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CLINICAL TRIAL REPORT

A phase I/II trial of vorinostat in combination with 5-fluorouracil in patients with metastatic colorectal cancer who previously failed 5-FU-based chemotherapy

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Abstract
Purpose We conducted a phase I/II clinical trial to determine the safety and feasibility of combining vorinostat with 5-fluorouracil (5-FU) in patients with metastatic colorectal cancer (mCRC) and elevated intratumoral thymidylate synthase (TS).
Methods Patients with mCRC who had failed all standard therapeutic options were eligible. Intratumoral TS mRNA expression and peripheral blood mononuclear cell (PBMC) histone acetylation were measured before and after 6 consecutive days of vorinostat treatment at 400 mg PO daily. 5-FU/LV were given on days 6 and 7 and repeated every 2 weeks, along with continuous daily vorinostat. Dose escalation occurred in cohorts of three to six patients.
Results Ten patients were enrolled. Three dose levels were explored in the phase I portion of the study. Two dose-limiting toxicities (DLTs) were observed at the starting dose level, which resulted in dose de-escalation to levels −1 and −2. Given the occurrence of two DLTs at each of the dose levels, we were unable to establish a maximum tolerated dose (MTD). Two patients achieved significant disease stabilization for 4 and 6 months. Grade 3 and 4 toxicities included fatigue, thrombocytopenia and mucositis. Intratumoral TS downregulation ≥50% was observed in one patient only. Acetylation of histone 3 was observed in PBMCs following vorinostat treatment.
Conclusions The study failed to establish a MTD and was terminated. The presence of PBMC histone acetylation indicates biological activity of vorinostat, however, consistent reductions in intratumoral TS mRNA were not observed. Alternate vorinostat dose-scheduling may alleviate the toxicity and achieve optimal TS downregulation.

Keywords HDACi · Colon · 5-Fluorouracil · Thymidylate synthase · Phase I

Abbreviations

CRC	Colorectal cancer
HDACi	Histone deacetylase inhibitor
TS	Thymidylate synthase
5-FU	5-Fluorouracil
LV	Leucovorin
DLT	Dose-limiting toxicity
MTD	Maximum tolerated dose
PBMC	Peripheral blood mononuclear cell
CBC	Complete blood count

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Introduction

Chemotherapy-based approaches for the treatment of metastatic colorectal cancer (mCRC) have improved significantly in recent years. The fluoropyrimidine 5-fluorouracil (5-FU) remains the most effective chemotherapeutic agent in the treatment of colorectal cancer and forms the central component in the FOLFIRI regimen with the addition of irinotecan, and the FOLFOX regimen with the addition of oxaliplatin. These combination chemotherapies have resulted in improved response rates of ~50% and significant improvements in progression-free and overall survival [1, 2]. Agents targeting the epidermal growth factor receptor (cetuximab and panitumumab) and the vascular endothelial growth factor (bevacizumab) have expanded the therapeutic options and further contributed to improved progression-free survival [3–6]. However, despite recent advances in mCRC chemotherapy, effective disease control remains hindered by the high occurrence of drug resistance, subsequent treatment failure and patient mortality. As median overall survival for patients with mCRC has surpassed 20 months, many patients fail standard therapeutic options, while still maintaining an excellent performance status and adequate organ function. This has resulted in a critical need to identify and exploit novel therapeutic strategies in patients who have failed 5-FU-based standard of care chemotherapies.

5-FU is reported to induce cellular toxicity by inhibiting the enzyme thymidylate synthase (TS) and through the incorporation of fluoronucleotides into RNA and DNA leading to thymidylate depletion, cell cycle arrest and apoptosis [7]. A number of clinical studies, conducted by independent laboratories have demonstrated that elevated TS gene expression is associated with resistance to 5-FU-based therapy [8–10]. A 3,000 patient meta-analysis demonstrated that high intratumoral TS expression was significantly associated with decreased overall survival [11]. Despite advances in our understanding of the molecular factors that contribute to the cytotoxicity of 5-FU, ~50% of mCRC patients do not respond to current 5-FU-based chemotherapy. Therefore, novel strategies to improve response rates and overcome 5-FU resistance are of great clinical importance.

