A Phase I Study of 5-Fluorouracil/Leucovorin and Arsenic Trioxide for Patients with Refractory/Relapsed Colorectal Carcinoma

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Abstract

Purpose: This Phase I study was designed to determine a safe combination dose of 5-fluorouracil (5-FU) and arsenic trioxide (ATO) to treat 5-FU–resistant relapsed/refractory colorectal cancer patients. We studied the effect of ATO in the downregulation of thymidylate synthase (TS) in peripheral blood mononuclear cells and in tumor biopsies.

Experimental Design: ATO was administered for 5 consecutive days during the first week and twice during weeks 2 to 3 and once on week 4. 5-FU/leucovorin (LV) was administered on days 8, 15, and 22. A modified accelerated titration design was used. 5-FU was dose escalated first followed by a planned dose increase for ATO.

Results: No dose-limiting toxicities were seen in seven patients who received 0.15 mg/kg ATO; grade 3 toxicities were as follows: neutropenia 1, diarrhea 1, and bowel obstruction 1. In patients receiving 0.20 mg/kg ATO, grade 3 toxicities were QTc prolongation 1, fatigue 4, alkaline phosphatase elevation 2, diarrhea 2, and peripheral edema 1. TS gene expression in peripheral blood mononuclear cell decreased in all patients. Eight tumors were biopsied, four showed TS downregulation, three showed upregulations, and one did not change. Estimated median progression-free survival and overall survival were 3.1 and 13.9 months, respectively. In patients who showed TS increase or no change versus TS reduction, estimated median progression-free survival was 2.6 versus 7.9 months (P = 0.188) and overall survival was 8.6 versus 11.7 months (P = 0.44), respectively.

Conclusions: Thus, we determined 0.20 mg/kg ATO, 2,600 mg/m² 5-FU, and 500 mg/m² leucovorin (LV) to be the recommended phase II dose. Clin Cancer Res; 16(11); 3019–27. ©2010 AACR.

For the last 40 years, 5-fluorouracil (5-FU) has been the most active chemotherapeutic agent for colorectal cancer (CRC). The reported response rate to single-agent 5-FU ranges from 10% to 30% (1). Yet, the median survival of patients with advanced CRC has remained ∼12 months (2). In the past decade, new agents, oxaliplatin, irinotecan (CPT-11), bevacizumab, panitumumab, and cetuximab, have been introduced into the clinical practice. Irinotecan as a single agent has shown a 10% response rate in patients with CRC (1, 3). However, the combination of Irinotecan, Fluorouracil, and leucovorin increased the response rate to ∼40% and the median survival to 14.8 months (4). FOLFIRI has shown an increase in median survival to ∼20 months. Similarly, oxaliplatin as a FOLFOX regimen resulted in an increase in median survival to 19.5 months (5). Thus, it is only when these agents are combined with 5-FU that significant improvements in response rates and median survival are seen. In the past 2 years, no new agents have been approved in the treatment of CRC. Therefore, it seems likely that the development of 5-FU resistance in CRC cells will have substantial and adverse effects on these promising combination regimens. A major mechanism of 5-FU resistance in CRC is overexpression of the dTMP-synthesizing enzyme thymidylate synthase (TS). Therefore, strategies to avoid cellular resistance to fluoropyrimidines may create new treatment options for 12,000 to 18,000 patients annually. We have found in vitro that low concentrations of arsenic trioxide (As2O3; ATO) effectively inhibited TS protein and gene expression, and reversed 5-FU resistance in CRC cell lines

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Translational Relevance

This is a phase I study designed to determine a safe combination dose of 5-fluorouracil (5-FU) and arsenic trioxide (ATO) to treat 5-FU-resistant relapsed/refractory colorectal cancer patients (CRC). Overexpression of the enzyme thymidylate synthase (TS) is correlated to the failure of 5-FU-based therapy. We hypothesize that the ATO downregulation of TS expression will lead to the re-sensitization of refractory CRC to 5-FU. We determined 0.20 mg/kg ATO, 2,600 mg/m² 5-FU, and 500 mg/m² leucovorin to be the recommended phase II dose. Manageable toxic side effects were seen in patients with metastatic CRC receiving the above regimen. There is a significant decrease in TS gene expression in correlative studies of peripheral blood mononuclear cells of all patients. Furthermore, four of eight tumor biopsies showed downregulation of TS gene expression. Successful outcome of this study will lead to a new treatment option for 5-FU-resistant relapsed/refractory colorectal cancer patients.

