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ORIGINAL ARTICLE

Pharmacological ascorbate with gemcitabine for the control of metastatic and node-positive pancreatic cancer (PACMAN): results from a phase I clinical trial

J. L. Welsh · B. A. Wagner · T. J. van't Erve · P. S. Zehr · D. J. Berg · T. R. Halfdanarson · N. S. Yee · K. L. Bodeker · J. Du · L. J. Roberts II · J. Drisko · M. Levine · G. R. Buettner · J. J. Cullen

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Abstract
Background Treatment for pancreatic cancer with pharmacological ascorbate (ascorbic acid, vitamin C) decreases tumor progression in preclinical models. A phase I clinical trial was performed to establish safety and tolerability of pharmacological ascorbate combined with gemcitabine in patients with biopsy-proven stage IV pancreatic adenocarcinoma.
Design Nine subjects received twice-weekly intravenous ascorbate (15–125 g) employing Simon's accelerated titration design to achieve a targeted post-infusion plasma level of ≥350 mg/dL (≥20 mM). Subjects received concurrent gemcitabine. Disease burden, weight, performance status, hematologic and metabolic laboratories, time to progression and overall survival were monitored.
Results Mean plasma ascorbate trough levels were significantly higher than baseline (1.46 ± 0.02 vs. 0.78 ± 0.09 mg/dL, i.e., 83 vs. 44 μM, $p < 0.001$). Adverse events attributable to the drug combination were rare and included diarrhea ($n = 4$) and dry mouth ($n = 6$). Dose-limiting criteria were not met for this study. Mean survival of subjects completing at least two cycles (8 weeks) of therapy was 13 ± 2 months.
Conclusions Data suggest pharmacologic ascorbate administered concurrently with gemcitabine is well tolerated. Initial data from this small sampling suggest some efficacy. Further studies powered to determine efficacy should be conducted.

Keywords Pancreatic neoplasm · Ascorbic acid · Clinical trial · Phase 1 · Gemcitabine · Drug toxicity

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J. L. Welsh · J. J. Cullen (✉)
Department of Surgery, 1528 JCP—UIHC,
The University of Iowa Carver College of Medicine,
Iowa City, IA 52242, USA
e-mail: joseph-cullen@uiowa.edu

B. A. Wagner · T. J. van't Erve · K. L. Bodeker · J. Du · G. R. Buettner · J. J. Cullen
Department of Radiation Oncology, The University of Iowa Carver College of Medicine, Iowa City, IA, USA

P. S. Zehr · D. J. Berg · T. R. Halfdanarson · G. R. Buettner · J. J. Cullen
The Holden Comprehensive Cancer Center,
The University of Iowa, Iowa City, IA, USA

D. J. Berg · T. R. Halfdanarson
Department of Internal Medicine, The University of Iowa Carver College of Medicine, Iowa City, IA, USA

N. S. Yee
Penn State Hershey Cancer Institute, Hersey, PA, USA

L. J. Roberts II
Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN, USA

J. Drisko
Integrative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

M. Levine
Molecular and Clinical Nutrition Section, Digestive Diseases Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

J. J. Cullen
The Veterans' Affairs Medical Center, Iowa City, IA, USA

Introduction

The incidence rate of pancreatic cancer is increasing, from 11.4 in 2000 to 12.0 per 100,000 in 2008 [1]. Of the 44,000 diagnosed with pancreatic cancer in 2011, just over 37,000 are expected to succumb to the disease that year—a mortality rate of over 80 % [2]. Despite being the fourth leading cause of cancer-related death in the United States, the National Cancer Institute reports that progress in pancreatic cancer treatment has not made the same advances as treatments for other cancers [3]. A significant therapeutic advance in pancreatic cancer was the initiation of the gemcitabine regimen as described by Burris et al. [4]. FOLFIRINOX, a multi-drug combination chemotherapy, was recently shown to improve overall survival in patients with metastatic pancreatic cancer when compared to gemcitabine alone, but with added toxicity [5]. Contributions to foster therapeutic breakthroughs for this disease are greatly needed.

Ascorbate (ascorbic acid, vitamin C) can be both an antioxidant and pro-oxidant; in the presence of catalytic metals, it typically exhibits pro-oxidant properties [6]. Pharmacological ascorbate is the use of high doses of ascorbate, administered IV, to achieve plasma levels of ascorbate on the order of 100–1,000 times that of healthy nutritional levels. At these high levels, pharmacological ascorbate has been proposed as a pro-drug for the delivery of H_2O_2 to tumors [7]. Pharmacological ascorbate, at doses achievable in humans, selectively kills pancreatic cancer cells via an H_2O_2-mediated mechanism, that is, acting as a pro-oxidant [7–9]. Additionally, preclinical data indicate that adding pharmacological ascorbate to the standard gemcitabine regimen may improve outcomes [10].

Reported clinical outcomes regarding the efficacy of high-dose ascorbate as a therapeutic agent are conflicting. The absence of benefit from studies of oral ascorbate is predictable and expected [11–13]. Considering the difference in route of administration, the decision was made to conduct a phase I trial to determine the safety and tolerability of pharmacological ascorbate when administered concomitantly with gemcitabine in pancreatic cancer patients. Given the recent preclinical study indicating that ascorbate has the potential to improve antitumor therapy, plasma levels of at least 20 mM were targeted [9]. Primary objectives of this study were to characterize the toxicity profile associated with IV ascorbate and the dose effect on plasma ascorbate levels when given twice weekly with gemcitabine to patients with metastatic pancreatic cancer.

Patients and methods

Regulatory requirements

An investigational new drug application (IND) was filed and obtained from the Center for Drug Evaluation and Research (CDER) of the FDA. The phase I protocol was reviewed and approved by CDER. Approval was sought and obtained from The University of Iowa IRB-01 (Biomedical), and the trial was listed on *clinicaltrials.gov* (NCT01049880). Good clinical practice consistent with ICH E8 was maintained, and protocol compliance was monitored quarterly by the Data and Safety Monitoring Board (DSMB) through an active audit process of all subjects. Annual reports compliant with 21CFR§312.33 were filed as required with the FDA.

