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Treatment of Pancreatic Cancer with Pharmacological Ascorbate

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Abstract

The prognosis for patients diagnosed with pancreatic cancer remains dismal, with less than 3% survival at 5 years. Recent studies have demonstrated that high-dose, intravenous pharmacological ascorbate (ascorbic acid, vitamin C) induces cytotoxicity and oxidative stress selectively in pancreatic cancer cells vs. normal cells, suggesting a promising new role of ascorbate as a therapeutic agent. At physiologic concentrations, ascorbate functions as a reducing agent and antioxidant. However, when pharmacological ascorbate is given intravenously, it is possible to achieve millimolar plasma concentration. At these pharmacological levels, and in the presence of catalytic metal ions, ascorbate can induce oxidative stress through the generation of hydrogen peroxide (H_2O_2). Recent *in vitro* and *in vivo* studies have demonstrated ascorbate oxidation occurs extracellularly, generating H_2O_2 flux into cells resulting in oxidative stress. Pharmacologic ascorbate also inhibits the growth of pancreatic tumor xenografts and displays synergistic cytotoxic effects when combined with gemcitabine in pancreatic cancer. Phase I trials of pharmacological ascorbate in pancreatic cancer patients have demonstrated safety and potential efficacy. In this chapter, we will review the mechanism of ascorbate-induced cytotoxicity, examine the use of pharmacological ascorbate in treatment and assess the current data supporting its potential as an adjuvant in pancreatic cancer.

INTRODUCTION

Pancreatic Cancer

Pancreatic adenocarcinomas, derived from the exocrine pancreas, represent 85% of all pancreatic neoplasms. Adenocarcinoma of the pancreas is the fourth leading cause of cancer related death in the United States and its incidence is steadily increasing [1]. Current National Cancer Institute (NCI) data estimate that 45,220 men and women (22,740 men, 22,480 women) will be diagnosed with pancreatic cancer in calendar year 2013 (age-adjusted incidence rate of 12.2 per 100,000 men and women per year) [2]. The median age at diagnosis for cancer of the pancreas is 71 years of age and the prognosis is dismal, with an overall 5-year relative survival rate of only 3%, and median age at death of 73 (age-adjusted death rate of 10.9 per 100,000 men and women per year) [2–4]. A recent NCI cancer bulletin

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CONFLICT OF INTEREST

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article on pancreatic cancer highlighted the aggressive nature and poor prognosis of pancreatic cancer by stating, “the slow but steady march toward more individualized care in cancer medicine has left pancreatic cancer behind. Patients diagnosed with this disease live no longer today than patients diagnosed two decades ago, despite more than a dozen large clinical trials. Even as many patients with other cancers have benefited from targeted drugs, pancreatic cancer remains as deadly as ever” [5].

Patients diagnosed with pancreatic cancer usually present late in the disease process with symptoms varying based on tumor location within the pancreas. The most commonly reported symptoms are abdominal/epigastric pain, jaundice, and anorexia/weight loss, and are most commonly associated with cancers located within the head of the pancreas (60–70% of pancreatic cancers) [6]. Currently, the only potentially curative treatment is surgical resection via pancreaticoduodenectomy, but only 15–20% of patients have surgically resectable disease at the time of presentation, with 40% of patients presenting with metastatic disease and the remaining patients presenting with locally advanced tumors.

Even after complete resection, prognosis is poor, with expected 5-year survival rates of approximately 25% [7–10]. For locally advanced and metastatic disease, median survival is approximately 10 and 6 months, respectively [11]. For the majority of patients with pancreatic cancer, adjuvant chemotherapy and combination radiotherapy remain the only treatment options with the goal of palliating symptoms [12]. For the last twenty years, gemcitabine has been the standard of care chemotherapy after a clear improvement in the one-year median survival rate in patients treated with gemcitabine, was demonstrated over those who had received 5-FU. Furthermore, treatment with gemcitabine was associated with a reduction in pain intensity scores, daily analgesic consumption, and an improvement in performance status [13]. A recent multi-site randomized control trial of a chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX), demonstrated significantly increased survival compared to patients treated with gemcitabine, as well as prolonged progression-free survival [14]. However, FOLFIRINOX was associated with more severe adverse events and added toxicity, which were not well tolerated by many patients. Despite this, FOLFIRINOX is now considered as the first line standard of care treatment for advanced pancreatic cancer in many patients. Even with the modest improvement in survival time attained with FOLFIRINOX, several clinical trials of multiple new therapies have been performed, and no drug or combination of drugs has significantly improved the overall prognosis [12, 15].

Ascorbate

Ascorbate (ascorbic acid, Vitamin C) is an essential nutrient for humans and plays a role in several important biochemical functions. Ascorbate is a water-soluble ketolactone with two ionizable hydroxyl groups, making it an excellent reducing agent and anti-oxidant at physiologic concentrations. Indeed, it readily undergoes two consecutive, one electron oxidations to form ascorbate radical ($Asc^{\bullet-}$) and dehydroascorbic acid (DHA), though the ascorbate radical is relatively unreactive due to resonance stabilization of the unpaired electron (Fig. 1) [16]. Though ascorbate oxidizes readily, its rate of oxidation is pH-dependent and can be further accelerated by the presence of catalytic metal ions [17]. At

physiological pH, the autoxidation of ascorbate is fairly slow, with a rate constant of approximately 300 M^{-1} s^{-1} [18].

Ascorbate functions across a various range of cell and organ systems as an anti-oxidant. In general, intracellular ascorbate concentrations are increased compared to extracellular fluids, reaching millimolar concentrations in blood components [19, 20]. However, erythrocytes have intracellular ascorbate levels similar to extracellular plasma [21, 22]. High levels of intracellular ascorbate are hypothesized to help maintain an intracellular reducing environment, but it has also been demonstrated that ascorbate can help reduce extracellular oxidants by transferring electrons across the plasma membrane, or through efflux of ascorbate itself [23, 24]. In addition, millimolar levels of ascorbate are found in the cornea and lens of the eye, which may help protect them from solar radiation damage [25, 26].

