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Ascorbic acid in cancer treatment: let the phoenix fly

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Abstract

Vitamin C (ascorbic acid, ascorbate), despite controversy, has re-emerged as a promising anti-cancer agent. Recent knowledge of intravenous ascorbate pharmacokinetics and discovery of unexpected mechanisms of ascorbate action have spawned many investigations. Two mechanisms of anti-cancer activity with ascorbate have gained prominence: hydrogen peroxide induced oxidative stress and DNA demethylation mediated by ten-eleven translocation enzyme activation. Here, we highlight salient aspects of the evolution of ascorbate in cancer treatment; provide insights into the pharmacokinetics of ascorbate; describe mechanisms of its anti-cancer activity in relation to the pharmacokinetics; outline promising pre-clinical and clinical evidence; and recommend future directions.

Background

Like a phoenix, ascorbic acid as an anti-cancer agent has had a spectacular rise, fall and re-emergence. In the 1970s, Ewan Cameron, joined by Linus Pauling, described retrospectively and in case reports that patients with advanced cancer had survival benefit and symptomatic relief using high dose ascorbate (10 g/day) (Cameron and Campbell, 1974; Cameron and Pauling, 1976; Cameron and Pauling, 1978). Subsequently, two rigorous double-blind placebo-controlled prospective trials performed at the Mayo Clinic using the same dose of ascorbate failed to confirm these results, and oral ascorbate as an anti-cancer agent was roundly dismissed (Creagan et al., 1979; Moertel et al., 1985; Wittes, 1985).

Pharmacologic Ascorbate and Hydrogen Peroxide (H_2O_2)

About two decades ago, new knowledge of ascorbate pharmacokinetics spawned discovery of anti-cancer mechanisms of ascorbate action (Table 1). To provide a foundational basis for dietary recommendations for ascorbic acid, healthy subjects underwent intensive clinical pharmacokinetics and physiology studies, the first studies of this kind for any vitamin (Levine et al., 1996; Levine et al., 2001). Findings were that oral doses of ascorbic acid over an ~ 80-fold dose range produced plasma concentrations that were tightly controlled by limited gastrointestinal absorption, saturated tissue transporters, and renal reabsorption and excretion (Corpe et al., 2013; Corpe et al., 2010; Levine et al., 1996; Levine et al., 2001; Padayatty et al., 2004; Sotiriou et al., 2002). Intravenous (IV) administration bypassed tight control until the kidney restored homeostasis. Depending on dose and infusion time, IV ascorbate produced plasma levels that were hundreds of fold higher than those produced by the maximum tolerated dose of oral ascorbate (Chen et al., 2008; Hoffer et al., 2008; Padayatty and Levine, 2000; Padayatty et al., 2004; Park et al., 2009). These pharmacokinetics data provided a potential explanation to the conflicting cancer treatment outcomes, because no one at the time knew that IV and oral ascorbic acid behaved so differently. Cameron administered 10 grams ascorbate intravenously as well as orally, while Mayo investigators used oral ascorbate alone. With pharmacokinetics as a foundation, it was shown that only ascorbic acid at pharmacologic concentrations from intravenous dosing, and that would not occur from oral dosing, acted as pro-drug for hydrogen peroxide (H_2O_2) formation in the extracellular space (Chen et al., 2005; Chen et al., 2007). Pharmacologic, but not physiologic, ascorbic acid was selectively toxic to cancer cells *in-vitro* and *in vivo* (Chen et al., 2005; Chen et al., 2008; Verrax and Calderon, 2009). Additionally, the requisite pharmacologic ascorbic acid concentrations are achieved predictably and safely in humans (Chen et al., 2008; Hoffer et al., 2008; Monti et al., 2012; Padayatty et al., 2010; Welsh et al., 2013). In animal models, pharmacologic ascorbate has anti-cancer activity either similar to conventional chemotherapy or synergizes with it (Espey et al., 2011; Ma et al., 2014; Serrano et al., 2015; Xia et al., 2017). Conversely, there are no data showing that

pharmacologic ascorbate interferes with chemotherapy. Early phase clinical trials indicate that IV ascorbate at 1g/kg over 90–120 minutes two to three times weekly is well tolerated and may enhance chemosensitivity as well as decrease chemotherapy related side effects.(Carr et al., 2014; Hoffer et al., 2015; Ma et al., 2014; Monti et al., 2012; Schoenfeld et al., 2017; Shim et al., 2014; Stephenson et al., 2013; Welsh et al., 2013).

