in 5-FU plasma levels seen after a small dose increase can be an indication of a reduction in 5-FU clearance. A similar conclusion can perhaps be made from the observations of increasing 5-FU plasma levels seen despite the continued use of therapeutic 5-FU dose or even dose reductions.

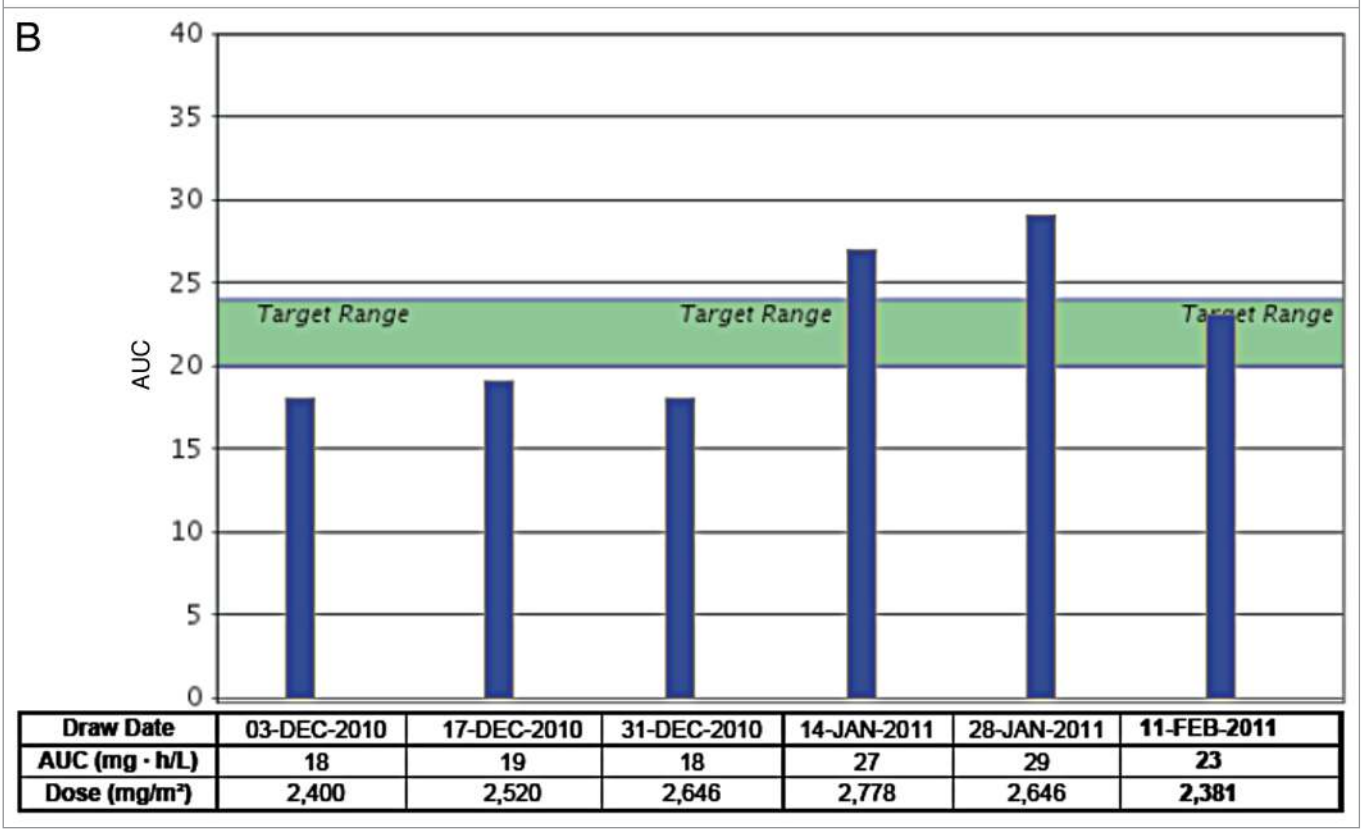
The elimination kinetics of 5-FU has been shown to be non-linear. When administered doses are increased, the half-life and bioavailability of 5-FU also increase,[9] and systemic clearance decreases. By contrast, the amount of 5-FU in the liver decreases.[10] The reduction in clearance with increased 5-FU doses is also seen when 5-FU is taken orally.[11] The mechanism behind the decrease in 5-FU clearance in response to 5-FU-based chemotherapy has not been elucidated. Although it has been proposed by Gamelin et al.[4] that this is due to a saturable metabolic process, the molecular basis for this saturation is not completely understood. In our study and in Gamelin's study, the change in clearance is observable after three cycles of chemotherapy. The time lag between the third and the fourth cycle is different between the patients in our study, and the population in Gamelin's study. Yet, the observations are similar. This raises the question on the impetus behind this change in 5-FU metabolism.