Besides its role as an antioxidant, ascorbate also functions as a co-factor in various enzymatic pathways. Examples include its roles as a reductant in the conversion of dopamine to norepinephrine, as a co-factor for peptidyl glycine α-amidating monooxygenase [26–29], and as a cofactor for Fe^{2+}-2-oxoglutarate dioxygenases, where it is necessary for maintaining iron in the ferrous state [30, 31]. Additionally, ascorbate is essential in the promotion of collagen synthesis and its proper assembly [32, 33], the regulation of hypoxia-inducible transcription factor (HIF) [34–37], and as a cofactor for histone demethylation [38–41].

Ascorbate can be synthesized from glucose in plants and most animals, however humans lack functional L-gulonolactone oxidase, the enzyme required for the last step of ascorbate synthesis, and are dependent on dietary intake to maintain essential stores [42]. Both ascorbate and dehydroascorbic acid (DHA) are absorbed via the enterocytes of the small intestine. Ascorbate absorption is mediated by Na^+-dependent vitamin C transporters (SVCTs), whereas DHA is absorbed by Na^+-independent facilitative glucose transporters (GLUTs) [43–46]. Because plasma glucose concentrations are much higher than plasma DHA concentrations the majority of intracellular ascorbate is absorbed via SVCTs, rather than indirectly through GLUT absorption of DHA [47, 48]. Besides the brush border of enterocytes, SVCTs are also found within renal tubular cells.

Plasma ascorbate concentrations are tightly regulated; both by the limited affinity of SVCT for ascorbate, as well as through a negative feedback loop in which high intracellular ascorbate concentrations leads to down-regulation of SVCT present on the enterocyte membranes [49–51]. Pharmacokinetic modeling has shown that it is a combination of saturable intestinal absorption, tissue accumulation, and renal re-absorption and excretion that determines the bioavailability of ascorbate [16, 52, 53]. As a consequence of this tight regulation, the bioavailability of oral ascorbate (micromolar concentrations) is much lower than what can be achieved when administered intravenously (millimolar concentrations) [53].

At physiologic concentrations and pH, ascorbate anion ($AscH^-$) comprises the major species of ascorbate and can readily donate an electron to free radicals such as hydroxyl radical ($HO^\bullet$), alkoxyl radical ($RO^\bullet$), peroxyl radical ($LOO^\bullet$), and thiol radical ($GS^\bullet$). It is also

known to play a pivotal role in the prevention of LDL and lipid peroxidation synergistically with vitamin E [16, 54–57]. The oxidation of AscH− produces ascorbate radical ($Asc^{-\bullet}$), which is relatively unreactive and is reduced back to ascorbate by NADH/NADPH reductases [58]. In the presence of catalytic metal ions, ascorbate can function as a pro-oxidant and induce oxidative stress as a pro-drug for the delivery of hydrogen peroxide [17, 59, 60]. Ascorbate reduces ferric iron to ferrous iron, which is then free to react with oxygen, producing superoxide ($O_2^{\bullet-}$). Superoxide then dismutates into hydrogen peroxide (H_2O_2), which in turn reacts with ferrous iron through the Fenton reaction, resulting in generation of reactive oxygen species. This process can also occur at a slower rate in the absence of catalytic metals through autoxidation of ascorbate.

Ascorbate as a Chemotherapeutic Agent

It has been greater than 50 years since it was first hypothesized that ascorbate may inhibit tumor growth [61] by inhibiting hyaluronidases; preventing the breakdown of the extracellular matrix and therefore cancer cell migration [62, 63]. A clinical benefit for ascorbate in the treatment of cancer was first demonstrated in the early trials of Cameron, Pauling, and Campbell in the 1970s in which increased survival was observed in patients receiving intravenous ascorbate [64–67]. Cameron and Campbell published case reports of 50 patients treated with high-dose ascorbate, with some report of benefit [64]. Cameron and Pauling then published a follow up study of 100 patients with terminal cancer who were given intravenous ascorbate demonstrating that patients who received ascorbate survived 300 days longer than retrospective control patients with similar disease [67]. However, ascorbate was dismissed as a chemotherapeutic agent following two randomized, double-blinded clinical trials in the 1980s utilizing in which orally administered ascorbate (10 g per day) demonstrated no difference in symptoms or survival between treatment groups and placebo controls [68, 69].

It was only later realized after pharmacokinetic studies were performed by Levine and colleagues, that the bioavailability of orally administered ascorbate is much lower than when administered intravenously [19, 70]. Oral doses of ascorbate ranging from 30 to 100 mg daily produced average fasting plasma concentrations of only 60 μM. Even doses of 1000 mg daily produced average fasting plasma concentrations of only 75–80 μM. As daily doses increased to 2500 mg, there were minimal further increases in plasma concentrations [19, 53, 70] due to a combination of saturable intestinal absorption, tissue accumulation, and renal re-absorption and excretion [16, 52, 53]. Riordan and associates found that intravenous administration of ascorbate produced peak plasma concentrations approximately 25-fold higher than that attained by the same dose given orally [71]. Thus, potentially therapeutic doses are attainable only through parenteral administration [72]. Animal studies using intraperitoneal dosing of ascorbate confirmed that only parenteral injection could produce pharmacologic ascorbate concentrations similar to those obtained by intravenous infusions in humans [73–75] These data suggest that the lack of efficacy of ascorbate as a cancer treatment as determined by the previous randomized controlled trials may have been due to the fact that patients were receiving sub-therapeutic doses. This knowledge of ascorbate's pharmacokinetics generated renewed enthusiasm into its potential in the treatment of cancer,

and several new studies into the efficacy and mechanisms of action of pharmacologic ascorbate were produced.

