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Pharmacologic ascorbate synergizes with gemcitabine in preclinical models of pancreatic cancer

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Abstract

Conventional treatment approaches have had little impact on the course of pancreatic cancer, which has the highest fatality rate among cancers. Gemcitabine, the primary therapeutic agent for pancreatic carcinoma, produces minimal survival benefit as a single agent. Therefore, numerous efforts have focused on gemcitabine combination treatments. Using a ratio design, this study established that combining pharmacologically achievable concentrations of ascorbate with gemcitabine resulted in a synergistic cytotoxic response in eight pancreatic tumor cell lines. Sensitization was evident regardless of inherent gemcitabine resistance and epithelial–mesenchymal phenotype. Our analysis suggested that the promiscuous oxidative actions of H₂O₂ derived from pharmacologic ascorbate can culminate in synergism independent of the cancer cell's underlying phenotype and resistance to gemcitabine monotherapy. Gemcitabine–ascorbate combinations administered to mice bearing pancreatic tumor xenografts consistently enhanced inhibition of growth compared to gemcitabine alone, produced 50% growth inhibition in a tumor type not responsive to gemcitabine, and demonstrated a gemcitabine dose-sparing effect. These data support the testing of pharmacologic ascorbate in adjunctive treatments for cancers prone to high failure rates with conventional therapeutic regimens, such as pancreatic cancer.

Keywords

Pancreatic cancer; Drug synergy; Ascorbate; Vitamin C; Gemcitabine; Free radicals

Pancreatic cancer is an aggressive malignancy that often develops with only nonspecific early symptoms. The prognosis is poor, with estimated 5-year survival rates of less than 5% [1]. In addition to late detection and fulminant disease course, the high mortality rate of pancreatic cancer is a consequence of disappointing treatment efficacy [2,3]. Standard therapy for patients with metastatic pancreatic cancer is gemcitabine (2',2'-difluoro-2'-deoxycytidine monohydrochloride), an analog of the nucleoside deoxycytidine [4]. Gemcitabine offers clinical benefit in an adjuvant setting for patients after resection for operable pancreatic cancer, with a median disease-free survival interval of 13.4 months in subjects treated with gemcitabine and 6.9 months for the untreated group [5]. Unfortunately,

little impact has been observed on median overall survival benefit for patients with locally advanced or metastatic disease, who comprise the majority of cases [6,7].

Numerous studies have attempted to optimize and improve the efficacy of gemcitabine monotherapy through combination with other agents. Despite these efforts, interindividual response variability, development of drug resistance, and overall poor efficacy continue to plague gemcitabine-combination treatments. For trials involving unresectable and metastatic cases, median survival often ranges between 6 and 7 months regardless of cytotoxic agents tested in combination with gemcitabine [6]. Given the urgent need to evaluate new effective agents, the aim of this preclinical study was to determine the potential for pharmacologic ascorbate to work in combination with gemcitabine.

With pharmacologic ascorbate treatment, parenteral administration of high doses of ascorbate bypasses limited intestinal absorption that occurs with oral ingestion. Maximal plasma ascorbate concentrations achievable with oral ingestion are approximately 220 μM [8,9]. In contrast, ascorbate concentrations approaching 30 mM are attained with parenteral regimens in rodents and humans [8–10]. When subjects are screened for normal renal function and glucose-6-phosphate dehydrogenase activity, pharmacologic ascorbate does not have evident toxicity, based on phase I data [10] and widespread use by complementary alternative medicine (integrative medicine) practitioners [11]. Antitumor effects with pharmacologic ascorbate have been observed against a wide variety of cancer cells both in vitro and as xenografts in mice [12–22], including pancreatic carcinoma [12,17]. The mechanism of pharmacologic concentrations of ascorbate involves oxidation through the intermediacy of ascorbate radical, leading to generation of the cytotoxic effector species hydrogen peroxide (H_2O_2) and subsequent Fenton chemistry [8,12,14,15,17].

We proposed that ideal clinical use of pharmacologic ascorbate would be in combination with other cancer chemotherapeutic agents [12,13]. In this study, we tested ratios of gemcitabine and ascorbate against a panel of established pancreatic carcinoma cell lines and evaluated the potential for combination treatments to translate into either improved or impaired treatment efficacy in mouse xenograft models.

Materials and methods

Cells and chemosensitivity assessment

Human pancreatic carcinoma cell lines BxPC-3, AsPC-1, and SU.86.86 were purchased from the American Type Culture Collection (Manassas, VA, USA). HPAF-II and Hs 766T cells were kindly donated by Dr. Raj Puri, FDA/CBER (Bethesda, MD, USA); MIA PaCa-2 by Dr. Joseph Cullen, University of Iowa (Iowa City, IA, USA); PANC-1 by Dr. Michael Brownstein, J. Craig Venter Institute (Rockville, MD, USA); and the murine line PAN-02 by Dr. Anthony Sandler, Children's Hospital Medical Center (Washington, DC, USA). Cell lines were independently authenticated where applicable (RADIL, Columbia, MO, USA). Gemcitabine (TRC, Toronto, ON, Canada) was prepared as 33.3 mM stock solution in sterile water. Gemcitabine concentrations were verified by UV absorption spectrum ($\epsilon_{268} = 9360 \text{ M}^{-1} \text{ cm}^{-1}$). Ascorbate (Sigma, St. Louis, MO, USA) was prepared as a 1 M stock solutions in sterile water, with sodium hydroxide added drop-wise to adjust the pH to 7.0. Aliquots stored frozen at -80°C were thawed for single use.

Cells (1×10^4) in exponential growth phase were cultured at 37°C in 5% CO_2 /95% air in the recommended growth medium containing 10% fetal calf serum and exposed to serial dilutions of gemcitabine and/or ascorbate. Cells were cultured an additional 72 h, the medium was exchanged, and the cells were incubated 4 h in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The colorimetric MTT assay

assessed relative proliferation, based on the ability of living, but not dead, cells to reduce MTT to formazan [23]. Cells did not reach plateau phase during the 62-h incubation period. Inhibitory concentration (IC_{50}) was defined as the median concentration of drug that inhibited cell growth by 50% relative to the untreated control. Pilot experiments for each cell line were performed to optimize assay duration and to center drug dilution series approximately on the IC_{50} .

Molecular analysis

RNA extracts were made from each human cell line using lysis and spin-column procedures per the manufacturer's protocol (Qiagen, Valencia, CA, USA). Relative expression of vimentin (VIM) and E-cadherin (CDH1) was determined after first-strand conversion to cDNA (Invitrogen) followed by RT-PCR using gene-specific primers (ABI, Foster City, CA, USA). Data were normalized to 18S RNA/sample.

Xenograft and treatment procedures

Either PAN-02 or PANC-1 tumor cells (1×10^6) suspended in normal saline solution were injected subcutaneously into the flank of female athymic mice (Ncr-nu/nu, age 5–8 weeks). When tumor volume reached ~30 to 40 mm³, mice were randomly assigned to treatment groups: control group received daily saline solution osmotically equivalent to ascorbate, ascorbate groups received 4 g ascorbate/kg body wt daily, and gemcitabine groups received either 30 or 60 mg gemcitabine/kg body wt every 4 days. All drugs were administered by intraperitoneal injection. For combinations, gemcitabine immediately preceded ascorbate. Longitudinal tumor volume was calculated from caliper measurements using volume = length \times width² \times 0.5. Body weight was measured on a digital pan balance. At the end of experiments, the mice were euthanized with final tumor weight assessed by gross necropsy. Blood was collected from the orbital sinus into heparinized hematocrit tubes (Fischer). Complete blood counts were performed in the Hematology Core Laboratory at the University of Kansas Medical Center.

