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ORIGINAL ARTICLE

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S-phase modulation by irinotecan: pilot studies in advanced solid tumors

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Abstract Two studies of irinotecan (CPT-11) followed 24 h later by an antimetabolite were conducted. The objectives of the studies were: (1) to determine whether the increase in S-phase in tumor cells seen 24 h after CPT-11 administration in animal studies is seen in advanced solid tumors in patients, (2) to determine the dose of CPT-11 required to produce this effect, (3) to compare two methods (immunohistochemistry, IHC, for cyclin A, and DNA flow cytometry, FC) for evaluating S-phase in tumor biopsies from patients, and (4) to establish the maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) of CPT-11, given 24 h before gemcitabine (GEM, 1000 mg/m^2). In one study CPT-11 was followed 24 h later by 5-fluorouracil (5-FU), 400 mg/m^2 per week for 4 weeks every 6 weeks. Tumor biopsies were obtained before and 24 h after CPT-11 administration before administration of 5-FU and assayed for S-phase by IHC for cyclin A and by FC. The starting dose of CPT-11 was 80 mg/m^2 per week with subsequent exploration of 40 and 60 mg/m^2 per week to establish the dose-effect relationship of the increase in tumor cells in S-phase. In the second study, CPT-11 was given 24 h before GEM 1000 mg/m^2 per week for 2 weeks every 3 weeks. Doses of 20–80 mg/m^2 were explored to establish the MTD and DLT and to study tumor cell S-phase in selected patients. CPT-11 80 mg/m^2 produced a mean increase in S-phase by IHC for cyclin A of 137%. Lesser increases were seen with 40 and 60 mg/m^2. CPT-11 followed 24 h later by 5-FU 400 mg/m^2 per week for 4 weeks was well tolerated. In the study of CPT-11 followed by GEM 1000 mg/m^2, 60 mg/m^2 of CPT-11 was the MTD.

Keywords Irinotecan · 5-Fluorouracil · Gemcitabine · S-phase modulation

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Introduction

Studies in the laboratory of one of us (Y.M.R.) have demonstrated that when irinotecan (CPT-11) is given with 5-fluorouracil (5-FU), the efficacy is affected by the order in which the drugs are administered [1, 2]. In rats bearing the Ward colon carcinoma, complete tumor regression (CTR) was achieved in approximately 60% of animals when the two drugs were given together at 50% of the maximum tolerated dose (MTD) of each drug (50 mg/kg). Giving 5-FU 24 h before CPT-11 reduced

the CTR rate to approximately 40%, while CPT-11 24 h before 5-FU gave nearly 100% CTR. Toxicity as measured by body weight decrease was not increased with the improvement in efficacy. For maximum antitumor response, the full dose of 5-FU (50% of the MTD) was required, but the dose of CPT-11 could be reduced to 12.5% of the MTD with only slight loss of activity.

Similar effects were seen with the HCT8, FaDu and A253 human tumor xenografts in nude mice. Time-course studies with the HCT8 xenografts in nude mice revealed that the optimum interval between CPT-11 and 5-FU was 24 h; the time intervals and percent of CTR were 0 h 2%, 6 h 40%, 24 h 80%, 48 h 60%, and 72 h 40%. A possible explanation for these observations was provided by cell cycle studies with HCT-8 in nude mice. Tumors were excised 24 h after intravenous injection of CPT-11 (50 mg/kg), evaluated by flow cytometry (FC) for the percentage of cells in different phases of the cell cycle, and compared to tumors from control mice. In control tumors 62.9% of the cells were in G_1 phase, 22.7% in S phase, and 14.4% in G_2 phase, and in treated tumors 39.5% were in G_1 phase, 57.1% in S phase, and 3.4% in G_2 phase. Thus the number of cells in S phase was increased by 152%, greatly increasing the number of cells sensitive to S phase-specific drugs. On the basis of these results, we performed pilot studies in which CPT-11 was given 24 h before either 5-FU or gemcitabine (GEM). The objectives of the studies were: (1) to determine whether the increase in S phase in tumor cells seen 24 h after CPT-11 administration in animal studies is seen in advanced solid tumors in patients, (2) to determine the dose of CPT-11 required to produce this effect, (3) to compare two methods (immunohistochemistry, IHC, for cyclin A, and FC) for evaluating S phase in tumor biopsies from patients, and (4) to establish the MTD and dose-limiting toxicity (DLT) of CPT-11, given 24 h before GEM (1000 mg/m^2).