Table 1:

Evolution of ascorbic acid studies in cancer

Cameron/Pauling studies	Mayo Clinic studies	Vitamin C pharmacokinetics and early phase clinical trials	Studies on H2O2 mechanism of anti-cancer activity	Studies on epigenetic mechanism of anti-cancer activity
Ewan Cameron, joined by Linus Pauling, described retrospectively and in case reports that patients with advanced cancer had survival benefit and symptomatic relief using high dose ascorbate (10g/day IV followed by oral) (Cameron and Campbell, 1974; Cameron and Pauling, 1976; Cameron and Pauling, 1978)	Two rigorous double-blind placebocontrolled prospective trials performed at the Mayo Clinic using the same dose of ascorbate, but orally only, failed to confirm these results, and oral ascorbate was dismissed as an anti-cancer agent. (Creagan et al., 1979; Moertel et al., 1985; Wittes, 1985)	Oral ascorbic acid over an 80-fold dose range was found to produce plasma concentrations that were tightly regulated by gastrointestinal absorption, but IV administration bypassed this control until the kidney restored homeostasis. Maximum tolerated doses of oral (~18g daily) ascorbate produced plasma concentrations of ~100–200uM. Intravenous ascorbate was found to produce plasma levels hundreds of fold higher than those produced by the maximum tolerated dose of oral ascorbate. (Hoffer et al., 2008; Padayatty and Levine, 2000; Padayatty et al., 2004; Park et al., 2009) Early phase clinical trials indicate that IV ascorbate at 1g/kg over 1½–2 hours two to three times weekly is well tolerated and may enhance chemosensitivity as well as decrease chemotherapy related side effects. (Carr et al., 2014; Hoffer et al., 2015; Ma et al., 2014; Monti et al., 2012; Schoenfeld et al., 2017; Shim et al., 2014; Stephenson et al., 2013; Welsh et al., 2013).	Plasma concentrations achieved by IV dosing found to act as a pro-drug for hydrogen peroxide (H2O2) in the extracellular space. Pharmacologic, but not physiologic, ascorbic acid was selectively toxic to cancer cells in-vitro and in vivo. (Chen et al., 2005; Chen et al., 2008; Verrax and Calderon., 2009; Espey et al., 2011; Ma et al., 2014; Serrano et al., 2015; Xia et al., 2017)	Ascorbate functions as a cofactor and increases the activity of the Ten Eleven Translocation (TET) enzymes causing DNA demethylation. This function has been found to result in the re-expression of tumor suppressor genes in cancer cells, promotion of stem cell differentiation and inhibition of leukemogenesis, and enhancement of DNA methyltransferase inhibitor (DNMTi)-induced immune signals via increased expression of endogenous retrovirus transcripts. (Agathocleous et al., 2017; Cimmino et al., 2017; Liu et al., 2016; Shenoy et al., 2017, Gustafson et al., 2015).

