Antioxidants and Redox Signaling

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Forum Review Article

Vitamin C and cell death

András Szarka[1], Orsolya Kapuy[2], Tamás Lőrincz[1], Gábor Bánhegyi[2]

[1]Department of Applied Biotechnology and Food Science, Laboratory of Biochemistry and Molecular Biology, Budapest University of Technology and Economics, Szent Gellért tér 4. H-1111 Budapest, Hungary

[2]Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary 1444

Running head: Vitamin C and cell death

*Corresponding author:

Dr. András Szarka
Laboratory of Biochemistry and Molecular Biology
Department of Applied Biotechnology and Food Science
Budapest University of Technology and Economics
Szent Gellért tér 4. Budapest H-1111 Hungary
Tel.: +36 1 4633858, e-mail: szarka@mail.bme.hu

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Antioxidants and Redox Signaling
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Abstract

Significance: Persistent oxidative stress is a common feature of cancer cells, giving a specific weapon to selectively eliminate them. Ascorbate in pharmacologic concentration can contribute to the suspended formation of hydroxyl radical via the Fenton reaction, thus it can be an important element of the oxidative stress therapy against cancer cells.

Recent Advances: The main components of ascorbate induced cell death are DNA double-strand breaks via the production of hydroxyl radical and ATP depletion due to the activation of PARP1. Presumably, DNA damage can be the primary contributor to the anti-cancer activity of pharmacologic ascorbate, as opposed to the rupture of bioenergetics. The caspase independency of high dose ascorbate induced cell death proposed the possible involvement of several types of cell death such as ferroptosis, necroptosis and autophagy.

Critical Issues: Ascorbate can target at least two key molecular features of cancer cells as a part of the anti-cancer therapy: the intrinsic or acquired resistance to cell death and the dysregulated metabolism of cancer cells. It seems probable that different concentrations of ascorbate alter the nature of induced cell death. Autophagy and necroptosis may play a role at intermediate concentrations, but caspase independent apoptosis may dominate at higher concentrations. However, ascorbate behaves as an effective inhibitor of ferroptosis that may have crucial importance in its possible clinical application.

Future Directions: The elucidation of the details and the links between high dose ascorbate induced cancer selective cell death mechanisms may give us a tool to form and apply synergistic cancer therapies.

Introduction

Ascorbate as a water-soluble antioxidant plays an integral part in the cellular oxidative metabolism, as it efficiently scavenges reactive oxygen and nitrogen species (ROS and RNS) produced under various stress conditions (72). Ascorbate has important role in the reduction of the tocopheroxyl radical to tocopherol, tocopherol-mediated protection against lipid peroxidation could be significantly enhanced by the presence of ascorbate (and glutathione) (25, 69). Furthermore, ascorbate is an essential cofactor for multiple enzymes involved in the biosynthesis of carnitine (39) and catecholamines (7). Ascorbate also maintains the catalytic activity of various Fe(II)-2-oxoglutarate-dependent dioxygenases during the posttranslational modification and folding of extracellular matrix proteins like collagen (67). In this regard, it was also shown that ascorbate can upregulate procollagen mRNA transcription primarily by stabilizing the mRNA transcript (55, 70). In another aspect of protein folding, it is presumed that ascorbate has an important role during disulfide bond formation in the lumen of the endoplasmic reticulum (67). Additional involvement of ascorbate in epigenetic regulation was found in specific histone lysine demethylases (termed JmjC-domain-containing histone demethylases) and DNA 5-methyl-cytosine hydroxylases (termed TET1-3) which possess an ascorbate-dependent hydroxylase activity (54). These hydroxylases were identified by the means of structure similarity to collagen hydroxylases as their catalytic domain is also organized in typical double-stranded β-helix fold containing two histidines and one carboxylate (aspartate or glutamate). This binds to Fe(II) which is a joint feature of Fe(II)-2-oxoglutarate-dependent dioxygenases.