Histone deacetylase inhibitors (HDACi) have recently emerged as potent and selective anticancer agents. These agents demonstrate anticancer activities through the inhibition of histone deacetylases (HDACs) resulting in the hyper-acetylation of both histone and non-histone proteins. [12–14]. HDAC inhibition is reported to modulate between ~2 and 10% of genes, promote differentiation, inhibit cell cycle progression, induce apoptosis and suppress angiogenesis [15]. The hydroxamic acid-based HDACi vorinostat (Zolinza, Merck) [16, 17] is a broad-spectrum inhibitor of class I and II HDACs that has demonstrated potent cytotoxicity in vitro against a variety of solid tumor cell lines. Vorinostat is currently approved for the treatment of cutaneous T-cell lymphoma (CTCL) and is currently in clinical investigation in a wide variety of hematologic and solid malignancies [18].

A number of studies have reported that HDACi alter the expression of key drug targets and/or metabolic pathways that are critical molecular determinants for other cancer therapeutics. This has resulted in synergistic growth inhibitory effects on cancer cells in vitro and in vivo when HDACi are combined with additional agents targeting these modulated pathways [19–27]. DNA microarray profiling initially identified the downregulation of the 5-FU target enzyme TS following treatment with multiple HDACi in bladder and breast cancer cell lines [28]. This observation was also observed in a heterogeneous panel of colon cancer cells [27, 29, 30]. Furthermore, it is reported that HDACi synergize with 5-FU in vitro and in vivo in colon cancer cell line models through HDACi-induced downregulation of TS, providing a mechanistic basis for the drug synergy [22, 27].

In light of the substantial pre-clinical data supporting the combination of HDACi and fluoropyrimidines, we conducted a phase I/II clinical trial to determine the safety and feasibility of combining vorinostat with infusional 5-FU in the treatment of mCRC patients who have progressed on standard 5-FU regimens. In addition, we sought to determine the objective response rate when vorinostat is administered in combination with 5-FU/LV and the efficacy of vorinostat alone to produce consistent decreases in intratumoral TS expression.

Materials and methods

This phase I/II dose escalation study of vorinostat in combination with infusional 5-FU was conducted at the University of Southern California Norris Comprehensive Cancer Center (Los Angeles, CA). The primary objective of the phase I study was to determine the safety and feasibility of combining daily oral vorinostat with infusional 5-FU chemotherapy in the treatment of stage IV colorectal cancer patients who have progressed on standard 5-FU regimens. In addition, the efficacy of vorinostat treatment alone to induce consistent decreases in intratumoral TS expression was examined. The phase II primary objective was to determine the objective response rate in patients treated with daily oral vorinostat combined with infusional 5-FU/LV. Secondary objectives of this clinical trial included determining the time to tumor progression, progression free and overall survival of patients with mCRC treated with vorinostat in combination with infusional 5-FU and

the determination of any link between TS repression by vorinostat and response to the combination treatment.

Patient criteria

Patients with histologically or cytologically confirmed advanced mCRC who had failed treatment with 5-FU, oxaliplatin and irinotecan are eligible for the study. Prior treatment with bevacizumab and/or cetuximab/panitumumab is allowed. Patients must have measurable disease, defined as at least one lesion that can be accurately measured in at least one dimension (longest diameter to be recorded) as >20 mm with conventional techniques or as >10 mm with spiral CT scan. Tumor must be accessible for core biopsy at the beginning of treatment and demonstrate a high intratumoral TS expression level (TS/β-actin $> 4.1 \times 10^{-3}$) as previously reported [31] prior to the beginning of treatment. Patients have to be ≥18 years of age, have an adequate Karnofsky performance status (>50%) and a life expectancy of >12 weeks. In addition, patients must have acceptable organ function as defined by: leukocytes > 3,000/μl, absolute neutrophil count > 1,500/μl, platelets > 100,000/μl, total bilirubin within 1.5 × normal institutional limits, aspartate aminotransferase/alanine aminotransferase ≤2.5 × institutional upper limit of normal (ULN), creatinine ≤ 1.5 × institutional ULN. Patients could not have received any systemic therapy within 4 weeks from initiation of study treatment. Patients with known brain metastases, uncontrolled intercurrent illness or HIV-positive patients receiving combination anti-retroviral therapy were excluded from the study because of possible pharmacokinetic interactions with vorinostat. All consenting patients with the potential to conceive agreed to the use of adequate contraception. The study and consent forms were approved by the institutional review board before the study was initiated. All patients provided signed informed consent before study entry.