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Ascorbate physiology and pharmacokinetics findings are the backbone of these observations. Nevertheless, extracellular H_2O_2 formation generated by pharmacologic ascorbate may not explain all pharmacology actions. As one example, recent findings implicate oxidized ascorbic acid, dehydroascorbic acid, as the mediator of cancer cell death in specific engineered cell lines (Yun et al., 2015). Although the conclusions are attractive, extracellular H_2O_2 generation from pharmacologic ascorbate could well account for the observations, a possibility not tested. Other investigators found no effects of dehydroascorbic acid on cancer cell using a variety of cell types (Chen et al., 2005; Ma et al., 2014; Schoenfeld et al., 2017). Clinically, dehydroascorbic acid currently would not be administered because of potential pancreatic toxicity (Patterson and Lazarow, 1950). As another example, we should not forget Ewan Cameron's cases. Prior to publications with Linus Pauling, Cameron reported a case series where unexpected tumor responses were observed, using intravenous ascorbate at 10 grams daily for 7–10 days followed by oral ascorbate at 10 grams daily (Cameron and Campbell, 1974). There were substantial limitations, including a Scottish patient population at substantial risk for severe vitamin C deficiency/scurvy that may have been corrected by treatment; lack of definitive pathology; retrospective selective bias; and lack of adequate controls. Even so, there remains at least one exceptionally well documented case report by Cameron and colleagues, of double complete regression (Cameron et

al., 1975; Campbell et al., 1991). We note that exquisite sensitivity to H_2O_2 generated by the initial IV ascorbate could still explain findings, and other limitations above may also be confounders. Although there are no direct measurements in humans that H_2O_2 is generated by pharmacologic ascorbate, there are direct rodent data (Chen et al., 2007; Chen et al., 2008). Supportive evidence that H_2O_2 is generated by pharmacologic ascorbate in humans is provided by reports of oxidative hemolysis in patients with glucose 6-phosphate dehydrogenase deficiency who received intravenous ascorbate (Huang et al., 2014; Rees et al., 1993). Indeed, it is for this very reason that patients who receive intravenous ascorbate must be pre-screened for glucose 6 phosphate dehydrogenase deficiency. Taken as a whole, Cameron's reported cases hint that there could be other H_2O_2-independent potential actions of pharmacologic ascorbate, because intravenous doses at those used are predicted to have relatively small effects on extracellular H_2O_2 formation.

Ascorbate and epigenetic regulation

In this vein, recent reports demonstrate that AA at physiologic concentrations from oral doses may exert its anticancer activity through an epigenetic mechanism (DNA demethylation), distinct from and mechanistically independent of H_2O_2 generation and pharmacologic ascorbate concentrations. Ascorbate functions as a cofactor and increases the activity of the Ten Eleven Translocation (TET) enzymes, causing DNA demethylation and increase in hydroxymethylation (Blaschke et al., 2013; Yin et al., 2013). This function has been found to result in re-expression of tumor suppressor genes in cancer cells, promotion of stem cell differentiation, and enhancement of DNA methyltransferase inhibitor (DNMTi)-induced immune signals via increased expression of endogenous retrovirus transcripts (Agathocleous et al., 2017; Cimmino et al., 2017; Liu et al., 2016; Shenoy et al., 2017). The comparative effect is currently unknown of IV versus physiologic, or oral, doses of Vitamin C on in vivo DNA demethylation and DNA hydroxymethylation. It is also unclear at this point to what extent other effects of ascorbic acid mediated through direct/ indirect cofactor functions, including Jumonji histone demethylase activity (Wang et al., 2011), contribute to its overall effect on established cancer. Despite these uncertainties, it is a reasonable assumption that with IV dosing, the impact on direct/ indirect cofactor functions of Vitamin C, including the TET mediated DNA demethylation effect, would be at least equal to, if not higher than, oral doses. Potential limiting factors to ascorbate uptake in cancers include tissue characteristics such as high cell density, poor perfusion, reduced expression/activity of transporters. Indeed, data from a three dimensional pharmacokinetic model to measure ascorbate diffusion suggests that ascorbate penetration and distribution in a poorly vascularized tumor may require higher than normal plasma concentrations.(Kuiper et al., 2014; Wohlrab et al., 2017) The IV dosing also provides the additional benefit of generating extracellular hydrogen peroxide, which can induce oxidative stress in cancer cells. With this background, there is potential to explore IV ascorbate as a therapeutic approach for subsets of malignancies with known aberrant hypermethylation such as chronic myelomonocytic leukemia (60–70% of cases with *TET2* mutations), myelodysplastic syndrome, *TET2* mutated together with either *IDH1* or *IDH2* mutated acute myelogenous leukemia (2-hydroxyglutarate mediated suppression of TET), clear cell renal cell carcinoma, and succinate dehydrogenase mutated paraganglioma. (Bejar et al., 2014; Figueroa et al., 2010; Hu et al., 2014; Jiang et al., 2009; Letouze et al., 2013; Patnaik and Tefferi, 2016; Rasmussen et al., 2015) Furthermore, loss of hydroxymethylcytosine, which has been reported in multiple cancers (Chen et al., 2013; Jin et al., 2011; Ko et al., 2010; Kudo et al., 2012; Mason and Hornick, 2013; Muller et al., 2012; Orr et al., 2012; Perez et al., 2012; Yang et al., 2013) and is an adverse prognostic marker, could be investigated as a potential biomarker for ascorbate treatment since ascorbate has been found to increase hydroxymethylcytosine levels *in vitro* in cancer cells via the activation of the TET enzymes.(Agathocleous et al., 2017; Gustafson et al., 2015; Shenoy et al., 2017) Given that most of the *TET2* inactivating mutations are uniallelic, it is possible that the activity of the wild-type copy could be optimized by pharmacologic ascorbate and produce therapeutic benefit. Furthermore, even if the TET enzyme is unmutated, its activity could still be suppressed in some malignancies by the abnormal accumulation of Krebs cycle intermediates. The relative importance of the two mechanisms of anti-cancer activity, i.e. epigenetic and H_2O_2 mediated, is yet to be established in humans.