Data analysis

MTT data were normalized to their corresponding untreated controls for each condition (drug, cell type) and were expressed as percentage fractional affect (f_a) so that all dose–response curves begin at 0% (0 representing unaffected). CalcuSyn 2.1 (Biosoft, Cambridge, UK) was used to fit dose–response curves across f_a (2 to 99%) based on data experimentally derived when gemcitabine was combined with ascorbate at a given constant molar ratio in accord with the isobolographic principle [24–26]. Median-effect plots were generated from dose–response data with the aid of GraphPad Prism 5.0 (La Jolla, CA, USA) as follows: $y = \log [f_a/\text{unaffected fraction } (f_u)]$ versus $x = \log (\text{dose})$ for gemcitabine and ascorbate exposure either alone or in combination at a given constant molar ratio. Dose-reduction index (DRI) values for gemcitabine were calculated by the equation $DRI_{IC_x} = (D_{\text{gem}}/D_{\text{gem} + \text{asc}})$, where D_{gem} is the dose of gemcitabine alone required to produce an IC_x level of cytotoxicity (e.g., IC_{50} , IC_{75} , IC_{90}) and the divisor $D_{\text{gem} + \text{asc}}$ is the dose of gemcitabine needed to produce the same IC_x level of cytotoxicity when it is combined with ascorbate (at a given molar ratio). DRI_{gem} is defined with respect to gemcitabine. CalcuSyn 2.1 was used to calculate combination index (CI) values by the equation $CI_{IC_x} = (D_{\text{gem} (+ \text{asc}) IC_x}/D_{\text{gem} IC_x}) + (D_{\text{asc} (+ \text{gem}) IC_x}/D_{\text{asc} IC_x}) + \alpha [(D_{\text{gem} (+ \text{asc}) IC_x})(D_{\text{asc} (+ \text{gem}) IC_x})/(D_{\text{gem} IC_x})(D_{\text{asc} IC_x})]$, where D is the dose of gemcitabine and ascorbate either alone or in combination at a given constant molar ratio required to produce an IC_x level of cytotoxicity [24–26]. The more conservative assumption of mutual exclusivity was adopted ($\alpha = 0$). GraphPad Prism 5.0 was used for additional statistical analysis.

Results

In vitro dose–response relationships of gemcitabine and ascorbate either alone or in combination

Dose–response relationships for either gemcitabine or ascorbate cytotoxicity were established in seven human (BxPC-3, AsPC-1, PANC-1, MIA PaCa-2, SU.86.86, HPAF-II, Hs 766T) and one murine (PAN-02) pancreatic cancer cell line. Initial studies established a dose range when cells were exposed to serial dilutions of either gemcitabine or ascorbate and assessed for chemosensitivity after 72 h by MTT assay. Dose–response curves and IC_{50} values for each cell line are shown in Fig. 1 ordered (1A–1H) according to relative sensitivity per cell type to gemcitabine alone. PAN-02, AsPC-1, and BxPC-3 were particularly sensitive to gemcitabine; PANC-1, MIA PaCa-2, and SU.86.86 showed intermediate sensitivities to gemcitabine; and HPAF-II and Hs 766T cells were resistant to gemcitabine. Compared to gemcitabine, ascorbate IC_{50} values were approximately 30- to 10,000-fold higher across most cell lines, the exception being Hs 766T cells, in which resistance to either gemcitabine or ascorbate was approximately equivalent.

A constant ratio design was used to systematically examine combination dose–response relationships between gemcitabine and ascorbate in pancreatic carcinoma [24–26]. Molar ratios of gemcitabine:ascorbate were chosen as follows: $1:1 \times 10^4$ and $1:2 \times 10^4$ for cells sensitive to gemcitabine, $1:1 \times 10^3$ and $1:5 \times 10^3$ for cells with moderate sensitivity, and 1:5 and 1:10 for cells with strong resistance to gemcitabine. Combination data are presented in terms of gemcitabine concentration. If the gemcitabine–ascorbate combinations were more potent than gemcitabine as a single agent, then the dose–response curves would be shifted leftward relative to curves generated with gemcitabine alone. Alternatively, a right shift would indicate that ascorbate pairing with gemcitabine was less potent (antagonistic) with respect to gemcitabine monotherapy.

The results showed a left shift in the dose–response curves in gemcitabine–ascorbate combinations for all cell lines compared to the corresponding curves with gemcitabine as a single agent (Fig. 1, red lines versus blue lines, respectively; symbols represent experimentally derived data points). Furthermore, as the ratio of ascorbate to gemcitabine was increased, cell death (f_a) elicited per mole of gemcitabine was increased (Fig. 1; higher ratio, solid red lines; lower ratio, dashed red lines; for simplicity, symbols for the lower ratio are not displayed). Although the maximal ascorbate dose tested in vitro was 10 mM, this concentration rapidly declines over several hours (unpublished observations). In mice and human subjects, peak plasma ascorbate concentrations of 25–30 mM, and sustained concentrations above 10 mM for 4 h, are readily achieved by parenteral administration [10,12].

Combination index and dose-reduction index of gemcitabine and ascorbate versus gemcitabine alone

The constant ratio design facilitates using dose–response values attained experimentally as the basis for calculating curves that approximate dose–responses across all levels of cytotoxicity (f_a) [24–26]. These data can be used to determine CI and DRI. The CI relies on the isobologram principle to examine whether drug combinations may be synergistic (<1), additive ($=1$), or antagonistic (>1). Related to CI is the DRI, which measures how the dose of one drug may be reduced when used in combination with another drug(s) while retaining the same therapeutic effect. DRI values of >1 indicate a favorable combination. The greater the magnitude of the DRI, the greater the synergistic drug combination. A DRI of <1 would be interpreted as an antagonistic combination.

CI and DRI values were calculated (see Materials and methods) using data obtained from the constant ratio experiments for each cell type (see Fig. 1). As seen in Table 1, CI values at IC₅₀, IC₇₅, IC₉₀ levels of cytotoxicity ranged from additive to highly synergistic. Table 2 provides DRI_{gem} values quantifying the degree to which the dose of gemcitabine specifically was reduced when used in combination with ascorbate versus the dose of gemcitabine alone. In all cell lines tested, DRI_{gem} values were >1 at the IC₅₀ and progressively increased at IC₇₅ and IC₉₀. Importantly, the magnitude of DRI_{gem} for all cell lines increased as the ratio of ascorbate to gemcitabine increased. Taking into consideration the four most gemcitabine-resistant cells tested (MIA PaCA-2, SU.86.86, HPAF-II, Hs766T), the DRI_{gem} values at the IC₉₀ level ranged from 100 to 10⁶, indicating that gemcitabine dose could be decreased 2 to 6 logs when combined with pharmacologic concentrations of ascorbate. Collectively, there was no evidence that ascorbate impaired gemcitabine efficacy. In contrast, these data unequivocally support the conclusion that the concentration of gemcitabine can be decreased to produce an equitoxic effect on pancreatic carcinoma cells when used in combination with pharmacologic concentrations of ascorbate.

Median–effect relationships of in vitro cytotoxicity elicited by gemcitabine and ascorbate either alone or in combination

Median–effect plots were generated to provide quantitative comparisons of the cause–consequence relationships between cell lines in their responses to gemcitabine and ascorbate (Fig. 2). The slope (m) of median–effect plots can provide information regarding cooperativity of drugs in their cytotoxic action [24–26]. Relatively steeper (higher m) and left-shifted (lower IC₅₀) median–effect regression lines are indicative of greater drug potency. Drugs potentially have similar modes of action if their median–effect regression lines are parallel [25].