Study design and treatment plan

Study design and statistical considerations

There were two stages in the study. The phase I portion of the study was designed to identify the maximum tolerated dose (MTD) and determine whether vorinostat resulted in TS expression downregulation. The standard 3 + 3 design was utilized for the phase I portion of this study. Once the MTD was identified, accrual was to continue at the MTD until a total of 20 patients have received vorinostat for 6 days and undergone a second biopsy on day 6. If 8 or more patients out of 20 experienced a 50% decrease in the inratumoral TS expression, this would be considered as evidence that vorinostat at the proposed dose is promising in terms of decreasing the TS expression. If the true chance of a 50% reduction in TS expression is 50%, then there is a 0.13 probability that we will observe 7 or fewer patients with a 50% decrease; and if the true chance of a 50% reduction is 25% (30%), then there is a 0.90 (0.77) probability that we will observe 7 or fewer patients with a 50% decrease.

Upon termination of the phase I portion of the study, progress to the phase II portion was conditional upon the identification of a MTD and the presence of a ≥50% decrease in TS expression levels in ≥50% of the patients (Fig. 1). A two-stage Simon optimal design was used to plan the phase II portion of the study. If none of the first 26 patients, who have been treated at the MTD, have experienced an objective response (complete or partial by RECIST criteria), then the study would be terminated with conclusion that the true overall response rate was less than 10%. If at least one objective response was observed, then accrual would continue until 59 patients have been treated. Three or more objective responses would be taken as evidence that this regimen has some activity in these patients. If the true response rate was 2%, there was a 10% chance (alpha or probability of a type I error) that we would conclude that the response rate was 10% or better; if the true response rate was 10%, there was a 90% chance (power or one minus the probability of a type II error) that we would conclude that the response rate was 10% or better.

Treatment plan

In the phase I portion of the study, all patients were screened by undergoing a CT-guided core biopsy, and the tumor specimen was analyzed for TS mRNA expression. Patients were eligible for treatment if they had high intratumoral TS expression level (TS/β-actin $> 4.1 \times 10^{-3}$) as defined in the inclusion criteria. Patients then received vorinostat alone at the dose of 400 mg daily for 6 days (days −6 to −1). A second CT-guided core biopsy was obtained after 6 days of treatment with vorinostat alone (day −1). The next day (day 1), patients continued vorinostat orally at the dose of 400 mg QD. In addition, patients received LV at 400 mg/m^2 over 2 h and 5-FU bolus 300 mg/m^2, followed by 5-FU continuous i.v. infusion at 1,800 mg/m^2 over 46 h on days 1 and 15 of each 28-day cycle dose level 1). All dose levels are given in Table 1.

Clinical evaluation and follow-up

A complete medical history, physical examination, complete blood count (CBC) and chemistry profile were