The cellular level of ascorbate is kept under strict control by its well-regulated transport. Vitamin C is absorbed along the entire length of the human intestine in the form of ascorbate and dehydroascorbate (DHA). The transport of both forms showed saturation with an apparent K_M of 267±33 μM for the reduced form and 805±108 μM for the oxidized form. The relatively low affinity of DHA transport compared with ascorbate transport indicates that the majority of vitamin C is absorbed in the reduced form. The transport of ascorbate is mediated by the members of the SVCT family (SVCT1 and 2) in a Na^+ dependent manner, while the uptake of DHA is mediated by different members of the facilitative glucose transporter family (GLUT1, 2, 3, 4, 8, and 10) with Na^+ independent

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

facilitated diffusion (72). Plasma ascorbate concentration reaches a plateau by increasing oral doses because of the following two factors: first, the capacity of the ascorbate transporters is limited. Second, the expression of SVCTs is fine-tuned by their ligand and by the redox state of the cell. Both ascorbate uptake and the expression of SVCTs are decreased at elevated ascorbate levels. Hence the plasma ascorbate concentration has a plateau at 80-100 µM (43, 72).

The reason of this well-regulated ascorbate level can easily be understood. The autoxidation of ascorbate results in the formation of ascorbyl radical and superoxide anion then H_2O_2. The autoxidation is pH-dependent and occurs relatively slowly under physiological conditions (64, 72). The relatively rare dianion form of ascorbate autoxidizes approximately six order of magnitude times faster than the much more abundant monoanion at physiological pH (Fig. 1) (64). In biological systems, in the course of the formation of ascorbyl radical, ascorbate can donate an electron to a transition metal such as iron or copper (Eq. 1). The reduced metal can react with oxygen forming superoxide anion (Eq. 2) and then H_2O_2 (Eq. 3) (Fig. 1). In the presence of higher, millimolar concentrations of ascorbate (pharmacologic ascorbate), H_2O_2 can readily react with further transition metal ions in the Fenton reaction to form the highly reactive, cytotoxic hydroxyl radical (Eq. 4) (Fig. 1) (72). This way the tight control of ascorbate concentration via its strictly regulated transport and the binding of the metal partner, iron to proteins, such as ferritin, that also strictly controls the level of its free or labile form (13, 66), help to avoid the continuous exposure of tissues to high concentrations of H_2O_2 and to its more reactive derivatives.

$Fe^{3+} + AscH^- \rightarrow Fe^{2+} + Asc^{\cdot -}$ Eq. 1

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -}$ Eq. 2

$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ Eq. 3

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$ Eq. 4

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Bypassing the tight control

This tight control of ascorbate level can be bypassed by the parenteral administration of ascorbate that gives the possibility to form H_2O_2 in discrete, well-defined time periods, decreasing the likelihood of harm, and it provides a pharmacologic basis for therapeutic use of ascorbate (60). Why can this loss of control be advantageous? Any difference between tumour and non-tumour cells gives the possibility of their selective elimination. In general, cancer cells can be characterized by elevated generation of ROS due to increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signalling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase or due to the crosstalk with infiltrating immune cells (5, 46) (Fig. 2). At the same time, it is hard to make clear statements on the antioxidant systems of cancer cells. Boosted antioxidant systems were reported in cancer cells to survive in a high oxidative stress environment and these boosted antioxidant systems were considered to be responsible for the difficult treatment of Ras- and Myc-driven cancer cells (41). Accordingly, some studies reported an increased catalase expression in tumours compared to normal tissues of the same origin. However, several others found the downregulation of catalase (36). Catalase activity was one to two orders of magnitude lower in several investigated cancer cell lines (such as human hepatocellular carcinoma, breast carcinoma and pancreas carcinoma) than in the normal rat liver or primary rat hepatocytes (62). The expression, the protein level and the activity of catalase in the cancer cells lag far behind the corresponding values of the primary rat hepatocytes and rat liver tissue. The fine balance of intracellular H_2O_2 level is controlled by a family of intracellular enzymes. The members of this family are the six peroxiredoxin enzymes, the glutathione peroxidase/glutathione system and catalase (33). At high H_2O_2 concentrations such as produced by pharmacologic ascorbate treatment the rate of removal of peroxide is, essentially, dominated by catalase (33). For details see the excellent work of Eruaditius et al. (33). The activity of the other H_2O_2 eliminating enzyme GPX showed analogous results having a 10-15-fold lower activity of the tumour cell lines than the primary ones (62). Therefore, they may have a decreased ability to cope with enhanced oxidative stress due to exposure of exogenous H_2O_2. Indeed, it was