Patients and methods

Patient eligibility criteria

Patients with histologically or cytologically documented advanced solid malignancies refractory to conventional therapy were candidates. An accessible, biopsiable tumor was required for the CPT-11/5-FU study and was optional for the CPT-11/GEM study. Additional entry criteria included age >18 years, performance status (ECOG) 0–2 for the CPT-11/5-FU study, 0–1 for the CPT-11/GEM study, life expectancy greater than 12 weeks, no treatment with antineoplastic agents in the previous 3 weeks (6 weeks for nitrosoureas or mitomycin C), no radiotherapy in the previous 6 weeks, adequate bone marrow function defined as a white blood cell count $\geq 3000/mm^3$, neutrophil count $\geq 1500/mm^3$, hemoglobin ≥ 10 g/dl, platelet count $>100{,}000/mm^3$, adequate renal function defined as serum creatinine <1.6 mg/dl or creatinine clearance ≥ 50 ml/min, adequate liver function defined as hepatic enzymes (AST, ALT) not more than 1.5 times the upper limit of normal, bilirubin ≤ 1.5 mg/dl, normal cardiac function with no history of uncontrolled heart disease, and a negative pregnancy test for patients for whom pregnancy could be a possibility. Prior CPT-11 was allowed but prior 5-FU (CPT-11/5-FU study) or prior GEM (CPT-11/GEM study) was not allowed. All patients were required to give written informed consent. The study protocols were approved by the Roswell Park Institutional Review Board as meeting all safety and ethical requirements. Patients were ineligible if they were lactating, had had a myocardial infarction within the previous 3 months, had congestive heart failure, unstable angina, active infection requiring systemic therapy, history of a second malignancy within the past 5 years other than non-melanoma skin cancer, or carcinoma in situ of the cervix.

Study design

CPT-11/5-FU study

The objective of the study was to establish the lowest dose of CPT-11 which would produce modulation of S phase in tumor tissue. It was not a phase I study and no attempt was made to establish an MTD. Modulation of S phase was arbitrarily defined as $>50\%$ increase in S phase after treatment in $>50\%$ of the patients evaluated at a given dose. A tumor biopsy was performed up to 1 week before treatment with CPT-11. CPT-11 was given over 90 min on day 1. On day 2, 24 h after CPT-11 administration, a second tumor biopsy was obtained. These biopsies were assayed for S phase by FC when sufficient tumor tissue was available and for cyclin A by IHC. Immediately following the second biopsy, 5-FU 400 mg/m^2 was given intravenously over 5 min. Treatment was given weekly for 4 weeks followed by a 2-week rest (one course). The starting dose of CPT-11 was 80 mg/m^2. Subsequent dose levels were determined by the results of the S-phase assay and toxicity. If modulation as defined above was seen at the starting dose, dose level 2 would be 50% of the starting dose (40 mg/m^2). If no modulation was seen, the subsequent CPT-11 dose would be 100 mg/m^2, unless DLT had been seen in more than one patient at 80 mg/m^2 in which case the study would be terminated. If the starting dose showed modulation but dose level 2 did not, an intermediate dose (60 mg/m^2) would be explored. Accrual of patients to each dose level continued until four patients were evaluable for S-phase modulation. (Since the two studies were run concurrently, and CPT-11 administration and biopsy evaluations were identical for both, data from both studies were used to satisfy this requirement for doses of 40 and 60 mg/m^2.)

Patients were evaluated for tumor response after two cycles. Patients with stable disease or response could continue with reassessment after each two cycles.

Patients with progressive disease were removed from the study.