Patient population

Patients were required to have histologically or cytologically confirmed unresectable, metastatic, or recurrent pancreatic adenocarcinoma. Pharmacological ascorbate infusions may cause red blood cell hemolysis in those deficient in the glucose-6-phosphate dehydrogenase (G6PD) enzyme. Thus, those with low G6PD levels were excluded (normal range 7–20.5 U/g hemoglobin, ARUP laboratories, Salt Lake, UT, USA). Other criteria included an ECOG performance status (PS) of 0, 1, or 2 with a life expectancy of at least 3 months or greater. Required initial laboratory values included a neutrophil count of $\geq$1,500/mm^3, platelet count of $\geq$100,000/mm^3, creatinine of $\leq$1.5 mg/dL or creatinine clearance of $\geq$60 mL/min, total bilirubin of $\leq$2$\times$ upper limit of normal (ULN), transaminases of $\leq$3 $\times$ ULN (<5 $\times$ ULN if liver metastases were present), and a prothrombin time or international normalized ratio (INR) within normal institutional limits. Key exclusion criteria included active comorbidities such as end-stage congestive heart failure, unstable angina, or a myocardial infarction within 6 months of enrollment. All patients were required to provide written informed consent. All enrollment eligibility was confirmed through a formal registration process utilizing an independent monitor provided by the Holden Comprehensive Cancer Center's DSMB.

Treatment plan

The treatment schema is provided in Fig. 1. Subjects received a test dose of 15 g ascorbate (Bioniche Pharma USA, Lake Forest, IL, USA) in 250 mL 5 % dextrose water (D5 W) infused over 30 min. If tolerated, a second test dose was administered within a calendar week. The dose of ascorbate was then increased weekly until a plasma level of $\geq$350 mg/dL (>20 mM) was achieved (Supplemental information Table 1). Once this level was attained, the ascorbate dose was adjusted as needed. Ascorbate infusions were given each week of the four-week cycle. For the purposes of this phase I study, dose-limiting toxicities (DLT) were defined as a grade 3 or greater non-

hematologic toxicity with attribution to the ascorbate. If a subject experienced a DLT, ascorbate treatment was terminated. In the absence of DLT, subjects continued ascorbate treatment until progression (defined by RECIST).

Gemcitabine was administered following the method established by Burris et al. [4]: IV infusion at a dose of 1,000 mg/m^2 over 30 min, with each cycle consisting of weekly gemcitabine infusions for 3 consecutive weeks with 1-week rest.

Blood samples were drawn pre- and post-infusion. Plasma was analyzed by a colorimetric/kinetic assay for ascorbate determination [14]. Each sample was divided into at least three aliquots and analyzed separately. Blood samples were stored on ice in a dark refrigerator until prepared for assay. No ascorbate degradation was expected to occur during processing and storage conditions. CT imaging was obtained at baseline and after completing every two cycles in accordance with standard of care at the University of Iowa Hospitals and Clinics.

Response and toxicity criteria

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events 3.0 (CTCAE). The RECIST guideline was used for the evaluation of antitumor activity. Toxicity attribution to ascorbic acid was initially determined by treating physician, reviewed by the study PI (JJC) and medical monitor (DJB), and confirmed by the DSMB.

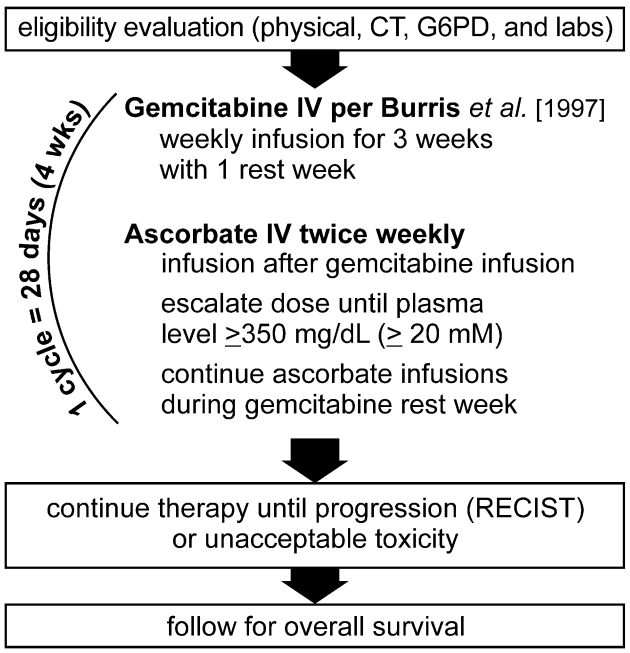

Fig. 1 Schematic of phase I clinical treatment plan. Red blood cell hemolysis may occur in people deficient in the G-6-PD enzyme, so this is an exclusion criterion. If the results of the G-6-PD laboratory test are negative, a test dose of 15 g of ascorbic acid was infused over 30 min. If tolerated throughout the first week, the dose of ascorbate was increased weekly (i.e., every 2 infusions) until the plasma level reaches at least 350 mg/dL (20 mM). Ascorbate infusions were given each week of the four-week cycle

Sample analysis

Sample preparation for plasma and RBCs

Whole blood (NaHeparin 75 USP units, BD Vacutainer® green top blood collection tube, 4 mL) was collected from clinical trial participants. Samples were centrifuged at 500*g* for 5 min, and then RBC-free plasma was collected for ascorbate and F_2-isoprostane measurements. Most samples were analyzed immediately; some plasma samples were frozen at −80 °C and analyzed within a week.