One possible mechanism behind the change in 5-FU clearance may involve the 5-FU catabolic enzyme dihydropyrimidine dehydrogenase (DPD). 5-FU is degraded via a 3-step catabolic pathway: a) conversion to 5-fluoro-5,6-dihydrouracil via dihydropyrimidine dehydrogenase; b) formation of α-fluoro-β-ureidopropionic acid via dihydropyrimidinase and c) conversion to α-fluoro-β-alanine via β-alanine synthase. These reactions occur primarily in the liver cytosol[12] (**Fig. 7**). The enzyme that catalyzes the rate-limiting step in this pathway is DPD.[13] The critical role that DPD plays in 5-FU metabolism is underscored by the deleterious effects in patients that have complete or near-complete deficiency of the enzyme.[14] Colorectal cancer patients exposed to a single bolus of 5-FU before surgery had significantly lower DPD mRNA levels in their primary tumors.[15] DPD activity in human PBMC's was decreased in colorectal cancer patients with intravenous treatment. This decrease was also seen in livers of rats that received a bolus injection of 5-FU.[16] An in vitro study on cervical carcinoma cells showed that extended exposure to 5-FU has a different effect on DPD expression than that of short-term exposure. With short-term 5-FU treatment, DPD mRNA is inhibited only by high 5-FU concentrations. On the other hand, treating for an extended period of time with lower 5-FU concentrations was sufficient to inhibit DPD mRNA expression.[17] In the case of nude mice with gastric cancer xenografts, a short-term exposure to 5-FU also resulted in a decrease in DPD activity.[18] Despite this mounting evidence that 5-FU treatment affects DPD, the impact on long-term 5-FU based chemotherapy on DPD has not been investigated.

Aside from potential differences in the rate-limiting enzyme of 5-FU catabolism, DPD, there are other plausible sources of variability that can explain the heterogeneity in 5-FU levels reported in this study. In a continuous infusion, the 5-FU levels of a patient depend on the time of day the blood sample was drawn. This is at least, in part due to a circadian variability in DPD activity.[19] However, it has been shown that the circadian variability of 5-FU levels did not account for most of the intra-patient variability observed in a study of 61 patients receiving 24 h continuous infusion.[20] Gender has been shown to influence 5-FU clearance, with women having a lower ability to clear 5-FU.[21-23] This could, at least in part, explain why 5-FU-related toxicities are more prevalent in women.[24] The influence of age on 5-FU clearance has been more equivocal. In one study, it was concluded that age did not affect clearance, as long as corrections



Draw Date	07-SEP-2010	06-OCT-2010	20-OCT-2010	03-NOV-2010	17-NOV-2010	01-DEC-2010
AUC (mg · h/L)	16	16	15	28	25	21
Dose (mg/m²)	2,400	2,520	2,520	2,646	2,514	2,439



Draw Date	03-DEC-2010	17-DEC-2010	31-DEC-2010	14-JAN-2011	28-JAN-2011	11-FEB-2011
AUC (mg · h/L)	18	19	18	27	29	23
Dose (mg/m²)	2,400	2,520	2,646	2,778	2,646	2,381

Figure 3. Ondose test results of two patients (A) and (B) whose 5-FU AUC did not respond with increases in administered doses in the first three cycles. After two adjustments, however, a small increase in the administered dose resulted in a marked increase in 5-FU AUC, exceeding the target range of 5-FU plasma level.