Ascorbate and cancer cell culture models: some limitations

Ascorbate modification of TET and Hypoxia Inducible Factor- Prolyl Hydroxylase (HIF-PHD) activities in cell culture models must also be considered in context of limitations of using ascorbate in these models. A reminder: cells in culture have no ascorbate unless it is purposely added to media. Most commercial media lack ascorbate because it easily oxidizes *in vitro*. When ascorbate is added to media, the extracellular concentration does not reflect the intracellular concentration that can be attained at homeostasis, when ascorbate concentrations are kept constant over time. Even acutely isolated cells from humans lose ~ 90 % of their intracellular ascorbate within 72 hours in culture (Bergsten et al., 1990). Excepting acutely isolated cells, ascorbate intracellular concentrations *in vitro* should be considered to begin at zero, a condition that is very difficult to reconcile with human biology. *In vitro*, estimates of steady-state intracellular concentrations of ascorbate for a given extracellular concentration depend not on simply one added extracellular concentration but rather on multiple factors, including oxidation of extracellular media ascorbate, replenishment of oxidized ascorbate, incubation times, contribution of dehydroascorbic acid transport, and ascorbate and GLUT transporter copy numbers. In addition, ascorbate-dependent enzyme activities and ascorbate transport mechanisms observed in cell lines might not fully reflect *in vivo* conditions. For these reasons, clinical trial designs testing ascorbate in cancer treatment cannot be based only on *in vitro* data, where adding ascorbate to cells changes enzyme activity, but rather on *in vivo* findings.

Physiologic ascorbate/cancer prevention; pharmacologic ascorbate/cancer treatment

