Science Translational Medicine
AAAS

High-Dose Parenteral Ascorbate Enhanced Chemosensitivity of Ovarian Cancer and Reduced Toxicity of Chemotherapy

Yan Ma *et al.*

Sci Transl Med **6**, 222ra18 (2014);
DOI: 10.1126/scitranslmed.3007154

Editor's Summary

Not-So-Sour Results for Cancer Patients

Ascorbic acid, or vitamin C, was first proposed as a cancer treatment decades ago. Unfortunately, despite anecdotal evidence for effectiveness of intravenous ascorbate, initial clinical trials used the oral form of the drug. On the basis of the results from these trials, ascorbate was determined to be ineffective, and its use for cancer was largely abandoned outside of alternative medicine. However, accumulating anecdotal evidence once again led scientists to reconsider the therapeutic potential of this compound.

Ma and colleagues investigated the use of intravenous ascorbic acid in conjunction with chemotherapy for ovarian cancer, starting from preclinical models and culminating in a human trial. The preclinical studies provided evidence of anticancer effects of ascorbate and demonstrated synergy with chemotherapeutic agents. The early-phase human trial was too small to statistically confirm efficacy, but it demonstrated a significant reduction in chemotherapy-induced adverse effects in patients receiving ascorbate. Although larger studies will be needed to confirm a direct anticancer effect of ascorbate, its ability to decrease chemotherapy-induced adverse effects should already make it a very valuable addition to chemotherapeutic regimens, because a reduction in toxicity would allow patients to tolerate higher (and potentially more effective) doses of chemotherapy.

Science Translational Medicine (print ISSN 1946-6234; online ISSN 1946-6242) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

CANCER

High-Dose Parenteral Ascorbate Enhanced Chemosensitivity of Ovarian Cancer and Reduced Toxicity of Chemotherapy

Yan Ma,[1,2] Julia Chapman,[3] Mark Levine,[4] Kishore Polireddy,[1,2] Jeanne Drisko,[2]* Qi Chen[1,2]*

Ascorbate (vitamin C) was an early, unorthodox therapy for cancer, with an outstanding safety profile and anecdotal clinical benefit. Because oral ascorbate was ineffective in two cancer clinical trials, ascorbate was abandoned by conventional oncology but continued to be used in complementary and alternative medicine. Recent studies provide rationale for reexamining ascorbate treatment. Because of marked pharmacokinetic differences, intravenous, but not oral, ascorbate produces millimolar concentrations both in blood and in tissues, killing cancer cells without harming normal tissues. In the interstitial fluid surrounding tumor cells, millimolar concentrations of ascorbate exert local pro-oxidant effects by mediating hydrogen peroxide (H_2O_2) formation, which kills cancer cells. We investigated downstream mechanisms of ascorbate-induced cell death. Data show that millimolar ascorbate, acting as a pro-oxidant, induced DNA damage and depleted cellular adenosine triphosphate (ATP), activated the ataxia telangiectasia mutated (ATM)/adenosine monophosphate–activated protein kinase (AMPK) pathway, and resulted in mammalian target of rapamycin (mTOR) inhibition and death in ovarian cancer cells. The combination of parenteral ascorbate with the conventional chemotherapeutic agents carboplatin and paclitaxel synergistically inhibited ovarian cancer in mouse models and reduced chemotherapy-associated toxicity in patients with ovarian cancer. On the basis of its potential benefit and minimal toxicity, examination of intravenous ascorbate in combination with standard chemotherapy is justified in larger clinical trials.

INTRODUCTION

Ascorbate (vitamin C) has long been used as an unorthodox therapy for cancer, even though the underlying scientific mechanisms were not well understood (*1*, *2*). Some early hypotheses were that cancer metastases spread through weakened collagen and that metastases could be blocked by vitamin C, which made collagen stronger (*3*), and ascorbate also inhibited the enzyme hyaluronidase, which otherwise destroyed collagen (*4*). Later in the 1970s, Cameron and Pauling reported that ascorbate (10 g/day) was effective in treating cancers (*5*, *6*), using intravenous ascorbate first and then followed by oral ascorbate. However, clinical trials conducted by Moertel and colleagues in Mayo Clinic found the same dose of ascorbate ineffective, by using it orally (*7*, *8*). It is now recognized that oral and intravenous ascorbate have different pharmacokinetics, mirroring those of vancomycin (*9*, *10*). With oral ascorbate, plasma and tissue concentrations are tightly controlled as a consequence of limited absorption, tissue transport, and renal excretion (*9*). Plasma concentrations rarely exceed 200 μM, even with oral supplementation of more than 100 times the recommended dietary allowance (*9*, *10*). By contrast, when ascorbate is injected intravenously, tight control is bypassed and pharmacologic concentrations of ascorbate are established until excess ascorbate is excreted by kidney. Plasma concentrations greater than 10 mM are safely sustained in humans for ~4 hours (*10*–*13*). When patients have normal renal function and glucose-6-phosphate dehydrogenase (G6PD) activity, toxicity is minimal even with intravenous doses as high as 1.5 g/kg, equivalent to 105 g for a 70-kg person (*2*, *12*). These data indicate that intravenous administration of pharmacologic ascorbate doses is safe and similar to drug administration. Therefore, the effect of ascorbate in cancer treatment is worth reexamining.

Recent studies revealed that, indeed, ascorbate could be a potential anticancer agent, when reaching pharmacological concentrations. Pharmacologic ascorbate concentrations, achieved through intravenous infusion, form ascorbate radicals and produce hydrogen peroxide (H_2O_2) in extracellular fluid at concentrations that are cytotoxic to many cancer cells but not normal cells (*11*, *14*, *15*). Likely, downstream reactive oxygen species (ROS) are also formed through trans-metal catalyzed reactions exemplified by Fenton chemistry and act as effectors (*16*, *17*). With rodent xenograft models, the growth of several cancers was inhibited by parenteral ascorbate treatment (*11*, *16*–*18*). High-dose intravenous ascorbate was also suggested to be effective in treating human patients, in some pathologically confirmed cancer cases (*19*, *20*). Two small clinical trials in pancreatic cancer demonstrated that 15 to 125 g per dose of intravenous ascorbate was well tolerated and suggested some efficacy (*13*, *21*). However, the reason for the selective cytotoxicity of ascorbate is not known because of a lack of understanding of the mechanism(s) of its actions. Given the current polarization of thought between the skepticism that surrounds therapeutic efficacy of vitamin C and the highly suggestive evidence in support of beneficial effects of pharmacologic ascorbate in cancer treatment, there is a critical need to establish a specific mechanism by which pharmacologic ascorbate manifests its therapeutic benefit in cancer. In the absence of such evidence, decisions concerning adoption of this innovative strategy for treatment of cancer will be very difficult. Here, we aimed to investigate

[1]Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, USA. [2]KU Integrative Medicine, University of Kansas Medical Center, Kansas City, KS 66160, USA. [3]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Kansas Medical Center, Kansas City, KS 66160, USA. [4]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
*Corresponding author. E-mail: qchen@kumc.edu (Q.C.); jdrisko@kumc.edu (J.D.).

the downstream mechanisms of ascorbate-induced cell death, and conducted an early-phase clinical trial examining safety and toxicity of high-dose intravenous ascorbate in ovarian cancer patients, combined with the conventional chemotherapeutic regimen paclitaxel and carboplatin.

RESULTS

Mechanism of ascorbate-induced cytotoxicity in ovarian cancer cells

We selected ovarian cancer as a model because of its poor prognosis in patients with metastases or chemo-resistant disease, and because of previously suggested ascorbate effect on this cancer (*20*, *22*). First, concentration-dependent cytotoxicity was tested in seven human ovarian cancer cell lines (Fig. 1A and table S1). All were susceptible to ascorbate with IC_{50}s of 0.3 to 3 mM, concentrations easily achievable by intravenous infusion. Meanwhile, an immortalized nontumorigenic ovarian epithelial cell line, HIO-80, was minimally affected. Catalase, a specific H_2O_2 scavenger, completely reversed cytotoxicity, confirming the necessity of H_2O_2 in mediating ascorbate-induced cell death.

DNA double-strand damage was observed in SHIN3 human ovarian cancer cells after ascorbate treatment, as shown by robust phosphorylation of histone 2AX (H2AX) (Fig. 1B). Phosphorylation was dependent on time and ascorbate concentration. DNA damage was assessed by quantifying DNA emitting from cell nuclei relative to remaining nuclear DNA ("Comet" assay) (*23*) (Fig. 1C and table S1). Ascorbate induced severe DNA damage alone. When ascorbate was combined with the DNA alkylating agent carboplatin, DNA damage increased. When a poly(adenosine diphosphate–ribose) polymerase (PARP) inhibitor was added to inhibit DNA repair, DNA damage further increased. DNA damage was prevented by catalase, whereas carboplatin or PARP inhibitor alone induced only minor changes. Cell death was enhanced when the PARP inhibitor olaparib was combined with ascorbate, compared to either agent alone, and was also fully reversed by catalase (Fig. 1D, fig. S1, and table S1).