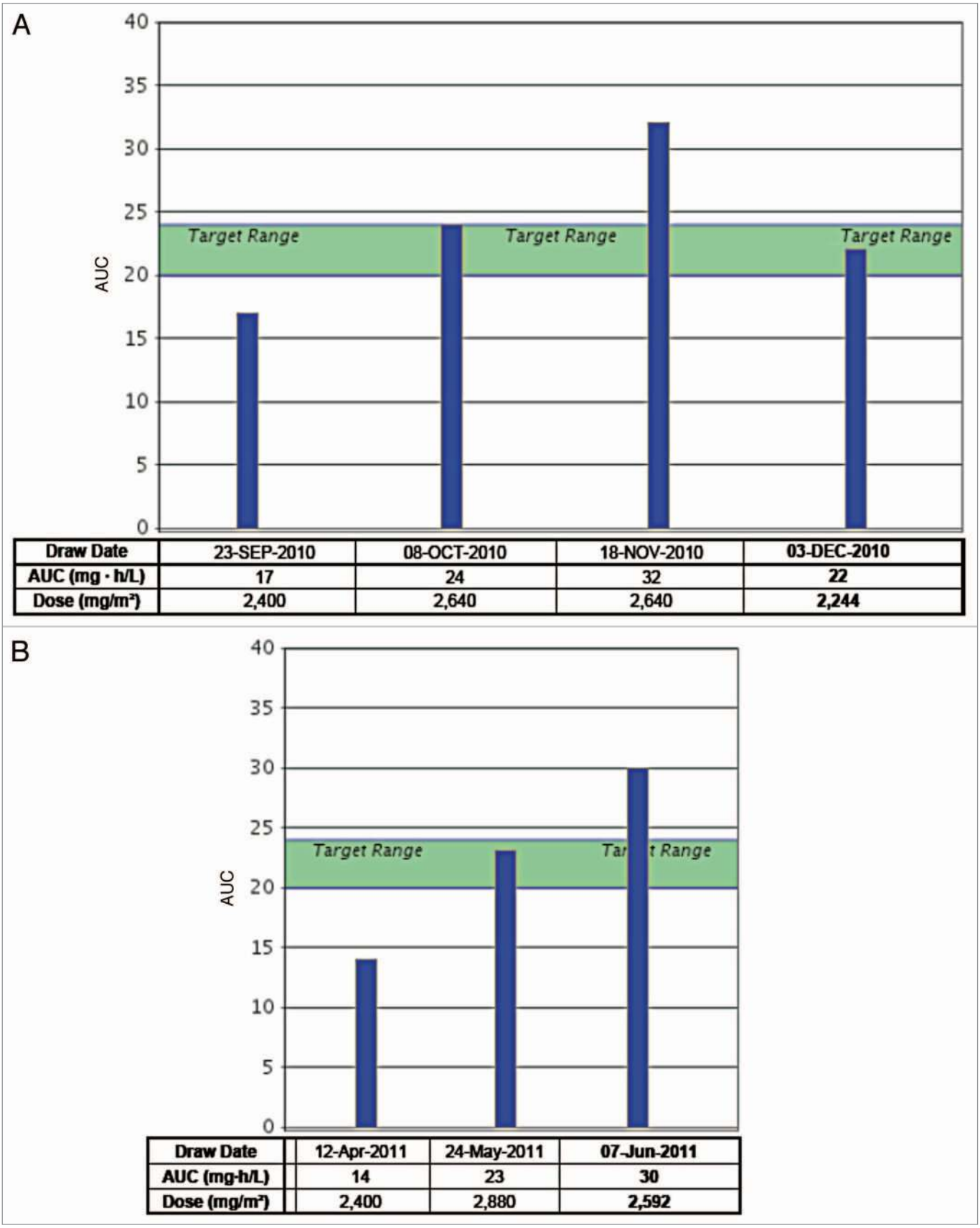
for gender and dose were made.[19] Yet, there are also indications that increasing age may reduce clearance.[23,25]

Treating patients with the regimen of 5-FU, leucovorin and oxaliplatin (FOLFOX) has proven to be more effective than FU/LV alone, in terms of a higher objective response rate, longer time to tumor progression, and a higher rate of relief from tumor-related symptoms.[26,27] Different concentrations of oxaliplatin have been combined with different modes of administration of 5-FU and leucovorin, leading to the development of different regimens.[28] Continuous infusion (CI) of 5-FU has been shown to have advantages possibly due to the short half-life of 5-FU and its cell cycle-dependent effect on thymidylate synthase.[29]