CPT-11/GEM study

This was a phase I study in which the primary objective was to establish the MTD and DLTs of CPT-11 when given 24 h before GEM 1000 mg/m^2 on a weekly for 2 weeks every 3 weeks schedule. A secondary objective was to provide data on S-phase modulation by CPT-11. The dose of GEM was fixed at 1000 mg/m^2. One patient was entered at the starting dose (20 mg/m^2) of CPT-11. If toxicity was more than grade II, two more patients would be entered at that dose level. If toxicity less than grade II was noted, the next patient would be entered at the next higher dose level. If no DLT was noted, escalation would proceed to the next higher dose level (CPT-11 dose levels 20, 40, 60, 80, and 100 mg/m^2). If a DLT was noted in one of the first three patients, the cohort would be expanded to a maximum of six patients. If a further DLT was seen, the previous cohort would be expanded to six patients. The MTD was defined as the highest dose in which fewer than two of six patients had DLT. The DLT was defined on the first cycle only. An attempt was made to recruit at least one patient having biopsiable disease at each dose level.

Dose-limiting toxicities

Non-hematologic DLT was defined as any non-hematologic toxicity of grade III or greater, except for nausea and vomiting controllable with medication. Hematologic DLT was defined as neutropenia grade IV for more than 7 days, thrombocytopenia grade IV or thrombocytopenic bleeding, febrile neutropenia (temperature >38.5°C) or grade IV anemia. The NCI CTC (version 2.0) were used.

On-study and follow-up investigations

Patients underwent a complete history and physical examination at the time of enrollment into the study and at the start of each new cycle. At these visits, performance status (ECOG) and weight were documented. A complete blood count with differential was performed at the beginning of each cycle and then weekly thereafter. Clinical chemistry analyses were performed weekly. In patients with liver metastases, the AST and ALT were repeated on the day of treatment to verify eligibility. Tumors were assessed radiologically before the patients were enrolled onto the study and then after even-numbered cycles.

Laboratory studies

Tumor biopsies were obtained before CPT-11 and 24 h after CPT-11 administration for measurements of the percentage of cells in S phase. S phase was measured by IHC for cyclin A and where possible by FC.

Immunohistochemistry for cyclin A

Antigen retrieval was achieved with citrate buffer (pH 6.0) in a microwave for 2×10 min before casein blocking. Sections were incubated with primary antibody (mouse antihuman cyclin A, 0.1 μg/ml, Novocastra Laboratories, Newcastle upon Tyne, UK) overnight at 4°C and with secondary antibodies from a Vectastain Universal Elite ABC peroxidase kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions. Slides were then incubated with chromogen containing H_2O_2 for 10 min at room temperature. Negative controls were isotype-matched mouse IgG1 at the same concentration as the primary antibody. Human tonsil was used as known positive (germinal centers) and known negative (lymphocytes) control. The pre- and posttreatment biopsies on a patient were prepared together. In biopsies where the amount of tumor tissue permitted, 300–400 tumor nuclei were read. Where fewer than this number was present, all of the tumor nuclei were read. The cyclin A index was defined as the number of positive cells divided by the total number of tumor cells evaluated [1].

DNA flow cytometry

Biopsy samples for FC were placed in ice-cold Hank's balanced salt solution and taken to the flow cytometry laboratory. They were disaggregated and divided into three aliquots. These were centrifuged at 3200 rpm at 4°C for 3 min, decanted, and vortexed. In preparation for data acquisition, aliquot I was stained by addition of 5 μl of 5 mg/ml ethidium monoazide, exposed to UV light (λ 350 nm) for 15 min, washed with phosphate-buffered saline and fixed with 2% formaldehyde. Aliquot II was analyzed unstained, for comparison, for calculation of the percentage of dead cells. Aliquot III was fixed in 70% ethanol for a minimum of 30 min at 4°C, centrifuged as above, decanted and vortexed, 1 ml of 0.5% bovine serum albumin added and again centrifuged as above. After decanting and vortexing, 1 ml of 0.05 mg/ml propidium iodide was added and staining carried out for a minimum of 30 min at 4°C. Data acquisition was on a FACScan (BD Biosciences) and analysis was carried out using the programs Winlist and Modfit (Verity Software House) and Multicycle (Phoenix Flow Systems).