TREATMENT OF PANCREATIC CANCER WITH PHARMACOLOGICAL ASCORBATE

In Vitro Studies

Selective cytotoxicity of pharmacologic ascorbate on cancer cells was first demonstrated *in vitro* by Chen *et al.* in 2005 [76]. In their study, cell viability was decreased in ten cancer cell lines and unchanged in four non-tumorigenic cell types after one-hour exposure to pharmacological ascorbate. Normal cells were unaffected by 20 mM ascorbate, while all but one cancer cell line showed decreased survival with ascorbate exposure; 5 of which had EC_{50} doses < 4 mM. Interestingly, cell death was suggested to be dependent upon H_2O_2 formation. Further work expanded these studies to 43 cancer cell lines [73]. Again, the results demonstrated similar results with the EC_{50} doses of ascorbate less than 10 mM for 34 of the 43 cell lines investigated, and with normal cells insensitive to concentrations of ascorbate exceeding 20 mM. Furthermore, the addition of catalase to the medium reversed the decrease in cell viability in multiple cancer types exposed to 10 mM ascorbate (1 h), supporting they hypothesis that cytotoxicity was mediated by H_2O_2. These studies demonstrated that pharmacological doses of ascorbate achievable by parenteral administration of the drug have selective toxicity against cancer cells *vs.* normal cells.

In the first study specific to pancreatic cancer cell susceptibility to ascorbate, Du *et al.* demonstrated selective sensitivity of three pancreatic cancer cell lines (MIA PaCa-2, AsPC-1, and BxPC-3) vs. a normal immortalized pancreatic ductal epithelial cell line (H6c7) to 1 hour treatment with both 5 and 10 mM ascorbate [77]. All of the pancreatic cancer cell lines demonstrated decreased cell metabolic viability as well as decreased clonogenic survival when treated with ascorbate [78, 79]. This study demonstrated a time and dose-dependent increase in measured H_2O_2 production with increased concentrations of ascorbate leading to the hypothesis that ascorbate associated H_2O_2 production induced oxidative stress within cancer cells, leading to caspase-independent cell death consistent with autophagy. Indeed, when cells were treated with scavengers of H_2O_2, ascorbate-associated decreases in clonogenic survival could be reversed. It was suggested that the difference in sensitivity observed in cancer cells vs. normal cells toward ascorbate might be explained by the low levels of antioxidant enzymes and high levels of endogenous ROS in cancer cells [16, 80–82].

In Vivo Studies

Initial studies using intraperitoneal dosing of ascorbate confirmed that only parenteral injection could produce pharmacologic ascorbate concentrations similar to those obtained by intravenous infusion in humans [73–75]. One study in mice showed that a bolus intraperitoneal injection of ascorbate at a dose of 1g kg^{-1} resulted in plasma concentration of 15 mM, whereas supplementation of the drinking water with the same dose increased plasma concentration to only 50 μM [75], similar to the results of human studies that found

that pharmacologic concentrations of ascorbate can only be attained via parenteral administration.

In 2008, Levine's group demonstrated [73] that in mice bearing glioblastoma xenografts, a single pharmacologic dose of ascorbate produced sustained concentrations of both $Asc^{\bullet-}$ and H_2O_2 within the interstitial fluids of the tumor. In contrast, the same pharmacologic ascorbate concentrations in whole blood generated little detectable $Asc^{\bullet-}$ and no detectable H_2O_2 [83]. This lack of $Asc^{\bullet-}$ and H_2O_2 in the blood leads to the hypothesis that erythrocytes were acting as a sink, actively recycling $Asc^{\bullet-}$ and H_2O_2 via more efficient and redundant H_2O_2 catabolic pathways relative to the extracellular fluid [84–89]. Therefore, pharmacologic ascorbate may serve as a pro-drug for H_2O_2 delivery to the tumor extracellular milieu, without accumulation in blood.

Tumor vessels are known to have a much higher permeability as compared to normal endothelium, which may permit the accumulation of macromolecules such as proteins in the interstitial fluid [90, 91]. Furthermore, iron, copper, and other catalytic metals are known to associate with damaged protein within the extracellular fluid [92–94]. Therefore, it was proposed that the catalytic activity needed for the generation of $Asc^{\bullet-}$ and H_2O_2 in the extracellular space and interstitial fluid of the tumor may be provided by an increased abundance of metal ions found there [76]. This hypothesis would explain why $Asc^{\bullet-}$ and H_2O_2 were found in sustainable concentrations in the interstitial fluids of the tumor after a single dose of pharmacologic ascorbate. Based on these considerations, the H_2O_2 generated by ascorbate oxidation in the extracellular space could accumulate to concentrations greater than found intracellularly, causing a net diffusion across the cell membrane and ultimately resulting in greater toxicity to tumor cells [16, 83].

Using this knowledge, the Cullen laboratory began focusing on pancreatic tumor xenografts [77]. It was soon demonstrated that mice receiving pharmacological ascorbate had significantly slower tumor growth when compared to control animals. Furthermore, there was a greater than 3-fold decrease in overall tumor volume in animals receiving ascorbate when compared to controls and a log-rank analyses of survival showed that animals receiving ascorbate had significantly increased survival. All mice that entered the study completed the treatment period, with none of the animals having to be sacrificed for continued weight loss or cachexia, further supporting the hypothesis that pharmacologic ascorbate is non-toxic to normal, healthy cells and tissues. Taken together these studies strongly suggested that pharmacologic doses of ascorbate achievable in humans might have potential for therapy in pancreatic cancer.

MECHANISM OF ASCORBATE INDUCED CYTOTOXICITY IN PANCREATIC CANCER

As noted above, the administration of pharmacologic ascorbate is associated with the formation of H_2O_2 in the extracellular fluid surrounding a tumor [17, 59, 60, 73, 76, 77, 83, 95]. In the presence of catalytic metal ions, ascorbate functions as a pro-oxidant and induces oxidative stress, but even in the absence of metals the autoxidation of ascorbate generates significant amounts of H_2O_2 when ascorbate is at millimolar concentrations [17, 59, 60].