Currently, the combination of short-term infusional 5-FU/LV and oxaliplatin (FOLFOX) is considered a standard first-line therapy for mCRC. At least seven modifications of this combination exist based on a difference in the dose intensity of 5-FU/LV and oxaliplatin. In the North America, FOLFOX6 is more commonly employed over FOLFOX4 largely due a convenience in the administration of chemotherapy in the community oncology practice. Both regimens use the same amount of oxaliplatin but with a significant difference in the dose intensity of short-term infusional 5-FU/LV (bolus 5-FU 400 mg/m^2 followed by 600 mg/m^2 of 22 h CI 5-FU on day 1 and day 2 vs. bolus 5-FU 400 mg/m^2 followed by 2400 mg/m^2 of 46 h CI 5-FU on day 1 only). Whether this difference in dose intensity translates into a meaningful clinical benefit is not clear as there are no prospectively designed clinical trials comparing the two regimens in a head to head fashion. In one small retrospective study of Japanese patients with refractory or advanced colorectal cancer treated with FOLFOX4 or mFOLFOX6 regimen, mFOLFOX6 produced a higher observed partial response rate (35.5 vs. 25%) without any significant differences in toxicity. However, this study was not primarily designed to compare the two regimens and only evaluated the efficacy, feasibility and tolerability of the two regimens in a Japanese cohort.[30] In the randomized OPTIMOX1 trial, FOLFOX4 was compared with FOLFOX7 in a stop and go fashion in mCRC patients as a novel strategy to mitigate the cumulative side effects of neurotoxicity resulting from oxaliplatin. The FOLFOX7 regimen contained the same amount of CI 5-FU/LV as FOLFOX6 without the 5-FU bolus but a higher oxaliplatin dose. Previously untreated patients were randomly assigned to either FOLFOX4 administered every 2 weeks until progression (arm A) or FOLFOX7 for six cycles, maintenance bolus and CI 5-FU/LV without oxaliplatin for 12 cycles, and reintroduction of FOLFOX7 upon progression. The two arms were equal in their duration of disease control (DDC), progression-free survival (PFS), overall survival (OS), objective tumor response and the toxicity profile. These two studies, although not primarily designed to compare the efficacy and toxicity of the two FOLFOX regimens, did not show a clinically meaningful difference in the outcomes such as overall survival, PFS and response rate based on a difference in 5-FU/LV intensity alone.[31]

While the outcomes data in the combination regimens of 5FU/LV with oxaliplatin is unequivocal in terms of difference in dose intensity, there is evidence that 5-FU/LV as a single agent does translate into clinical benefit if delivered at an optimum dose based on pharmacokinetic adjustments vs. conventional body-surface-area calculations. Gamelin et al.[2] compared in a multicenter Phase III randomized study conventional dosing of fluorouracil (FU) plus folinic acid with pharmacokinetically guided 5-FU dose adjustment in terms of response, tolerability and survival. The 5-FU doses were adjusted weekly until the patients reached the therapeutic plasma range (AUC 20–24 mg.h/L). In the intent-to-treat analysis of the 208 patients, objective response rate was 18.3% in arm A, in which the 5-FU dose was calculated based on body-surface area, and 33.7% in arm B ($p = 0.004$) in which the 5-FU dose was individually determined using pharmacokinetically guided adjustments. Median overall survival was 16 mo in arm A and 22 mo in arm B ($p = 0.08$). The higher median OS of 22 mo reached in the pharmacokinetically adjusted arm was comparable to the other commonly used 5-FU/LV combination regimens, with a low toxicity and financial costs. Whether this clinical benefit resulting from the optimization of a weekly single agent 8 h infusion of 5-FU (1500 mg/m^2) dose can be reproduced in FOLFOX4 or FOLFOX6 regimen with 5-FU dose optimization to an AUC of 20–24 is currently unknown. Furthermore, there is limited evidence assessing the optimum AUC needed for oxaliplatin and irinotecan based regimens. These studies are critical given the observation that oxaliplatin can actually reduce the clearance of 5-FU.[32] There is limited evidence that suggests with a modified FOLFOX4 regimen (resembling FOLFOX4 with oxaliplatin 85 mg/m and leucovorin 200 mg/m^2 day 1, however 5-FU comparable to FOLFOX6 with fluorouracil 400 mg/m^2 bolus day 1 and 2500 mg/m^2 over 44 h) that over 80% of patients required a 5-FU infusion increase targeting a concentration of 0.6 mg/mL to achieve an AUC of 28.8 mg.h/L. Despite this titration there was a smaller number of patients reported to have grade ¾ toxicity (diarrhea/mucositis) and suggestive increase in median overall survival (22 vs. 28 mo).[6] A single-arm trial involving 90 patients treated with FOLFIRI with dose optimization of 5-FU CI starting at 2500 mg/m^2 over 46 h and modified to target an AUC of 25–30 mg.h/L (Css 0.55–0.65 mg/mL) reported a median overall survival of 28 mo with minimal grade ¾ toxicity.[33]