For glutathione redox determinations, RBCs were collected and then washed twice with cold isotonic saline solution. After washing, an aliquot was taken for CBC

Table 1 Patient characteristics

Characteristic	Number of patients
Sex	
Male	6
Female	5
Age (years)	
Median	62
Range	50–69
ECOG score	
0	3
1	7
2	1
Smoking history	
Never	3
Former	6
Current	2
Clinical stage	
IV	11
T Stage	
T3	8
T4	3
N stage	
N1	11
M stage	
M1	11
Histological type	
Adenocarcinoma	11
CA 19-9	
<40	3
>40	8

analysis (Sysmex XE-2100™ Automated Hematology System). RBCs were lysed with 5 % perchloric acid/100 μM diethylenetriaminepentaacetic acid (DETAPAC; Sigma-Aldrich Chemical Co, St Louis, MO, USA); this precipitates the protein and preserves glutathione (GSH) and glutathione disulfide (GSSG). The sample was centrifuged to pellet the protein (4,000*g*, 5 min). The clean supernatant was stored at −80 °C or immediately analyzed using HPLC.

Measurement of plasma ascorbate

Ascorbate levels in patient plasma samples were estimated with a plate reader-based assay as described in Vislisel et al. [14] with minor modifications to accommodate the 1,000-fold differences in ascorbate concentrations seen under nutritional (μM) as opposed to the post-IV ascorbate infusion levels (mM). Briefly, plasma samples were extracted with a buffer containing 90 % methanol and 10 % water with 250 μM DETAPAC (90:10, v/v), mixed, and incubated on ice for 10 min to precipitate the protein. The sample was then clarified by centrifugation, 10 min at 16*g* with an Eppendorf model 5415D Microfuge.

Specifically for this study, we standardized the extraction procedure so that plasma samples from the enrollment screen and pre-infusion were diluted using 150 μL of the plasma and 600 μL (5× dilution) of the extraction buffer. In contrast, the post-IV ascorbate samples were diluted at a ratio of 50 μL of the plasma to 450 μL (10× dilution) of the extraction buffer. The screen and pre-IV ascorbate plasma samples were further diluted another 3× (15× overall) into a buffer containing 72 % methanol and 28 % water with 250 μM DETAPAC (72:28, v/v). Post-intravenous ascorbate infusion plasma samples were diluted an additional 77.5- to 105-fold in this same buffer. Overall, the dilutions of the screen and pre-infusion samples were 5- and 15-fold (final), whereas the post-infusion samples were diluted from 775- to 1,050-fold (final). This protocol ensured that all samples assayed had the same methanol content (72 %): a critical factor for the success of this assay [14]. The diluted plasma samples were then assayed immediately without further storage. All samples were placed as 100-μL aliquots in 96-well optical bottom black plates (Thermo Fisher Scientific, Rochester, NY, USA). Authentic L-ascorbic acid (Macron Chemicals, Avantor Performance Materials, Center Valley, PA, USA) standards in 72 % methanol and 28 % water with 250 uM DETAPAC (72:28, v/v) were included in the assay as 100-μL aliquots with stock solution concentrations ranging from 2.5 to 50 μM: Again, the 72 % methanol content in the assay was maintained in the standards. The assay was initiated at room temperature by adding 100 μL of a 2.3 mM 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxyl (Tempol; Sigma-Aldrich, St. Louis, MO, USA) in 2 M sodium acetate dihydrate (Sigma-Aldrich) buffer previously adjusted to pH 5.5 with acetic acid. The samples were then incubated for 10 min in the dark, during which two equivalents of Tempol oxidized one ascorbic acid by two separate 1-electron oxidations to dehydroascorbic acid (DHA). After 10 min, 42 μL of 5.5 mM *ortho*-phenylenediamine (*o*PD) in 2 M sodium acetate buffer (pH 5.5) was added to the samples. Kinetic measurements were initiated and collected every 22 s with a TECAN SpectraFluor Plus plate reader (Tecan, Research Triangle Park, NC, USA) using a 345-nm bandpass filter for excitation and a 425-nm bandpass filter for emission. The condensation product of the reaction of *o*PD with DHA is the fluorescent 3-(dihydroxyethyl)furo[3,4-b]quinoxaline-1-one; this product was monitored under these instrumental settings. Plasma sample concentrations were determined by comparison with the standard curve established from the rates of the DHA-*o*PD condensation product formation from the authentic ascorbate standards using linear portions of the progress curves: This linear portion occurs in the first four kinetic time points (<90 s) under the concentrations and conditions described here. Full kinetic curves were collected and examined to ensure no abnormalities.

Ascorbate radical in blood

Whole blood was drawn into 100-mm Hirschmann melting point determination tubes (Z61174, Sigma-Aldrich) by capillary action; breaking off the sealed end of the capillary tubes allowed 20-50 μL of the whole blood to flow freely into the tubes without air bubbles. The tubes were then sealed at one end with capillary tube sealant. The capillary tube, with the sealed end down, was placed into the bottom of a 250 mm × 3 mm (ID) thin-walled quartz EPR tube (707-SQ-250 M Wilmad—Lab Glass, Vineland, NJ, USA). The EPR tube was then placed into a Bruker HS EPR cavity (Bruker, Billerica, MA, USA) so that the blood sample was centered within the cavity without air bubbles filling the spectral acquisition volume and that any sample leakage and capillary sealant being outside the spectral acquisition volume. EPR spectra were collected with a Bruker EMX EPR spectrometer: room temperature; microwave power, 20 mW; frequency, 9.853 GHz; scanning 10 G with a sweep time of 20.972 s; receiver gain, 5.02×10^4; modulation frequency, 100 kHz; modulation amplitude, 0.70 G; and signal channel time constant, 327.680 ms. All spectra were collected in the additive mode using five scans. Estimates of $[Asc^{\cdot -}]_{ss}$ in whole blood were made using 3-carboxy-PROXYL (3-CP) (Sigma-Aldrich) as a standard; the concentration of 3-CP in the standard was verified using $\varepsilon_{234} = 2{,}370 \pm 50\ M^{-1}\ cm^{-1}$ [15]; signals were double integrated using the Bruker WIN-EPR software with corrections for differences in saturation behavior [16].