Excessive oxidative stress can deplete cellular adenosine triphosphate (ATP) (*24*). In ascorbate-treated (3 mM) SHIN3 cells, there was a sharp ATP decrease within 1 hour, with gradual recovery after 6 hours, similar to findings with H_2O_2 (Fig. 1E and table S1). This transient fall of intracellular ATP is associated with cell death induced by the same concentration of ascorbate (Fig. 1A and table S1), and is consistent with autophagy and lack of caspase activity in ascorbate-induced cell death as previously reported (*16*, *25*, *26*). By contrast, in the nontumorigenic ovarian epithelial cell HIO-80, an ATP drop was absent with the same ascorbate treatment (Fig. 1E and table S1), and these cells were much more resistant to ascorbate-induced death (Fig. 1A and table S1). Different responses between cancerous and noncancerous cells might be explained by the Warburg effect (*27*). Some cancer cells rely primarily on glycolysis for ATP production so that their ATP synthesis is inefficient compared with that in normal cells that use oxidative phosphorylation. As observed here, some cancer cells are more sensitive to the metabolic stress induced by ascorbate, leading to selective cytotoxicity as a consequence of metabolic pathways characteristic of cancer cells.

Another key mediator of cellular response to DNA damage, protein kinase ATM (ataxia telangiectasia mutated), was activated by phosphorylation within 15 min of ascorbate treatment in SHIN3 cells (Fig. 1B). Phosphorylation was dependent on both ascorbate concentration and time (Fig. 1B). Phosphorylated ATM can inhibit mammalian target of rapamycin (mTOR), a central regulator of protein synthesis and cell proliferation, by activating AMPK [adenosine monophosphate (AMP)–activated protein kinase] via phosphorylation, in response to challenge by ROS (*28*). As a cell energy sensor, AMPK is also activated by a decreased ATP/AMP ratio (*29*). After ascorbate treatment, AMPKα phosphorylation was observed in a time- and concentration-dependent manner, downstream of ATM phosphorylation (Fig. 1F). Phosphorylated mTOR (p-mTOR) decreased in proportion to ascorbate concentration and time (Fig. 1F), consistent with ATM/AMPK activation. Ascorbate also decreased the total concentration of mTOR in a dose- and time-dependent manner. H_2O_2 induced similar effects in SHIN3 cells, stimulating phosphorylation of H2AX, ATM, and AMPK, and decreasing phosphorylation of mTOR (Fig. 1, B and F). Together, these data indicate that pro-oxidant ascorbate induced genotoxic (DNA damage) and metabolic stress (decreased ATP), with an end result of decreased mTOR (Fig. 1G).

Synergistic action of ascorbate with carboplatin and paclitaxel in preclinical ovarian cancer models

Building on this mechanism of action for pharmacologic ascorbate, combination treatment approaches were designed for synergy. We examined the effect of ascorbate in combination with carboplatin, the first-line chemotherapy for ovarian cancer. Carboplatin induces DNA damage by reaction of the platinum molecule with nucleophilic sites on DNA (*30*), a mechanism not directly based on ROS. As shown in Fig. 1C (table S1), addition of carboplatin to ascorbate enhanced DNA damage. To expand this finding, we examined cell survival. Using constant ratio design (*31*), we exposed human ovarian cancer cells OVCAR5, OVCAR8, and SHIN3 to ascorbate and carboplatin combinations (AA + Cp) at three different molar ratios (Fig. 2 and table S1). For comparison, the nontumorigenic ovarian epithelial cell HIO-80 was treated using the same combination ratios. AA + Cp together displayed greater cell killing [increased fraction affected (f_a)] in all three cancer cell lines compared to either drug alone (Fig. 2, A and B, and tables S1 and S2). The higher the ratio of ascorbate to carboplatin was in the combination, the larger the increase in f_a (Pearson $r = 0.861$). Whereas the ovarian epithelial cell HIO-80 was sensitive to carboplatin treatment, the addition of ascorbate to carboplatin at any tested combination ratio did not induce more cell death than carboplatin alone. Because synergy was indicated specifically in cancer cells but not normal cells, combination index (CI) for the three cancer cell lines was calculated using isobologram principles (*31*) to determine synergism ($CI < 1$), additive effect ($CI = 1$), or antagonism ($CI > 1$). An additive to synergistic effect was shown for ascorbate and carboplatin in OVCAR5 and SHIN3 cells at all combination ratios, and in OVCAR8 cells at a high ascorbate ratio (Fig. 2C and table S1). Fold decrease in carboplatin dose (DRI) increased as f_a increased, and also as the AA/Cp ratio increased (Fig. 2D and table S1). These data show that when ascorbate is added to carboplatin, the concentration of carboplatin that produces cell killing can be decreased, and synergy favors a higher ratio of ascorbate in the combination.

Synergy was further tested in an intraperitoneally implanted SHIN3 ovarian cancer in athymic mice (Fig. 3). High-dose parenteral ascorbate had no discernible adverse effects alone or with chemotherapy.