Pharmacokinetic methods

Plasma samples were collected before treatment and at 1.5, 1.75, 2.0, 2.5, 3.5, 5.5, 8.5, 10.5, and 25.5 h in the CPT-11/5-FU study and before treatment and at 0.5, 1.0, 1.5,

2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 24.0, and 32 h in the CPT-11/GEM study with time 0 being the beginning of the 90-min infusion of CPT-11. Samples were analyzed by high-performance liquid chromatography (HPLC) for CPT-11 and SN-38 concentrations using a validated HPLC method with fluorescence detection. The method was a modification of that described by Warner and Burke [9].

CPT-11 and SN-38 were extracted from plasma with acidified methanol. The residue after evaporation of methanol was dissolved in mobile phase and injected onto the HPLC. Separation was carried out on a Waters Nova Pak C18 column equipped with a μBondapak C18 guard column. The mobile phase consisted of 20% acetonitrile and 80% triethylamine acetate. Detection was by fluorescence, with excitation at a wavelength of 370 nm and emission at 510 nm. Standards of CPT-11 and SN-38 were prepared in human plasma and processed in an identical manner to the unknowns. The HPLC system consisted of a Waters Associates M600 pump, M717 autosampler, M474 fluorescence detector operated by a PC with Waters Millennium software.

Camptothecin (CPT) was used as internal standard. The ratios of the peak areas for the CPT-11 and CPT and that for SN-38 and CPT were used for quantitation. The limits of quantitation for both CPT-11 and SN-38 were 2.5 ng/ml. Quality assurance was maintained by simultaneously assaying the quality control samples prepared in bulk prior to assay validation.

Pharmacokinetic parameters were determined by standard non-compartmental methods using NONLIN (version 2.1). AUC was computed using the linear/log trapezoidal method, and half-life was calculated as ln 2/Ke, where Ke is the rate constant for elimination.

Results

A total of 36 patients (17 in the CPT-11/5-FU study and 19 in the CPT-11/GEM study) were enrolled. In the CPT-11/5-FU study, there were 15 evaluable patients. Their median age was 59 years (range 43–69 years), and they had the following cancer diagnoses: non-small-cell lung carcinoma (five patients), colorectal carcinoma (four patients), esophageal carcinoma (two patients), and miscellaneous (four patients). All patients had received prior chemotherapy (more than three regimens in eight patients). The starting dose was 80 mg/m^2 per week of CPT-11 followed by 400 mg/m^2 of 5-FU. Eight patients were entered. No grade IV toxicity was seen. Two patients had grade III toxicity as their greatest toxicity on course 1. All four patients whose tumor biopsies were evaluable for S phase met the criteria for S-phase modulation. A dose of 40 mg/m^2 of CPT-11 with 400 mg/m^2 of 5-FU was therefore evaluated. This failed to meet the criteria for modulation (see below, S-phase modulation); a dose of 60 mg/m^2 CPT-11 was therefore explored.

In the CPT-11/GEM study, of 19 patients entered, 18 were evaluable. Their median age was 57 years (range 44–73 years), and they had the following cancer diagnoses: non-small cell lung carcinoma (nine patients), colorectal carcinoma (two patients), and miscellaneous (seven patients). All patients except one had prior chemotherapy (more than three regimens in six patients). DLTs in the form of grade IV anemia (one patient) and grade III hypotension and fatigue (one patient) were noted in two of six patients at 80 mg/m^2. The MTD of CPT-11 was 60 mg/m^2, followed 24 h later by GEM 1000 mg/m^2 weekly for 2 weeks every 21 days.

Hematologic toxicity

In the CPT-11/5-FU study, no patient had grade IV neutropenia. Two patients treated at the 80 mg/m^2 of CPT-11 dose level had grade III neutropenia.

In the CPT-11/GEM study, significant myelosuppression was noted at all dose levels. Grade IV toxicities were noted in 10 of 18 evaluable patients at CPT-11 doses of 40 mg/m^2 (one of six patients), 60 mg/m^2 (five of six patients), and 80 mg/m^2 (four of six patients). The grade IV toxicities were neutropenia (ten patients) and anemia (one patient). The neutropenia lasted less than 7 days; there was no febrile neutropenia. The neutropenia was therefore not a DLT. The grade IV anemia was a DLT.