demonstrated that the rate constants for overall removal of extracellular H_2O_2 by tumour cells are, on average, half of the rate constants for normal cells (64) (Fig. 2). Although there are conflicting results on the reprogramming of antioxidant defence in tumour cells, the persistent oxidative stress is not debated in cancer cells and it is evidenced by 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in various tumours (15). ROS are used by cancer cells to stimulate proliferation, invasion, migration and angiogenesis. It seems that cancer cells are able to adjust oxidative stress to a level which is sufficient to maintain their survival but not to initiate their elimination (15). This adaptation of cancer cells to elevated ROS level treads a very fine line between their proliferation and cell death. Therefore, increased ROS levels are frequently used as cytotoxins in cancer patients (57).

As it was outlined above reduced vitamin C can donate an electron to a transition metal such as iron or copper, then the reduced metal is capable of reacting with O_2 forming superoxide anion and then H_2O_2. Finally, the produced H_2O_2 in the presence of pharmacologic concentrations of ascorbate can readily react with further transition metal ions to form the highly reactive, cytotoxic hydroxyl radical in the Fenton reaction (Fig. 1) (72). The pharmacologic ascorbate induced cell death of cancer cells was clearly related to its ROS generating property and depended on H_2O_2 production (72) since it can be avoided by exogenous catalase or adenoviral-mediated overexpression of catalase or GPX1 (64, 65). Furthermore, ascorbate toxicity could be completely suspended by enzymes that metabolize H_2O_2 *in vitro* (22, 31, 56, 65).

On the mechanism of ascorbate toxicity

Albeit the potential role of pharmacologic ascorbate in cell death was proposed more than 40 years ago (19), the exact mechanism of its action has not been elucidated up to this day. Necessarily several important discoveries have been made on the type of cell death and on the role of transition metals or ROS. We try to give a short overview of these findings and discuss their relationship with each other and with the altered metabolism of cancer cells.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

The possible involvement of ferroptosis in ascorbate induced cancer cell death

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Since the nature of the cytotoxicity induced by pharmacologic ascorbate is oxidative, that involves the production of ROS and lipid peroxides (LOOX) the involvement of ferroptosis, the only unequivocally oxidative stress and lipid peroxidation driven cell death, would be obvious.

Ferroptosis was induced by a group of chemical compounds such as erastin and RSL3 in tumour cells harbouring RAS mutation (inter alia in *NRAS* mutant HT-1080 cells) (29, 76) (Fig. 3). The novel cell death type could be inhibited by the iron chelator deferoxamine, the water soluble antioxidant GSH and by the lipophilic antioxidant, α-tocopherol, suggesting that ferroptosis is related to intracellular iron and ROS (76) (Fig. 2). Due to the iron-dependency of the novel cell death type, it was latter called ferroptosis. The specific inhibitors of ferroptosis, such as Ferrostatin-1 and Liproxstatin-1, inhibit the accumulation of lipid hydroperoxides underlining the role of lipid peroxidation in the induction of ferroptosis (78) (Fig. 3). Both inhibitors seem to behave as radical trapping antioxidants. All the characteristic features of the novel cell death type such as its morphology, biochemistry and genetics differ considerably from all other already known cell death types (28, 76). It is proposed that autophagy via the degradation of the iron binding protein ferritin contributes to the elevation of the labile iron pool and to the consequent induction of ferroptosis (35). The oncogenic kinase inhibitor, sorafenib was reported to induce ferroptosis in hepatocellular carcinoma cells (48) and in different solid tumours (42). Ascorbate could act synergistically with sorafenib, moreover, it broadened the therapeutic range of sorafenib (62). Cell deaths induced by the known ferroptosis inducers, such as erastin and RSL3 or by pharmacologic ascorbate show very similar characteristics. These features are 1. iron-dependent, ROS mediated cell death mechanisms, which could be suppressed by co-treatment with the iron chelator deferoxamine (28, 75); 2. caspase-independency (28, 50); 3. elevation of labile iron pool via autophagy mediated ferritin degradation (in ferroptosis) (35) and increased labile iron pool via ascorbate induced disrupted cellular iron metabolism (6); 4. pharmacologic ascorbate or erastin, RSL3 treatment caused increased LC3-II level and formation of autophagosomes (21, 26, 34). All