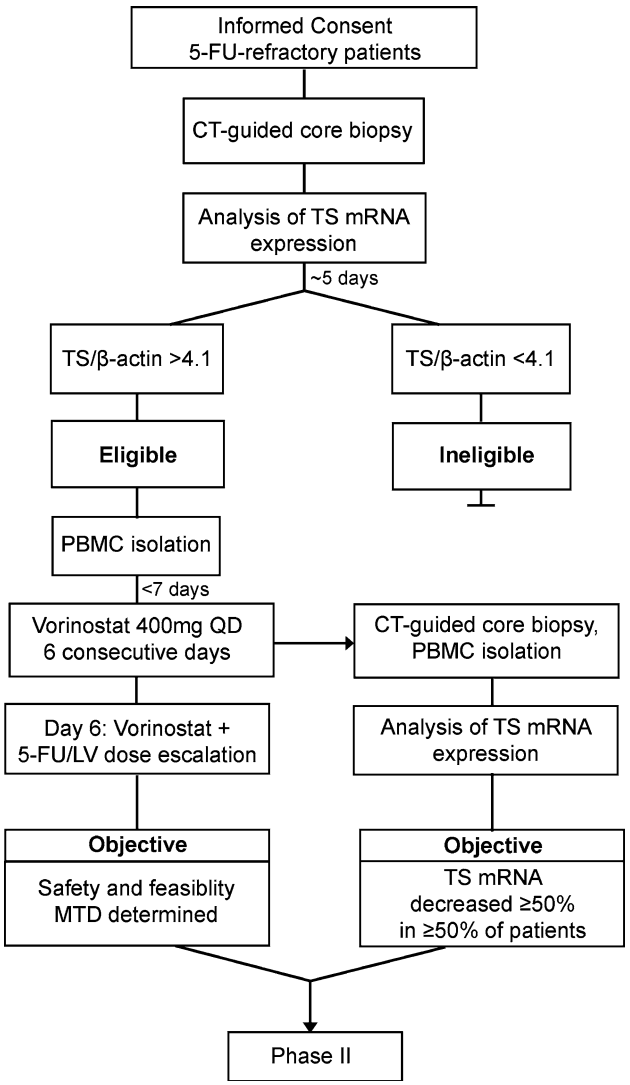

Fig. 1 Simplified schematic outlining the phase I/II clinical trial design and stages. Briefly, patients who have previously failed 5-FU chemotherapies will provide informed consent and meet inclusion criteria outlined in "Patient criteria". Within 7 days, the eligible patient will have a CT-core guided biopsy and real-time RT-PCR analysis of TS mRNA expression will be performed. Patients with TS mRNA expression above the > 4.1 TS/β-actin ratio cut-off are deemed eligible. Patients will then have research blood drawn for PBMC isolation and histone acetylation analysis prior to initiation of vorinostat treatment. Patients will then receive vorinostat at 400 mg QD for 6 consecutive days at the conclusion of which the CT-guided core biopsy will be repeated and analyzed for TS mRNA expression and research blood drawn for histone acetylation analysis of PBMCs to determine vorinostat biological activity. 5-FU/LV will then be administered (day 6 and 7, repeated every 2 weeks) and vorinostat treatment continued at 400 mg QD at the starting dose level 1 (Table 1). Upon determination of the MTD and the conclusion that vorinostat at the proposed dose causes a $\geq$50% reduction in intratumoral TS mRNA expression, the trial will proceed to phase II as described in the "Study design and statistical considerations"

obtained within 1 week of treatment initiation. Baseline computed tomographic (CT) scans were obtained within 4 weeks before commencing treatment. CBC and comprehensive chemistry profile were repeated on a weekly basis. Medical history, physical examination and toxicity assessment as per National Cancer Institute Common Toxicity Criteria 3.0 were done weekly during the first cycle and every cycle thereafter. CT scans were repeated every 4 cycles (8 week) to assess response. Responses were categorized according to RECIST 1.0.

Dose-limiting toxicities

A dose-limiting toxicity was defined as any of the following conditions attributable to study treatment in cycle 1: grade 4 neutropenia or grade 3 neutropenia with fever, grade 4 thrombocytopenia, any grade 4 non-hematologic toxicity or any grade 3 non-hematologic toxicity with the following exceptions: grade 3 dehydration, diarrhea, or nausea and vomiting, in the absence of adequate therapy, or grade 3 electrolyte changes that respond to supplementation. Any toxicity resulting in a delay of treatment lasting longer than 3 weeks was considered a DLT.

Dose modifications

When a patient experienced $\geq$grade 3 non-hematologic toxicity ($\geq$grade 2 for diarrhea) or $\geq$grade 3 neutropenia or thrombocytopenia, vorinostat and 5-FU/LV were withheld for up to 3 weeks until the toxicity was resolved to $\leq$grade 1 at which point vorinostat and 5-FU were restarted at the next reduced dose level. If grade 3 toxicity did not resolve to $\leq$grade 1 within 3 weeks, the patient was removed from the study. If a patient again experienced $\geq$grade 3 toxicity, treatment was withheld for up to 3 weeks until the toxicity resolved to $\leq$grade 1 and vorinostat and 5-FU/LV were restarted at two dose levels below the original starting dose. If $\geq$grade 3 toxicity recurred for the third time, the patient was removed from the study**.** Patients requiring dose modifications below the lowest dose level were taken off study.

Pharmacodynamic analyses

Tumor biopsies

Pre-treatment and on-treatment tumor samples were collected from patients with metastases accessible to CT-guided biopsies. The same target lesion was biopsied before and during vorinostat treatment. On-treatment samples were collected 2–6 h following the previous dose of vorinostat. Tumor biopsy samples were immediately subject to formalin fixation and paraffin embedding after the procedure in preparation for subsequent gene expression analysis.