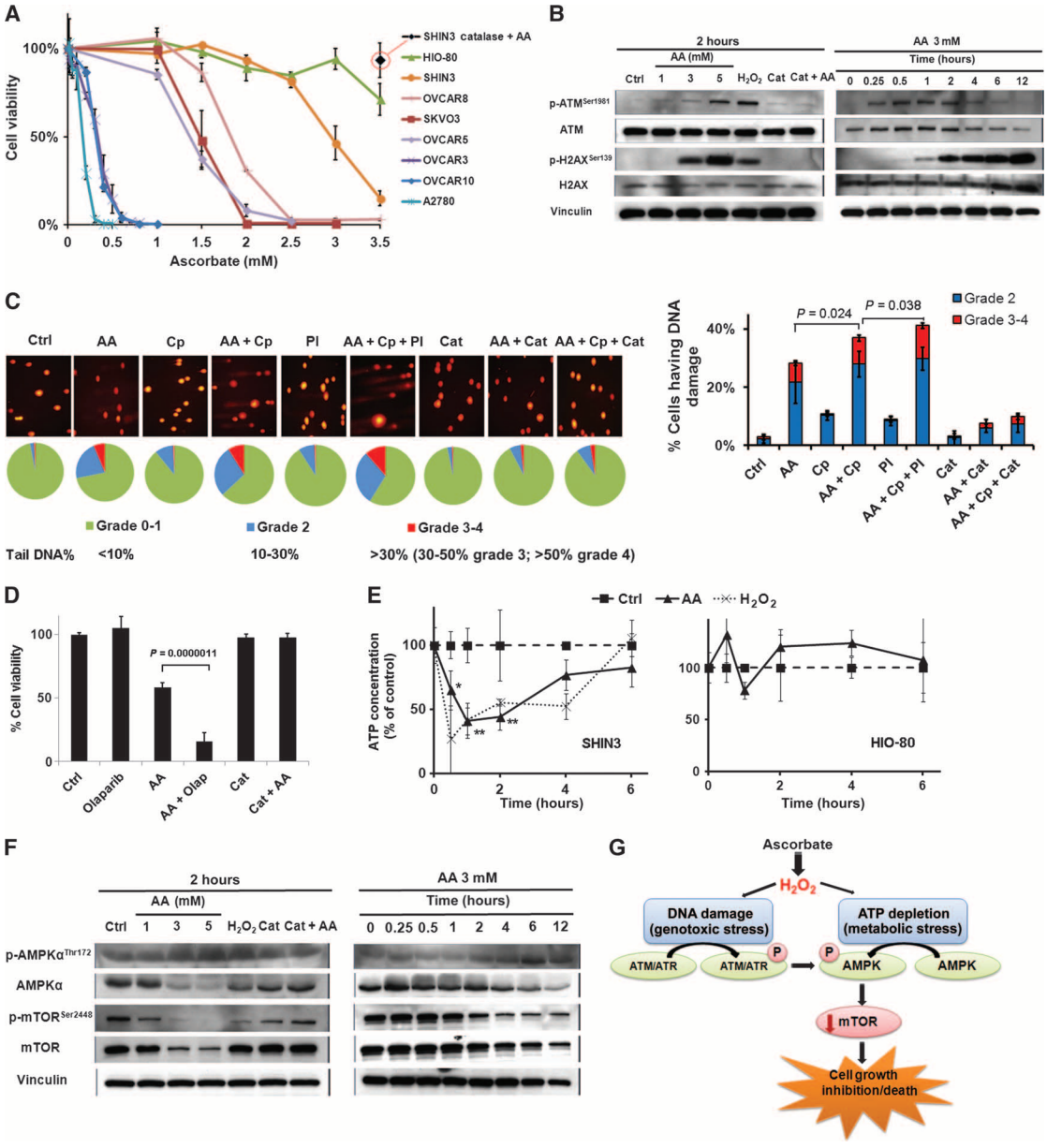

Fig. 1. Mechanisms by which ascorbate induces ovarian cancer cell death. (**A**) Cytotoxicity of pharmacologic ascorbate on human ovarian cancer cells (OVCAR10, SKVO3, OVCAR3, A2780, OVCAR5, OVCAR8, and SHIN3) and an immortalized, nontumorigenic human ovarian epithelium cell (HIO-80). Addition of catalase (600 U/ml) before ascorbate (3.5 mM) reversed the cytotoxicity of ascorbate in SHIN3 cells (diamond with red circle). Cell viability was measured at 48 hours of treatment. Data are means ± SD of two to eight independent experiments each in triplicate. (**B**) Western blot analysis showing phosphorylation of ATM and H2AX induced by ascorbate in a dose-dependent (left) and time-dependent (right) manner in SHIN3 cells. Vinculin served as a loading control. Ctrl, control; H_2O_2, hydrogen peroxide (1 mM); Cat, catalase (600 U/ml). (**C**) Grading of DNA damage (pie graphs), representative images (upper left), and percentage of cells having DNA damage (bar graphs) from single-cell DNA electrophoresis (Comet assay) showing DNA damage induced by ascorbate (AA; 2 mM, 3 hours), carboplatin (Cp; 0.8 mM, 3 hours), PARP inhibitor (PI; phenanthridine, 20 μM, 3 hours), catalase (Cat; 100 U/ml, 3 hours), and the indicated combinations. Tail DNA% was defined as 100 × tail DNA intensity/cell DNA intensity and was used for grading. At least 150 cells were graded per sample. Data in the pie graphs and the bar graphs represent an average of three independent experiments done in triplicate. The *P* values in the bar graphs are statistical comparison (*t* test) of grade 3 to 4 DNA damage. (**D**) PARP inhibitor enhanced ascorbate-induced cytotoxicity in SHIN3 cells, and catalase-reversed the cytotoxicity. AA, ascorbate (2.5 mM, 24 hours); Olap, olaparib (20 μM, 24 hours); Cat, catalase (100 U/ml, 24 hours). Data are means ± SD of five repeats. Data for longer exposure (48 hours) are shown in fig. S1. (**E**) Pharmacologic ascorbate depleted ATP in SHIN3 cells but not in HIO-80 cells. Cells were treated with 3 mM ascorbate or 500 μM H_2O_2. ATP was analyzed by an HPLC assay coupled with ultraviolet (UV) detection, normalized first to the total cellular protein in each sample, and then compared to the untreated cells (Ctrl) at the respective time point to express the result as a percentage of the control. Data are means ± SD ($n = 3$). $^*P = 0.050$, $^{**}P = 0.0038$ at 1 hour and $P = 0.0049$ at 2 hours comparing AA to control by *t* test. (**F**) Western blot analysis showing the phosphorylation of AMPKα and decrease in both mTOR and p-mTOR by ascorbate in a dose- and time-dependent manner. Vinculin served as a loading control. (**G**) Scheme of proposed mechanisms by which pharmacologic ascorbate leads to cancer cell death.

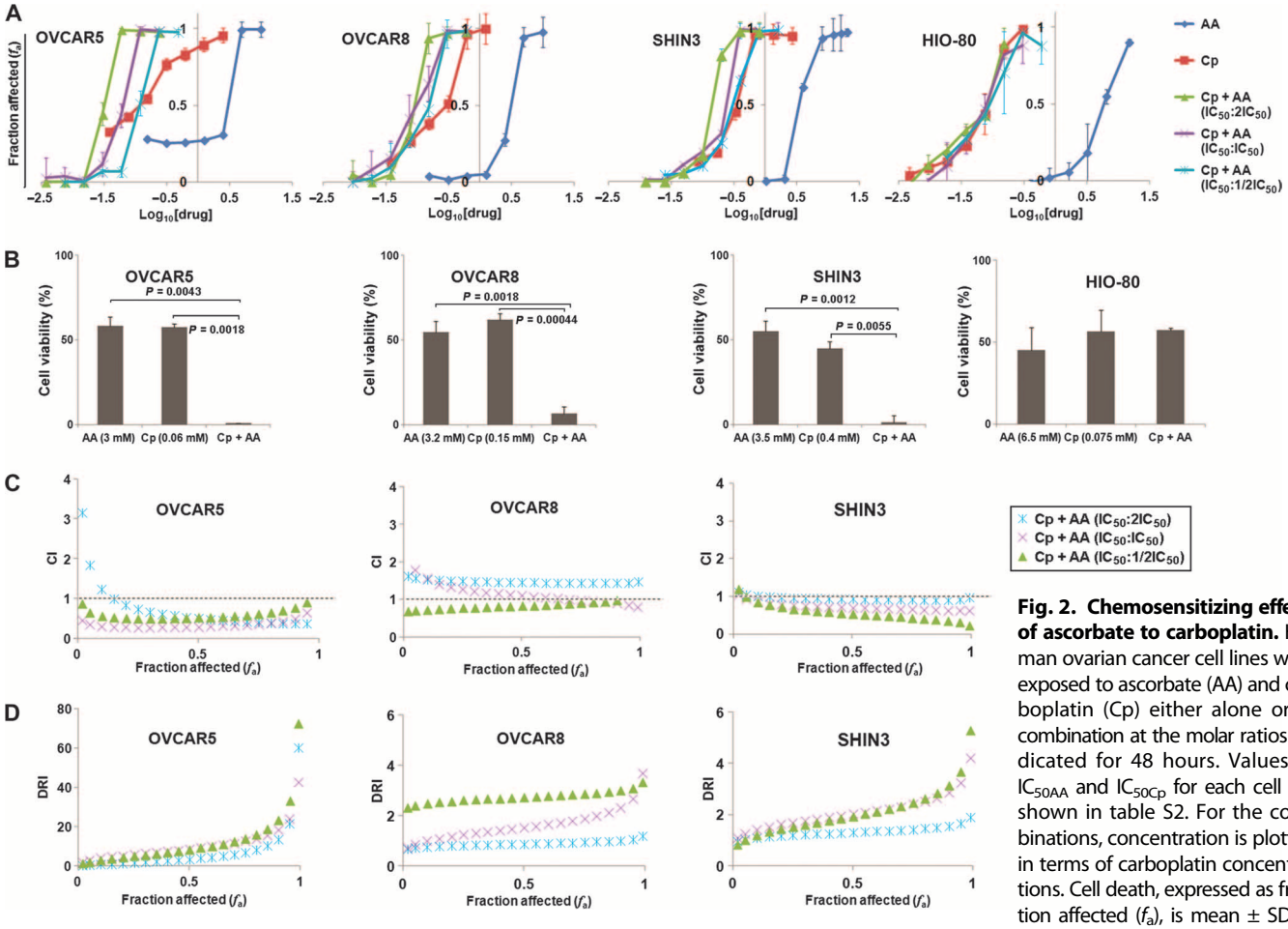

Fig. 2. Chemosensitizing effects of ascorbate to carboplatin. Human ovarian cancer cell lines were exposed to ascorbate (AA) and carboplatin (Cp) either alone or in combination at the molar ratios indicated for 48 hours. Values of IC_{50AA} and IC_{50Cp} for each cell are shown in table S2. For the combinations, concentration is plotted in terms of carboplatin concentrations. Cell death, expressed as fraction affected (f_a), is mean ± SD of two to six individual experiments per cell type, each done in triplicate. (**A**) Fraction affected (f_a) as a function of drug concentrations (Log$_{10}$[drug]). Ascorbate concentrations ranged from 0 to 20 mM, and carboplatin concentrations ranged from 0 to 3 mM. (**B**) Inhibitory effects of representative concentrations. (**C** and **D**) Combination index (CI) (C) and dose reduction index (DRI) (D) across the fraction affected (f_a) from 0.01 to 0.99, calculated by CalcuSyn 2.1 software (Biosoft), using the equation $CI = (D_{Cp(+AA)}/D_{Cp}) + (D_{AA(+Cp)}/D_{AA}) + \alpha\,[(D_{Cp(+AA)})(D_{AA(+Cp)})/(D_{Cp})(D_{AA})]$, where D is the concentration of carboplatin and ascorbate either alone or in combination at a given molar ratio to achieve a given f_a. The more conservative assumption of mutual exclusivity was adopted ($\alpha = 0$). CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. DRI values for carboplatin were calculated across the f_a by the equation $DRI = (D_{Cp}/D_{Cp+AA})$, where D_{Cp} is the concentration of carboplatin alone required to produce a given f_a, and D_{Cp+AA} is the concentration of carboplatin needed to produce the same f_a when it is combined with ascorbate. DRI > 1 indicates dose-reducing effect for carboplatin.