Patients and Methods

Eligibility criteria

Patients 18 years and older with histologically confirmed stage IV (TanyNanyM1), advanced, refractory, metastatic CRC were eligible. CRC patients with k-ras wild-type who initially responded and then failed at least three different standard chemotherapy regimens were considered refractory. In patients with mutated k-ras, failure to respond to at least two different standard chemotherapy regimens was considered refractory. In our institution, traditionally, patients diagnosed with advanced CRC received Avastin and FOLFIRI. Avastin and FOLFOX combination was used as second line therapy. When both the regimens proved ineffective, patients were treated with Erbitux and CPT-11. ATO and 5-FU combination therapy was offered only to patients who failed at least all three or two regimens, respectively. Other eligibility criteria included disease progression, bidimensionally measurable disease, biopsiable tumor, life expectancy of ≥2 months, and accessibility for the placement of a central venous catheter (port-a-cath) for the administration of chemotherapy and patient consent to undergo two separate biopsies for the determination of TS tumor signatures. Patients required an Eastern Cooperative Oncology Group performance status of 0 to 2, WBC of ≥3,000 cells/mm³, and platelet count of ≥100,000 cells/mm³. Patients must have shown adequate renal function as documented by serum creatinine ≤1.5 × ULN, bilirubin ≤1.5 mg/dl, and aspartate aminotransferase and alkaline phosphatase level ≤5 times the normal limit.

Patients receiving an investigational drug within 4 weeks before the start of study were ineligible. Metastatic disease to the central nervous system, history of prior neurologic disorders (grade 3 or higher by the National Cancer Institute (NCI) Common Toxicity Criteria, in particular seizure disorders), and/or peripheral neuropathy of ≥grade 2 also excluded patients from study participation. Patients who were of childbearing age and refused pregnancy testing, or were pregnant, were not able to participate in this study. All patients gave informed consent before undergoing protocol-specific evaluations and treatment.

Treatment plan and study design

One treatment cycle spanned 5 weeks, which included 4 weeks of treatment followed by 1 week of rest. A fixed dose of ATO was administered on days 1 to 5, 8, 11, 15, 18, and 22 through i.v. infusion over 1 to 4 hours. A loading dose was administered on days 1 to 5 to downregulate TS expression levels. After the first week, the administration of ATO twice weekly was sufficient to maintain TS downregulation. The assigned dose of 5-FU and 500 mg/m² of leucovorin were administered over 24 hours by i.v. infusion on days 8, 15, and 22 following ATO administration. At the discretion of the principal investigator, patients were given subsequent cycles of treatment provided they had recovered from toxicity and established evidence of treatment benefit.

Dose escalation

Dose escalation was conducted for the two main drugs: 5-FU and ATO. Dose escalation was based on the occurrence of dose-limiting toxicity (DLT) during the first-treatment cycle (5 wk, including the 1 wk of rest). DLT was defined as grade 3 or higher nonhematologic toxicity, or grade 4 hematologic toxicity as per NCI Common Terminology Criteria for Adverse Events v3. Initially, ATO was administered at a fixed dose of 0.15 mg/kg, whereas 5-FU was escalated. The doses of 5-FU tested were 1,600, 2,000, 2,300, and 2,600 mg/m². Dose escalation began with an accelerated phase; each cohort consisted of one patient per dose level. In the absence of DLT in the first treatment cycle, the starting dose of 5-FU for each subsequent cohort was one dose level higher than that used for the previously treated patient. Therefore, enrollment of patients was spaced at least 5 weeks apart, allowing for the evaluation of first-cycle dose-limiting toxicities. The accelerated phase of 5-FU was concluded at 2,600 mg/m², which was earlier reported by us to be the recommended phase II dose for
5-FU (8). No patient experienced any DLT at 0.15 mg/kg ATO in combination with an escalated dose of 5-FU. We then proceeded with the escalation of ATO to 0.20 mg/kg in combination with 5-FU at 2,600 mg/m².