Measurement of isoprostanes

Isoprostanes are a unique series of prostaglandin-like compounds formed in vivo via the non-enzymatic free radical-initiated peroxidation of arachidonic acid, a ubiquitous polyunsaturated fatty acid. The F_2-isoprostanes have become the biomarker of choice for assessing endogenous oxidative stress because these molecules are chemically stable and have been detected in all biological fluids and tissues analyzed [17]. Blood from subjects was collected before and after ascorbate infusion to assess parameters indicative of oxidative stress. To quantify F_2-isoprostane levels, gas chromatography–mass spectrometry was utilized. This assay was chosen because it has been shown to be capable of detecting changes indicative of in vivo oxidative stress in plasma samples [17] and is verifiable [17–19].

Measurement of GSH and GSSG in RBCs with HPLC-BDD

To determine the status of the GSSG, $2H^+$/2GSH couple in red blood cells, we used HPLC with electrochemical detection following the protocol outlined by Park et al. [20]. The method is based on an electrochemical detection (ECD) system using a boron-doped diamond (BDD) electrode (Model 5040, ESA Biosciences, Chelmsford, MA, USA). The BDD electrode is an excellent detector for thiol and disulfide compounds as these analytes require a high electrode potential. Derivatization of the sample is not required allowing higher throughput. With the minimal sample processing required, there is less opportunity for the sample to oxidize, which would lead to overestimation of GSSG [21]. The results from the HPLC/BDD along with the information from the complete blood count allow determination of the intracellular concentrations of GSH and GSSG. These concentrations in conjunction with the Nernst equation (pH 7.40) were used to determine the intracellular redox status of the couple in red blood cells, E_{hc} [22].

Study design and statistical analysis

This phase I study was designed as a single-institution, prospective, open-label study of safety and tolerability of ascorbate when administered concurrently with gemcitabine. Enrollment followed a two-stage design with influence from Simon's accelerated titration design. Three subjects were accrued in the first stage of this study and completed at least 1 month of study treatment. If $\geq$2 subjects experienced a DLT, a stopping rule would be invoked and the study stopped. If <2 subjects experience a DLT, the study would progress to the second stage, where up to seven additional subjects would complete at least 1 month of therapy. If $\geq$3 dose-limiting toxicities occur in the combination of stage 1 and 2 subjects, the study will be terminated and the treatment deemed unsafe. If less than three subjects experienced DLT, the treatment would be considered tolerable and investigated in a phase II study powered for efficacy.

Results

Patient population

A total of 15 patients were enrolled from April 2010 to November 2011, with 14 of these patients eligible. One patient was found to be ineligible due to a high G6PD. Two patients withdrew due to the travel required for treatment. One patient withdrew due to insurance denial.

Treatment delivery and protocol compliance

Of the 11 subjects treated with ascorbate, two discontinued protocol treatment the first month due to progressive disease necessitating palliative treatment. The characteristics of the subjects are listed in Table 1. The remaining nine completed at least 1-month protocol therapy, making them evaluable for statistical purposes. Quarterly audits identified no compliance deficiencies. Adverse events were reviewed monthly at investigator's meetings and reviewed against protocol requirements for dose-limiting toxicities. Individual ascorbate doses for maintenance of 350 mg/dL ranged from 50 to 125 g per infusion. Ascorbate levels ranged from 20 to 25 mM in the first hour post-infusion, levels shown in preclinical studies to provide antitumor effects [9].

Six of the nine treated subjects maintained or improved their performance status. Average treatment duration was 6 months (177 days, range 69–556 days) during which mean weight loss was 5.0.3 $\pm$ 1.6 kg while receiving study treatment (Supplemental Fig. 1). No subjects were lost to follow-up. Of the nine patients who completed at least 1 month of protocol therapy, time to progression was 26 $\pm$ 7 weeks, while overall survival was 13 $\pm$ 2 months (mean $\pm$ SEM) (Fig. 2).

Safety and toxicity

No dose-limiting toxicities or serious adverse events (as defined by 21CFR312.32) occurred. Toxicities were comparable with published trials of gemcitabine regimens [4]. Table 2 provides the maximum grade for toxicities in the initial treatment cycle, while Table 3 provides the same data for all treatment cycles. The increased toxicities with repeated doses were attributable to the gemcitabine