Compared with saline treatment controls, ascorbate alone reduced tumor burden (Fig. 3A and table S1). Ascorbate and carboplatin combination treatment (AA + Cp) was more effective compared to either ascorbate or carboplatin alone. Similar potentiation was seen with an ascorbate and paclitaxel combination (AA + Pax). Because paclitaxel and carboplatin are often combined in treating ovarian cancer patients, we tested AA + Cp + Pax. Triple combination treatment reduced tumor weight by 94% compared to controls and completely abrogated ascites formation, an effect significantly better than Cp + Pax ($P = 0.0062$ for tumor weight and $P = 0.048$ for ascites volume) (Fig. 3, A to C, and table S1). Combinations of AA + Cp, AA + Pax, and AA + Cp + Pax all showed improved effects compared to treatment with chemotherapeutic drugs alone. Inhibition of mTOR and p-mTOR in tumors was detected when ascorbate was present in any combination group (Fig. 3D), with the most inhibition in the AA + Cp + Pax group. Consistently, AMPK phosphorylation was enhanced in tumors treated with ascorbate, either alone or in combination with chemotherapeutic drugs (Fig. 3D). Although Cp + Pax treatment induced p-AMPK, the strongest induction was in the AA + Cp + Pax group. Robust increases in H2AX (a marker for DNA damage) and its phosphorylated form (pH2AX) were also evident in the AA + Cp + Pax group (Fig. 3D). Lack of difference among the other groups might be due to low sensitivity in the detection assay because tumor tissues are composed of impure cell populations and because the time for tissue collection was limited by euthanasia at 24 hours after the last drug administration, which was not ideal to capture H2AX phosphorylation.

The chemotherapeutic drugs at the doses used here did not cause observable toxicity or pathological changes in the liver, kidney, or

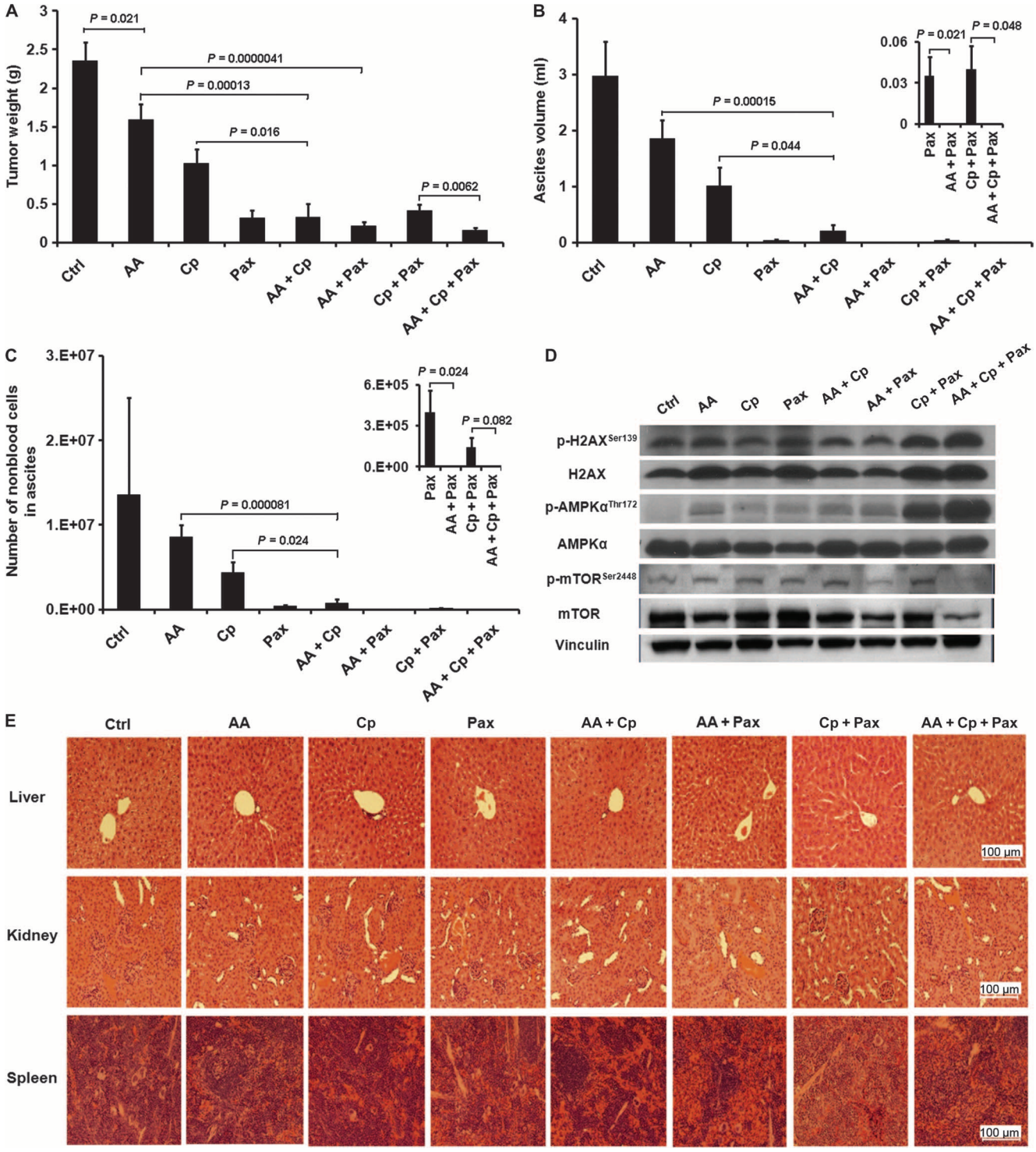

Fig. 3. Effects of ascorbate alone and in combination with chemotherapy in an ovarian cancer xenograft model. SHIN3 cells (2×10^6) were intraperitoneally inoculated into NCr-*nu*/*nu* mice. Fourteen days after tumor inoculation, treatment commenced with intraperitoneal injection of ascorbate [AA; 4 g/kg, equivalent to ~1.3 g/kg intravenously (*15*), twice every day, $n = 16$], carboplatin (Cp; 20 mg/kg per week, $n = 10$), paclitaxel (Pax; 5 mg/kg per week, $n = 10$), ascorbate and carboplatin (AA + Cp; $n = 10$), ascorbate and paclitaxel (AA + Pax; $n = 10$), ascorbate, carboplatin, and paclitaxel (AA + Cp + Pax; $n = 15$), or osmotically equivalent saline ($n = 18$). (**A** to **C**) After 25 days of treatment, mice were euthanized, and total tumor weight (A), volume of ascites (B), and nonblood cell number in ascites (C) were measured. Data are means ± SEM. (**D**) Western blot analysis showing inhibition in mTOR and p-mTOR in tumors of combination treatment groups. Vinculin served as a loading control. (**E**) Histological analysis of major organs. Liver, kidney, and spleen were fixed in 4% formaldehyde and sliced and subjected to hematoxylin and eosin (H&E) staining (magnification, ×200; scale bars, 100 μm).

spleen. Ascorbate alone or in combination with chemotherapy also did not cause any pathologic changes in the liver, kidney, or spleen (Fig. 3E).

Reduction of chemotherapy-associated toxicity by ascorbate in ovarian cancer patients

A pilot phase 1/2a clinical trial was conducted in patients with newly diagnosed stage III or IV ovarian cancer. High-dose intravenous ascorbate was added to conventional paclitaxel/carboplatin therapy, and toxicity was assessed. Twenty-seven participants were randomized into either the standard Cp + Pax arm or the Cp + Pax + AA arm. Cp + Pax chemotherapy was administered for the initial 6 months, and AA treatment for 12 months. Participants were followed for survival for 5 years. Two subjects in the Cp + Pax arm voluntarily withdrew because they wanted intravenous vitamin C, and they were excluded from data analysis. Data on 25 participants (12 in Cp + Pax arm and 13 in Cp + Pax + AA arm) were evaluated for untoward events using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events version 3 (CTCAEv3). No treatment-related grade 5 toxicity (death) occurred. Ascorbate treatment did not increase the rate of grade 3 or 4 toxicity. Moreover, grade 1 and 2 toxicities were substantially decreased in the Cp + Pax + AA group versus the Cp + Pax group (Fig. 4A and tables S1 and S3). Compared with participants treated with Cp + Pax, participants treated with Cp + Pax + AA had decreases in almost all the categories of toxicity evaluated, including neurotoxicity, bone marrow toxicity, infection, hepatobiliary/pancreatic toxicity, toxicities in the renal/genitourinary, pulmonary, and gastrointestinal systems, and dermatology (Fig. 4B and tables S1 and S4). Overall survival trended toward improvement with ascorbate addition to standard chemotherapy (Fig. 4C and table S1), and the median time for disease progression/relapse was 8.75 months longer in the Cp + Pax + AA arm than in the Cp + Pax arm (Fig. 4D and table S1), although neither one achieved statistical significance because the trial was not statistically powered to detect efficacy. These results might also have improved with more frequent ascorbate dosing (*13*).