Table 1 Dose levels of vorinostat and 5-FU/LV

Dose level	Vorinostat	LV (mg/m^2)[a]	5-FU bolus (mg/m^2)	5-FU infusion (mg/m^2)[b]
2	400 mg PO QD 14 days	400	400	2,400
1	400 mg PO QD 14 days	400	300	1,800
−1	400 mg PO QD 7 days, every 2 weeks	400	300	1,800
−2	400 mg PO QD 5 days, every 2 weeks	400	225	1,350

5-FU/LV repeated every 2 weeks. PO QD; orally, once daily

[a] LV i.v. over 2 h

[b] 5-FU infusion over 46 h

Laser capture microdissection

The biopsies and tumor specimens were delivered to Response Genetics, Inc. for gene expression analysis. A pathologist reviewed paraffin-embedded tumor blocks for quality and tumor content. Ten sections—one micrometer thick—were obtained from the identified areas with the highest tumor concentration. Sections were mounted on uncoated glass slides. For histological diagnosis, three sections representative of the beginning, the middle and the end of the tissue were stained with H&E using the standard method. Slides were prepared as previously described and laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed in all the tumor samples to ensure that only tumor cells were dissected [32, 33].

RNA isolation and cDNA synthesis

RNA isolation and cDNA synthesis was performed using the method developed by Dr. Danenberg at USC (US Patent #6248535) as previously described [34, 35]. The dissected particles of tissue were transferred to a reaction tube containing 400 µl of RNA lysis buffer containing 4 M guanidine isothiocyanate with 0.5% sarcosine and dithiothreitol, homogenized and heated to 95°C for 30 min. The sample was then extracted with 2 M sodium acetate solution, pH 4.0 and phenol/chloroform/isoamyl alcohol solution (10:1.93:0.036). After centrifugation, the RNA in the supernatant was precipitated, washed in 70% ethanol, dried briefly and finally resuspended in 5 mM Tris chloride (pH = 8). RNA-containing solution was then reverse transcribed using random hexamers.

Real-time PCR analysis of intratumoral TS expression

TS mRNA copy numbers and the internal reference gene β-actin were quantified using TaqMan® real-time PCR on board an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). A usable range of TaqMan® cycle thresholds (C_Ts) were established by processing dilutions of identical specimens multiple times on multiple days. A series of control RNAs were purchased from Stratagene to use as PCR plate calibrators. The mean relative TS expression in the standards was determined ($n = 20$) and included on every plate. The PCR mixture consisted of 1200 nM of each primer, a 200 nM probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nM of dATP, dCTP, dGTP, dTTP; 3.5 mM $MgCl_2$ and 1× Taqman Buffer A containing a reference dye added to a final volume of 20 µl (all reagents from PE Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. TS gene expression values are expressed as ratios (differences between the C_T values) between TS and β-actin that provides a normalization factor for the amount of RNA isolated from a specimen. All patient specimens were measured in triplicate and an average value determined for the ratio of TS to β-actin. The statistical significance of changes in TS expression was assessed using the Wilcoxon exact signed ranks test.

Sample collection for analysis of histone acetylation

Pretreatment and on-treatment whole blood samples were collected to analyze the effects of vorinostat on histone acetylation in peripheral blood mononuclear cells (PBMCs). At the specified time point, an 8 cc whole blood sample was collected and deposited in Ficoll-Plaque (Pharmacia, Peapack, NJ) collection tubes and PBMCs isolated according to the manufacturers guidelines. PBMCs were subsequently washed in ice-cold PBS, centrifuged for 15 min at ×300*g* and the supernatant aspirated. The cell pellet obtained was frozen at −80°C until processed for Western blot analysis.

Antibodies and Western Blotting

Total cellular protein was isolated from PBMC pellets and analyzed by standard Western blotting technique as described previously [36, 37]. Western blots were probed overnight at 4°C to detect acetylated histone 3 (Ac-H3)

Table 2 Baseline patient characteristics $N = 10$

	N
Age (year)	
Median (range)	60 (40–73)
Sex	
Male/female	4/6
Race	
White/Asian/Hispanic	8/1/1
N of previous treatments for mCRC	
Median (range)	5 (3–8)
Karnofsky performance status (%)	
90	6
80	4
Prior chemotherapy[a]	
Capecitabine	7
Gemcitabine	5
Panitumumab	2
Prior radiation therapy	2

[a] In addition to 5-FU, oxaliplatin, irinotecan, cetuximab and bevacizumab

protein using anti-acetyl-H3 rabbit monoclonal antibody (Millipore, Billerica, MA) and 2 h at room temperature with goat-anti-rabbit HRP. Blots were re-probed for monoclonal anti-β-tubulin (1:4,000; Sigma, St. Louis, MO) to control for loading. HRP signal was detected using HyGlo and Hyblot film (Denville Scientific, Metuchen, NJ) and developed on a Hope-Micromax film processor (Hope X-Ray, Warminster, PA). Western bands were quantified using Scion Image Software (Scion Corporation, Frederick, MD). Relative fold-increase in Ac-H3 expression was determined by calibrating the pixel intensity for the Ac-H3 bands versus their respective β-tubulin bands from a representative Western blot. The ratio of the post-vorinostat Ac-H3 signal was then directly compared to pre-vorinostat control Ac-H3 signal for each patient and presented as fold-change in histogram format.