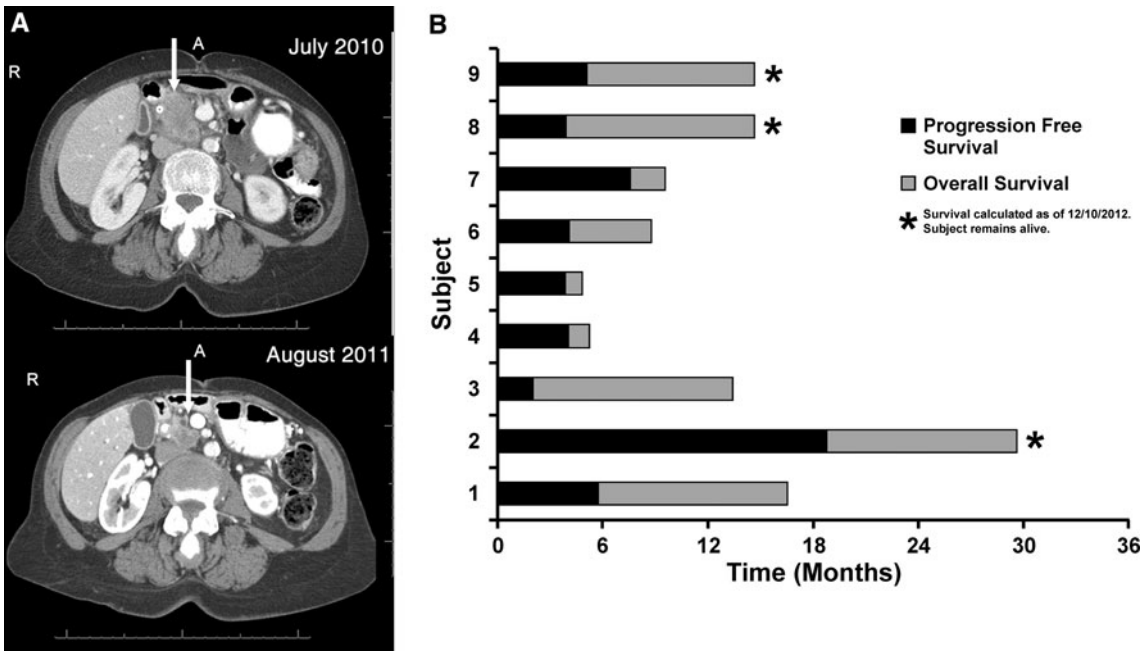

Fig. 2 Response to therapy. **a** Baseline and post-treatment CT scans in a patient receiving ascorbate plus gemcitabine. The patient tolerated dose escalation of ascorbate, which stabilized at 75 g twice a week. Post-infusion analysis demonstrated plasma ascorbate concentrations of 22–27 mM (390–475 mg dL^{-1}) at 1 h. The patient had a ninefold decrease in the primary tumor size within 4 months of treatment. **b** Overall survival. Our phase I trial was designed to determine the effect of escalating doses of ascorbate when combined with gemcitabine in stage IV pancreatic cancer patients. The trial utilized a modified Burris regimen, administering gemcitabine for 3 weeks for each cycle of therapy along with ascorbate given twice weekly for every week. Historic median survival for gemcitabine-treated patients is 5.65 months [4]. The mean survival is 13 months

treatment. In the nine subjects treated, few grade 3 and 4 toxicities were observed, and none were attributed to ascorbate. Grade 3 laboratory toxicities were limited to elevated GGT ($n = 2$) and hypokalemia ($n = 1$), both likely related to the disease process. There were no grade 4 laboratory toxicities. Grade 3 and 4 hematologic toxicities included leukopenia ($n = 1$), lymphopenia ($n = 1$), neutropenia ($n = 2$), and thrombocytopenia ($n = 1$), consistent with the percentages reported for these toxicities with gemcitabine alone [4]. Constitutional toxicities (Table 2) possibly attributable to ascorbate included nausea ($n = 6$) and diarrhea ($n = 4$). Thirst/dry mouth was definitively attributed to ascorbate ($n = 4$), a transient symptom alleviated within the same day as infusion.

Pharmacology

In all subjects treated, ascorbate levels were measured before and immediately after infusions. Figure 3a demonstrates peak plasma ascorbate levels achieved with increasing doses for all subjects. Pre- and post-infusion ascorbate levels in a typical patient in the dose escalation scheme are depicted in Fig. 3b. For this subject, a 75-g dose yielded peak plasma levels ranging between 320 and 630 mg/dL. Interestingly, once targeted levels were achieved, ascorbate mean trough levels were significantly higher than baseline in all patients (1.46 ± 0.02 vs. 0.78 ± 0.09 mg/dL; i.e., 83 vs. 44 μM; $p < 0.001$) (Fig. 3c). In addition to measuring plasma ascorbate levels, generation of ascorbate radical was measured via EPR in whole blood of both pre- and post-infusion samples to examine the degree of ascorbate oxidation occurring in the blood with pharmacological dosing. The level of ascorbate radical in pre-infusion samples at nutritional levels of ascorbate was below the limit of detection (Fig. 3d). However, in post-infusion samples at pharmacological levels of ascorbate, the rate of ascorbate oxidation is greatly increased as seen by readily detectable ascorbate radical [23].

Our hypothesis is that ascorbate is a pro-drug for the delivery of H_2O_2 to pancreatic tumors to induce cytotoxicity [9]. Lipids are readily attacked by oxidative cascades initiated by H_2O_2 resulting in the formation of a number of oxidation products [17]. F_2-isoprostanes have become the biomarker of choice for assessing endogenous oxidative stress; these molecules are chemically stable and have been detected in all biological fluids and tissues analyzed [17]. Measurement of F_2-isoprostanes levels before and after ascorbate infusions demonstrated a decrease in F_2-isoprostane levels in all patients when compared to baseline levels (Fig. 4). These data suggest that these high levels of ascorbate do not induce systemic oxidative stress. We hypothesize that the antioxidant properties of ascorbate play

Table 2 Summary of maximum grade for laboratory and constitutional toxicities (initial treatment cycle only)

Toxicity	Number of subjects[b]				
	Grade of adverse event[a]				
	0	1	2	3	4
Metabolic					
Hypoalbuminemia	6	0	3	0	0
Alkaline phosphatase, elevated	5	2	2	0	0
Gamma-glutamyl transpeptidase, elevated	1	0	1	1	0
Aspartate aminotransferase, elevated	5	2	2	0	0
Alanine aminotransferase, elevated	1	6	2	0	0
Hyperbilirubinemia	7	2	0	0	0
Creatinine, elevated	9	0	0	0	0
Hypocalcemia	6	2	1	0	0
Hypercalcemia	9	0	0	0	0
Hypokalemia	4	5	0	0	0
Hyperkalemia	7	2	0	0	0
Hyponatremia	6	3	0	0	0
Hypernatremia	9	0	0	0	0
Hematologic					
Anemia	0	6	3	0	0
Leukopenia	2	4	2	1	0
Lymphopenia	6	0	2	0	0
Neutropenia	1	2	2	2	0
Thrombocytopenia	1	7	0	1	0
Constitutional					
Nausea/vomiting	4	5	0	0	0
Diarrhea	8	1	0	0	0
Constipation	9	0	0	0	0
State of consciousness	9	0	0	0	0
Pain	7	2	0	0	0
Fever	7	2	0	0	0
Cutaneous	7	2	0	0	0
Oral	8	1	0	0	0
Hemorrhage	9	0	0	0	0
Infection	8	1	0	0	0
Pulmonary	9	0	0	0	0
Hair	9	0	0	0	0
Peripheral neurotoxicity	9	0	0	0	0
Proteinuria	9	0	0	0	0
Cardiac rhythm	9	0	0	0	0
Allergic	9	0	0	0	0
Hematuria	9	0	0	0	0