We noted a suboptimal 5-FU AUC of 10 in one patient who received FOLFOX4 regimen. This observation is not surprising given that CI 5-FU is half of what is used in FOLFOX6. Similar data has been observed in other reports of patients undergoing the FOLFOX4 regimen with an average AUC of 12.4 compared with an average AUC of 20.4 for FOLFOX6 regimen.[34] Given the prevalence of this observation across the patient population receiving FOLOFX4 regimen, it cannot be entirely attributed to individual variability in 5-FU metabolism. At the same time it does pose an important question regarding achieving an optimal dose with FOLFOX4 regimen given the correlation of higher 5-FU AUC of 20–24 levels with therapeutic efficacy. Our observations in a patient treated with FOLFOX4 showing a level of 5-FU that is half of the minimum value for the therapeutic range brought to our attention an important issue as various 5-FU containing regimens are tested and combined with novel targeted agents in patients with colorectal cancer. It would seem to be



Draw Date	23-SEP-2010	08-OCT-2010	18-NOV-2010	03-DEC-2010
AUC (mg · h/L)	17	24	32	22
Dose (mg/m²)	2,400	2,640	2,640	2,244

Draw Date	12-Apr-2011	24-May-2011	07-Jun-2011
AUC (mg-h/L)	14	23	30
Dose (mg/m²)	2,400	2,880	2,592

Figure 4. Ondose test results of two patients (A) and (B) that demonstrate that 5-FU levels can be supertherapeutic in subsequent cycles even if a previously established effective dose is administered.

Draw Date	20-MAY-2010	03-JUN-2010	17-JUN-2010
AUC (mg · h/L)	9	10	10
Dose (mg/m²)	1,200	1,200	1,440

Figure 5. Ondose test results of a patient that received the FOLFOX4 regimen.

important to ensure that 5-FU levels are optimized in such trials in order to not overestimate the benefit from targeted therapy. For example, if 5-FU levels are sub-optimal with regard to therapeutic range in a regimen such as FOLFOX4 one might expect a lesser effect in terms of response rate or patient survival for cohorts treated with FOLFOX4 and such result might contribute to a greater apparent benefit from regimens where 5-FU is more optimally dosed or from those regimens that involve the addition of a combined targeted agent.

Aside from analyzing 5-FU levels of the patients as they undergo repeated cycles of chemotherapy, we also took note of the ratios of administered-dose: AUC in the patients. In ten out of 15 patients (67%), the respective ratio of administered-dose: AUC had a decreasing trend as a patient underwent chemotherapy. This indicates that as a patient goes through multiple cycles of chemotherapy, they may need to receive less 5-FU during treatment to continue to have therapeutic plasma 5-FU levels. The decrease in 5-FU metabolism that we have observed with chemotherapy is supported by a recent study by Ibrahim et al.[35] They reported that 5-FU clearance is decreased even in the course of one cycle of chemotherapy. They compared 5-FU clearance on day 1 to that on day 5 of the first chemotherapy cycle of 81 patients. In another study, an increase in AUC was observed in patients given weekly doses of 5-FU.[21] The mechanism behind the decrease in 5-FU clearance in response to 5-FU based chemotherapy needs to be further elucidated. Low 5-FU clearance has been shown to be a predictor of severe toxicity.[36] This research problem is significant given the current practice that doses given to patients are only reduced when toxic side-effects have been experienced. These side-effects can be avoided with the understanding that 5-FU-based chemotherapy may decrease the ability of patients to metabolize/clear 5-FU.