Non-hematologic toxicity

In the CPT-11/5-FU study, no grade IV toxicity was seen. At 80 mg/m^2, the maximum toxicity during course one was grade III in two, grade II in four, and grade I in two patients; grade III toxicities included nausea, anorexia, diarrhea, and electrolyte disturbances. At 60 mg/m^2 of CPT-11, one of four patients developed grade III toxicity (fatigue). Grade III toxicity was not seen at 40 mg/m^2 of CPT-11.

In the CPT-11/GEM study, grade III hyponatremia was noted in one of six patients at 60 mg/m^2 of CPT-11. At 80 mg/m^2, grade III hypotension and dehydration noted in one of six patients was a DLT.

Antitumor activity

There were no complete or partial responses noted in either study. In the CPT-11/5-FU study, 3 of 15 patients had stable disease after one course of therapy (one non-small-cell lung carcinoma, one gastric carcinoma, and one esophageal carcinoma). In the CPT-11/GEM study, 7 of 19 patients had stable disease after two cycles of treatment (range 9.7 weeks to 125+ weeks). One patient with metastatic gastric carcinoma continued on the study with stable disease at the time of this report.

S-phase modulation

Initially, tumor was obtained by fine needle aspiration or tapping of malignant effusions. The former yielded insufficient material for analysis and the latter yielded

mostly dead cells. Subsequent biopsies were core biopsies. A total of 36 biopsies, 18 each before and after CPT-11 administration were obtained. Of these, 12 pairs were evaluable by IHC and 5 by FC for increase in S phase. The reasons for lack of evaluability of specimens by IHC were as follows. In two patients the pretreatment biopsy showed $<1\%$ of positive nuclei. One of these (after 60 mg/m^2 CPT-11) showed no increase in S phase, and the other (after 80 mg/m^2 of CPT-11) showed a 200% increase (increase in this sample by FC was 52%). However, it was felt that at this level of positivity, the reliability of the data would have been poor, so these data were not included with the calculations of modulation. In two patients there was no tumor in the posttreatment biopsy. In one the posttreatment biopsy had a large amount of necrosis and could not be evaluated and in the other the whole specimen was used for FC. Thus, data on cyclin A index were based on 24 biopsies (four pretreatment and four posttreatment at each of the doses of 40, 60, and 80 mg/m^2; Table 1).

Data from FC were more difficult to obtain. Only five pairs of biopsies, two each at 40 and 80 mg/m^2 and one at 60 mg/m^2, were evaluable for increase in S phase. The principal reasons that samples were not evaluable were (number of samples in parentheses): no aneuploid fraction seen (eight), S-phase confidence poor (six), and insufficient material to perform flow (four). The data for IHC in patients with evaluable pre- and posttreatment biopsies are shown in Table 1 and an example is shown in Fig. 1. It can be seen from Table 1 that there was substantial variability in the increases in cyclin A index but there was a trend for them to be dose-related with mean values for the increase of 30%, 47%, and 134% for CPT-11 doses of 40, 60, and 80 mg/m^2, respectively. By the criteria established at the start of the study there was modulation in one of four patients at 40 mg/m^2, two of four at 60 mg/m^2, and four of four at 80 mg/m^2. The difference between the 80 and 40 mg/m^2 doses was significant at the $P<0.05$ level by the Wilcoxon rank sum test. The differences between 80 and 60 mg/m^2 and between 60 and 40 mg/m^2 were not significant. Data from FC pre- and posttreatment were available in four of these patients and are shown in Table 2. These showed the same direction of change as IHC but the increases were quantitatively larger. In one patient at 40 mg/m^2 the whole sample was used for FC. It showed an increase of 25% in the percentage of cells in S phase. In four patients (two at 40 mg/m^2 and two at 80 mg/m^2) data were available on the G_2 phase before and after CPT-11. Three of these samples showed a decrease in G_2 after CPT-11 administration and one showed an increase (Table 2).