Results

Patient characteristics, treatment administration and toxicities

Patient characteristics

Ten patients with mCRC were enrolled into the phase I portion of the study (Table 2). Nine out of the 10 patients were evaluable for DLT. The median age was 60 (40–73), and the median number of prior chemotherapy regimens was 5 (3–8). All patients had previously failed 5-FU, oxaliplatin, irinotecan, bevacizumab and cetuximab or panitumumab. In addition, 3 patients had received gemcitabine and other experimental treatment on a phase I study.

Treatment administration

All patients received the 6 days of vorinostat alone prior to the second biopsy. All patients then proceeded to receive the combination of 5-FU/LV and vorinostat. The median number of cycles was 1.5 (range 1–6). Ten patients received a total of 21 cycles of vorinostat/5-FU. The median number of treatment cycles received by patients who experienced stable disease was 5 (range 4–6).

Dose-limiting toxicities and maximum tolerated dose

Three dose levels were explored in the phase I portion of the study. At starting dose level 1, two of the 3 patients experienced DLTs consisting of grade 3 fatigue and grade 4 thrombocytopenia (Table 3). Five patients were accrued to dose level −1, but one was inevaluable for DLT due to not receiving 80% of the planned dose of vorinostat. Two of the 4 evaluable patients experienced DLT consisting of grade 3 and 4 fatigue. Two patients were then accrued to dose level −2, both of whom experienced DLT consisting of grade 3 mucositis and grade 4 thrombocytopenia (Table 3). The MTD could not be determined due to the

Table 3 Adverse events

Patient	Dose level	Cycles received	DLT yes/no	Description of DLT
1	1	2	Yes	Grade 3 fatigue
2	1	2	No	N/A
3	1	1	Yes	Grade 4 thrombocytopenia
4	−1	6	No	N/A
5	−1	1	Yes	Grade 3 fatigue
6	−1	4	No	N/A
7	−1	1	Yes	Grade 3 fatigue
8	−1	2	I.E	I.E
9	−2	1	Yes	Grade 4 thrombocytopenia
10	−2	1	Yes	Grade 3 mucositis

I.E inevaluable, *N/A* not applicable

presence of 2 DLTs at the lowest planned dose (dose level −2).

Other toxicities

Grade 3 or 4 hematologic toxicities were limited to the grade 4 thrombocytopenia noted in 2 patients. In addition to the dose-limiting fatigue and mucositis noted earlier, cycle 1 non-hematologic toxicities included grade 3 anorexia, nausea, vomiting, dizziness, hypophosphatemia and dehydration, which occurred in one patient each at the rate of 10%. There were no grade 3 or 4 toxicities in cycle 2 or beyond (Table 3).

Antitumor activity

Given the inability to identify a MTD and the absence of TS expression downregulation in 50% of the patients, the study was terminated after the phase I portion. Five of the 10 patients completed ≥4 cycles (8 weeks) of treatment and were therefore evaluable for response. There were no objective responses. Two patients had stable disease lasting for 6 and 4 months at dose level −1, respectively. One of these patients had resolution of grade 2 abdominal pain and fatigue while on treatment. All other evaluable patients developed progressive disease within 2 months of study initiation.

Pharmacodynamics

Intratumoral TS expression by real-time RT-PCR

Following six consecutive days of vorinostat treatment, four of ten patients demonstrated reductions in TS mRNA relative to β-actin expression as measured in their pre- and post-treatment biopsies (Table 4). Reductions in TS mRNA ranged from −19.5 and −17.3% up to −40.5 and −51.7%. In the remaining 5 patients, TS expression increased between 7.9 and 97.3% (Table 4). No relationship was found between change in TS mRNA expression and TS mRNA expression level prior to vorinostat treatment. The median change in TS expression after six consecutive days of vorinostat treatment (400 mg QD) was +3.1% (range: −51.7 + 97.3) and only one patient achieved the target of a 50% reduction in TS expression. According to the study design, a Wilcoxon signed rank test determined that there was no association between vorinostat treatment and TS down-regulation or between changes in TS expression and clinical outcome.