DISCUSSION

Conventional chemotherapies have little impact on the outcomes for relapsed and chemo-resistant ovarian cancers, but there is potential to improve the treatment outcome using the CAM (complementary and alternative medicine) regimen of high-dose intravenous ascorbate as adjuvant. We investigated this possibility, and results showed that ascorbate induced ovarian cancer cell death at concentrations easily achievable clinically by intravenous infusion (*10*–*13*, *21*). Ascorbate worked synergistically in vitro and in vivo with the first-line chemotherapeutic drugs carboplatin and paclitaxel. In patients with advanced ovarian cancer, treatment with ascorbate reduced toxicities associated with chemotherapy. Because the study was not powered for detection of efficacy, statistical improvement in survival was not

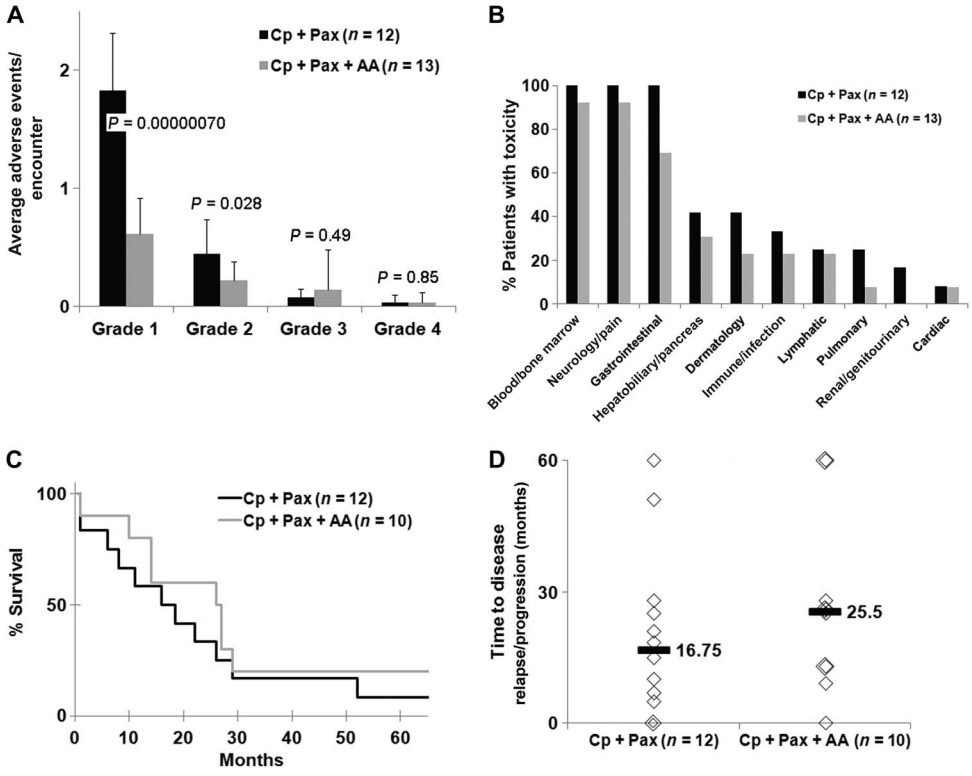

Fig. 4. Reduction of toxicity in ovarian cancer patients after adding ascorbate to chemotherapy. Cp + Pax arm: participants received standard of care chemotherapy for 6 months. Cp + Pax + AA arm: in addition to the Cp + Pax treatment, participants received intravenous AA using a dose-escalating protocol, with final dose of either 75 or 100 g per infusion depending on peak plasma concentration of each individual. The target peak plasma concentration of ascorbate was 350 to 400 mg/dl (20 to 23 mM). Once the dose was determined, participants received ascorbate infusion two times per week for a total of 12 months. The first 6 months were in conjunction with the Cp + Pax chemotherapy. Fourteen subjects were randomized to Cp + Pax arm. Two voluntarily withdrew before receiving any treatment and were excluded from data analysis. Thirteen subjects were randomized to the Cp + Pax + AA arm. Two were noncompliant with tobacco use and were removed from the arm, and another one was removed after in vitro assays detected that the subject was resistant to all chemotherapy. These three subjects received doses of chemotherapy and ascorbate, so their adverse events were counted, but they were excluded from survival follow-up. (**A**) Average adverse events per encounter for all participants and all toxicity categories. Any and all unwanted events were counted and graded for severity according to NCI CTCAEv3. Records for adverse events include patient interviews, emergency room visits, patients' oncologist visits, and hospitalization records. The number of adverse events in each grade for each participant was divided by the number of encounters of that participant, and then the adverse events per encounter were averaged in the Cp + Pax arm and the Cp + Pax + AA arm, respectively. (**B**) Percentage of participants who had toxicities in each arm. Toxicities were categorized by anatomic organ/system according to NCI CTCAEv3. All grades of toxicities were counted. More detailed data on patient toxicities are included in table S1. (**C**) Kaplan-Meier curves of overall survival at 60 months after diagnosis. (**D**) Time to disease progression or relapse for each subject. The bars represent median time of each arm.

observed. Given the advantage of low toxicity of ascorbate, larger clinical trials need to be done to definitively examine the benefit of adding ascorbate to conventional chemotherapy.

It is accepted that the antitumor effect of pharmacologic ascorbate is mediated by generation of sustainable ascorbate radical and H_2O_2 in the extracellular space (*11*, *14*, *15*). However, because of the inherent promiscuity of ROS effects on cells, it has been difficult to define a general molecular mechanism in all types of susceptible cancer cells (*32*, *33*). Different studies have suggested a variety of mechanisms in different cellular systems, including apoptosis (*34*–*36*), nonapoptotic cell death (*14*, *25*, *37*–*39*), autoschizis (*40*–*43*), ATP depletion (*26*, *44*, *45*), cell cycle arrest (*46*, *47*), and autophagy (*16*, *26*, *48*). It remains inconclusive how ascorbate exerts its selective antitumor effects (*49*). Here, we revealed that pharmacologic ascorbate-generated ROS induced DNA damage and ATP depletion, and thus triggered a series of cell responses including activation of ATM/AMPK and inhibition of mTOR. We do not exclude other molecular mechanisms because H_2O_2 could generate downstream ROS and affect various cellular and molecular targets. Different cells could have different responses. Our mechanistic data in the tested ovarian cancer cells provide new information to understand the various cellular phenomena observed previously, because it is well known that DNA damage, ATP depletion, and the ATM/AMPK/mTOR pathways are linked to suppression of cell proliferation and cell cycle arrest, as well as apoptosis, necrosis, and autophagy (*50*–*54*). Enhanced understanding of the mechanism provides rationale and strategies for synergy using pharmacologic ascorbate together with other chemotherapeutic agents. As shown in this study, the Cp + Pax + AA combination was tested clinically as a proof of concept. The mechanisms unveiled here provide many possibilities for synergy of ascorbate with targeted cancer therapies, such as PARP inhibitors and/or mTOR inhibitors.

Advantages of using ascorbate as a cancer treatment include its low toxicity and availability. The safety of high-dose intravenous ascorbate was reported in a survey of CAM practitioners (*2*) and three independent phase 1 clinical trials (*12*, *13*, *21*). Our trial demonstrated safety of longer use for 1 year. Remarkably, addition of ascorbate reduced toxicities induced by standard chemotherapy in almost all evaluated categories, without decreasing survival. On the basis of this information and positive cellular and animal data showing tumor inhibition, the efficacy of ascorbate treatment in ovarian cancer is worth investigating in larger clinical trials.

High-dose parenteral ascorbate is currently administered to thousands of patients by practitioners of CAM (*2*). With enhanced understanding of anticancer action presented here, plus a clear safety profile, biological and clinical plausibility have a firm foundation. Together, our data here provide strong evidence to justify larger and robust clinical trials for detection of efficacy combining ascorbate with conventional chemotherapy.