To construct median–effect plots for each cell line, dose–response data (Fig. 1) were log transformed as $y = \log (f_a/f_u)$ versus $x = \log (\text{dose})$. As seen in Fig. 2, the anti-log of the x intercept denotes the IC₅₀ (values given in Fig. 1), which were clearly lower (left-shifted) when ascorbate was used in combination with gemcitabine versus gemcitabine monotherapy in all cell lines tested. In each case, the median–effect slope for ascorbate was greater than the slope for gemcitabine as a single agent ($m_{\text{asc}} > m_{\text{gem}}$). In six of the eight cell types examined, m_{asc} trended toward parallelism with the median–effect slope of its respective gemcitabine and ascorbate combinations ($m_{\text{gem} + \text{asc}}$). The exceptions, which displayed a mixed pattern, were the strongly gemcitabine-resistant HPAF-II and Hs 766 T cells.

A limitation of these data obtained by MTT assay is an inability to discern changes in f_a at both the low and the high extremes of the dose–response curves. The findings that $m_{\text{asc}} > 1$ and $m_{\text{gem} + \text{asc}} \gg 1$ (Fig. 2) indicated greater sigmoidal dose–response relationships in the presence of ascorbate and may in fact underestimate combined effects for the mutually nonexclusive condition (each drug acting independent of the other) as defined by Chou and Talalay [26]. As $m_{\text{gem}} \quad m_{\text{asc}} \quad m_{\text{gem} + \text{asc}}$, exclusivity of effects cannot be firmly ascertained. Therefore, the more conservative assumption of mutual exclusivity was used in calculating CI values. The mean linear regression coefficient (r^2) values for gemcitabine, ascorbate, and collective gemcitabine + ascorbate dose responses (independent of ratio) were 0.969, 0.977, and 0.969, respectively. Linear conformity of experimental data indicated that the responses were directly related to the dose, consistent with applicability of the median–effect principle [24].

Contribution of H₂O₂ to the chemosensitization effect of pharmacologic ascorbate

Prior studies have demonstrated that H₂O₂ produced through the oxidation of ascorbate was a critical mediator of ascorbate-induced cytotoxicity [8,12,14,15,17]. To determine the

degree to which H_2O_2 mediated the effect of ascorbate in sensitizing pancreatic cancer cells to gemcitabine, MIA PaCa-2 and PANC-1 cells were treated in either the presence or the absence of catalase in the culture medium. In both cell lines, exogenously added catalase (600 U/ml) reversed the cytotoxic effect of ascorbate and gemcitabine combination by approximately 75% (Fig. 3; $**P < 0.001$ for MIA PaCa-2, $P = 0.006$ for PANC-1). A significant difference in cell viability between the ascorbate + gemcitabine + catalase group and the untreated group was still evident ($P = 0.014$ for MIA PaCa-2 and $P = 0.002$ for PANC-1). These data indicate that although H_2O_2 was the major contributor of the chemosensitization effect of ascorbate, cytotoxicity (~25%) may be associated with other factors such as ascorbate radical, superoxide, and/or other reactive species.

Relationship between epithelial-to-mesenchymal phenotype and drug sensitivity

Prior studies have shown sensitivity of pancreatic cancer cell lines to chemotherapeutic agents correlated in part with epithelial-to-mesenchymal transition (EMT) phenotype [27]. EMT in cancer cells is characterized as a change from an epithelial-like morphology to a more fibroblast-like state borne by alterations in cytoskeleton, adhesion, and motility [28]. Emblematic of this progression are both suppression of CDH1 and induction of VIM expression. To examine whether EMT phenotype underlies cytotoxic behaviors toward gemcitabine and ascorbate combination treatment, the human cell lines in our panel were characterized according to CDH1 and VIM expression levels by qRT-PCR. Using these criteria, an EMT phenotype pattern (mesenchymal to epithelial) of MIA PaCa-2 > PANC-1 > AsPC-1 > Hs 766T > SU.86.86 > BxPC-3 > HPAF-II was observed (Fig. 4A), largely consistent with previous studies [27]; PAN-02 was not tested. Relative gene expression data were transformed to graphically separate cell lines along the abscissa according to EMT phenotype versus IC_{50} response to gemcitabine and ascorbate, either alone or in combination (Fig. 4B). These plots did not reveal an association between cellular EMT phenotype and cytotoxic behaviors of gemcitabine and ascorbate.

Relationship between growth rate and drug sensitivity

To determine whether pancreatic cancer cell growth rate dictated sensitivity to gemcitabine and ascorbate, in vitro doubling times were analyzed. Data shown in Table 3 are ordered according to gemcitabine sensitivity. No correlation between growth rate and ascorbate sensitivity (IC_{50}) was observed (Pearson $r = 0.658$). In contrast, a positive correlation with growth rate was evident in the case of gemcitabine IC_{50} ($r = 0.774$) and gemcitabine–ascorbate combination IC_{50} ($r = 0.760$).

Evaluation of gemcitabine and ascorbate therapeutic combination effect in vivo

To examine the efficacy of gemcitabine and ascorbate combination treatment in vivo, PAN-02 and PANC-1 cell lines were chosen to xenograft into mice as representative gemcitabine-sensitive and gemcitabine-resistant pancreatic carcinomas. Mice were treated by intraperitoneal injection of gemcitabine, either 30 or 60 mg/kg every 4 days; ascorbate, 4 g/kg daily; corresponding combinations of both gemcitabine and ascorbate; or saline control. Similar to their in vitro counterparts, the growth rate and gemcitabine sensitivity of PAN-02 xenografts were greater than those of PANC-1 (Figs. 5A and 6A). After 21 days, PAN-02 tumor volume was decreased 38% in the ascorbate group, 74.5 and 84% in the gemcitabine groups (30 or 60 mg/kg respectively), and 84 and 90% in the gemcitabine (30 or 60 mg/kg) + ascorbate groups, each relative to saline-treated control (Figs. 5A–D). Paired two-tailed t test showed a significant difference between gemcitabine (30 mg/kg) and gemcitabine (30 mg/kg) + ascorbate treatment ($P = 0.038$), as well as between gemcitabine (60 mg/kg) and gemcitabine (60 mg/kg) + ascorbate treatment ($P = 0.021$; Fig. 5E). In Tukey's multiple comparison test (post-repeated-measures ANOVA, days 0–21), the mean difference in PAN-02 tumor volume between gemcitabine (30 mg/kg) and gemcitabine (30 mg/kg) +

ascorbate was 164 mm³ (not significant). The mean difference in tumor volume between gemcitabine (60 mg/kg) versus gemcitabine (60 mg/kg) + ascorbate was 185 mm³ ($P < 0.05$). Using paired two-tailed t test comparisons, the differences in PAN-02 tumor volume or weight in mice receiving gemcitabine alone versus gemcitabine + ascorbate treatment was significant (Figs. 5E and F).