In this study, the possibility that tumor TS expression levels might correlate with the variability of 5-FU plasma levels was explored. Patients that had higher tumor TS levels tended to have lower AUC's, although the difference between the distribution of the AUC levels of the high and low TS patient groups was not statistically significant. This lack of statistical significance may be due to the limited sample size. On the other hand, it may also be an indication that TS level is not a major determinant of 5-FU AUC's. In cells, including in tumor cells, 5-FU is metabolized in a two-step reaction to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP).[37] FdUMP forms a complex with the cofactor 5,10-methylenetetrahydrofolate and TS.[38-40] This makes TS less available to catalyze the formation of thymidylate, a critical step in DNA synthesis. The inhibition of thymidylate synthase is one of the primary modes of action of 5-FU. Thus, studies have been done to correlate TS expression with survival[41-44] and response.[45] These studies show that low TS expression is predictive of better survival and response to 5-FU-based chemotherapy. Polymorphisms of the TS gene, have also been analyzed and correlated with response to 5-FU. The TS gene has

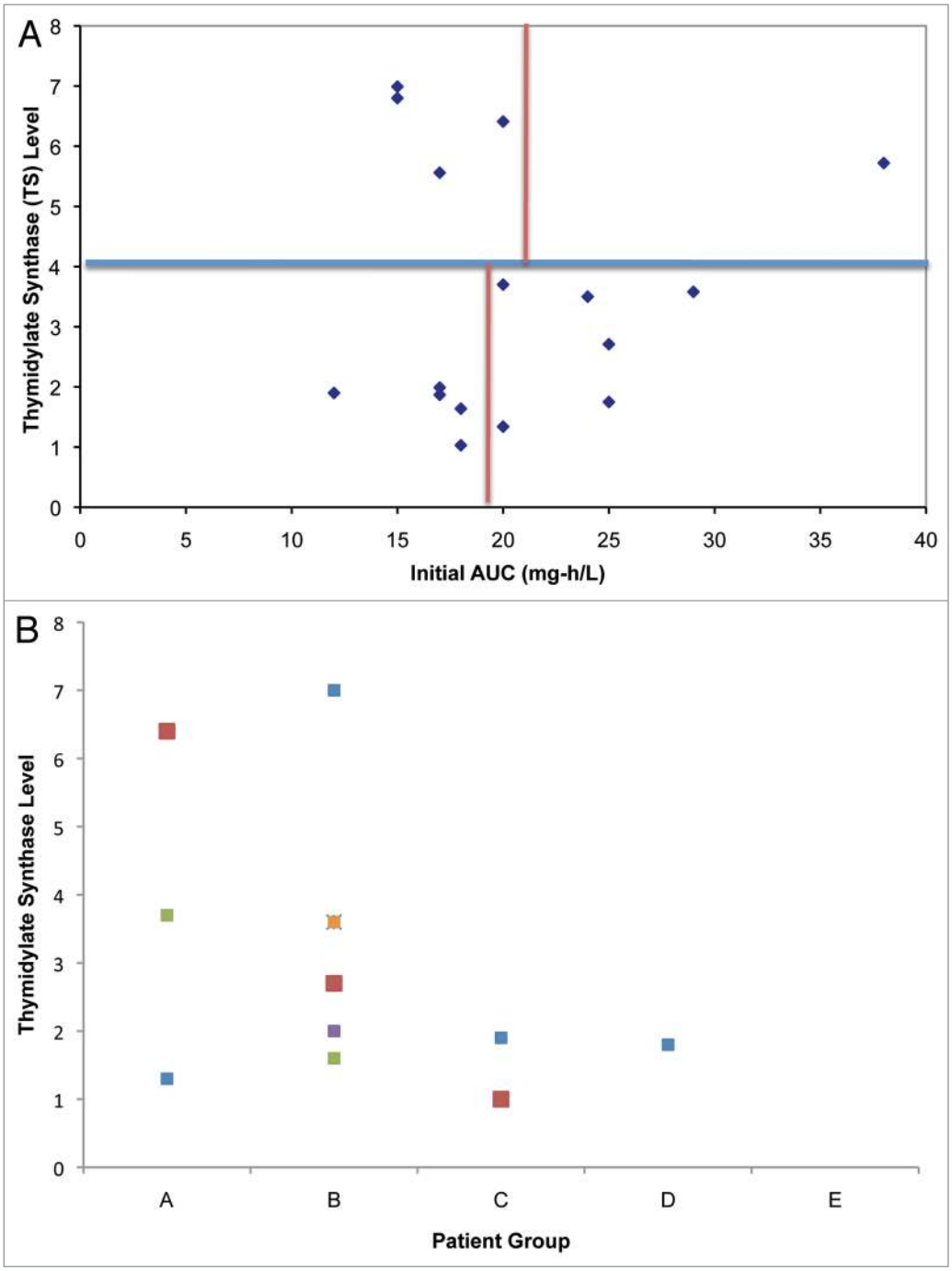


Figure 6. Thymidylate synthase (TS) levels of patients in this study. (A) Thymidylate synthase mRNA expression levels of the tumors of the patients were plotted against the initial 5-FU AUC values of the patients. The horizontal line indicates the normalized mRNA cut-off value of 4, with TS values higher than 4 indicative of high TS. Vertical lines arbitrarily separate AUC levels based on TS expression into potentially meaningful groups as described in the text. (B) TS levels of the patients in different groups based on the pattern of variability of their 5-FU levels, as described in **Figure 1** and in the Results section.

a tandem repeat in its 5′-untranslated region. The number of repeats is polymorphic.[46] This polymorphism has been correlated with response and toxicity of 5-FU chemotherapy, with patients having the triple repeat experiencing fewer side effects. These same patients, however, also had a low response rate. Although 5-FU AUC's were not reported in the study, it is possible that these patients had low 5-FU AUC's. Thus, patient AUC's may be influenced more by TS gene polymorphisms than TS expression levels. This, however, needs further elucidation.

In the future, in addition to greater attention to 5-FU levels, it will be important to gain a better understanding of what physiological and genetic differences might underlie the observed