these observations and similar features propose that ferroptosis may play role in pharmacologic ascorbate induced cancer cell death.

The above hypothesis was investigated on HT-1080 cell line, since ferroptosis was originally described on these cells (28). The EC50 value of pharmacologic ascorbate on HT-1080 cells was 0.5 mM that is in the range of the most sensitive cell lines such as human lymphoma (JLP-119), glioblastoma (S635) cell lines, murine neuroblastoma (2a), breast cancer (TS1A), (4 T1) cell lines and rat glioblastoma (RG2), (9 L), (C6) (22, 47, 72). The ineffectiveness of the specific ferroptosis inhibitors (ferrostatin-1 and liproxstatin-1) on pharmacologic ascorbate treated HT-1080 (a cell line harbouring *NRAS* mutation) and MCF-7 (a cell line without RAS mutation) cell lines to elevate their viability suggests that ferroptosis is not involved in the pharmacologic ascorbate induced cell death (Fig. 4) (47). This assumption was further strengthened by the different morphology of HT-1080 cells due to pharmacologic ascorbate or RSL3 treatment and by the ineffectiveness of the lipophilic antioxidant α-tocopherol (47), that could effectively elevate the viability of erastin and RSL3 treated HT-1080 cells (28, 48). On the contrary to the earlier observed similar features of pharmacologic ascorbate and erastin, RSL3 induced cell deaths ascorbate at lower, near physiological concentration behaved as an inhibitor of both RSL3 and erastin induced ferroptosis (Fig. 3 and Fig. 4) (47). At higher concentrations, the cytotoxic effect of ascorbate exceeded its protective properties hence its interference with ferroptosis could only be investigated in the presence of pyruvate. As it was shown, pyruvate countered the cytotoxic effect of ascorbate without affecting ferroptosis. Consequently in the presence of pyruvate elevated concentrations of ascorbate resulted in increased protection of cells from ferroptosis (47). The investigation of the production of ROS in the form of hydroxyl radical and lipid peroxides due to pharmacologic ascorbate and ferroptosis inducers (erastin, RSL3) revealed that both pharmacologic ascorbate and RSL3 induced the formation of ROS and lipid peroxides. According to the protective role of ascorbate, the co-treatment of RSL3 treated cells with low dose ascorbate significantly decreased the level of both ROS and lipid peroxides (47) (Fig. 3). On the base of these observations it can be presumed that the anti-ferroptotic effect of ascorbate may originate from its antioxidant property.

Furthermore, the demonstrated anti-ferroptotic effect of ascorbate – which is also present to some extent at physiological concentrations should also be considered in the course of the possible clinical application of ferroptosis inducers.