Pharmacokinetic results

Both the AUC and C_{max} of CPT-11 increased linearly with dose, and there was no trend for half-life to either increase or decrease with dose. A similar profile was observed for SN-38. However, dose was less predictive of SN-38 pharmacokinetic parameters than for CPT-11. The mean half-life of CPT-11 and SN-38 across all patients was 7.9 h (34%CV) and 15.0 h (53%CV), respectively. The mean clearances of CPT-11 and SN-38 were 17.1 (25%CV) and 227.0 l/h/m^2 (66%CV), respectively. There were no statistically significant differences in pharmacokinetic parameters of either CPT-11 or SN-38 between day 1 and day 8 of the GEM study ($P>0.05$, paired t-test on logarithmically transformed data). Similarly, there were no statistically significant differences in pharmacokinetic observed when the GEM study was compared with the 5-FU study ($P>0.05$, t-test on logarithmically transformed data).

Discussion

GEM and 5-FU are nucleoside analogs whose cytotoxic activities are related to inhibition of DNA synthesis by

Table 1 Cyclin-A index by IHC before and after CPT-11 administration in 24 tumor biopsies

CPT-11 dose (mg/m^2)	Diagnosis	Biopsy site	Cyclin A index		Change (%)
			Pretreatment	Posttreatment	
40	NSCLC	Subcutaneous	18	30	+67
	Adenocarcinoma, prostate	Lymph node	7	9	+29
	Adenocarcinoma, colon	Liver	18	26	+44
	Squamous cell carcinoma, larynx	Subcutaneous	29	23	−21
Mean (40 mg/m^2)			18	22	+30
60	Adenocarcinoma, colon	Subcutaneous	13	23	+77
	Squamous cell carcinoma, larynx	Subcutaneous	26	23	−12
	NSCLC	Lymph node	22	22	0
	Adenocarcinoma, esophagus	Liver	19	42	+121
Mean (60 mg/m^2)			20	23	+47
80	NSCLC	Subcutaneous	21	41	+95
	Adenocarcinoma, esophagus	Liver	3	9	+200
	NSCLC	Adrenal	22	44	+100
	NSCLC	Bronchus	17	41	+141
Mean (80 mg/m^2)			16	34	+134*

*$P<0.05$ vs 40 mg/m^2, Wilcoxon rank sum test (two-tailed test).

Table 2 Percentage of tumor cells in S phase and G_2 phase before and after CPT-11 administration by DNA FC

CPT-11 dose (mg/m^2)	Diagnosis	Biopsy site	S phase			G_2 phase		
			Pretreatment	Posttreatment	Change (%)	Pretreatment	Posttreatment	Change (%)
40[a]	NSCLC	Subcutaneous nodule	16	20	+25	10	6	−40
40	NSCLC	Subcutaneous nodule	11	21	+91	6	17	+183
60	Esophagus	Subcutaneous nodule	9	49	+444	–	–	–
80	NSCLC	Subcutaneous nodule	14	51	+264	17	0	−100
80	NSCLC	Adrenal	14	32	+129	14	10	−29

[a]Whole sample used for FC

different mechanisms. Both agents are active in a variety of solid tumors. Since they are S-phase specific, increasing the number of cells in S phase should enhance cytotoxic activity. In preclinical studies, CPT-11 enhanced 5-FU activity in the Ward colon carcinoma model in the rat at doses well below its MTD and without an increase in toxicity when given 24 h before 5-FU. In a similar fashion, in preclinical models, sequential administration of CPT-11 followed 24 h later by GEM resulted in improved antitumor activity with decreased toxicity at doses below the MTD of either drug. Preclinical data also suggest that the basis for these observations might be an accumulation of tumor cells in S phase 24 h after CPT-11 administration.