[a] Graded using common terminology criteria for adverse events v.3

[b] Represents the number of subjects (of total $N = 9$) experiencing adverse event during initial treatment cycle with ascorbate + gemcitabine

Table 3 Summary of maximum grade for laboratory and constitutional toxicities (aggregate for all treatment cycles)

Toxicity	Number of subjects[b]				
	Grade of adverse event[a]				
	0	1	2	3	4
Metabolic					
Hypoalbuminemia	6	0	3	0	0
Alkaline phosphatase, elevated	0	6	3	0	0
Gamma-glutamyl transpeptidase, elevated	4	2	1	2	0
Aspartate aminotransferase, elevated	2	4	3	0	0
Alanine aminotransferase, elevated	0	4	5	0	0
Hyperbilirubinemia	7	2	0	0	0
Creatinine, elevated	8	1	0	0	0
Hypocalcemia	4	4	1	0	0
Hypercalcemia	8	1	0	0	0
Hypokalemia	3	5	0	1	0
Hyperkalemia	6	3	0	0	0
Hyponatremia	4	5	0	0	0
Hypernatremia	9	0	0	0	0
Hematologic					
Anemia	0	3	6	0	0
Leukopenia	2	3	3	1	0
Lymphopenia	4	0	3	1	1
Neutropenia	1	1	3	2	2
Thrombocytopenia	1	7	0	1	0
Constitutional					
Nausea/vomiting	3	4	2	0	0
Diarrhea	5	2	1	1	0
Constipation	9	0	0	0	0
State of consciousness	9	0	0	0	0
Pain	2	1	5	1	0
Fever	7	2	0	0	0
Cutaneous	7	2	0	0	0
Oral	9	0	0	0	0
Hemorrhage	9	0	0	0	0
Infection	4	1	4	0	0
Pulmonary	8	0	1	0	0
Hair	9	0	0	0	0
Peripheral neurotoxicity	9	0	0	0	0
Proteinuria	9	0	0	0	0
Cardiac rhythm	9	0	0	0	0
Allergic	9	0	0	0	0
Hematuria	9	0	0	0	0

[a] Graded using common terminology criteria for adverse events v.3

[b] Represents the number of subjects (of total $N = 9$) experiencing adverse event during entire course of treatment with ascorbate + gemcitabine