The impact of gemcitabine and ascorbate combination treatment on mice bearing gemcitabine-resistant PANC-1 xenografts was prominent. After 33 days, PANC-1 tumor volume was decreased 36% in the ascorbate group, 4% in the gemcitabine (30 mg/kg) group, and 52% in the gemcitabine (30 mg/kg) + ascorbate group; each relative to untreated (Figs. 6A and C). Similar results were observed with the 60 mg/kg gemcitabine groups in which treatment with gemcitabine alone decreased tumor volume only 10% compared to a 55% decrease with gemcitabine + ascorbate (Figs. 6B and D). These data were highly significant by paired two-tailed t test comparing gemcitabine (either 30 or 60 mg/kg) to gemcitabine + ascorbate treatment ($P = 0.003$ for 30 mg/kg and $P = 0.002$ for 60 mg/kg). In Tukey's multiple comparison test (post-repeated-measures ANOVA, days 0–33), the mean difference in PANC-1 tumor volume for gemcitabine (30 mg/kg) versus gemcitabine (30 mg/kg) + ascorbate was 26 mm³ ($P < 0.001$). A similar difference (21 mm³) was observed between gemcitabine (60 mg/kg) and gemcitabine (60 mg/kg) + ascorbate ($P < 0.001$). These data were consistent with paired two-tailed t test comparisons, which reached $P = 0.009$ significance for PANC-1 tumor volume in mice receiving gemcitabine (60 mg/kg) alone versus gemcitabine (60 mg/kg) + ascorbate (Fig. 6E). The difference between PANC-1 tumor weight in mice receiving gemcitabine alone versus gemcitabine + ascorbate treatment was significant only in the 60 mg/kg comparison (paired two-tailed t test, $P = 0.04$; Fig. 6F).

Collectively, these data support that the combination of gemcitabine and pharmacologic ascorbate produced a treatment effect on the growth rate of PANC-1 and PAN-02 tumor xenografts in mice that was significantly greater than gemcitabine monotherapy. It was notable that despite differences in tumor xenograft gemcitabine resistance, ascorbate treatment alone exhibited ~40% inhibitory effect on the growth rate of either gemcitabine-responsive tumor PAN-02 or gemcitabine-nonresponsive tumor PANC-1. These results were consistent with previous work [12,17] and with current data showing ascorbate alone has a partial inhibitory effect on pancreatic cancers (Figs. 1, 5, and 6). No untoward side effects were observed, with the exception of osmotic load stress in some mice due to the sodium component of pharmacologic ascorbate administration into the peritoneal cavity. Mice showing dehydration signs were easily restored with a subcutaneous injection of half-normal saline. Complete blood counts did not show any significant difference among treatment groups (data not shown). Body weight did not significantly vary between groups (all mice; PAN-02, 21.0 ± 0.8 ; PANC-1, 29.3 ± 1.5). In summary, these *in vivo* data showed that combinations of gemcitabine and pharmacologic ascorbate were superior in reducing tumor volume and weight relative to gemcitabine treatment alone.

Discussion

Our strategy herein was to apply standard pharmacologic principles to evaluate the chemotherapy pairing of gemcitabine and ascorbate. The findings showed that combining clinically achievable concentrations of pharmacologic ascorbate with gemcitabine increased chemosensitivity across the spectrum of malignant phenotypes represented in our panel of pancreatic cancer cells (Figs. 1–4, Tables 1 and 2). Using a combination ratio design [24,25], this study provides evidence that pharmacologic ascorbate synergized with gemcitabine to improve therapeutic efficacy. These results were consistent with growth

inhibition responses of pancreatic carcinoma xenografts in mice that received combined gemcitabine and pharmacologic ascorbate treatment (Figs. 5 and 6).

Pharmacologic ascorbate demonstrated several novel and useful qualities that support its potential as an adjuvant to regimens for pancreatic carcinoma treatment. The combination of gemcitabine and pharmacologic ascorbate may be similar mechanistically to the pairing of gemcitabine and platin-alkylating agents such as oxaliplatin, which was shown to improve efficacy in patients with advanced pancreatic cancer [29]. In addition to forming DNA adducts, oxaliplatin is thiophilic and can generate oxidative stress [30,31], analogous to H_2O_2 produced by catalysis of pharmacologic ascorbate [12,15,32]. An important distinction is that pharmacologic ascorbate does not suffer from dose-limiting toxicity inherent to alkylating agents and other traditional chemotherapeutics. A key finding was that DRI_{gem} was consistently and substantially elevated as the ratio of ascorbate to gemcitabine was increased, in some cases by several orders of magnitude (Table 2, Fig. 1). The converse of raising the ascorbate:gemcitabine ratio is to lower the concentration of gemcitabine to produce an equitoxic effect when combined with pharmacologic concentrations of ascorbate. These in vitro findings were corroborated by data showing a gemcitabine dose-sparing effect in mice bearing PAN-02 carcinomas, in which tumor weight in the gemcitabine (30 mg/kg) + ascorbate treatment group was comparable to that from treatment with gemcitabine alone at twice the dose (60 mg/kg, Fig. 4E). The gemcitabine dose-lowering influence of pharmacologic ascorbate may lessen side effects and thereby serve to prolong gemcitabine treatment cycles and improve its efficacy.

Across the tested cell lines, Hs766T exhibited the strongest resistance to ascorbate alone, with an IC_{50} of 3.6 mM (Fig. 1). Normal pancreatic ductal epithelial cells in vitro showed no significant sensitivity to 10 mM ascorbate [16]. Moreover, toxicity was not associated with treatment of mice with pharmacologic ascorbate (4 g/kg ip daily), consistent with the lack of toxicity toward normal human tissues observed with complementary use of high-dose intravenous ascorbate [10,11]. The basis for disparate sensitivity among cancer cells, and collectively between cancer cells and normal cells, may be related to unique differences in the metabolism of glucose, iron, and reactive oxygen species [37–54].

Transformation of dose–response data obtained in a constant ratio design into median–effect plots affords the opportunity to examine potential similarities in mechanisms of action between drugs [24,25]. The pattern that emerged in this analysis (*m*; Fig. 2) suggested that the cooperativity of gemcitabine and ascorbate may be similar mechanistically to ascorbate alone. In our cell culture experiments, the oxidative cytotoxicity elicited by pharmacologic ascorbate was highest within 24 h of exposure, whereas gemcitabine took several days (48 to 72 h) to optimally manifest cell death. Although the direct congeners of ascorbate and gemcitabine were temporally disjointed, their apparent synergism demonstrated herein predicts that downstream consequences persisted far beyond the lifetime of the pharmacologic ascorbate and its transient effectors. It is tempting to link H_2O_2 produced by pharmacologic ascorbate catalysis [12,15,32] with genomic instability induced by gemcitabine. The phosphorylated metabolites of gemcitabine act as competitive inhibitors of the ribonucleotide reductase complex, which leads to a decrease in intracellular deoxyribonucleotide pools necessary for DNA replication and cell proliferation [4]. In addition to DNA synthesis, increased supplies of deoxyribonucleotides are essential for repair of DNA damage after oxidative lesioning caused by agents such as H_2O_2 [33–35]. Mutual exclusivity could not be established in this study (Fig. 2) and it was likely that a multiplicity of actions accounted for the cytotoxic behaviors of gemcitabine and ascorbate, either alone or in combination, which may be unique to each cell type.