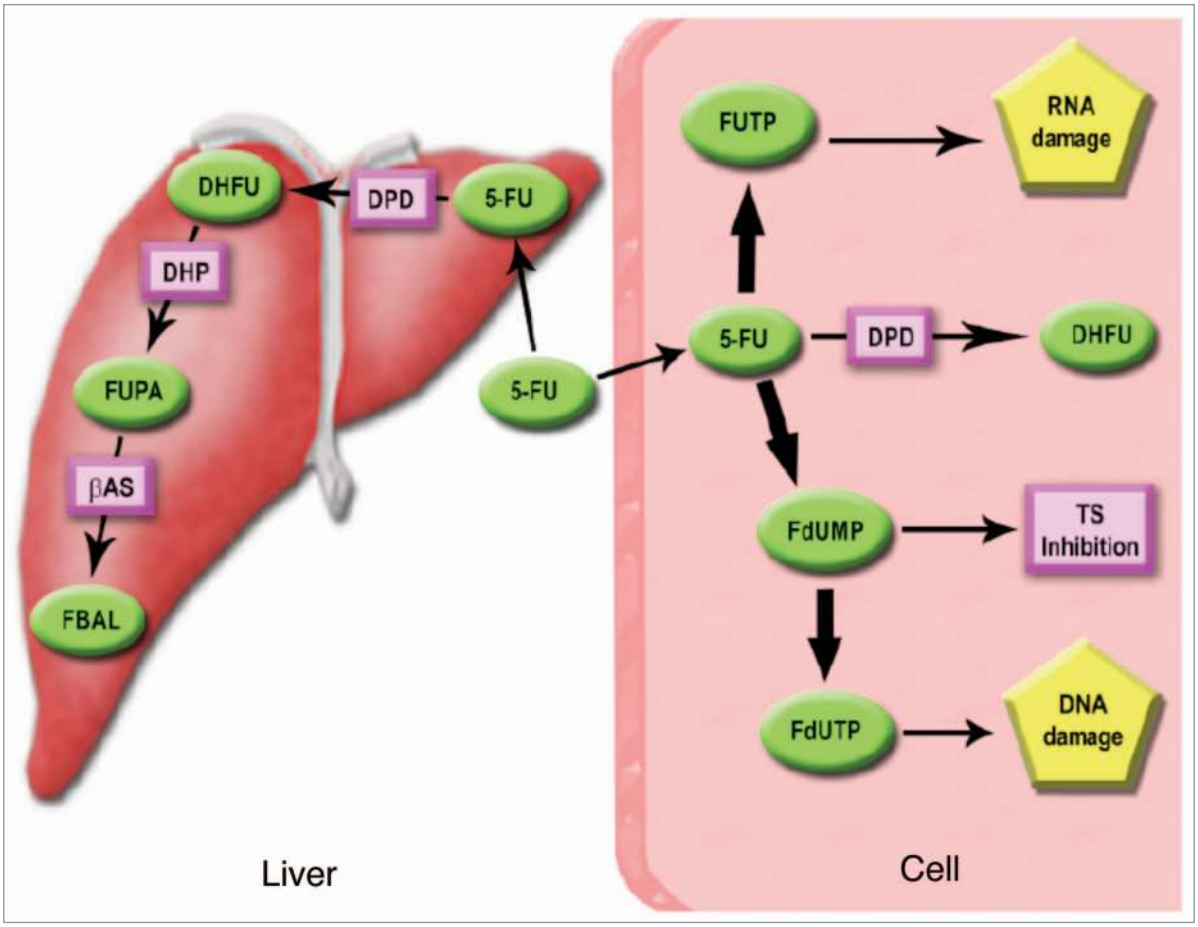


Figure 7. 5-FU is largely metabolized in the liver. The rate-limiting enzyme for 5-FU, DPD, is shown. In the liver and in cells, 5-FU is converted by DPD to dihydrofluorouracil (DHFU). In the cell, 5-FU undergoes catabolic reactions and conversion reactions, resulting in thymidylate synthase inhibition (TS), DNA and RNA damage. Only a subset of the reactants and enzymes involved in the 5-FU metabolic pathways are shown. For an excellent review of these processes, the readers are referred to Longley et al.[47] Ovals denote the reactants/products of the reactions and rectangles enclose the enzymes. Abbreviations: 5FU, 5-fluorouracil; DPD, Dihydropyrimidine dehydrogenase; DHFU, dihydrofluorouracil; DHFU, 5-fluoro-5,6-dihydrouracil; DHP, dihydropyrimidinase; FUPA, α-fluoro-β-ureidopropionic acid; βAS, β-alanine synthase; FBAL, α-fluoro-β-alanine; FUMP, fluorouridine monophosphate; FdUMP, fluorodeoxyuridine monophosphate; FUTP, fluorouridine triphosphate; FdUTP, fluorodeoxyuridine triphosphate. Thick arrows denote multiple reactions are necessary to convert the reactant to the product shown. Walt Kline II prepared the artwork for this Figure.

heterogeneity among patients during optimization of infusional 5-FU dose. Larger studies will need to examine patient demographics including gender, age, ethnicity and disease state and it would be of interest to examine single nucleotide polymorphisms in DPD, TS, as well as other genes. It will also be of interest to more closely examine in vivo gene expression changes in liver to unravel other potential pathways that might impact on 5-FU levels over time after exposure to 5-FU therapy. The liver studies are particularly important given that 5-FU catabolism occurs mainly in the liver.[12]

Materials and Methods