The role of iron in ascorbate induced cell death

As it was stated in the Introduction, transition metals are key players in the cytotoxicity of ascorbate. The cell permeable metal chelator deferoxamine could prevent the loss of viability of DU145 tumour cells exposed to high concentrations of ascorbate (75). High-dose ascorbate induced DNA breakage in lymphocytes could also be inhibited by both iron and copper chelators. The potential role of chromatin-bound copper was also proposed by the observed similar inhibitory effect of neocuproine (a cell membrane–permeable Cu (I)-specific chelator) in nuclei (74). However, it should be noted that the specificity of neocuproine towards copper ions is not absolute, it can also chelate iron with non-negligible efficiency (68). The experiments with different chelators show exceedingly the high toxicity of free or labile (loosely bound) iron thus it is usually bound to proteins, such as ferritin in the cells (13, 66). Ferritin behaves as a natural chelator or an iron scavenger to prevent oxidative damage (6). If iron is released from ferritin it can catalyse the formation of hydroxyl radicals. It was shown that ascorbate can induce the release of iron from ferritin (6). Both increased ferritin release and increased lactate production could be observed in Kelly and SK-N-SH neuroblastoma cell lines. Ascorbate mediated H_2O_2 production can be fuelled by the elevated ferritin production and secretion that can serve as a continuous iron source (27). Elevated levels of ROS and ascorbate can disrupt the cellular iron metabolism leading to increased labile iron pool (Fig. 3) (6, 18, 40). One of the general features of cancer cells is their elevated intracellular labile iron levels (71). In addition to the higher basal level of labile iron of glioblastoma and non-small cell lung cancer cells, they also showed a significant dose-dependent ascorbate induced elevation in cellular labile iron pool. This could not be observed in normal human astrocytes or in normal human bronchial epithelial primary cells (65). The loss of Fe-S cluster protein activity was accompanied by the elevation of the labile iron pool. Both could be prevented by the overexpression of catalase implicating H_2O_2 as the causative agent in ascorbate-induced elevation of the labile iron pool (Fig. 3) (65). The elevated mitochondrial ROS

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

triggered the elevation of the labile iron pool, that could result in more pronounced oxidation of cellular ascorbate. This induced the production of more H_2O_2, causing a further elevation of the labile iron pool of cancer cells compared to normal cells (Fig. 3 and 4). The high concentration of H_2O_2 in the presence of high-labile iron promotes the Fenton reaction generating the highly toxic hydroxyl radical (Fig. 3) (Schoenfeld 2017). The non-cancerous cells can be characterized by lower basal and ascorbate-mediated H_2O_2 and labile iron levels. In these cells, H_2O_2 is metabolized quickly before it can take part in pro-oxidant reactions. Therefore, ascorbate is not toxic for non-cancerous cells (22, 64, 65). This phenomenon could be the reason for the observed safety of pharmacologic ascorbate in animal xenograft (23, 51, 75) and human (3) studies.

DNA double-strand breaks or bioenergetic attack?

It is generally accepted that the generation of H_2O_2 gives the background for the anti-cancer activity of pharmacologic ascorbate (22, 23, 64, 72). As we stated in the previous chapter, H_2O_2 is produced in the presence of transition metals and a high dose of ascorbate in the extracellular space of tumours (64, 72). However, the H_2O_2 produced in the blood is immediately scavenged by the catalase and GSH peroxidase of red blood cells (75), thus a higher level of H_2O_2 generated by pharmacologic administration could only be measured in the extracellular fluid (60). H_2O_2 then passes through the membrane by the mediation of specific aquaporins (Fig. 5) (11, 12). This role of aquaporins is underlined by the observation that peroxiporin expression is important in the modulation of the susceptibility of cancer cells to high dose ascorbate treatment (32).

DNA is considered to be the primary target of hydroxyl radical formed in the reaction of H_2O_2 and Fe^{2+} associated with DNA (16). DNA double-strand breaks (DSB) could be generated via the production of H_2O_2 in pharmacologic ascorbate treated neuroblastoma, pancreatic and ovarian cancer cells (Fig. 5) (24, 51). As a reinforcement of the role of H_2O_2, DSB could be prevented by extracellular catalase (30). In a recent study enhanced markers for DNA damage such as the phosphorylation of histone H2AX and CHK1 were reported in MIA PaCa-2 and PANC-1 pancreatic cancer cells due to pharmacologic ascorbate treatment (16). Extracellular catalase co-treatment prevented the formation of phosphorylated H2AX

and CHK1 in both cell lines. This also strengthens the role of extracellular H_2O_2 in the genotoxicity of pharmacologic ascorbate treatment (Fig. 5).