Drugs with different mechanisms of action are desirable for use in combination chemotherapy. In contrast to the vogue for focus on a specific molecular target, the promiscuity of H_2O_2 produced by pharmacologic ascorbate catalysis has the potential to render cytotoxic effects across a wide assortment of cellular targets (DNA, protein, lipid, etc.). This may be a particularly important advantage in cancers of the pancreas, which encompass a range of malignant pancreatic neoplasms with diverse precursor origins and differentiation patterns [36]. Resistance to gemcitabine may develop at a variety of levels (nucleoside transport, phosphorylation, catabolic enzymes) [4] that are unrelated to H_2O_2 /oxidative defense, minimizing the development of cross-resistance. The broad applicability of the gemcitabine and pharmacologic ascorbate combination was illustrated by the finding that sensitization occurred with gemcitabine and ascorbate combination regardless of the epithelial–mesenchymal transition phenotype of the cells in our panel (Fig. 4). Patients with malignant cells that have progressed to the mesenchymal-like molecular phenotype typically face an aggressive and metastatic disease that is more resistant to standard therapy [27,28]. Therefore, it may be of translational importance that pharmacologic ascorbate facilitates chemosensitization responses in mesenchymal-like cell types equivalent to less invasive epithelial cell types. Our in vitro data were corroborated in mice bearing the highly mesenchymal-like PANC-1 xenografts that did not respond to gemcitabine monotherapy, whereas ~50% growth inhibition was observed in the corresponding gemcitabine + ascorbate treatment groups ($P < 0.001$; Figs. 6C and D).

Obloquy for ascorbate lingers among oncologists who cite the failed double-blind placebo-controlled trials at the Mayo Clinic in which oral administration of 10 g ascorbate daily had no effect on cancer progression [55,56]. It was not recognized at the time that intestinal absorption of ascorbate after oral ingestion becomes saturated at >0.2 g [9,57]. Only parental administration of ascorbate can provide pharmacologic concentrations and anticancer activity. Data in this study support the rationale for testing the utility of parentally administered pharmacologic ascorbate in adjuvant regimens, particularly in cancers with a relatively fulminant course. The emerging ability to decipher links between genetics, diet, and pancreatic carcinogenesis offers a promising new framework for understanding the etiology(ies) of this disease [58]. However, a striking 96% of individuals currently diagnosed with pancreatic adenocarcinoma have nonresectable, locally advanced, or metastatic disease [7], for whom few therapeutic options are available to abate a rapid disease progression. Although some have suggested interference of vitamin C with chemotherapy [59], investigators in that supporting study used the ascorbate metabolite dehydroascorbic acid, at concentrations that are toxic in humans and could not be produced from either physiologic or pharmacologic ascorbate [13,59,60]. This study clearly provides evidence that pharmacologic ascorbate can improve gemcitabine therapeutic efficacy. The optimal schedule for this and other possible drug combinations to translate pharmacologic ascorbate into the clinic requires further examination. The current data support testing of pharmacologic ascorbate in combination with therapeutic modalities including agents such as gemcitabine in double-blind placebo-controlled trials.

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Abbreviations

Asc	ascorbate
CDH1	E-cadherin

DRI	dose-reduction index
EMT	epithelial-to-mesenchymal transition
Gem	gemcitabine, or 2',2'-difluoro-2'-deoxycytidine monohydrochloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
VIM	vimentin

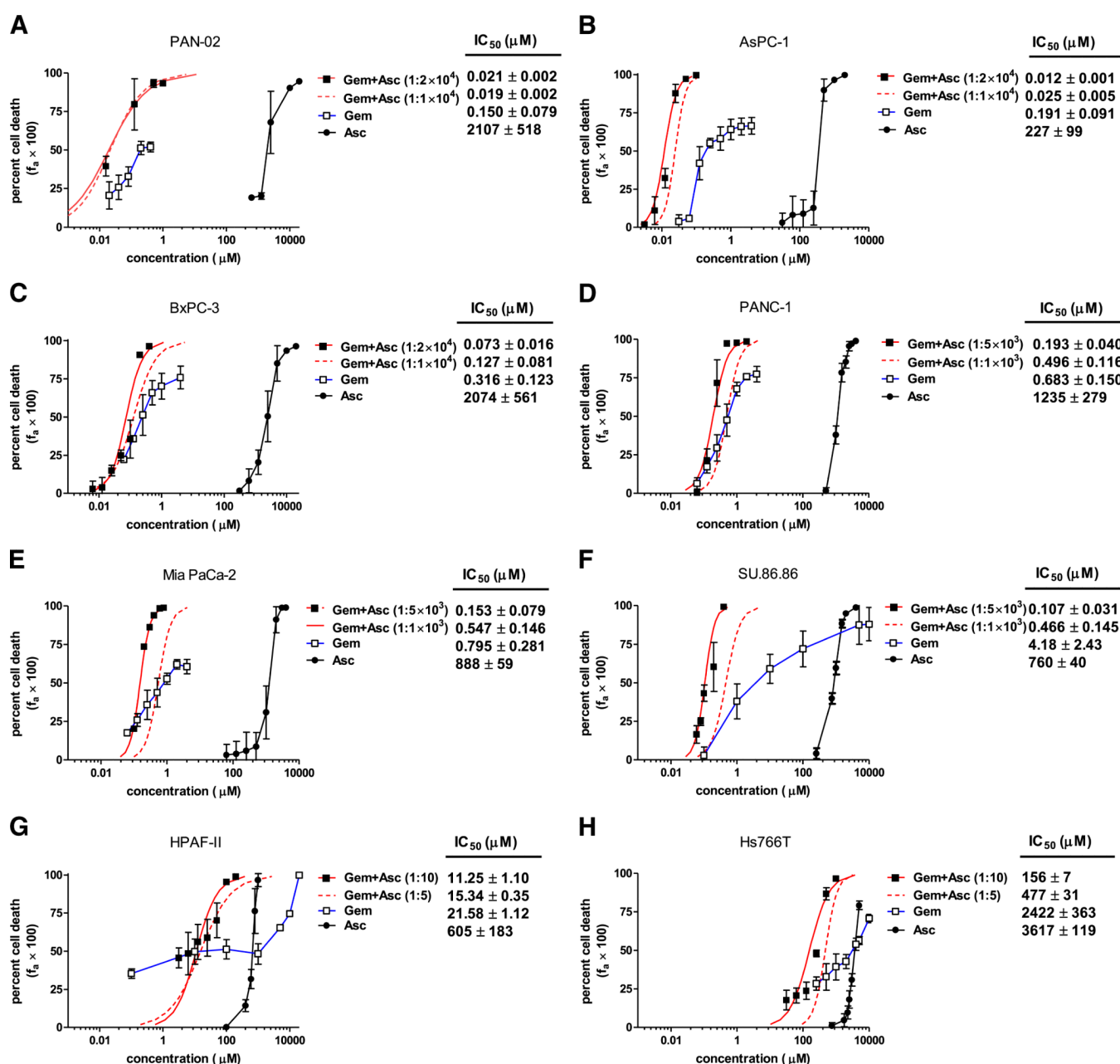
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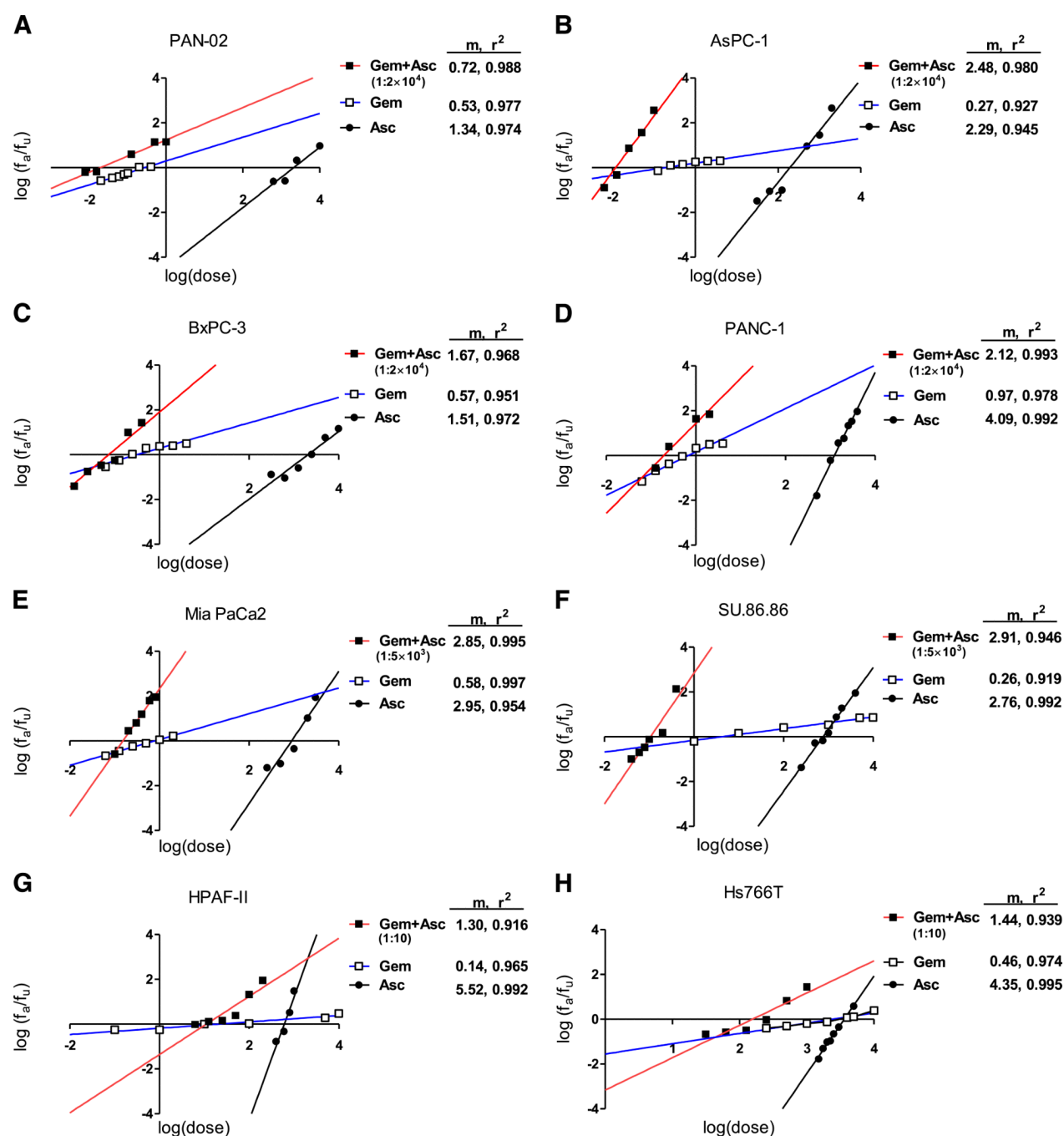
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**Fig. 1.**