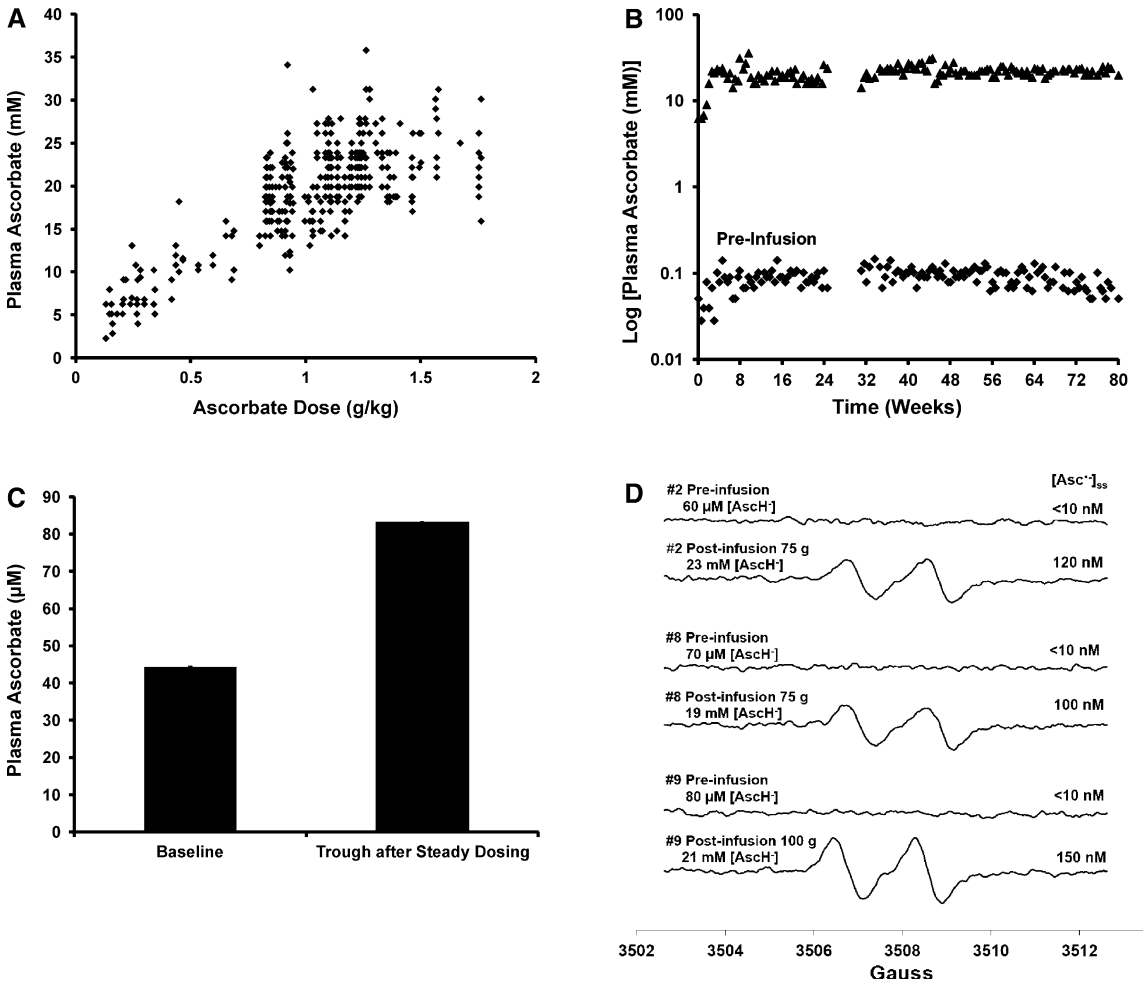

Fig. 3 Ascorbate infusions achieve plasma millimolar levels. Dose escalation of ascorbate infusions was administered to achieve a target level of 350–450 mg/dL (20–25 mM). Once subjects achieved these levels, the dose of ascorbate was not changed. **a** Peak ascorbate levels of 20–30 mM were reached with doses ranging from 0.75 to 1.75 g/kg. **b** Typical plasma levels of ascorbate achieved in a single patient over time. Peak ascorbate levels post-infusion were approximately 500-fold higher than both baseline and pre-infusion trough ascorbate levels. **c** Trough ascorbate levels after steady dosing regimen were significantly higher than baseline screening ascorbate levels. **d** Ascorbate radical is observed in whole blood only with high levels of ascorbate. Ascorbate radical is below the limit of detection (<10 nM under these experimental conditions) in pre-infusion samples of whole blood that have typical nutritional levels of ascorbate, here 60–80 µM. Ascorbate radical (100–150 nM) is easily detectable in post-infusion samples that have very high levels of ascorbate (19–23 mM). The presence of ascorbate radical indicates the ongoing oxidation of ascorbate in whole blood [23]

a role in the systemic decrease in the F_2-isoprostanes levels, while the local pro-oxidant effects induce tumor cell killing. Similarly, glutathione and its half-cell reduction potential are other measurable markers indicative of the oxidation state of the redox buffer. In severe systemic oxidative stress, glutathione (GSH) may become depleted when the degree of oxidative stress overwhelms the capability of glutathione disulfide reductase (GR) and the glutathione redox cycle with cell death occurring when the cell becomes overly oxidized [24]. As seen in Fig. 5, the intracellular concentrations of glutathione (GSH) and glutathione disulfide (GSSG) in red blood cells pre- and post-infusion of ascorbate and the calculated half-cell reduction potentials (E_{hc}) demonstrate no significant change in red blood cell intracellular concentration of GSH or E_{hc}, suggesting that although pharmacological ascorbate is a pro-oxidant toward tumor cells, there is no evidence of oxidative stress to red blood cells as seen by the stability of the intracellular redox buffer.

Discussion

Our results are compared to other trials of pharmacological ascorbate in advanced malignancies. In a phase I toxicity study, Hoffer et al. [25] treated patients with advanced cancers or hematologic malignancies with ascorbate infused at doses of 0.4–1.5 g/kg three times weekly with five subjects receiving the highest dose. All tolerated the ascorbate infusions well. While two subjects had

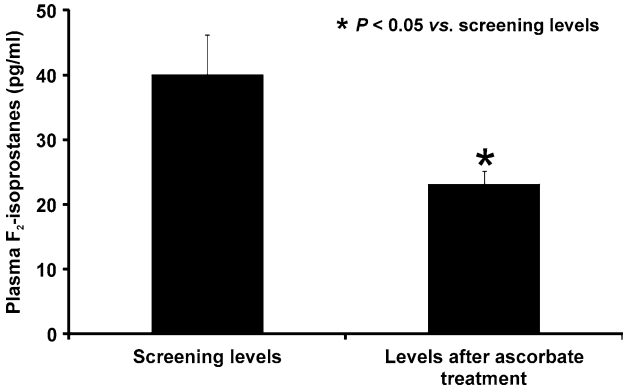

Fig. 4 Pharmacological ascorbate does not increase markers of systemic oxidative damage. Baseline and post-treatment F_2-isoprostane levels in five patients receiving gemcitabine + ascorbate twice weekly to achieve peak plasma levels ≥20 mM (≥350 mg dL^{-1}). In all patients tested, the F_2-isoprostane level decreased after ascorbate infusions

unexpected stable disease, all eventually succumbed. The investigators concluded ascorbate may need to be combined with cytotoxic or other redox-active molecules to be an efficacious treatment.

Recently, Monti et al. [26] conducted a trial of pharmacological ascorbate plus gemcitabine and erlotinib in patients with metastatic pancreatic cancer. A dose escalation design was used with maximum ascorbate dose of 100 grams three times weekly but for only 8 weeks. Gemcitabine and erlotinib were administered per standard treatment regimens. Findings included fifteen non-serious adverse events and eight serious adverse events in 14 patients. All of the adverse events were attributed to the chemotherapy regimen or progression of disease. Eighty-two percent of evaluable patients had weight loss of approximately 5.5 kg.

Adverse events in the present study attributable to ascorbate were rare and included diarrhea ($n = 4$) and dry