While our focus is on cancer treatment with ascorbate, findings in mice suggest a role of oral ascorbate in delaying cancer development, based on ascorbate dependent activities of TET and inhibition of hypoxia inducible factor by prolyl hydroxylases (HIF-PHD) enzymes (Agathocleous et al., 2017; Campbell et al., 2016; Gao et al., 2007). TET experiments used the *Gulo*$^{-/-}$ mouse model that lacks the endogenous ascorbate synthesis pathway resulting from the gene encoding L-gulonolactone oxidase (*Gulo*), which encodes a key enzyme

of the pathway, being knockout. Like humans, whose L-gulonolactone oxidase is non-functional, *Gulo*$^{-/-}$ mice are ascorbate requiring. Recent elegant experiments utilizing bone marrow transplantation in ascorbate deficient *Gulo*$^{-/-}$ mice with TET2 deficiency indicated that there is enhanced development of acute myelogenous leukemia. In these mice, oral ascorbate postponed progression of genetically susceptible transplanted hematopoietic cells to acute myelogenous leukemia (Agathocleous et al., 2017). Oral ascorbate was shown to increase TET activity and hydroxymethylcytosine levels. However, there was no evidence of a decrease in blast percentage in the same animals once acute myelogenous leukemia was established. Similarly, in a lymphoma xenograft model tumor development was decreased by oral ascorbate, which had HIF-1-dependent anti-tumorigenic properties (Gao et al., 2007). Here too, oral ascorbate was introduced before tumor development, seven days before inoculation of cancer cells. For comparison purposes, tumor treatment to reduce tumor growth in many rodent models was mediated by parenteral ascorbate to produce pharmacologic concentrations (Chen et al., 2008; Espey et al., 2011; Ma et al., 2014; Rouleau et al., 2016; Serrano et al., 2015; Verrax and Calderon, 2009; Xia et al., 2017)). In a hepatoma xenograft model, parenteral ascorbate inhibited tumor growth, but oral ascorbate had no treatment effect (Verrax and Calderon, 2009). In other experiments *Gulo*$^{-/-}$ mice were implanted with various tumors. Tumor growth was faster in ascorbate deficient compared to mice repleted with ascorbate by mouth (Campbell et al., 2015; Campbell et al., 2016; Cha et al., 2013). Considered as a whole, the animal data suggest that oral ascorbate may be effective as an agent to prevent or postpone the development of certain types of malignancy in genetically pre-disposed individuals. Possibilities include individuals having hematopoietic cells with a *TET2* mutation, with or without low grade dysplasia, by optimizing activity of the wild-type allele; *VHL* haplodeficient individuals predisposed to renal cell carcinoma by optimizing HIF-PHD activity; and succinate dehydrogenase/fumarate hydratase deficient individuals predisposed to renal cell carcinoma or pheochromocytoma/paraganglioma, by optimizing activity of the metabolically inhibited TET and HIF-PHD enzymes. In contrast, to our knowledge, there are no experiments in *Gulo*$^{-/-}$ mice and in other rodent models that show that varying oral ascorbate is effective for treatment of pre-existing solid or hematologic malignancies. Some investigators have noted that *Gulo*$^{-/-}$ mice tolerate much lower ascorbate concentrations than humans (Campbell et al., 2015; Tu et al., 2015) i.e. < 1 mM. *Gulo*$^{-/-}$ mice that had ascorbate concentrations approximating those of scurvy in humans for one year neither had symptoms of scurvy during their life span nor significant evidence of cancer at necropsy (Tu et al., 2017). For these reasons, we believe that caution is appropriate in extending findings of *Gulo*$^{-/-}$ mice models to humans.

Ascorbic acid concentrations in cancer patients are often at deficiency concentrations (Huijskens et al., 2016; Liu et al., 2016; Mayland et al., 2005), the reason for which is still uncertain. Possibilities include generalized cancer cachexia, enhanced consumption of ascorbate by tumors, or downregulation of ascorbate transporters. It is therefore reasonable to consider whether there is a role for oral ascorbic acid as a treatment modality by correction of deficiency of ascorbate, independent of pharmacologic administration. In humans, results of randomized treatment trials were negative in unselected advanced cancers and advanced colorectal cancer (Creagan et al., 1979; Moertel et al., 1985). There are rare case reports where oral ascorbic acid may have been useful, but this was in conjunction with intravenous as well as oral ascorbate. Based on recent in vitro evidence, correction of ascorbate deficiency may aid conventional hypomethylating agents i.e. DNA methyltransferase inhibitors to exert maximal effect (Liu et al., 2016).