Evaluation of the chemosensitizing effects of ascorbate and gemcitabine. (A–H) Pancreatic cancer cell lines were exposed to gemcitabine (Gem) and ascorbate (Asc) either alone or in combination at molar ratios, as indicated, for 72 h. For Gem + Asc combinations, concentration is plotted in terms of Gem dose. Cell death, expressed as the percentage fractional affect ($f_a \times 100$), are means \pm SD of three to six individual experiments per cell type, each done in triplicate. Symbols are experimentally derived data points. Red lines (solid and dashed) are calculated approximations from 2 to 99% f_a at the Gem + Asc ratios indicated: higher ratio, solid red lines; lower ratio, dashed red lines. Note. For simplicity, experimentally derived data points for the lower ratio were omitted. IC₅₀, concentration of drug that inhibited cell growth 50% relative to untreated control.

**Fig. 2.**

Median-effect relationships of ascorbate and gemcitabine. (A–H) Dose-response data for each pancreatic cancer cell line were transformed as $y = \log(f_a/f_u)$ versus $x = \log(\text{dose})$ for gemcitabine (Gem) and ascorbate (Asc) exposure either alone or in combination at the molar ratio as indicated for 72 h. For Gem + Asc combinations, concentration is plotted in terms of Gem dose. Anti-log of the x intercept denotes IC_{50} (see Fig. 1) [24,25]. Slope (m) and linear regression coefficient (r^2) values are shown.

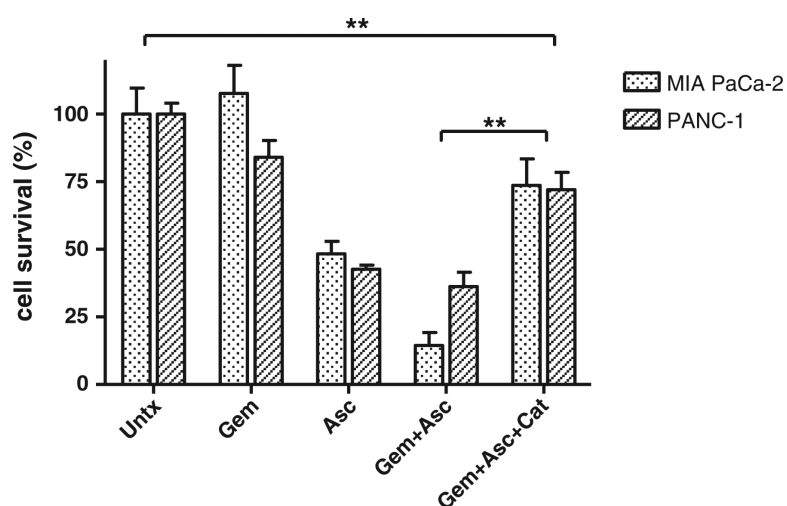


Fig. 3.

Contribution of H_2O_2 to the chemosensitization effect of gemcitabine and ascorbate combination. MIA PaCa-2 cells and PANC-1 cells in log-phase growth were either untreated (Untx) or incubated with gemcitabine (Gem; MIA PaCa-2, 0.5 μ M; PANC-1, 0.25 μ M), ascorbate (Asc; 1.25 mM), or the respective combination of Gem + Asc. Catalase (Cat; 600 U/ml) was added immediately before the drug combinations (Gem + Asc + Cat). Viability per cell type was determined from triplicate MTT assay determinations at 72 h postexposure. Data were normalized to respective Untx cells as 100% survival. ** $P < 0.01$; paired two-tailed t test.