Upon activation, CHK1 is released from the chromatin and halt cell cycle progression to allow repair. In response to genotoxic stress and DNA damage it ensures the fidelity of DNA repair checkpoints (52). The inhibition of CHK1 resulted in elevated cytotoxicity due to high dose ascorbate treatment (16). Lesions could be observed both in the nuclear (nDNA) and mitochondrial DNA (mtDNA) immediately after treatment with pharmacologic ascorbate. The repair of mtDNA is known to be less efficient to that of nDNA. Accordingly, it was found that after 1 h of pharmacologic ascorbate treatment mtDNA possessed a higher number of lesions as opposed to nDNA and the number of these were repaired more slowly compared to control (16). Poly (ADP-ribose) polymerase 1 (PARP1) marks DNA damage by extended poly (ADP-ribose) synthesis. Extended DNA damage results in extended activation of PARP1 which in turn leads to intensive NAD^+ consumption and in the consequent depletion of ATP (Fig. 5). This way PARP1 activation makes a tight relationship between DNA damage, NAD^+ metabolism, cellular energetic status and finally to cell survival or death (49, 63). Reasonably, the role of this bioenergetic rupture in pharmacologic ascorbate induced cell death has also been emerged. The depletion of ATP was not due to changes in the rate of production, but due to its increased consumption (16). Both the pharmacological inhibition and the genetic deletion of PARP1 verified that pharmacologic ascorbate induced PARP1 over-activation is responsible for the depletion of NAD^+ and ATP. The decrease in NAD^+ and ATP pools was almost prompt, but both of them restored to near basal levels within 12 to 24 h after the suspension of ascorbate treatment (16). Both the pharmacologic inhibition and the genetic deletion of PARP1 prevent the depletion of NAD^+ and ATP but did not inhibit cell death. Moreover, the rate of cell death due to high dose ascorbate treatment was enhanced in PARP1 knockout cells or in PARP1 inhibitor (olaparib) pre-treated cells (16) presuming that the DNA damage could be the primary contributor for the anti-cancer activity of pharmacologic ascorbate, but the rupture of bioenergetics is likely not.

Although hydroxyl radical induced cell death was caspase independent, it could be characterized by the typical morphological and biochemical changes of apoptosis (61). Not

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

surprisingly, high dose ascorbate induced cell death was considered to be apoptosis in the studies published before 2010 (Fig. 4) (72). Since that time several new forms of cell death were described, thus attempts were taken to clarify the potential role of apoptosis and to elucidate the exact mechanism of ascorbate induced cell death. The pan-caspase inhibitor Z-VAD did not have any effect on high dose ascorbate induced cell death of neuroblastoma cell lines indicating that it was independent of caspase activation (50). According to the ineffectiveness of Z-VAD, caspase-3 cleavage was also absent in neuroblastoma cells treated with high dose ascorbate. Similar results were gained in the case of other cell lines (47). The caspase independency of high dose ascorbate induced cell death proposed the possible involvement of necroptosis. The necroptosis inhibitor, necrostatin-1 showed a modest inhibitory effect but only against a moderate concentration of ascorbate (0.5 mM), the protective effect of the inhibitor was lost above 1.0 mM (47). The increase of the protein level of the necroptosis marker, RIPK1 at both 0.1 and 0.6 mM of ascorbate treatment (47) further suggested the possible role of necroptosis in moderate concentrations of ascorbate induced cell death (Fig. 4).