The present studies were conducted in an attempt to validate these observations clinically. The CPT-11/5-FU study was designed as a pilot study to establish the lowest dose of CPT-11 which would give a consistent increase in S phase in tumor tissue without prohibitive toxicity. The study was therefore designed to start at a dose level expected to give significant but tolerable toxicity, with subsequent exploration of lower doses of

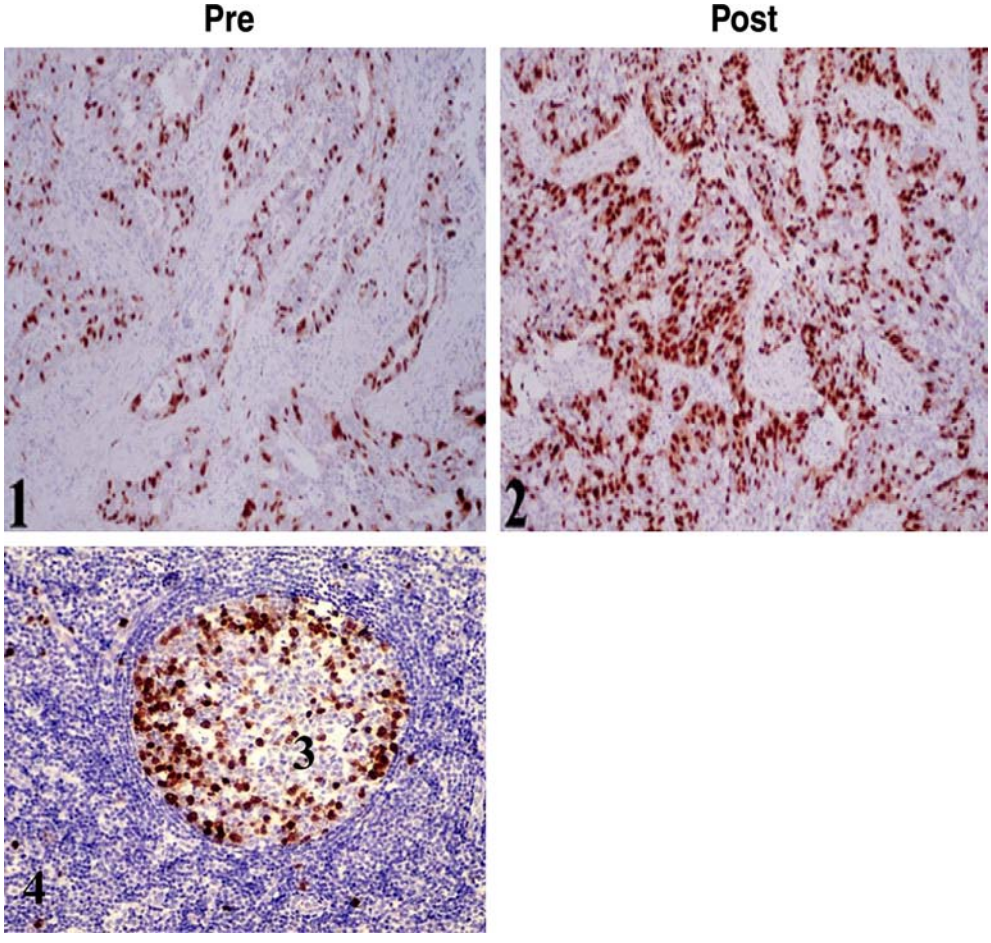

Fig. 1 Intratumoral cyclin-A expression before (1) and after (2) CPT-11 administration in a patient with metastatic non-small-cell lung cancer treated with CPT-11 at 80 mg/m^2. The positive control was the germinal center in human tonsil (3) and the negative control was the surrounding lymphocytes (4)

CPT-11 if the starting dose gave the anticipated modulation of S phase. For this study, all patients were required to have two tumor biopsies, one before and one after CPT-11 administration. Two methods were used to measure the S phase, the cyclin A index (ratio of the number of tumor cells staining positive for cyclin A to the total number of tumor cells counted) and FC. The temporal relationship between cyclin A accumulation and the onset of S phase has been analyzed in detail by Erlandsson et al. [3]. Using simultaneous incorporation of BrdU and staining for cyclin A and cyclin E in nine transformed and nine untransformed cell lines in culture, they demonstrated that cyclin A accumulation starts simultaneously or nearly simultaneously with DNA synthesis and continues through S phase. However, it also continues into the G_2 phase. This is discussed below.