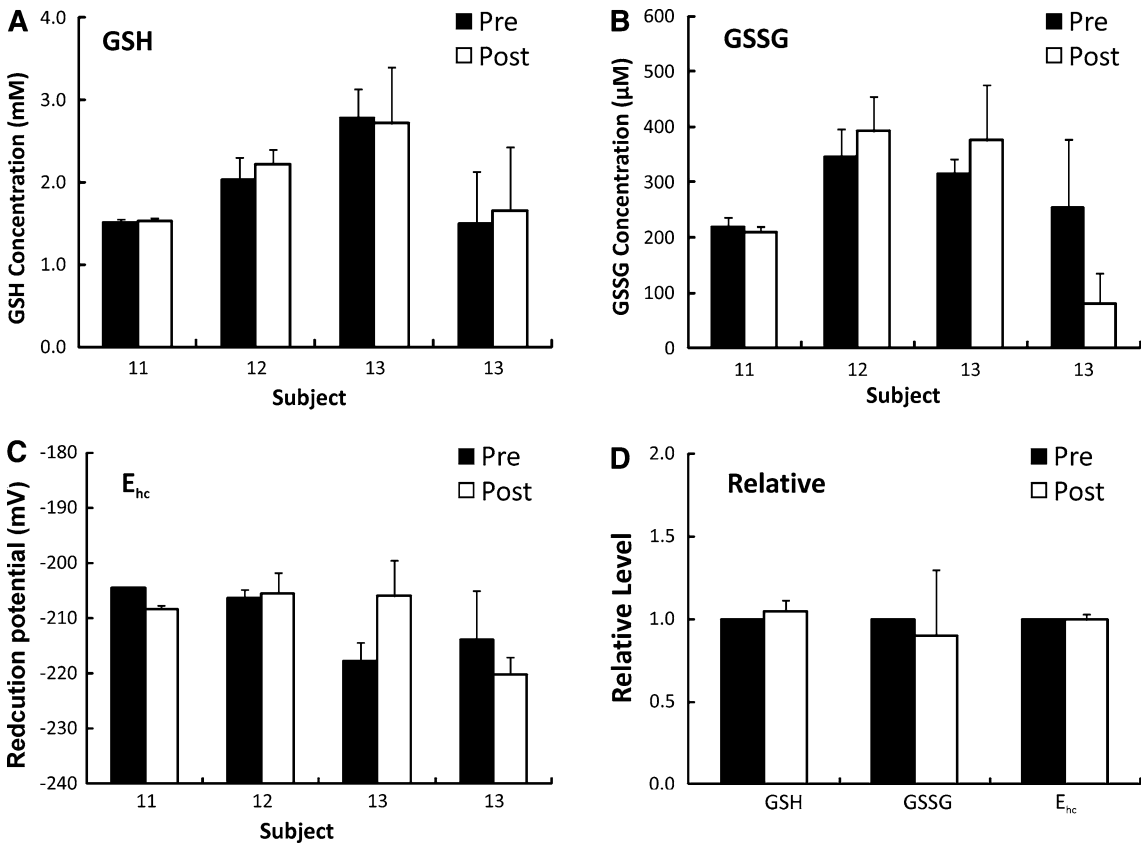

Fig. 5 Pharmacological ascorbate does not alter the GSSG/2GSH redox buffer of RBCs. Blood was collected from patients before and immediately after infusion and assayed for the intracellular concentrations of GSH and GSSG in the RBCs; this information was used to assess the intracellular redox status as manifest by E_{hc}. Plotted in **a**, **b**, and **c** are the intracellular concentrations for samples from three individuals and the corresponding calculated half-cell reduction potential (E_{hc}); the *error bar* represents measurement variability from triplicate determinations. In **d** the relative average levels for all analytes are presented. Pre-infusion concentrations for each individual are normalized to 1, and then the corresponding relative values for the post-sample are calculated. The *error bars* represent differences between patients

mouth ($n = 6$) while subjects maintained performance status and lost minimal weight, only 5.3 ± 1.6 kg on average over 6 months of evaluation. Observed adverse events appear to be less severe when compared to the adverse events published in the literature for the gemcitabine regimen. While such a small sample size ($n = 9$) limits broader applicability, these results are encouraging and may warrant further investigation.

In contrast to the Monti's study, we infused ascorbate doses based on the plasma levels of achieved after infusion was completed. Our goal was to achieve a targeted peak ascorbate level of ≥350 mg/dL (≥20 mM), which was determined as antitumoral based on the findings of Du et al. [9]. Maximum doses ranged from 50 g ($n = 1$), 75 g ($n = 3$), 100 g ($n = 2$), and 125 g ($n = 3$).

The Monti's study had a short ascorbate treatment of only 8 weeks to assess safety and tolerability. Even so, during that time, eight of the nine patients had a decrease in the size of the primary tumors. Monti et al. [26] report an estimated mean progression-free survival (PFS) of 12.7 weeks (89 days) and overall survival (OS) of 6 months (182 days). In contrast, our study strategy was to continue treatment until progression per RECIST. Our mean PFS was 26 weeks and OS of 12 months ($n = 9$). While neither study was powered to determine therapeutic efficacy, results are striking when compared to those of Burris et al., who reported a PFS of 9 weeks and OS of approximately 6 months [4].

In a recent multi-site trial, patients treated with FOLFIRINOX increased survival by 4.3 months compared to gemcitabine [5]. Median PFS was nearly double, and the response rate nearly tripled with the FOLFIRINOX regimen. However, quality of life was significantly lower, and serious grade 3 and 4 adverse events were significantly increased in the FOLFIRINOX group of patients.

Further studies with pharmacological ascorbate will be needed to demonstrate efficacy and confirm safety, but adverse events attributable to ascorbate were uncommon in our current study. Ascorbate infusions significantly increased the trough levels of ascorbate prior to intravenous administration. In addition, the half-cell reduction potential of the intracellular redox buffer of red blood cells remained stable. Furthermore, plasma levels of F_2-isoprostanes were decreased in subjects post-infusion, suggesting ascorbate may be protective to normal tissue. This may explain the apparent decrease in adverse event frequency observed during this trial, the reduced weight loss throughout treatment, and the maintained PS scores.

In summary, IV administration of ascorbate 50–125 g twice weekly produced plasma levels of at least 350 mg/dL (20 mM) with no significant adverse events or shift in a toxicity profile. The use of pharmacological ascorbate in combination with gemcitabine in patients with metastatic or unresectable pancreatic adenocarcinoma should be safe and well tolerated. A phase II clinical trial powered to determine efficacy of concomitant ascorbic acid combined with gemcitabine is warranted.

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Conflict of interest The authors have declared no conflicts of interest.

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