**Patient evaluation and follow-up**

All patients underwent prestudy evaluations that included medical history, a serum-human chorionic gonadotropin pregnancy test for women of childbearing age, a physical examination with Eastern Cooperative Oncology Group performance status score, hematology and chemistry profiles, a urinalysis and a carcinoembryonic antigen measurement. A cardiac profile (electrocardiogram) and initial imaging/diagnostic studies with corresponding tumor assessment were done within 4 weeks before initiating therapy.

Evaluations were done at different intervals throughout the study. Electrocardiogram, history, physical, and hematology and biochemistry parameters were obtained before therapy on days 1, 8, 15, and 22, as well as when clinically indicated. Plasma arsenic levels were drawn on preinfusion days 1, 5, 8, 11, 15, 18, and 22. Carcinoembryonic antigen measurements were obtained on week 4 of every cycle and as clinically warranted. Imaging studies and tumor response were assessed after the completion of every two cycles. Response and progression were evaluated using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee (9). Those patients who achieved complete, partial, or stable disease had confirmatory tumor assessment 4 weeks postresponse. Posttreatment evaluations namely history, physical exam, hematology and biochemistry profiles, carcinoembryonic antigen measurement, imaging and diagnostic studies, and tumor response assessments were carried out on all patients no longer receiving therapy. These were carried out at the end of months 3, 6, and 12 and then annually.

**Pharmacokinetics and pharmacodynamics**

The goal of the correlative studies was to determine the effect of ATO on TS expression in tumor and in PBMCs, on serum plasma arsenic levels, and the relationship between these biological markers. TS and β-actin (internal control) expressions in PBMC were obtained before treatment start (baseline) and on days 1, 5, 8, 11, 15, 18, and 22. Fine needle aspirates of liver or lung metastases were obtained within 1 week before the start of treatment and on day 23 of cycle one. All fine needle aspirates were confirmed for malignant cells before further processing.

Peripheral blood samples (PAXgene Blood RNA tube, Qiagen) were obtained preinfusion on day 1 and 1 hour post-ATO infusion on days 1, 5, 8, 11, 15, 18 and 22. RNA isolation, processing, and gene expression analysis were as detailed under Subbarayan et al. (7).

It is possible that concurrent 5-FU administration may alter ATO pharmacokinetics. The plasma elemental arsenic levels were monitored (Nichols Laboratory) 1 hour preinfusion on day 1 and 1 hour post-ATO administration on days 1, 5, 8, 11, 15, 18, and 22, in royal blue top tubes. Pharmacokinetic analysis was done using PK Solutions v2.0 software. Arsenic parameters were correlated with TS levels, disease response, and recurring toxicities.

**Statistical analysis**

Patient characteristics were summarized using descriptive statistics: counts, percentages for categorical variables, median, and range for continuous variables. Response rates were reported with corresponding 95% confidence intervals (95% CI) computed by the exact binomial method. Progression-free survival (PFS) was measured from date of enrollment in the study to the earliest date of relapse, progression, or death from any cause. Overall survival (OS) was measured from the time of enrollment to the study till death. Event-free patients were censored at the last documented date of event-free status. PFS and OS were estimated by the Kaplan-Meier method. The log-rank test was used to evaluate the potential prognostic effect of TS changes. Serial elemental arsenic levels were obtained before and post-ATO injection twice weekly for the first 3 weeks of the treatment cycles with an additional evaluation on week 4. The corresponding data were fit to a single-compartmental model with exponential washout. Statistical analysis was done in SAS version 9.2.