A difficulty with working with human tumor samples from patients is that when a change is seen, it is unclear how much of the change may be due to the intrinsic variability of the method and/or to tumor heterogeneity and how much is a real change caused by the intervention used. The variability due to the method and to tumor heterogeneity would normally be measured by assaying repeated samples from the same tumor at the same time. However, in the clinical situation this is not often possible. As an indirect approach to this problem, we studied the intrinsic variability due to the method and/or to tumor heterogeneity in human tumor xenografts where it was possible to obtain multiple samples from the same tumor at the same time. We used these data to place an upper limit on that part of the change observed in patients which was likely to be due to expected variability. In these studies, female athymic nude (nu/nu) mice at 8–12 weeks of age and weighing 20–25 g were implanted with approximately 50 mg of one of four human tumor xenografts (HCT8, HT-29, A253, Fadu). CPT-11 was administered by tail vein injection at a dose of 100 mg/kg. Three biopsies were obtained from each xenograft 24 h later. These were evaluated for cyclin A index in the same way as the human tumor biopsies. The results were (cyclin A index $\pm$SD before and after CPT-11 administration): $37 \pm 2\%$ and $70 \pm 1.8\%$ in HCT8 xenografts; $29 \pm 1\%$ and $55 \pm 2.0\%$ in HT29 xenografts; $22 \pm 0.8\%$ and $37 \pm 3.0\%$ in A253 xenografts; and $36 \pm 0.8\%$ and $56 \pm 5.2\%$ in Fadu xenografts [6]. The SD thus ranged from 2.2 to 9.3% of the measured value. Although the variability in advanced human tumors may be considerably greater than in xenografts, these observations indicate that only a small proportion of the large increases seen in patients at 80 mg/m^2 are likely to be due to variability in the IHC methodology or to tumor heterogeneity.

The results from DNA FC were limited, as relatively few of the samples were assessable for S phase by FC. The reasons for this are outlined above. However, all five patients showed an increase in the percentage of cells in S phase after CPT-11 administration and in three patients the increase was considerably greater than that shown by the cyclin A index (Table 2). Since cyclin A continues to accumulate into G_2 phase this is an important observation. It is also important to note the reduction in the number of cells in G_2 phase seen in three of four patients, indicating that the cyclin A index increase was not due to accumulation of cells in G_2 phase.

CPT-11 has been successfully combined with 5-FU as well as with GEM in several tumor types including colorectal and non-small-cell lung cancer [5, 7, 8]. The focus of our pilot studies was on perturbation of S phase by CPT-11 as a means to improve the response rates to subsequently administered antimetabolites that are mostly S-phase specific. Two clinical studies have attempted to correlate response with the particular sequence of administration of CPT-11 and GEM [4, 6]. However, in these studies GEM was administered either immediately before or after CPT-11 and no biopsy correlations were done to specifically look at S-phase perturbation. In preclinical systems, the maximum accumulation of cells in S phase does not occur until 24 h after dosing with CPT-11 [1].

The CPT-11/GEM study was designed to establish an MTD of CPT-11 given 24 h before GEM at a dose of 1000 mg/m^2. Myelosuppression was noted at most of the dose levels in this study, raising the possibility that modulation of the bone marrow might be occurring with CPT-11 pretreatment. However, no unexpected bone marrow toxicity was seen in the CPT-11/5-FU study even at the 80 mg/m^2 dose of CPT-11. The question of the effect of the increase in S phase on the toxicity and antitumor efficacy of the combination could not be answered in the present studies and will require randomized comparisons. The effect of CPT-11 on S phase and its relation to dose will also need to be evaluated further. The individual variability in the increase in this study was large and the discrepancy in the magnitude of the effect between the IHC data and the FC data was considerable. With the small numbers studied, a significant difference in S-phase increase could be demonstrated between the 40 and the 80 mg/m^2 of CPT-11 but not between the 60 and the 80 mg/m^2 doses. These areas will be explored in an ongoing phase I study of CPT-11 and capecitabine in breast carcinoma and in a planned phase II study of CPT-11 followed 24 h later by GEM in patients with non-small-cell lung carcinoma who have failed a platinum-containing regimen.

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