MATERIALS AND METHODS

Clinical trial

A prospective randomized phase 1/2a pilot trial was conducted at two sites: University of Kansas Medical Center Cancer Center in Kansas City, Kansas, and Research Medical Center Resource Center–Gynecologic in Kansas City, Missouri, between 2002 and 2007. The institutional review boards for each site approved the protocol, and all participants provided written informed consent. Oversight was provided by the U.S. Food and Drug Administration's Center for Drug Evaluation and Research, Division of Oncology Drug Products, with an Investigational New Drug assignment for injectable ascorbic acid. The trial was registered with http://www.ClinicalTrials.gov and assigned identifier NCT00228319. The primary objective was to determine the safety of high-dose intravenous ascorbate when combined with first-line chemotherapy paclitaxel and carboplatin in the treatment of advanced-stage ovarian cancer. Endpoint analysis was by NCI CTCAEv3. Patients with newly diagnosed stage III or IV ovarian cancer were subject to eligibility screening, requiring them to be ambulatory with Eastern Cooperative Oncology Group (ECOG) performance status 0 to 2; have normal G6PD status; have adequate renal, hepatic, and hematologic function; be able to receive first-line chemotherapy for duration prescribed; and not use tobacco products. Of the 27 subjects randomized, 22 completed the trial (81.5%). Two subjects voluntarily withdrew from the Cp + Pax arm before treatment commenced because they wanted intravenous vitamin C, and they were excluded from data analysis. Two subjects were removed from the Cp + Pax + AA arm because they were noncompliant with tobacco use, and one was removed from the Cp + Pax + AA arm after in vitro cytotoxic assays detected that her tumor cells were resistant to all chemotherapy. These three subjects received doses of chemotherapy and ascorbate, so their adverse events were counted, but they were excluded from the survival report (table S3). Double blinding was used at enrollment and randomization, but was not maintained during the treatment because no placebo control was used.

All participants received Cp + Pax chemotherapy according to standard of care, and all doses were administered in either the University of Kansas Cancer Center or the oncology clinic of the oncologist coinvestigator. Ascorbate dose for the Cp + Pax + AA arm was established via dose escalation initiated at 15 g per infusion titrated up to a therapeutic range of 75 or 100 g per infusion, with a target peak plasma concentration of 350 to 400 mg/dl (20 to 23 mM) (*12*, *13*). The ascorbate infusion was given at a rate of 0.5 g/min, and 400 mg of magnesium chloride (Wellness Pharma) was supplemented into each infusion. Once the therapeutic dose was established, the Cp + Pax + AA group received ascorbate two times per week in conjunction with chemotherapy for 6 months, and injectable ascorbate was continued for another 6 months after chemotherapy completion.

Mouse xenografts and treatment

All procedures were conducted under an Animal Care and Use Protocol approved by the Animal Care and Use Committee of the University of Kansas Medical Center. Two million SHIN3 cells were injected intraperitoneally into 5-week-old female NCr-*nu*/*nu* mice (National Cancer Institute–Frederick). Two weeks after tumor inoculation, mice were randomized into eight groups and treatment commenced with intraperitoneal injection as follows: (i) control, saline twice daily; (ii) AA, ascorbate at 4 g/kg twice daily; (iii) Cp, carboplatin at 20 mg/kg once per week; (iv) Pax, paclitaxel at 5 mg/kg once per week; (v) AA + Cp; (vi) AA + Pax; (vii) Cp + Pax; and (viii) AA + Cp + Pax. For combination treatments, carboplatin was injected immediately after ascorbate. Paclitaxel was injected the day after ascorbate and carboplatin injection, and ascorbate was skipped on the day paclitaxel was given. Twenty-five days after the initiation of treatment, all mice were euthanized and gross necropsy was performed, with determination of tumor weights, ascites volumes, and the number of nonblood cells in ascites fluid. Nonblood cell counts in ascites reflect ascites tumor cell numbers. Three major organs (liver,

kidney, and spleen) were subjected to histological analysis using H&E staining. Frozen tumor tissues were then subjected to Western blot analysis.

In vitro drug combination evaluation

On the basis of Chou-Talalay's median-effect plots and isobologram principles (*31*), ascorbate and carboplatin combinations were examined at three molar ratios: IC_{50Cp}:(1/2 × IC_{50AA}), IC_{50Cp}:IC_{50AA}, and IC_{50Cp}:(2 × IC_{50AA}). Cells were exposed to serial dilutions of each single drug or their combinations at the set molar ratios. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) data were normalized to their corresponding untreated controls for each condition (drug, cell type) and were expressed as percentage fractional affect (f_a). CalcuSyn 2.1 (Biosoft) was used to calculate CI values across f_a (0.02 to 0.99) by the equation $CI = (D_{Cp(+AA)}/D_{Cp}) + (D_{AA(+Cp)}/D_{AA}) + \alpha[(D_{Cp(+AA)})(D_{AA(+Cp)})/(D_{Cp})(D_{AA})]$, where D is the concentration of carboplatin and ascorbate either alone or in combination at a given molar ratio to achieve a given f_a. The more conservative assumption of mutual exclusivity was adopted ($\alpha = 0$). DRI values for carboplatin were calculated across the f_a by the equation $DRI = (D_{Cp}/D_{Cp+AA})$, where D_{Cp} is the concentration of carboplatin alone required to produce a given f_a, and D_{Cp+AA} is the concentration of carboplatin needed to produce the same f_a when it is combined with ascorbate.

Comet assay

Comet assay was performed under neutral conditions as described (*55*) with minor modifications. Briefly, 2×10^4 cells in 10 μl of phosphate-buffered saline (PBS) buffer were mixed 1:9 with 0.5% low–melting point agarose (Promega), pipetted onto a slide precoated with 1% normal–melting point agarose, and allowed to solidify at 4°C. Solidified slides were immersed in cell lysis solution [2.5 M NaCl, 0.1 M EDTA, 10 mM tris-HCl, 1% Triton X-100, and 10% dimethyl sulfoxide (DMSO), pH 10.0] at 4°C for 1.5 hours, followed by DNA denaturation solution (300 mM NaOH, 1 mM EDTA, pH >13) for 40 min, and tris-base buffer (89 mM tris-base, 89 mM boric acid, 2 mM EDTA) at 4°C for 10 min. Electrophoresis was performed for 20 min at 1 V/cm. Slides were washed, and then gels were dehydrated in cold 70% ethanol for 5 min, air-dried, and stained with ethidium bromide (5 μg/ml). Slides were immediately examined under an Olympus IX71 fluorescence microscope equipped with a DP71 charge-coupled device camera (Olympus). Images were acquired using DP controller software (Olympus). At least 150 cells in 20 randomly selected fields were evaluated per sample. DNA damage was graded by tail DNA%, which is defined as 100 × tail DNA intensity/cell DNA intensity (grade 0 = 0%; grade 1 = 0 to 10%; grade 2 = 10 to 30%; grade 3 = 30 to 50%; grade 4 >50%).

ATP detection

Cellular ATP was extracted by rapidly lysing cells in 0.05 M KOH and then immediately neutralizing to pH 6 with 0.1 M KH_2PO_4. The supernatant was analyzed using a gradient high-performance liquid chromatography (HPLC) method on a Waters e2695 HPLC with UV detection at 254 and 340 nm (Waters 2489 diode array UV detector). Reversed-phase chromatography was performed with an XBridge C18 column 3.5 mm (Waters). The mobile phase (pH 6) contained acetonitrile (2% for solvent A and 30% for solvent B), 0.1 M KH_2PO_4, and 0.008 M tetrabutylammonium hydrogen sulfate. The ratios of solvent A to solvent B at 0, 4, 7, 12, 15, and 22 min were 100:0, 90:10, 80:20, 60:40, 0:100, and 100:0, respectively. With 8-min post-run wash, the total run time was 30 min per sample. Empower II software (Waters) was used for instrument control and data analysis. All values were normalized to the protein content of the whole-cell lysate detected by the bicinchoninic acid protein method (Pierce Biotechnology).

Reagents, cells, and chemosensitivity assay

Clinically used ascorbic acid injection was purchased from Mylan (previously Bioniche Pharma). L-Ascorbic acid (Sigma-Aldrich) for laboratory use was prepared as 1 M stock solution in sterile water, with sodium hydroxide added dropwise to adjust pH to 7.0. Aliquots stored at −80°C were thawed for single use. Catalase, carboplatin, and phenanthridine were purchased from Sigma-Aldrich; olaparib was from Selleck; and paclitaxel was from LC Laboratories. All other reagents and chemicals were obtained from Fisher Scientific unless specifically indicated.

Human ovarian cancer cell lines OVCAR8 and SHIN3 were provided by P. Eck of the University of Manitoba, Canada; OVCAR3, OVCAR5, OVCAR10, SKVO3, A2780, and HIO-80 (an immortalized, nontumorigenic human ovarian epithelium cell line) were provided by A. Godwin of the University of Kansas Cancer Center. All cancer cell lines were cultured in medium recommended by the provider (RPMI 1640 or Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS). HIO-80 was cultured in M199/MCDB105 medium (volume ratio, 1:1) containing 4% FBS, insulin (0.3 U/ml), and 2 mM L-glutamine.

Cell viability was detected by MTT assay using 96-well plates (*56*). Seeded cell density was 1×10^4 per well. Cells were treated with ascorbate, chemotherapeutic drugs, or a combination of the two as indicated in each figure for 48 hours, washed with PBS, and incubated with MTT for 4 hours. Formazan crystals that formed were dissolved in DMSO, and absorbance at 492 nm was determined with BioTek Synergy 4 microplate reader. Inhibitory concentration (IC_{50}) was defined as the median concentration of drug that inhibited cell growth by 50% relative to the untreated control.