Patient characteristics. The observations described here involved the study of existing data, including patient medical records, blood test results, pathological specimens and diagnostic images in the course of routine clinical care in a colorectal cancer clinic. The information in this manuscript was recorded in such a manner that subjects could not be identified. Patient confidentiality was maintained and the work was performed in compliance with institutional and federal guidelines, and with approval from our Institutional Review Board.

A total of 21 patients with colorectal cancer as part of routine clinical care had 5-FU pharmacokinetic testing using the commercially available OnDose test (Myriad Genetic Laboratories Inc., Salt Lake City, UT) to target a plasma AUC level of 20–24 mg.h/L. The 21 subjects ranged in age from 34–76 y old, included 11 men and ten women, and included 16 patients with colon cancer and five with rectal cancer. The patients included nine with Stage IV disease, including one with Stage IV and no evidence of disease (NED) following resection, and 12 patients with Stage III disease.

Chemotherapy regimens. Regimens used included FOLFOX6 + Avastin for the eight patients with measurable Stage IV disease, and FOLFOX6 for 11 patients with Stage III or Stage IV NED disease. One patient with Stage III disease

received the FOLFIRI regimen and one patient received the FOLFOX4 regimen.

Blood collection for 5-FU AUC determination. Routine blood collection for 5-FU levels was drawn from an upper extremity peripheral vein on the contra-lateral side where the patients had a port for infusional 5-FU. Blood was collected at the 26th hour of the continuous 5-FU infusion of FOLFOX6. In the case of the patient receiving FOLFOX4, blood was drawn at 23 ± 3 h of infusion.

KRAS, BRAF mutation status and thymidylate synthase expression level. The analyses of the K-Ras and B-Raf mutation status, and the thymidylate synthase expression level in patient tumor tissue were performed by Response Genetics Inc. (Los Angeles, CA).

Disclosure of Potential Conflicts of Interest statement

No potential conflicts of interest were disclosed.

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