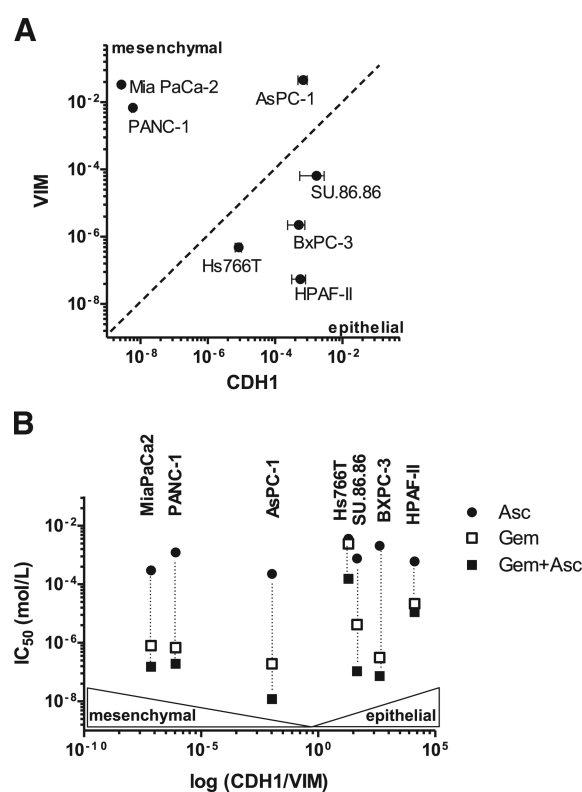
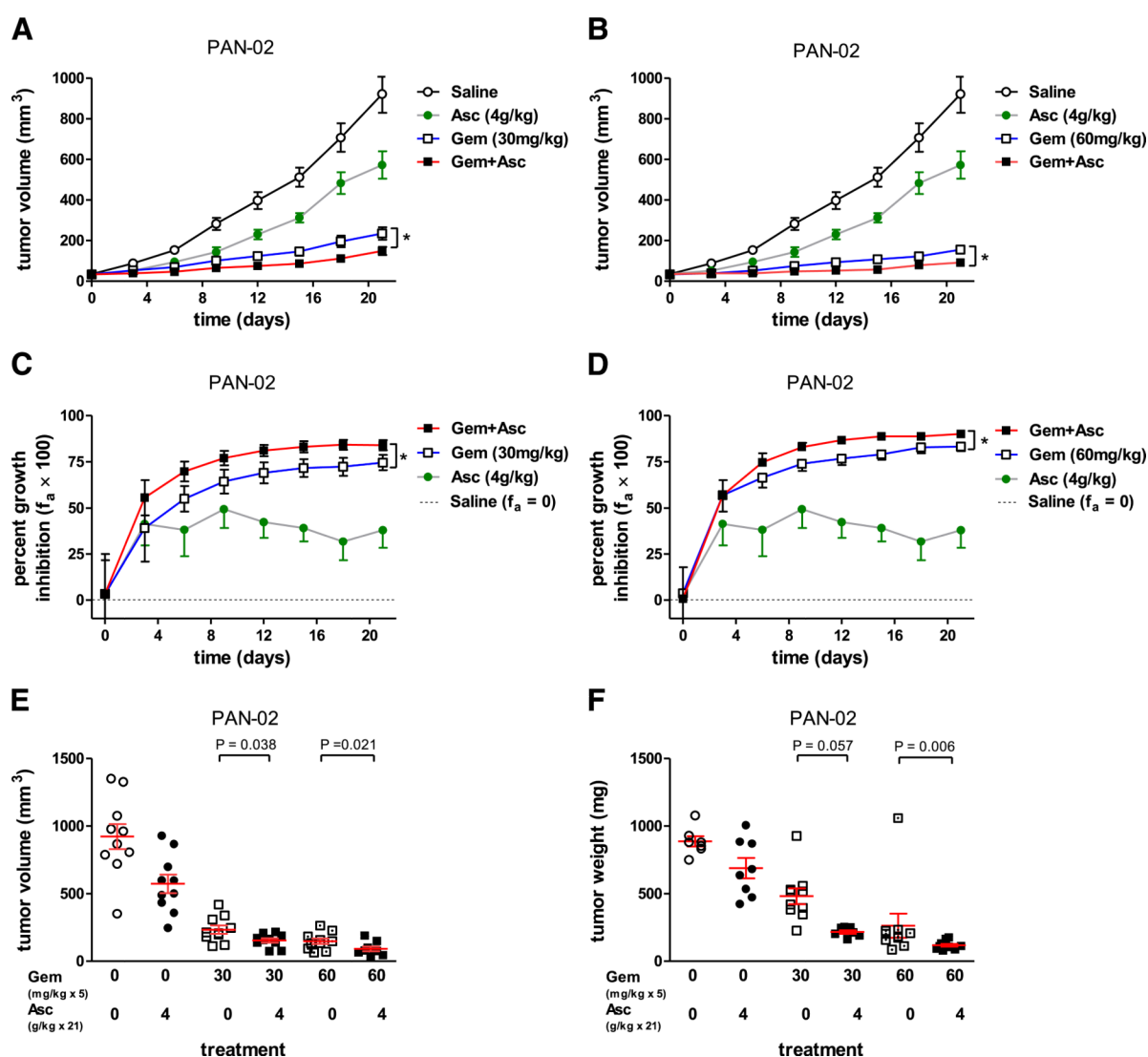
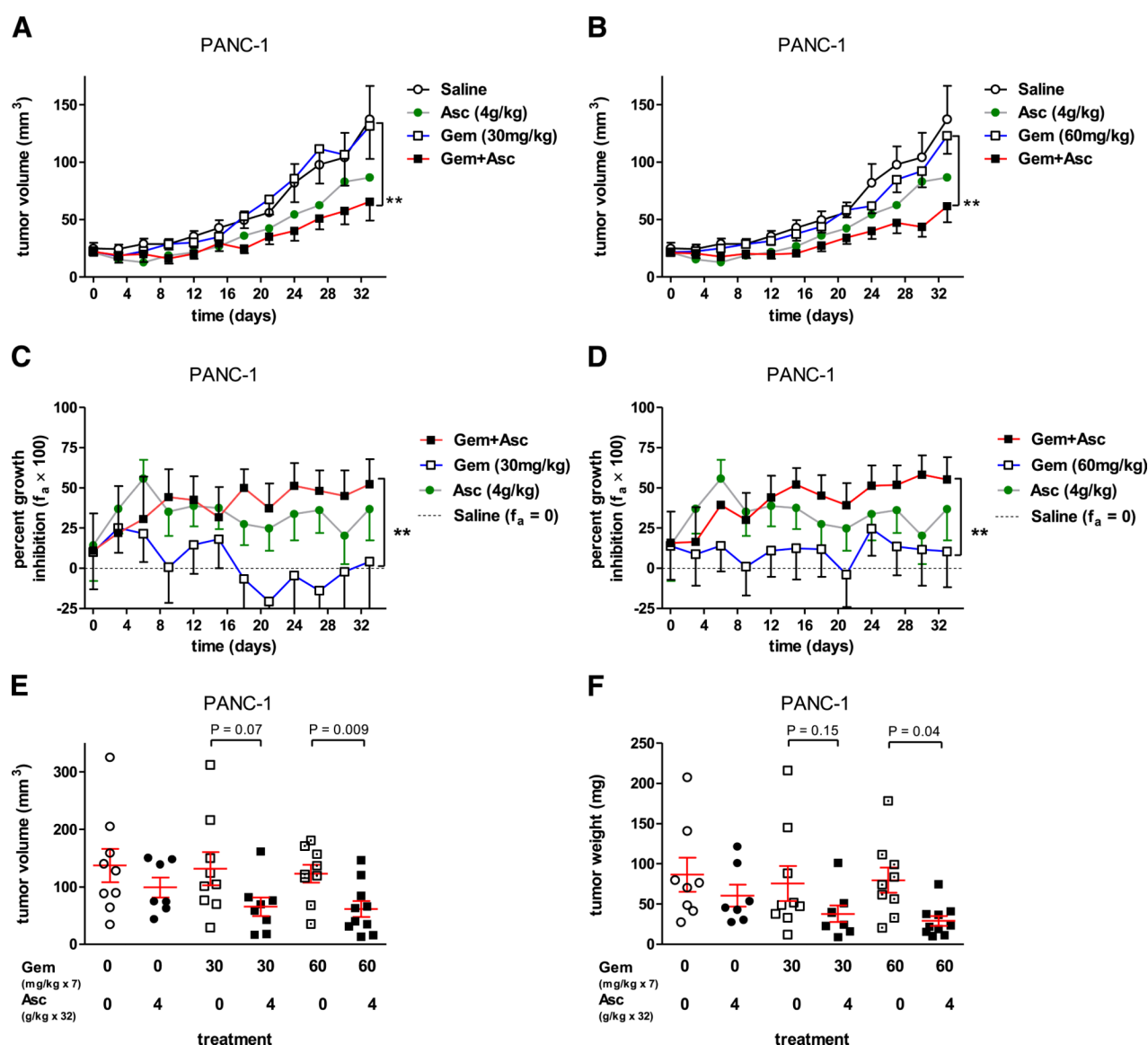