Western blots

Cells or mouse tumor tissues were lysed on ice in radioimmunoprecipitation assay buffer [25 mM tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1× Pierce protease and phosphatase inhibitor], followed by homogenization and sonication, and then centrifugation at 20,000*g* for 10 min at 4°C. SDS–polyacrylamide gel electrophoresis (12%) was used, and 45 μg of protein was loaded per sample. Antibodies were from Cell Signaling Technology except for anti–phospho-$H2AX^{Ser139}$, which was from Millipore. Dilutions were as follows: anti-ATM, 1:1000; phospho-$ATM^{Ser1981}$, 1:500; H2AX, 1:500; phospho-$H2AX^{Ser139}$, 1:1000; AMPKα, 1:1000; phospho-$AMPK\alpha^{Thr172}$, 1:500; mTOR, 1:1000; phospho-$mTOR^{Ser2448}$, 1:500; vinculin, 1:1000; and all secondary antibodies, 1:5000. Each immunoblot was performed three times to confirm results. Images were analyzed with the National Institutes of Health (NIH) ImageJ software (version 1.46).

Statistical analysis

Two-tailed Student's *t* test was performed for comparison of treated groups to control group in the cell and animal experiments, as well as for toxicity comparison between chemotherapy group and chemotherapy + ascorbate group. Welch's *t* test was used when the variances in the two compared populations were unequal. A log-rank test (*57*) was performed for comparison of the survival curves between the chemotherapy group and the chemotherapy + ascorbate group.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/222/222ra18/DC1

Fig. S1. Effects of PARP inhibitor and catalase on ascorbate-induced cytotoxicity in ovarian cancer cells.

Table S1. Original data (provided as an Excel file).

Table S2. IC_{50} of ascorbate and carboplatin as single-drug or combination treatment.

Table S3. Number of patient encounters, adverse events, and duration of adverse event record.

Table S4. Number of patients showing adverse events in each category.

REFERENCES AND NOTES

1. M. J. González, J. R. Miranda-Massari, E. M. Mora, A. Guzmán, N. H. Riordan, H. D. Riordan, J. J. Casciari, J. A. Jackson, A. Román-Franco, Orthomolecular oncology review: Ascorbic acid and cancer 25 years later. *Integr. Cancer Ther.* **4**, 32–44 (2005).
2. S. J. Padayatty, A. Y. Sun, Q. Chen, M. G. Espey, J. Drisko, M. Levine, Vitamin C: Intravenous use by complementary and alternative medicine practitioners and adverse effects. *PLOS One* **5**, e11414 (2010).
3. W. J. McCormick, Cancer: The preconditioning factor in pathogenesis; a new etiologic approach. *Arch. Pediatr.* **71**, 313–322 (1954).
4. E. Cameron, D. Rotman, Ascorbic acid, cell proliferation, and cancer. *Lancet* **1**, 542 (1972).
5. E. Cameron, L. Pauling, Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3685–3689 (1976).
6. E. Cameron, L. Pauling, Supplemental ascorbate in the supportive treatment of cancer: Reevaluation of prolongation of survival times in terminal human cancer. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4538–4542 (1978).
7. E. T. Creagan, C. G. Moertel, J. R. O'Fallon, A. J. Schutt, M. J. O'Connell, J. Rubin, S. Frytak, Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. *N. Engl. J. Med.* **301**, 687–690 (1979).
8. C. G. Moertel, T. R. Fleming, E. T. Creagan, J. Rubin, M. J. O'Connell, M. M. Ames, High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. *N. Engl. J. Med.* **312**, 137–141 (1985).
9. M. Levine, C. Conry-Cantilena, Y. Wang, R. W. Welch, P. W. Washko, K. R. Dhariwal, J. B. Park, A. Lazarev, J. F. Graumlich, J. King, L. R. Cantilena, Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3704–3709 (1996).
10. S. J. Padayatty, H. Sun, Y. Wang, H. D. Riordan, S. M. Hewitt, A. Katz, R. A. Wesley, M. Levine, Vitamin C pharmacokinetics: Implications for oral and intravenous use. *Ann. Intern. Med.* **140**, 533–537 (2004).
11. Q. Chen, M. G. Espey, A. Y. Sun, C. Pooput, K. L. Kirk, M. C. Krishna, D. B. Khosh, J. Drisko, M. Levine, Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 11105–11109 (2008).
12. L. J. Hoffer, M. Levine, S. Assouline, D. Melnychuk, S. J. Padayatty, K. Rosadiuk, C. Rousseau, L. Robitaille, W. H. Miller Jr., Phase I clinical trial of i.v. ascorbic acid in advanced malignancy. *Ann. Oncol.* **19**, 1969–1974 (2008).
13. D. A. Monti, E. Mitchell, A. J. Bazzan, S. Littman, G. Zabrecky, C. J. Yeo, M. V. Pillai, A. B. Newberg, S. Deshmukh, M. Levine, Phase I evaluation of intravenous ascorbic acid in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. *PLOS One* **7**, e29794 (2012).
14. Q. Chen, M. G. Espey, M. C. Krishna, J. B. Mitchell, C. P. Corpe, G. R. Buettner, E. Shacter, M. Levine, Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 13604–13609 (2005).
15. Q. Chen, M. G. Espey, A. Y. Sun, J. H. Lee, M. C. Krishna, E. Shacter, P. L. Choyke, C. Pooput, K. L. Kirk, G. R. Buettner, M. Levine, Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8749–8754 (2007).
16. J. Du, S. M. Martin, M. Levine, B. A. Wagner, G. R. Buettner, S. H. Wang, A. F. Taghiyev, C. Du, C. M. Knudson, J. J. Cullen, Mechanisms of ascorbate-induced cytotoxicity in pancreatic cancer. *Clin. Cancer Res.* **16**, 509–520 (2010).
17. J. Verrax, P. B. Calderon, Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects. *Free Radic. Biol. Med.* **47**, 32–40 (2009).
18. H. B. Pollard, M. A. Levine, O. Eidelman, M. Pollard, Pharmacological ascorbic acid suppresses syngeneic tumor growth and metastases in hormone-refractory prostate cancer. *In Vivo* **24**, 249–255 (2010).
19. S. J. Padayatty, H. D. Riordan, S. M. Hewitt, A. Katz, L. J. Hoffer, M. Levine, Intravenously administered vitamin C as cancer therapy: Three cases. *CMAJ* **174**, 937–942 (2006).
20. J. A. Drisko, J. Chapman, V. J. Hunter, The use of antioxidants with first-line chemotherapy in two cases of ovarian cancer. *J. Am. Coll. Nutr.* **22**, 118–123 (2003).
21. 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, Pharmacological ascorbate with gemcitabine for the control of metastatic and node-positive pancreatic cancer (PACMAN): Results from a phase I clinical trial. *Cancer Chemother. Pharmacol.* **71**, 765–775 (2013).
22. B. J. Monk, R. L. Coleman, Changing the paradigm in the treatment of platinum-sensitive recurrent ovarian cancer: From platinum doublets to nonplatinum doublets and adding antiangiogenesis compounds. *Int. J. Gynecol. Cancer* **19** (Suppl. 2), S63–S67 (2009).
23. N. P. Singh, M. T. McCoy, R. R. Tice, E. L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**, 184–191 (1988).
24. Y. J. Lee, E. Shacter, Oxidative stress inhibits apoptosis in human lymphoma cells. *J. Biol. Chem.* **274**, 19792–19798 (1999).
25. J. Verrax, M. Delvaux, N. Beghein, H. Taper, B. Gallez, P. Buc Calderon, Enhancement of quinone redox cycling by ascorbate induces a caspase-3 independent cell death in human leukaemia cells. An in vitro comparative study. *Free Radic. Res.* **39**, 649–657 (2005).
26. P. Chen, J. Yu, B. Chalmers, J. Drisko, J. Yang, B. Li, Q. Chen, Pharmacological ascorbate induces cytotoxicity in prostate cancer cells through ATP depletion and induction of autophagy. *Anticancer Drugs* **23**, 437–444 (2011).
27. O. Warburg, F. Wind, E. Negelein, The metabolism of tumors in the body. *J. Gen. Physiol.* **8**, 519–530 (1927).
28. A. Alexander, S. L. Cai, J. Kim, A. Nanez, M. Sahin, K. H. MacLean, K. Inoki, K. L. Guan, J. Shen, M. D. Person, D. Kusewitt, G. B. Mills, M. B. Kastan, C. L. Walker, ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4153–4158 (2010).
29. S. B. Rothbart, A. C. Racanelli, R. G. Moran, Pemetrexed indirectly activates the metabolic kinase AMPK in human carcinomas. *Cancer Res.* **70**, 10299–10309 (2010).
30. K. C. Micetich, D. Barnes, L. C. Erickson, A comparative study of the cytotoxicity and DNA-damaging effects of *cis*-(diammino)(1,1-cyclobutanedicarboxylato)-platinum(II) and *cis*-diamminedichloroplatinum(II) on L1210 cells. *Cancer Res.* **45**, 4043–4047 (1985).
31. T. C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* **58**, 621–681 (2006).
32. M. G. Espey, P. Chen, B. Chalmers, J. Drisko, A. Y. Sun, M. Levine, Q. Chen, Pharmacologic ascorbate synergizes with gemcitabine in preclinical models of pancreatic cancer. *Free Radic. Biol. Med.* **50**, 1610–1619 (2011).
33. M. Levine, S. Padayatty, M. Espey, Vitamin C: A concentration-function approach yields pharmacology and therapeutic discoveries. *Adv. Nutr.* **2**, 78–88 (2011).
34. R. Carosio, G. Zuccari, I. Orienti, S. Mangraviti, P. G. Montaldo, Sodium ascorbate induces apoptosis in neuroblastoma cell lines by interfering with iron uptake. *Mol. Cancer* **6**, 55 (2007).
35. S. W. Hong, D. H. Jin, E. S. Hahm, S. H. Yim, J. S. Lim, K. I. Kim, Y. Yang, S. S. Lee, J. S. Kang, W. J. Lee, W. K. Lee, M. S. Lee, Ascorbate (vitamin C) induces cell death through the apoptosis-inducing factor in human breast cancer cells. *Oncol. Rep.* **18**, 811–815 (2007).
36. S. Y. Lin, W. W. Lai, C. C. Chou, H. M. Kuo, T. M. Li, J. G. Chung, J. H. Yang, Sodium ascorbate inhibits growth via the induction of cell cycle arrest and apoptosis in human malignant melanoma A375.S2 cells. *Melanoma Res.* **16**, 509–519 (2006).
37. J. S. Kang, D. Cho, Y. I. Kim, E. Hahm, Y. Yang, D. Kim, D. Hur, H. Park, S. Bang, Y. I. Hwang, W. J. Lee, L-Ascorbic acid (vitamin C) induces the apoptosis of B16 murine melanoma cells via a caspase-8–independent pathway. *Cancer Immunol. Immunother.* **52**, 693–698 (2003).
38. S. Ohtani, A. Iwamaru, W. Deng, K. Ueda, G. Wu, G. Jayachandran, S. Kondo, E. N. Atkinson, J. D. Minna, J. A. Roth, L. Ji, Tumor suppressor 101F6 and ascorbate synergistically and selectively inhibit non–small cell lung cancer growth by caspase-independent apoptosis and autophagy. *Cancer Res.* **67**, 6293–6303 (2007).
39. J. Verrax, J. Cadrobbi, C. Marques, H. Taper, Y. Habraken, J. Piette, P. B. Calderon, Ascorbate potentiates the cytotoxicity of menadione leading to an oxidative stress that kills cancer cells by a non-apoptotic caspase-3 independent form of cell death. *Apoptosis* **9**, 223–233 (2004).
40. J. M. Jamison, J. Gilloteaux, H. S. Taper, J. L. Summers, Evaluation of the in vitro and in vivo antitumor activities of vitamin C and K-3 combinations against human prostate cancer. *J. Nutr.* **131**, 158S–160S (2001).
41. J. Gilloteaux, J. M. Jamison, D. Arnold, E. Ervin, L. Eckroat, J. J. Docherty, D. Neal, J. L. Summers, Cancer cell necrosis by autoschizis: Synergism of antitumor activity of vitamin C: Vitamin K3 on human bladder carcinoma T24 cells. *Scanning* **20**, 564–575 (1998).
42. J. Gilloteaux, J. M. Jamison, D. Arnold, H. S. Taper, J. L. Summers, Ultrastructural aspects of autoschizis: A new cancer cell death induced by the synergistic action of ascorbate/menadione on human bladder carcinoma cells. *Ultrastruct. Pathol.* **25**, 183–192 (2001).
43. H. S. Taper, J. M. Jamison, J. Gilloteaux, C. A. Gwin, T. Gordon, J. L. Summers, In vivo reactivation of DNases in implanted human prostate tumors after administration of a vitamin C/K_3 combination. *J. Histochem. Cytochem.* **49**, 109–120 (2001).