**Results**

**Patient characteristics**

Twelve patients participated in this phase I study. All patients had metastatic CRC that had initially responded and subsequently progressed through the following regimens: FOLFOX or FOLFIRI, and in combination with bevacizumab, panitumumab, cetuximab, and irinotecan. Patients' ages ranged from 32 to 81 years, with a median of 56 years. The majority of patients were white (67%), male (83%), and non-Hispanic (67%). Patient characteristics are detailed in Table 1. The first seven patients received 0.15 mg/kg ATO and patients 8 to 12 received 0.20 mg/kg ATO (Table 1). Accordingly, patient no. 1 received 1,600 mg/m² 5-FU, patient no. 2 started with 2,000 mg/m² 5-FU, patient no. 3 started with 2,300 mg/m² 5-FU, and patient no. 4 and subsequent patients were administered 2,600 mg/m² 5-FU. The average number of treatment cycle received per patient was 6 with a range of 2 to 10 cycles.

**Toxicities**

All patients were monitored for signs of cardiac toxicity (persistent hypertension, arrhythmias, and weight gain), neuropathy (lethargy and peripheral neuropathy), hepatotoxicity, nephrotoxicity, and skin toxicity. During cycle one, there were one episode each of grade 2 abdominal pain and peripheral edema. No DLTs (nonhematologic grade 3 or all grade 4 toxicities) were observed in any of the seven patients who were treated with 0.15 mg/kg of ATO (Table 2).

Five patients were treated with 0.20 mg/kg ATO and 2,600 mg/m² 5-FU. Only one of these five patients...
Pharmacokinetics

Serial elemental arsenic levels were obtained before and post-ATO injection twice weekly for the first 3 weeks of the treatment cycles, with an additional evaluation on week 4. The data were fit to a single-compartmental model with exponential washout. Plasma arsenic levels peaked at the conclusion of week 1, consisting of 5 consecutive days of loading with ATO. At 0.15 mg/kg ATO, the peak concentration reached ~100 μg/L and total area under the curve equaled 831 (r² = 0.84; Fig. 1A and B). With 0.20 mg/kg ATO, the peak concentration reached ~160 μg/L and total area under the curve equaled 1,429 (r² = 0.96; Fig. 1C and D). As seen in Fig. 1, serum arsenic levels remained higher throughout the course of treatment in patients who received 0.20 mg/kg ATO compared with those who received 0.15 mg/kg.

Response

TS mRNA expression was measured by real-time PCR in PBMCs. All 12 patients, regardless of receiving the lower 0.15 mg/kg or the higher 0.20 mg/kg ATO, showed a significant decrease in TS gene expression in PBMC (proof of our hypothesis). These levels remained low throughout the entire treatment (Fig. 2A). Within a week of cessation of treatment, there was partial recovery of TS mRNA in the PBMC (data not shown).

Tumor biopsies were obtained from eight patients with metastatic liver or lung disease, and measured for TS mRNA levels. Results showed that by the end of cycle one of treatment, ATO suppressed the tumor TS mRNA level by an average of 66% in four patients (Fig. 2B). The TS mRNA levels increased in three patients by an average of 71.2% and showed no change in one patient. Based on radiological findings, two patients showed stabilization of disease without clear evidence of tumor shrinkage. The two patients with stabilization of disease showed TS downregulation in tumor tissues (−62.2, −40.1; Fig. 2B). Because patients failed numerous previous chemotherapy regimens, we have considered the stabilization of disease as a response, yielding to a response rate of 16.7% (Table 3).