Analysis of histone acetylation in PBMCs

Peripheral blood samples were analyzed for evidence of histone acetylation in PBMCs to determine whether a once-daily 400-mg vorinostat dose had measurable biological activity in these patients. PBMCs were isolated from whole blood specimens sampled prior to the first dose of vorinostat treatment and on the sixth consecutive day within 6 h of the final vorinostat dose. From ten patients, seven of these matched samples contained material of sufficient quality for reproducible comparative Western blot analysis. A significant increase (≥twofold) in the accumulation of acetylated histone 3 was observed in 6 of the 7 patients following Western blot analysis and subsequent densitometric normalization (Fig. 2). These data would indicate that a 400-mg once-daily dose of vorinostat resulted in measurable biological activity.

Discussion

In this phase I clinical trial, we evaluated the combination of the histone deacetylase inhibitor vorinostat with 5-FU/LV in patients with advanced colorectal cancer, who had failed standard therapies. The rationale for this drug combination stems from a substantial body of data in preclinical models, indicating that the combination of HDACi and the fluoropyrimidines interact synergistically to inhibit

Table 4 TS mRNA expression pre- and post-vorinostat

Patient	Pre[a]	Post[a]	Change (post–pre)	% Change	Up- or down-regulated
1	6.4	7.51	1.11	17.3	↑
2	**10.33**	**4.99**	**−5.34**	**−51.7**	↓
3	5.12	10.1	4.98	97.3	↑
4	5.89	7.54	1.65	28.0	↑
5	16.46	21.56	5.1	31.0	↑
6	4.7	5.07	0.37	7.9	↑
7	9.28	9.12	−0.16	−1.7	↓
8	6.52	5.25	−1.27	−19.5	↓
9	6.84	4.07	−2.77	−40.5	↓
10	7.33	6.04	−1.29	−17.6	↓

[a] TS mRNA expression relative to β-actin

Bold values denote ≤50% reduction in relative TS mRNA

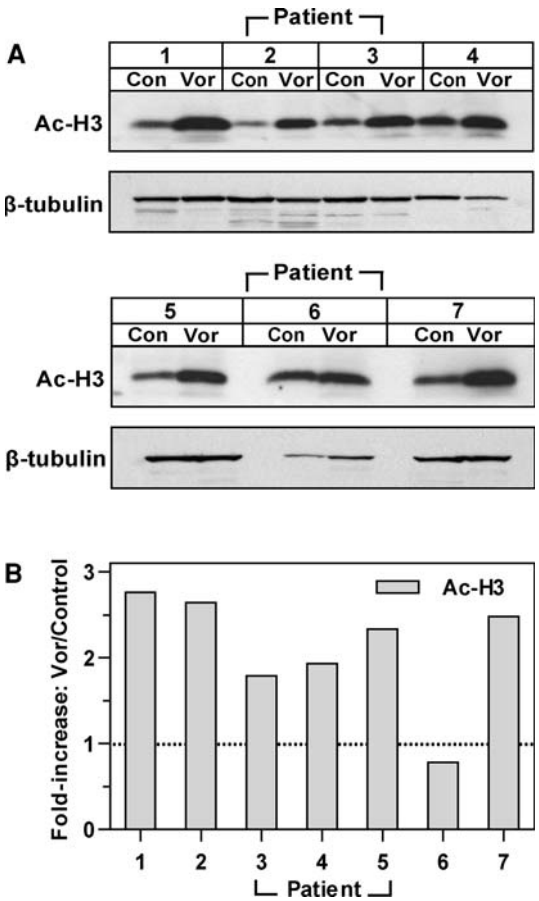

Fig. 2 **a** Western blot analysis of acetyl-H3 (*Ac-H3*) and *β*-tubulin expression. Proteins were extracted from peripheral blood mononuclear cells (*PBMCs*) isolated from matched whole blood samples obtained prior to (control) and after 6 consecutive days of vorinostat (*Vor*) treatment at 400 mg QD in seven patients. Proteins were subject to SDS–PAGE, transferred to PVDF membrane and probed using monoclonal anti-Ac-H3 and anti-*β*-tubulin as outlined in "Materials and methods". **b** Histogram illustrating the fold increase in Ac-H3 in PBMCs post-vorinostat treatment when compared to pre-treatment following densitometric analysis and normalization as described in "Materials and methods"

proliferation and induce apoptosis in vitro and in vivo in colon cancer models. The molecular basis of this proposed synergy is through the HDACi-induced downregulation of TS at the mRNA and protein levels [21, 22, 27]. Therefore, the hypothesis was that vorinostat would reverse 5-FU resistance through the downregulation of TS mRNA in patients whose tumors harbor elevated TS expression, thereby re-sensitizing them to 5-FU.