Since ascorbate readily oxidizes to produce H_2O_2, pharmacologic ascorbate has been proposed as a pro-drug for the delivery of H_2O_2 to tumors [16, 73, 76, 77, 83]. H_2O_2 generation is dependent on ascorbate concentration and incubation time, and has a linear relationship with ascorbate radical formation [83]. H_2O_2 can affect both extracellular and intracellular targets, as it is permeable across lipid membranes [96, 97]. Extracellular H_2O_2 may attack membrane lipids forming lipid hydroperoxides and causing leaky membranes. Intracellularly, oxidative stress and DNA damage promote cell death, possibly via autophagy [73, 76, 77, 83].

Interestingly, it has been observed that increasing intracellular levels of ascorbate does not enhance cytotoxicity. L-Ascorbate 2-phosphate (A2P) is a form of ascorbate that is protected from oxidation by the presence of a phosphate moiety. It binds to the extracellular cell surface where the phosphate group is then hydrolyzed by cell membrane associated esterases, resulting in the transport of the ascorbate into the cell [98–100]. The addition of A2P to cell culture media increases intracellular ascorbate to millimolar concentrations [101] but the addition of A2P to cells being treated with extracellular pharmacological ascorbate does not enhance the cytotoxicity in pancreatic cancer cells [102]. Indeed, cells treated with A2P showed no changes in clonogenic survival when compared to controls, while cells treated with ascorbate had significant decreases in clonogenic survival. Additionally, clonogenic survival was similar in cells treated with the combination of A2P and ascorbate when compared to ascorbate alone, suggesting that increases in intracellular ascorbate are not responsible for the ascorbate-induced cytotoxicity observed when ascorbate is added to the extracellular medium. These data further support the hypothesis that ascorbate-induced cytotoxicity is due to the formation of extracellular H_2O_2, which then diffuses into the cell and causes cytotoxicity as opposed to intracellular ascorbate oxidation.

Further evidence supporting the importance of H_2O_2 production in selective cancer cell cytotoxicity is offered by experiments that have investigated the neutralization of H_2O_2 by endogenous and exogenous catalase. Klingelhoeffer *et al.* demonstrated differential sensitivity to ascorbate and H_2O_2 by pretreatment with extracellular exogenous catalase, which was also correlated with endogenous intracellular catalase production in numerous cancer cell lines [103]. Sensitivity of three pancreatic cancer cell lines to pharmacologic ascorbate was reversed both by the pretreatment of cells with exogenous catalase (extracellular), as well as by overexpression of intracellular catalase by transfecting cells using adenovirus containing catalase cDNA [77]. Additionally, ascorbate toxicity has been enhanced in Ehrlich ascites carcinoma cells *in vitro* by treatment with the catalase inhibitor aminotriazole [104]. Taken together, these studies suggest that extracellular pharmacologic ascorbate mediates cancer cell death by H_2O_2 formation [53].

The role of catalytic metal ions in the formation of H_2O_2 via pharmacologic ascorbate cannot be understated. In the presence of iron, ascorbate is oxidized rapidly as it reduces Fe^{3+} to Fe^{2+}, which is then free to react with oxygen, producing superoxide radical ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O^{\bullet -}$). Superoxide radical then dismutates into hydrogen peroxide ($O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$), which in turn reacts with ferrous iron through the Fenton reaction producing hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet}$). The presence of ascorbate can

allow the recycling of Fe^{3+} to Fe^{2+}, which in turn will catalyze the formation of highly reactive oxidants from H_2O_2 [16].

The quantity and availability of catalytic metal ions may also play a significant role in ascorbate-induced toxicity. Normally, iron is not free in solution but is sequestered by iron binding proteins such as transferrin and ferritin [105, 106]. Transferrin is the major iron transport protein, moving iron absorbed in the diet from enterocytes to the cell surface where it binds to transferrin receptor, promoting endocytosis and internalization of the protein. Once intracellular, the iron is released from transferrin where it is bound to ferritin, the iron storage protein. In pathological states, such as thalassemia and hemochromatosis, free iron is found in the plasma and extracellular fluid. Chronic inflammatory diseases are also associated with increased oxidative stress and levels of catalytic metal ions in tissues. For example, increased deposition of iron protein has been demonstrated in the synovial membranes in patients with rheumatoid arthritis [107, 108]. Additionally, septic patients have been found to have increased catalytic iron levels [109]. In these situations the administration of ascorbate without an iron chelator could potentially lead to extensive oxidative tissue damage, similar to what is observed in ischemia/reperfusion injury [17, 110, 111]. Elevated serum levels of ferritin also occur in various malignancies [112–114]. Tumor vessels have increased permeability compared to normal endothelium, permitting the accumulation of macromolecules such as protein into the interstitial fluid [90, 91]. As discussed above, the presence of extracellular metal-containing proteins is thought to be essential for the pro-oxidant effects of ascorbate [59, 83]. Given the increase in ferritin in cancer, and the potential for its extracellular accumulation, iron-saturated ferritin may be the source of catalytic iron required for the selective ascorbate-induced cytotoxicity observed in pancreatic and other cancers [16]. Indeed, ferritin staining was detected in the stroma surrounding the neoplastic cells of breast cancer tissue [115], and serum ferritin levels have been shown to correlate to the tumor stage and volume in cervical cancer [116]. Furthermore, iron can be released from ferritin by biological reductants, including ascorbate [67] and increased ferritin may provide a continuous source of catalytic iron required for pharmacologic ascorbate-mediated H_2O_2 production and cytotoxicity.