Based on all 12 patients, the estimated median PFS was 3.1 months (95% CI, 2.1-11.9 mo). The median OS was 13.9 months (95% CI, 3-19.3 mo; Fig. 3A and B). Additionally, we compared PFS and OS between four patients who showed TS reduction and four patients with TS increase or no change. In patients who showed a decrease in TS mRNA levels, the estimated median PFS was 7.9 months (95% CI, 2.1-11.9 mo), whereas median PFS was 2.6 months (95% CI, 2.1-4.5 mo) in patients with TS increase or no change (Fig. 3C). However, given the small number of patients, the difference in PFS was not statistically significant (log-rank P = 0.188). We also examined the OS between patients in whom TS mRNA was reduced and not reduced. The estimated OS medians were 11.7 months (95% CI, 5-16.2 mo) and 8.6 months (95% CI, 3.0-13.9 mo), respectively. In this small cohort of patients, the observed survival advantage was not statistically significant (log-rank P = 0.44; Fig. 3D).

Discussion

5-FU has been the most active chemotherapeutic agent for CRC and the mainstay of treatment for the last 40 years. However, development of 5-FU resistance in CRC patients is a major challenge. Strategies to reverse cellular resistance of plasma arsenic reached ~100 μg/L and total area under the curve equaled 831 (r² = 0.84; Fig. 1A and B). With 0.20 mg/kg ATO, the peak concentration reached ~160 μg/L and total area under the curve equaled 1,429 (r² = 0.96; Fig. 1C and D). As seen in Fig. 1, serum arsenic levels remained higher throughout the course of treatment in patients who received 0.20 mg/kg ATO compared with those who received 0.15 mg/kg.
resistance to 5-FU by ATO may create a treatment choice for 12,000 to 18,000 patients annually, who otherwise have no alternative options. We and others have shown that the development of 5-FU resistance in metastatic CRC patients is highly associated with gene amplification (2) and overexpression of intratumoral TS mRNA (11). Thus, strategies to reduce TS overexpression are likely to restore 5-FU sensitivity.

ATO was recently rediscovered in the modern era as a highly active agent for the treatment of chemotherapy/ATRA-resistant acute promyelocytic leukemia (12, 13). Clinical trials done in the United States resulted in 92% complete response rates (14). Thereafter, ATO was approved by the Food and Drug Administration and has become treatment of choice for acute promyelocytic leukemia patients. ATO is now widely studied in the treatment of multiple myeloma. We recently reported in a pilot study that ATO (0.25 mg/kg) plus ascorbic acid, a glutathione-depleting agent, was well tolerated in patients with refractory/relapsed multiple myeloma (15). We found that ATO kills myeloma cells through the generation of reactive oxygen species involving both caspase-dependent and caspase-independent pathways (15, 16). However, it has been observed that ATO has diverse mechanisms of antineoplastic effect in a wide range of tumors (16). Recently, ATO has been examined for potential treatment options in several solid tumor cell lines targeting various mechanisms (17, 18). It is important to realize the above in vivo arsenic concentrations compare favorably to our in vitro concentrations. Evaluation is based only on the limited number of patients in our study. It is our understanding that ATO is a cytostatic agent and promotes downregulation of the tumor TS gene expression.

Although all these studies have shown successful results, no trial had yet studied the effects of ATO on TS gene expression and 5-FU sensitivity. Previously, ATO has only been used as a single agent and, as a result, has proved ineffective in the treatment of CRC. We found that ATO reduces PBMC and tumor TS mRNA levels.

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NOTE: Patient may have multiple episodes of toxicity. DLT was defined as grade 3 or higher nonhematologic toxicity, or grade 4 hematologic toxicity as per NCI Common Terminology Criteria for Adverse Event v3.
for better efficacy of treatment (19). Biochemical modulation is a method that leads to a “biochemical switch,” e.g., in pyrimidine biosynthetic pathway leading to de novo shutdown, therefore augmenting the salvage pathway. This will lead to the further production of FdUMP, which could bind to “wild-type” TS and inhibit DNA synthesis. TS is shown to auto regulate at the translational level. Sequestration of TS by FdUMP eliminates this autoregulation, thereby resulting in increased TS production. Additionally, during cell cycle, fresh TS is continuously formed. Both the above mechanisms could “override” the FdUMP present in the cells and allow DNA synthesis to resume, preventing biochemical modulation to be successful.