44. I. O. Farah, V. L. Lewis, W. K. Ayensu, O. Mahmud, Assessing the survival of MRC-5 and A549 cell lines upon exposure to ascorbic acid and sodium ascorbate. *Biomed. Sci. Instrum.* **47**, 201–206 (2011).
45. J. Verrax, N. Dejeans, B. Sid, C. Glorieux, P. B. Calderon, Intracellular ATP levels determine cell death fate of cancer cells exposed to both standard and redox chemotherapeutic agents. *Biochem. Pharmacol.* **82**, 1540–1548 (2011).
46. A. Frömberg, D. Gutsch, D. Schulze, C. Vollbracht, G. Weiss, F. Czubayko, A. Aigner, Ascorbate exerts anti-proliferative effects through cell cycle inhibition and sensitizes tumor cells towards cytostatic drugs. *Cancer Chemother. Pharmacol.* **67**, 1157–1166 (2011).
47. P. M. Herst, K. W. Broadley, J. L. Harper, M. J. McConnell, Pharmacological concentrations of ascorbate radiosensitize glioblastoma multiforme primary cells by increasing oxidative DNA damage and inhibiting G2/M arrest. *Free Radic. Biol. Med.* **52**, 1486–1493 (2012).
48. J. J. Cullen, Ascorbate induces autophagy in pancreatic cancer. *Autophagy* **6**, 421–422 (2010).
49. N. Parrow, J. Leshin, M. Levine, Parenteral ascorbate as a cancer therapeutic: A reassessment based on pharmacokinetics. *Antioxid. Redox Signal.* **19**, 2141–2156 (2013).
50. J. Wendt, S. Radetzki, C. von Haefen, P. G. Hemmati, D. Güner, K. Schulze-Osthoff, B. Dörken, P. T. Daniel, Induction of $p21^{CIP/WAF-1}$ and G2 arrest by ionizing irradiation impedes caspase-3-mediated apoptosis in human carcinoma cells. *Oncogene* **25**, 972–980 (2006).
51. O. Surova, B. Zhivotovsky, Various modes of cell death induced by DNA damage. *Oncogene* **32**, 3789–3797 (2013).
52. A. T. Vessoni, E. C. Filippi-Chiela, C. F. Menck, G. Lenz, Autophagy and genomic integrity. *Cell Death Differ.* **20**, 1444–1454 (2013).
53. Q. Huang, Y. T. Wu, H. L. Tan, C. N. Ong, H. M. Shen, A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. *Cell Death Differ.* **16**, 264–277 (2009).
54. M. T. Hayashi, J. Karlseder, DNA damage associated with mitosis and cytokinesis failure. *Oncogene* **32**, 4593–4601 (2013).
55. M. Wojewódzka, I. Buraczewska, M. Kruszewski, A modified neutral comet assay: Elimination of lysis at high temperature and validation of the assay with anti-single-stranded DNA antibody. *Mutat. Res.* **518**, 9–20 (2002).
56. F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**, 271–277 (1986).
57. J. Crowley, N. Breslow, Statistical analysis of survival data. *Annu. Rev. Public Health* **5**, 385–411 (1984).

Acknowledgments: We thank A. Godwin (University of Kansas Cancer Center) and P. Eck (University of Manitoba, Canada) for providing the cell lines. We thank the Center for Biostatistics and Advanced Informatics at the University of Kansas Medical Center for overseeing randomization and designed data intake databases for the clinical trial. We thank R. Wesley (Biostatistics and Clinical Epidemiology Service, NIH) for advice on statistical analysis. Special thanks are given to clinical trial team members D. Khosh, J. Weed, V. Hunter, and E. Schrick. **Funding:** This work was financially supported by Gateway for Cancer Research Foundation (formerly Cancer Treatment Research Foundation, Schaumburg, IL), a grant from the University of Kansas Endowment provided by the L. Charles Hilton Family Foundation, a bridging grant from the University of Kansas Research Institute, and the Intramural Research Program, National Institute of Diabetes and Digestive and Kidney Diseases, NIH. **Author contributions:** Q.C. and Y.M. designed and performed all the cellular and animal experiments. J.D. and J.C. designed and performed the clinical trial. Q.C., J.D., M.L., and Y.M. analyzed the data and wrote the manuscript. K.P. generated part of the data on Comet assay and combination treatment on cells. All authors participated in the revising of the manuscript. **Competing interests:** The authors declare that they have no competing interests.

Submitted 26 July 2013
Accepted 27 December 2013
Published 5 February 2014
10.1126/scitranslmed.3007154

Citation: Y. Ma, J. Chapman, M. Levine, K. Polireddy, J. Drisko, Q. Chen, High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy. *Sci. Transl. Med.* **6**, 222ra18 (2014).