The differential toxicity of pharmacological ascorbate between cancerous and normal cells may be due to a combination of low levels of antioxidant enzymes and high levels of endogenous ROS in cancer cells [16, 80–82]. Additionally, erythrocytes may act as a sink for $Asc^{\bullet-}$ and especially H_2O_2 due to their redundant H_2O_2 catabolic pathways relative to the extracellular fluid, preventing peroxide buildup in the blood and delivery to healthy tissues, while ensuring H_2O_2 delivery to the extracellular milieu. However, the selective toxicity of ascorbate still needs to be clarified and at this time the real reasons for the selectivity are not clear.

ENHANCEMENT OF ASCORBATE-INDUCED CYTOTOXICITY IN PANCREATIC CANCER

Catalytic metals are known to increase the rate of ascorbate-induced H_2O_2 formation and, potentially, oxidative stress-mediated cytotoxicity. Porphyrin-based superoxide dismutase

(SOD) mimetics contain a catalytic redox-active metal (Mn, Fe, or Cu) coordinated within a stable porphyrin ring. In the presence of a reducing agent such as ascorbate, manganese porphyrins function as superoxide reductases rather than dismutases, with Mn(III) reduced to Mn(II) by ascorbate, allowing Mn(II) to react with O_2 forming superoxide, which can then form H_2O_2 [16]. Several studies have demonstrated enhanced ascorbate oxidation using manganese porphyrins *in vitro*, with synergistic killing of pancreatic cancer cell lines *vs.* pharmacologic ascorbate alone [117, 118]. In addition, manganese porphyrins have already been tested alone *in vivo* and have minimal toxicity even at increased doses [119]. Thus, porphyrins could be used as adjuvants to enhance the efficacy of pharmacologic ascorbate in the treatment of pancreatic cancer.

Oxidative stress has also been shown to increase the levels of catalytic iron in tissues along with ionizing radiation and some chemotherapeutic drugs [120–122]. Thus, combination therapies, such as chemotherapy plus pharmacologic ascorbate, and radiation therapy plus pharmacologic ascorbate may act synergistically to potentiate ascorbate-induced cytotoxicity. To test for synergism between chemotherapy and ascorbate, Espey and colleagues demonstrated that pharmacologic ascorbate in combination with gemcitabine, the standard of care chemotherapeutic in pancreatic cancer, significantly enhanced gemcitabine cytotoxicity [123]. Furthermore, as the ratio of ascorbate to gemcitabine was increased, cytotoxicity increased proportionally, suggesting that when used in combination, a lower gemcitabine dose could be used to obtain the same antitumor activity [53]. Finally, treatment of pancreatic cancer xenografts with the ascorbate + gemcitabine combination *in vivo* resulted in significant inhibition of tumor growth *vs.* ascorbate or gemcitabine alone. Thus, pharmacologic ascorbate synergizes with gemcitabine to improve therapeutic efficacy.

As previously discussed, the cytotoxicity of pharmacologic ascorbate is directly related to the production of H_2O_2 in the extracellular space, and its flux into cancer cells, resulting in oxidative stress, DNA damage, and ultimately cell death. In a recent study, Olney *et al.* proposed that inhibiting intracellular hydroperoxide removal during the administration of pharmacologic ascorbate would increase the cytotoxicity observed in pancreatic cancer cells [102]. Human pancreatic cancer cells were treated with ascorbate alone or in combination with intracellular inhibitors of H_2O_2 removal, including the glutathione disulfide reductase inhibitor 1,3 *bis* (2-chloroethyl)-1-nitrosurea (BCNU), siRNA targeted to glutathione disulfide reductase (siGR), and an inhibitor of glucose metabolism, 2-deoxy-D-glucose (2DG). Also, endogenous catalase (removal of H_2O_2 via reduction to water) activity was inhibited using aminotriazole so that ascorbate-induced changes in intracellular H_2O_2 could be determined (Fig. 2).

BCNU is a clinically used chemotherapeutic agent that causes DNA alkylation but is also known to inhibit glutathione disulfide reductase (GR). With GR inhibited, cells have a reduced ability to remove H_2O_2 via the glutathione peroxidase system. 2DG is a glucose analog and a competitive inhibitor for uptake via the glucose transporters. Competition between 2DG and glucose is thought to cause inhibition of glucose metabolism, thereby creating a chemically induced state of glucose deprivation resulting in inhibition of hydroperoxide detoxification [79]. Likewise, knockdown of endogenous GR protein levels using siRNA could also prevent H_2O_2 via the glutathione peroxidase system.

When inhibitors of peroxide removal were added to pharmacological ascorbate, both ascorbate-induced cytotoxicity, and intracellular H_2O_2 concentrations were increased. The combination of BCNU and ascorbate dramatically decreased clonogenic survival, while genetically inhibiting GR levels by >50% also decreased clonogenic survival. Finally, using the glucose analog 2DG, experiments demonstrated that combination treatment with 2DG and ascorbate enhanced cytotoxicity with decreased clonogenic survival and cell viability in pancreatic cancer cell lines [102]. Endogenous catalase activity inhibition with aminotriazole significantly increased intracellular H_2O_2 concentrations using all three methodologies of inhibiting hydroperoxide removal discussed above. Together these data demonstrate that inhibitors of H_2O_2 removal enhance both ascorbate-induced intracellular steady-state concentrations of H_2O_2 and cytotoxicity. Thus, treatments consisting of a combination of ascorbate and inhibitors of the removal of H_2O_2 may potentially be an effective therapy for pancreatic adenocarcinoma.