On the other hand TS gene suppression shuts down transcription. This mechanism works regardless of wild-type or mutated TS. We have shown ATO to inhibit TS at mRNA level, remaining suppressed for the duration of therapy. At reduced TS levels, tumors are very sensitive to small quantities of FdUMP, inhibiting DNA synthesis for prolonged periods. Lower TS levels create the prime environment for 5-FU to be used, thus effectively maximizing tumor response. Therefore, we decided to perform a study combining ATO with 5-FU to suppress TS gene expression for the treatment of CRC.

Previously, we completed in vitro studies assessing the sensitivity of a panel of CRC cell lines [HT29, SW480 sensitive to 5-FU, and HT29FU (resistant to 5-FU)] to different concentrations of ATO. We assessed the effects of ATO concentration on TS expression. Downregulation of TS gene expression in HT29FU cells is most marked between 0.2 and 0.6 \( \mu \)mol/L. This downregulation in TS mRNA is similarly reflected in TS protein levels.

**Fig. 1.** Mean serum arsenic levels. A, predicted and observed serum arsenic levels at ATO dose of 0.15 mg/kg; pretreatment and posttreatment. B, correlation between predicted and observed serum arsenic levels at ATO dose of 0.15 mg/kg. C, predicted and observed serum arsenic levels at ATO dose of 0.2 mg/kg; pretreatment and posttreatment. D, correlation between predicted and observed serum arsenic levels at ATO dose of 0.2 mg/kg.
Cell detachment, viability assays, and molecular marker studies suggested the efficacy of ATO as an anti-CRC agent, particularly against 5-FU–resistant cells. This formed the rationale for our previous pilot study of ATO plus ascorbic acid as a single-agent regimen in 5-FU–resistant relapsed/refractory CRC patients (7). No significant disease responses were seen in that study. However, TS mRNA expression in the PBMCs was significantly reduced (\(P = 0.03\), one-sided paired \(t\) test) following treatment in the four patients whom blood samples were obtained. Only buffy coat was analyzed for TS regulation; no tumor biopsies were taken in that study (7). In our current NCI-funded phase I study, TS gene expression decreased in PBMC samples in all patients who received either 0.15 mg/kg or 0.20 mg/kg ATO.

In our previous study, using ATO at 0.25 mg/kg as a single agent, we observed a higher number of grade 3 toxicities (7). In our current phase I study, we have encountered less toxicities with repeated treatments at 0.20 mg/kg ATO and 5-FU at 2,600 mg/m\(^2\). Therefore, we have determined 0.20 mg/kg ATO, 2,600 mg/m\(^2\) 5-FU, and 500 mg/m\(^2\) leucovorin to be the recommended phase II dose.

Table 3. Response (best overall response by Response Evaluation Criteria in Solid Tumors criteria)

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<th>%</th>
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<td>1</td>
<td>8.3</td>
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Pharmacokinetic studies were also done on plasma arsenic levels. Treatment regimen maintained serum arsenic levels at the therapeutic range over the course of treatment. When these results were analyzed in relation to patient toxicity, a correlation could be seen between higher serum arsenic levels and increased toxicity.
The conduct of phase I trial showing the combination of ATO and 5-FU has acceptable toxicities and has led to the suppression of TS mRNA in PBMC (proof of our hypothesis). Furthermore, we observed the suppression of TS mRNA in 50% of the patients examined. Two of the four patients whose TS tumor mRNA were reduced showed clinical response (stable disease). Although this is a small phase I study, we do not typically report disease-free survival or OS. However, it is of great interest to observe an increase in OS benefit in all the patients enrolled in this study. This was more pronounced in patients whose tumor TS level decreased ($P = 0.44$). To confirm the results of this small cohort of patients, we plan to conduct a phase II trial to examine the combination of 0.20 mg/kg ATO, 2,600 mg/m$^2$ 5-FU, and 500 mg/m$^2$ leucovorin in relapsed/refractory CRC patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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