The rationale for the use of high dose intravenous ascorbate in cancer treatment is not to correct plasma deficiency, but rather to induce an oxidative stress on cancer cells (Chen et al., 2005; Chen et al., 2007; Chen et al., 2008) and to ensure adequate delivery of ascorbate within tumors for optimal cofactor function (Kuiper et al., 2014). As a way forward, it is essential to acknowledge that only pharmacologic, i.e. parenteral ascorbate, to date has produced clearly documented treatment efficacy in early phase human trials. In rodent models of treatment of established cancers, only pharmacologic ascorbate is effective regardless of the model: nude-mouse, syngeneic, specific for dehydroascorbic acid/GLUT transport mechanisms, or coupled to TET actions. These data provide guidance for clinical trials. Pharmacologic ascorbate is the standard to which other ascorbate dosing schemes should be compared to. There is promising efficacy in small human studies, and a patient safety profile similar to that of an intravenous antibiotic rather than systemic chemotherapy, but many unknowns remain. Dosing information is limited, including dosing amount, dosing frequency, ascorbate administration timing with conventional agents, and treatment duration. Clinical evidence is that at a minimum 1 g/kg should be administered intravenously over 2 hours twice weekly, with more frequent administration perhaps better (Hoffer et al., 2008; Ma et al., 2014; Monti et al., 2012; Polireddy et al., 2017; Schoenfeld et al., 2017; Welsh et al., 2013). Based on the small datasets available, the absolute minimum treatment duration is 2 months before efficacy is assessed, and probably 3–4 months (Drisko et al., 2018; Monti et al., 2012; Nielsen et al., 2017; Rouleau et al., 2016). These considerations place burdens on both trialists and patients. For patients, it may not be appealing to have to sit for treatment 2 hours three times weekly, for months if not longer. For trialists, problems can be anticipated with initial recruitment and treatment continuity. Nevertheless, we should not decrease dosing, frequency, or duration without clear supportive data. Basing decisions on *in vitro* data will perhaps irrevocably harm the emerging efforts to test ascorbate in cancer treatment.

The Phoenix Can Rise

The implications are substantial of a therapeutic agent that is universally available and accessible, with the potential of anti-cancer activity across multiple tumor types as well as reducing chemotherapy toxicity. Should the oncology research community invest in the necessary randomized trials to test whether there is benefit of IV ascorbate? We firmly recommend yes, utilizing well-designed randomized (and where possible, placebo-controlled) trials. Establishing biomarkers for selection of patients who benefit from IV ascorbate would enable us to tailor treatment more effectively and efficiently. Mechanism bases should be explored, with caveats. However, despite intentions to pursue well-defined biomarkers of response, it may be difficult to zero in on a single, highly predictive biomarker. This is because pharmacologic ascorbate can be considered a promiscuous agent mechanistically with respect to generation of extracellular H_2O_2 as well as its varied cofactor functions (Levine and Violet, 2017). Once extracellular H_2O_2 is present, myriad mechanisms can mediate cancer cell death. While these aspects may not be in keeping with the “targeted therapy” era in oncology, they

should not deter exploration of ascorbate's potential as a therapeutic agent. Primary endpoints may be tumor response rate or survival. Secondary endpoints include adverse effect profiles and quality of life. We strongly encourage the field to explore ascorbate's potential, but also send a cautionary note that new studies need to be done properly. Let's not shoot down the re-rising ascorbate treatment phoenix.

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ABBREVIATIONS

IV	intravenous
H_2O_2	hydrogen peroxide
HIF PHD	Hypoxia-inducible factor, prolyl hydroxylase dependent
TET	Ten- Eleven Translocation

Footnotes

Disclosure of Potential Conflicts of Interest: The authors declare no competing interests

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