The combination of 5-FU, LV and vorinostat was poorly tolerated, resulting in the need to de-escalate the doses of the combination twice. Given the inability to establish a MTD and the absence of TS downregulation in 50% of patients, the study was terminated without proceeding to the phase II portion. Even though the toxicities observed, including the DLTs of fatigue and thrombocytopenia, are consistent with those previously reported for vorinostat (34, 35), the poor tolerability noted in our study may be due to the continuous dosing schedule of vorinostat at the starting dose level. This is consistent with another study that evaluated the same combination of 5-FU and vorinostat and which determined the MTD to be 1,700 mg of vorinostat for 3 days on an every 2 week cycle [38]. However, since the regimen was still difficult to administer at dose level −2, other factors are likely to have contributed to the overall toxicity in our study, such as the fact that all patients were heavily pre-treated.

Despite a substantial body of compelling pre-clinical in vitro data with HDACi and 5-FU combinations, no objective responses were observed in this trial. However, 2 patients, neither of which demonstrated any vorinostat-induced downregulation of TS mRNA, had stable disease of 4 and 6 months. While it is not possible to positively attribute the observed disease stabilization to the addition of vorinostat to 5-FU/LV, it is worth noting that the clinical benefit of 5-FU alone in 5-FU-refractory colon cancer patients is negligible [39]. Furthermore, as dosing was de-escalated to dose level −2, with significantly reduced dosing of 5-FU/LV, the biological activity in these patients may have been inadvertently diminished. Of note, Fakih et al. [38] have reported anti-tumor activity with this combination administered on a different schedule than ours. In 21 CRC patients, 11 patients developed SD and 1 patient experienced a PR. The preliminary evidence of anti-tumor activity reported in both studies is worth noting, and the challenge lies in determining the mechanism by which the addition of a HDACi, such as vorinostat, to 5-FU results in tumor stabilization.

One of the objectives of this clinical trial was to determine whether vorinostat could induce intratumoral downregulation of TS mRNA in patients with elevated TS expression. While several patients did have reductions in their TS mRNA expression, the median overall change was +3.1%, and it was concluded that the evaluated dose of vorinostat was not sufficient to induce a 50% downregulation. A recent clinical trial utilizing a twice-daily 300-mg vorinostat schedule reported that peak serum concentrations did not exceed 2 μmol/l and no significant downregulation in TS expression was observed by both IHC and RT-PCR [40]. While pharmacokinetic analyses were not performed in our clinical trial, it is plausible that serum concentrations did not peak high enough with a once-daily 400-mg vorinostat schedule or persist long enough to induce effective intratumoral TS mRNA downregulation. Most in vitro analyses utilized a continuous vorinostat exposure to demonstrate downregulation of TS expression at the mRNA and protein level [21, 22, 27]. This fails to take into consideration the effects of vorinostat half-life and rapid systemic clearance. The 30- to 90- min half-life

($T_{1/2}$) of vorinostat results in a rapid decrease in serum concentrations, particularly with once-daily administrations [40–42]. In addition, the effects of HDACi on pan-acetylation are rapidly reversed as cells re-establish acetylation homeostasis [43]. A modified vorinostat schedule designed to attain higher serum peak concentrations or to maintain adequate serum concentrations for longer tumor cell exposure may be more effective at downregulating intratumoral TS expression. The clinical activity of the combination of 5-FU and vorinostat at 1,700 mg/daily for 3 days on an every 2 week schedule as reported by Fakih et al. lends support to the notion that higher doses of vorinostat may be needed to achieve sufficient and prolonged serum concentrations. It is plausible that a threshold exists whereby a higher serum concentration and longer duration of tumor cell exposure to HDACi are needed to induce the downregulation of TS mRNA. However, the presence of detectable histone acetylation in six out of seven patients in our study indicates that vorinostat did induce biological activity in vivo. It is also possible that the clinical benefit observed with vorinostat and 5-FU is independent of any effects on TS expression and may include modulation of other 5-FU-metabolizing enzymes.

As an alternate approach to the dosing schedule manipulation, novel HDACi that are currently in development are demonstrating increased potency in pre-clinical analyses [27, 44] and more favorable pharmacokinetic properties in the clinic [45] and may therefore be more effective at downregulating intratumoral TS mRNA expression and re-sensitizing patients to 5-FU-based therapy.

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