PHASE I CLINICAL TRIALS OF PHARMACOLOGICAL ASCORBATE

Theoretically pharmacological ascorbate should be safe in patients. However, given that ascorbate acts as a pro-drug for the delivery of H_2O_2, there is a risk that at high concentrations, it could potentially induce intravascular hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency that cannot efficiently regenerate glutathione (Fig. 1) [124–126]. Additionally, in patients with pre-existing renal insufficiency or nephrolithiasis, there is a risk of forming calcium oxalate crystals within the urinary space, as oxalic acid is one of the end products of ascorbate oxidation [127]. Furthermore, because of the rapid oxidation of ascorbate into H_2O_2 by catalytic metals, the administration of pharmacologic ascorbate to individuals with iron overload (hemochromatosis, thalassemia, and other conditions requiring multiple transfusions) is likely contraindicated [71, 128]. In the early trials of Cameron and Campbell, there were also rare cases of massive tumor hemorrhage reported after the administration of high-dose ascorbate in patients with advanced cancers [64, 129]. At this time, several Phase I clinical trials of intravenous ascorbate in patients with advanced cancers have been performed [74, 130–133]. The main focus of these initial trials has been pharmacokinetics, dosing, resulting ascorbate plasma concentrations, and safety. In addition, some trials have also examined the clinical consequences of the pharmacologic ascorbate therapy.

In 2004, Riordan *et al.* published case reports of patients with renal cell carcinoma, breast cancer, pancreatic cancer and lymphoma in which I.V. ascorbate was administered in doses of 10 to 100 grams/day, up to three times per week, with improved patient well-being, and in some cases reduced tumor size, with minimal toxicity [134]. Based on these reports, Riordan and colleagues performed a phase I clinical trial the following year in which 24 late stage terminal cancer patients were given continuous infusions of ascorbate at doses of 150 to 710 mg/kg/day for up to eight weeks [131]. During therapy, serum ascorbate concentrations ranged from 0.28 to 3.8 mM. The most commonly reported adverse events during the study were minor and included nausea, dry mouth, dry skin and minor edema. Grade 3 adverse events were minor and few, demonstrating that pharmacological ascorbate was well-tolerated with caution in patients with a history of nephrolithiasis.

Later, Hoffer and colleagues performed a phase I dose-escalating and pharmacokinetic study of intravenous ascorbate in patients with advanced malignancies with the primary objective of determining a recommended dosage for phase II trials, and secondary objectives of determining adverse effects, anti-tumor effects, and patient quality of life during treatment [74]. Ascorbate was administered three times per week at fixed doses of 0.4, 0.6, 0.9, and 1.5 g/kg. During therapy, serum ascorbate concentrations ranged from 2.4 to 26 mM, and at the highest dosage (1.5 g/kg) pharmacokinetic studies determined that plasma ascorbate concentrations exceeding 10 mM were achievable for approximately 4.5 hours; levels that are known to induce cytotoxicity in a variety of cancer cells [73, 76, 77]. Adverse effects reported were mild and were consistent with the side effects expected from the rapid infusion of any high osmolarity solution. The symptoms were preventable by encouraging patients to drink fluids before and during the infusion. No unusual biochemical or hematologic abnormalities were observed during the study, and patients whose ascorbate dose was ≥ 0.6 g/kg maintained their physical quality of life throughout the trial, whereas those at the lowest dose did not. None of the patients were found to have objective tumor responses to the treatment, and all patients eventually experienced disease progression. The authors recommended 1.5 g/kg as the starting dose for future phase II trials and concluded that ascorbate might need to be combined with cytotoxic or other redox-active molecules to be an efficacious treatment [74].

Pharmacological ascorbate may also affect quality of life in cancer patients [133]. In 60 patients with various newly diagnosed malignant cancers who were receiving adjuvant intravenous ascorbate, quality of life was assessed before beginning treatment, and then at weeks 2 and 4 of therapy [135–137]. In this study, patients received pharmacologic ascorbate in biweekly infusions, with target blood ascorbate concentrations of 350–400 mg/dL immediately following infusion [138]. The study did not address peak plasma ascorbate levels or clinical disease progression. Interestingly, the QOL scores significantly improved at both 2 weeks ($p < 0.05$) and 4 weeks ($p < 0.01$) of treatment, compared to initial scores. Patients also showed significant increases in physical, emotional, cognitive and social functioning after 4 weeks of I.V. ascorbate therapy. Furthermore, patients showed significant relief of fatigue, pain, insomnia, constipation and financial difficulties scores. Nearly half of the patients at two weeks and 60% of their patients at four weeks had minimally or much improved quality of life. At the conclusion of their study, the authors proposed that pharmacologic ascorbate can safely improve the quality of life of cancer patients, and suggested its further use as a palliative care therapy, focusing on improving quality of life, and in particular, relief from fatigue.

Recently, Stephenson *et al.*, completed another phase I trial to evaluate the safety, tolerability, and pharmacokinetics of high dose pharmacologic ascorbate as a monotherapy in patients with advanced cancers that were refractory to standard therapy [132]. If the relatively poor anticancer response observed in the prior phase I studies with ascorbate was due to inadequate concentrations of ascorbate achieved within the systemic compartment, or failure to achieve the adequate duration of elevated concentration necessary for cytotoxicity then higher blood levels, longer exposure times, and higher dose intensity should increase both toxicity and antitumor efficacy. In the study, five cohorts of three patients received intravenous ascorbate for 4 consecutive days per week, for 4 weeks. The ascorbate was

administered at 1 g/min starting at a total dose of 30 g/m^2 in the first cohort with subsequent cohorts having the dose increased by 20 g/m^2 until a maximum tolerated dose was found. Dose-limiting toxicity (DLT), defined as any reversible adverse event ≥ grade 3 was monitored. If a DLT was observed in ≥ 2 patients, then the maximum tolerated dose (MTD) would be defined as the dose just below the dose at which the DLT was observed. Data were obtained for cohorts treated with 30, 50, 70, 90, and 110 g/m^2.