Fig. 4. Relationship between cancer cell mesenchymal–epithelial phenotype and chemosensitivity. (A) Differential expression of vimentin (VIM) and E-cadherin (CDH1) in the human pancreatic cancer cell lines indicated was determined by RT-PCR. Data were normalized to 18S mRNA/sample and represent the $2^{-\Delta\Delta C_t}$ mean of triplicate determinations of two individual experiments \pm SD. Dashed line: above, mesenchymal-like; below, epithelial-like. (B) Data are plotted as log transformation of VIM/CDH1 expression versus concentration of drug, gemcitabine (Gem) and/or ascorbate (Asc), that inhibited cell growth by 50% relative to untreated control (IC₅₀). Abscissa wedges indicate relative mesenchymal-to-epithelial phenotypes. Dashed line: length equals the magnitude of the IC₅₀ difference with Gem alone or in combination with Asc (ratios indicated in Fig. 1). For Gem + Asc combinations, concentration is plotted in terms of Gem dose.

**Fig. 5.**

Evaluation of gemcitabine and ascorbate therapeutic combination effect on PAN-02 tumors in vivo. Mice were treated by intraperitoneal injection with gemcitabine (Gem), either (A, C) 30 or (B, D) 60 mg/kg every 4 days; ascorbate (Asc), 4 g/kg daily; or saline solution osmotically equivalent to Asc as indicated. Data are expressed as the mean \pm SD of tumor volume (A, B, E), percentage of growth inhibition (treatment/saline \times 100; C, D), and weight (F) of 8–10 mice per group. * P = 0.038 (A, C), * P = 0.021 (B, D), paired two-tailed t test.

**Fig. 6.**

Evaluation of gemcitabine and ascorbate therapeutic combination effect on PANC-1 tumors in vivo. Mice were treated by intraperitoneal injection with gemcitabine (Gem), either (A, C) 30 or (B, D) 60 mg/kg every 4 days; ascorbate (Asc), 4 g/kg daily; or saline solution osmotically equivalent to Asc as indicated. Data are expressed as the mean \pm SD of tumor volume (A, B, E), percentage of growth inhibition (treatment/saline \times 100; C, D), and weight (F) of 8–10 mice per group. ** $P = 0.003$ (A, C), ** $P = 0.002$ (B, D), paired two-tailed t test.

Table 1

Combination index values for gemcitabine-ascorbate regimens in pancreatic carcinoma cells.

Cell	Gem:Asc ratio	Combination index (IC ₅₀)	Combination index (IC ₇₅)	Combination index (IC ₉₀)
PAN-02	1:1 × 10 ⁴	0.18	0.20	0.29
PAN-02	1:2 × 10 ⁴	0.25	0.41	0.78
AsPC-1	1:1 × 10 ⁴	1.20	0.98	0.89
AsPC-1	1:2 × 10 ⁴	1.10	1.01	0.97
BxPC-3	1:1 × 10 ⁴	1.01	0.87	0.92
BxPC-3	1:2 × 10 ⁴	0.94	0.72	0.63
PANC-1	1:1 × 10 ³	1.12	0.90	0.87
PANC-1	1:5 × 10 ³	1.06	1.19	1.45
MIA PaCa-2	1:1 × 10 ³	0.85	0.85	0.80
MIA PaCa-2	1:5 × 10 ³	0.91	0.91	0.89
SU.86.86	1:1 × 10 ³	0.72	0.71	0.83
SU.86.86	1:5 × 10 ³	0.72	0.69	0.67
HPAF-II	1:5	0.83	0.35	1.00
HPAF-II	1:10	0.70	0.35	0.67
Hs 766T	1:5	0.85	0.83	0.99
Hs 766T	1:10	0.49	0.72	1.19

Combination index is a quantitative measure of the degree of gemcitabine:ascorbate interaction in terms of synergism (CI < 1), additive effect (CI = 1), or antagonism (CI > 1) for a given measurement of cytotoxicity (IC₅₀, IC₇₅, IC₉₀ shown). Cytotoxicity was determined in at least three individual experiments per cell type by triplicate MTT assay determinations at 72 h postexposure.

Table 2

Gemcitabine dose reduction index (DRI) as a function of ascorbate molar ratio in pancreatic carcinoma cells.

Cell line	Gem:Asc molar ratio	DRI _{gem} IC ₅₀ (fold decrease)	DRI _{gem} IC ₇₅ (fold decrease)	DRI _{gem} IC ₉₀ (fold decrease)
PAN-02	1:1 × 10 ⁴	12.9	27.4	58.2
PAN-02	1:2 × 10 ⁴	14.4	24.8	43
AsPC-1	1:1 × 10 ⁴	7.9	288	1.0 × 10 ⁴
AsPC-1	1:2 × 10 ⁴	16.0	558	1.9 × 10 ⁴
BxPC-3	1:1 × 10 ⁴	2.5	7.0	19.6
BxPC-3	1:2 × 10 ⁴	4.3	15.4	55.4
PANC-1	1:1 × 10 ³	1.4	2.5	4.7
PANC-1	1:5 × 10 ³	3.5	6.4	11.5
MIA PaCa-2	1:1 × 10 ³	1.4	6.0	25.4
MIA PaCa-2	1:5 × 10 ³	5.2	23.9	109.6
SU.86.86	1:1 × 10 ³	8.9	349	1.4 × 10 ⁴
SU.86.86	1:5 × 10 ³	39	1813	8.4 × 10 ⁴
HPAF-II	1:5	1.4	9.88	7.0 × 10 ⁵
HPAF-II	1:10	1.9	1988	2.0 × 10 ⁶
Hs 766T	1:5	5.0	35.0	242.4
Hs 766T	1:10	15.5	79.2	403.6

DRI_{gem} represents the *fold decrease* in gemcitabine doses required to produce IC₅₀, IC₇₅, IC₉₀ levels of cytotoxicity when combined with ascorbate (at the indicated molar ratio, $D_{\text{gem}} + \text{asc}$) compared with the dose of gemcitabine alone (D_{gem}): $\text{DRI}_{\text{IC}_x} = D_{\text{gem}}$ divided by $D_{\text{gem}} + \text{asc}$. Cytotoxicity was determined in at least three individual experiments per cell type by triplicate MTT assay determinations at 72 h postexposure.

Table 3

Growth rates of pancreatic carcinoma cells.

Cell line	Doubling time (h)
PAN-02	18
AsPC-1	28
BxPC-3	42
PANC-1	31
MIA PaCa-2	24
SU.86.86	36
HPAF-II	24
Hs 766T	55

Doubling times were extrapolated from the growth rates of exponentially growing cells in culture over a period of 3 to 7 days. Cells are ordered with regard to gemcitabine sensitivity (high to low).