Pharmacokinetics showed that ascorbate was eliminated by simple first-order kinetics and did not accumulate to any significant level during consecutive daily administrations. The half-life and clearance rate values of ascorbate were similar for all patients across all the cohorts. The maximum plasma concentration increased proportionately with increasing ascorbate doses before maxing out at 70 g/m^2. Each of the three highest doses maintained plasma ascorbate levels between 10–20 mM for 5–6 hours. Even at the higher doses, intravenous ascorbate was well tolerated, again with the most common side effects being headache and nausea. None of the patients experienced any objective tumor response with 3 patients having stable disease and the rest with tumor progression. Collectively, the studies discussed above have demonstrated that pharmacologic ascorbate is safe and well tolerated. The relatively mild side effects related to its administration are thought to be due to its high osmolality and include nausea, vomiting, headache, dry mouth, flushing, diarrhea and minor edema [74, 130–132]. These symptoms were widely preventable by encouraging patients to drink fluids before and during the infusion [74]. In addition, pharmacologic ascorbate may improve quality of life and relieve certain symptoms related to cancer burden, including fatigue [133]. The one major side effect directly attributable to pharmacologic ascorbate seems to be the risk of subsequent kidney stone formation in patients with prior history of stones [131]. Importantly plasma ascorbate concentrations up to 49 mM are attainable and levels of 10–20 mM are sustainable for 5–6 hours.

CLINICAL TRIALS OF PHARMACOLOGICAL ASCORBATE IN THE TREATMENT OF PANCREATIC CANCER

In 2012, Monti *et al.* published the results of a phase I clinical trial specifically aimed at examining the safety and efficacy of intravenous ascorbate in the treatment of patients with metastatic stage IV pancreatic ductal adenocarcinoma [130]. Fourteen patients were enrolled to receive intravenous ascorbate of 50, 75, or 100 grams three times per week for 8 weeks, combined with the standard of care chemotherapy gemcitabine and erlotinib. The primary aim of the trial was assessing safety with a secondary aim of assessing response to treatment. Out of 14 patients who enrolled in the study, 9 patients completed the treatment. Two subjects chose to discontinue the study because it was too difficult for them to come into the hospital for treatments, and three subjects died from rapid disease progression before completing therapy. Adverse events including nausea and mild lightheadedness and serious adverse events were attributable to chemotherapy or progression of disease. In patients receiving 100 grams of ascorbate, peak plasma levels of between 25 to 32 mM were reported. At the end of the study, 8 out of 9 patients who completed 8 weeks of treatments had reduction in the size of their primary tumor, and one patient’s tumor was stable in size. By RECIST criteria, 7 patients had stable disease and 2 patients had progressive disease

[139]. Progression-free survival time was similar to previously reported data from patients treated with gemcitabine/erlotinib alone. Most importantly, this study demonstrated that there was no increased toxicity when pharmacologic ascorbate was added to gemcitabine and erlotinib treatments in patients with metastatic pancreatic cancer.

More recently in 2013, the University of Iowa group completed a phase I clinical trial examining the toxicity and efficacy of pharmacological ascorbate combined with gemcitabine in patients with stage IV pancreatic adenocarcinoma [140]. Fourteen subjects were enrolled to receive 15–125 g of ascorbate per infusion, twice weekly for 4 week cycles. The dose of ascorbate escalated each week and was tailored to each patient to achieve plasma ascorbate concentrations of ≥ 20 mM, In addition, each patient received gemcitabine once weekly for 3 weeks followed by one week of rest. Ascorbate treatments cycles continued until a patient experienced a ≥ grade 3 dose limiting toxicity or until disease progression, as defined by the RECIST criteria [139]. Nine subjects completed at least two cycles of treatment. No dose limiting toxicities or serious adverse effects attributable to intravenous ascorbate occurred during the study. The most common side effects reported were nausea, diarrhea and dry mouth. Pharmacologically, plasma ascorbate levels ranged from 20 to 25 mM in the first hour post-infusion, and mean trough levels remained significantly elevated compared to baseline levels in all subjects. $Asc^{\bullet -}$ was measured in pre and post-infusion blood samples, and was only detectable during pharmacological levels of ascorbate, consistent with a greatly increased rate of ascorbate oxidation [141]. Additionally, F_2-isoprostane levels, a marker of systemic oxidative stress [142], decreased after ascorbate infusions, suggesting that ascorbate does not induce systemic oxidative stress but may act as an antioxidant systemically while simultaneously acting as a pro-oxidant at the tumor. Two-thirds of the patients improved their overall performance status, while the mean time to disease progression was 26 ± 7 weeks. As of August 2014 mean overall survival was 15 ± 2 months, a significant improvement compared to trials of gemcitabine alone for advanced pancreatic cancer (mean progression free survival and mean overall survival of 9 weeks and 6 months, respectively) (Fig. 3) [13]. This study again demonstrated that the use of pharmacological ascorbate in combination with gemcitabine is safe and well tolerated. Combined, these studies provide a strong justification for the establishment of a phase II clinical trial sufficiently powered to determine the efficacy of pharmacological ascorbate combined with gemcitabine.

PHARMACOLOGICAL ASCORBATE AS AN ADJUVANT TO RADIOTHERAPY

For the majority of patients with pancreatic cancer, adjuvant chemotherapy and radiotherapy remain the only treatment option with the goal of palliating symptoms and improving quality of life [12]. Radiation has been shown to increase the levels of catalytic iron in tissues, which increases the rate of ascorbate-induced peroxide formation and cytotoxicity [17, 121]. The sensitivity of glioblastoma multiforme cells to radiation was enhanced by exposure to pharmacological ascorbate [143]. Combination treatment was shown to increase the number of double stranded DNA breaks relative to that produced by radiation alone. Similar findings were demonstrated in the human leukemia cell line, HL60 [144]. Therefore, investigation into whether pharmacologic ascorbate may acts as synergistically with radiation therapy to increase radiation-induced pancreatic cancer cell death is of interest.

CONCLUSIONS

It has been over fifty years since ascorbate was described as a potential therapy for cancer. Parenteral ascorbate first demonstrated clinical benefit in the treatment of cancer in the early trials of Cameron, Pauling, and Campbell in the 1970s. Randomized, double-blinded clinical trials at the Mayo Clinic utilizing orally administered ascorbate; neither study showed any benefit and ascorbate was dismissed as a potential chemotherapeutic agent in the treatment of cancer by the research and medical communities. Till recently, that studies showed that oral and intravenous ascorbate have strikingly different pharmacokinetic properties and that the bioavailability of orally administered ascorbate is much lower than when administered intravenously. Clinical data show that when ascorbate is given orally, fasting plasma concentrations are tightly controlled at <100 μmol/L. However we now know that intravenous doses of ~ 80 g/m^2 are able to achieve plasma concentrations of up to 49 mmol Thus, interest into the clinical potential of pharmacological ascorbate in the treatment of cancer was rekindled. Interestingly, at pharmacologic concentrations and in the presence of catalytic metal ions, ascorbate functions as a pro-oxidant and can induce oxidative stress via the generation of extracellular H_2O_2.

The selective cytotoxicity of pharmacologic ascorbate to tumor cells has been confirmed in several cancer cell lines in both *in vitro* and *in vivo* models in several laboratories. Several phase I trials have demonstrated that pharmacological ascorbate is safe and well tolerated, with mild side effects and possible improvement in quality of life during treatment. Two recent phase I clinical trials investigating the safety and efficacy of pharmacologic ascorbate in combination with gemcitabine chemotherapy in the treatment of stage IV pancreatic adenocarcinoma demonstrated safety without increased toxicity, with promising and possible efficacy.

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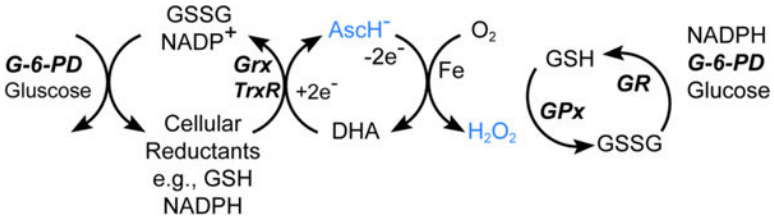

Fig. 1.

Ascorbate oxidation results in a 2-electron reduction of dioxygen forming H_2O_2. G-6-PD = glucose-6-phosphate dehydrogenase; GPx = glutathione peroxidase; GR = glutathione disulfide reductase; Grx = glutaredoxin; GSH = glutathione; GSSG = glutathione disulfide; Trx = thioredoxin.

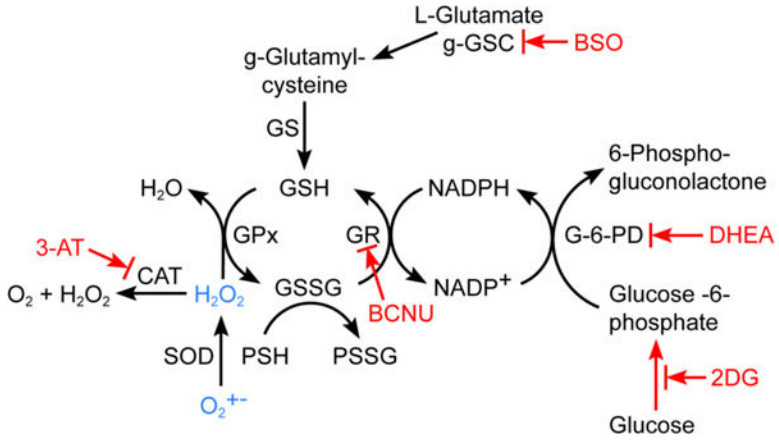

Fig. 2.

Antioxidant enzyme schematic. GSH = glutathione; GSSG = glutathione disulfide; GR = glutathione disulfide reductase; G-6-PD = glucose-6-phosphate dehydrogenase; $^{\gamma}$GCS = gamma-glutamylcysteine synthetase; GPx = glutathione peroxidase; GS = glutathione synthetase. Inhibitors of the pathway are: 3-AT= 3-amino-1,2,4-triazole; BCNU = 1,3 bis (2-chloroethyl)-1-nitrosurea; BSO = buthionine sulfoximine; 2DG = 2-deoxy-D-glucose.

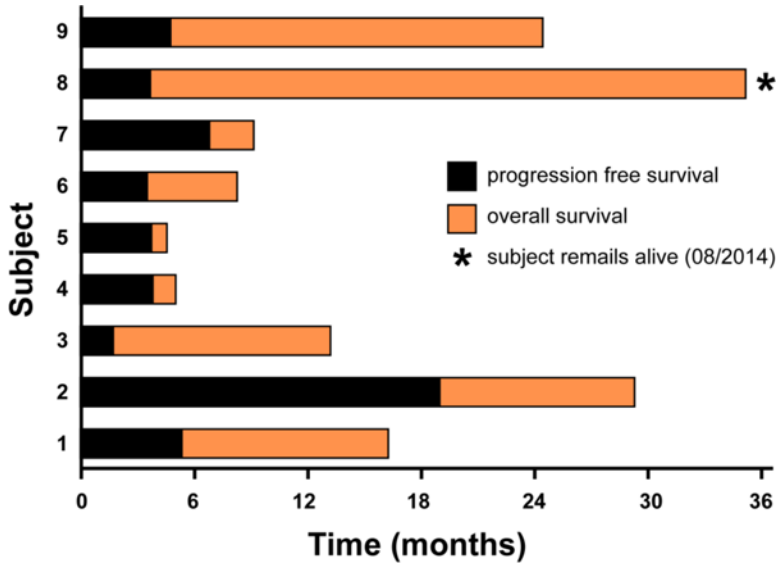

Fig. 3.

Progression free and overall survival. This phase I trial was designed to determine the effect of escalating doses of ascorbate when combined with gemcitabine in stage IV pancreatic cancer patients. The trial utilized a modified Burris regimen, administering gemcitabine for 3 weeks for each cycle of therapy along with ascorbate given twice weekly for every week. Historic median survival for gemcitabine-treated patients alone is 5.65 months. The mean survival in our study currently stands at 15 months.