The role of ascorbate in autophagy regulation in cancer cells

Several studies published since 2010 found that ascorbate promoted cell death by the activation of the caspase-independent pathway of autophagy (72). The well-known autophagy inhibitors wortmannin and bafilomycin A1 showed an inhibitory effect to some extent against a moderate concentration of ascorbate (0.5 mM), but interestingly this protective effect was lost at higher concentrations (above 1.0 mM) (47). The physiologic and higher concentration of ascorbate (0.1 and 0.6 mM) promoted higher ratio of LC3II/LC3I further strengthened the potential role of autophagy in ascorbate induced cancer cell death. The pre-treatment of the cells with bafilomycin A1 further increased the ratio of LC3II/LC3I due to ascorbate treatment supposing the presence of autophagic response during ascorbate treatment (Fig. 4) (47).

By using MIA-PaCa-2 human pancreatic cell line it was recently confirmed that ascorbate promoted a caspase-independent self-cannibalism in a dose-dependent manner, while catalase or trolox (a water-soluble derivative of Vitamin E) significantly suppressed the

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

ascorbate induced cell death. To verify the importance of autophagy the changes in Beclin1 (a key inducer of autophagosome formation) and LC3II/LC3I protein levels were detected by western blot. Furthermore, anti-LC3 immunocytochemical staining was also performed upon ascorbate treatment. Beclin1 level did not change significantly, while the increasing ratio of LC3II/LC3I supposed autophagosome formation (Fig. 4). In the absence of Beclin1, by performing siRNA-generated knock-down of Beclin1 protein expression, the ascorbate-dependent cell death was also suppressed. Since the Bcl2 protein family (such as Bcl2, Bax and Bcl-XL) has an essential role in the regulation of autophagy (45), their protein levels were also detected upon treatment. While the level of Bax and Bcl-XL did not change significantly, the expression of Bcl2 was diminished. By using immunocoprecipitation it was also proved that Beclin1 could not interact with Bcl2 in ascorbate treated pancreatic cells. All these data suggested that Beclin1 was involved in ascorbate-promoted autophagic cell death in the human pancreatic cancer cell line. In addition, co-treatment of ascorbate with rapamycin (an mTOR inhibitor / autophagy activator) enhanced the anti-cancer activity of ascorbate. To confirm their *in vitro* scientific data an *in vivo* study was also performed by using the growth of human pancreatic cancer cell xenografts in athymic nu/nu mice (34).

The effect of pharmacological concentrations of ascorbate on autophagy induction was studied using various human prostate cancer lines. IC50 values ranged from 1.9 to 3.5 mM, which concentrations are referred to as clinical usage of intravenous ascorbate. All androgen-independent cells were sensitive to ascorbate treatment due to the alteration of H_2O_2 level in the cell, while ascorbate-induced cell death was attenuated by the H_2O_2 scavenger catalase. In these cancer cells, addition of ascorbate was able to diminish ATP levels and it was confirmed that ascorbate generated ROS-induced autophagy-dependent cell death (21).

The potential role of S-glutathionylation in pharmacologic ascorbate induced cell death

The oxidation of the cysteine thiolate in specific protein residues by ROS results in the formation of a sulfenic acid moiety (R-SOH). This reaction can be reversed by glutathione giving rise to an intermolecular disulfide in a process termed S-glutathionylation.

S-glutathionylation of proteins has a marked impact on function and is thought to be a physiologically relevant mechanism in redox sensing and response. Several enzymes linked to the process were identified: the forward direction is mostly attributed to the π isoform of glutathione-S-transferase (GSTp) while the reverse direction is governed by glutaredoxins (53).

Ultimately these enzymes can link oxidative stress through S-glutathionylation to programmed cell death. As it was recently shown during oxidative stress in the central nervous system GSTp is responsible for the S-glutathionylation of Keap1 leading to Nrf2 activation and neuronal protection (20). S-glutathionylation together with GSTp can also modulate apoptotic cell death through affecting JNK activity and the binding properties of several members of the apoptotic machinery (4). Furthermore, as it was previously uncovered due to the metabolic stress-induced oxidation of crucial cysteine residues the absence of prenylation of HRas can also drive the cell towards apoptosis (17).

Similarly, pharmacologic ascorbate derived oxidative stress resulted in the inactivation of GAPDH by S-glutathionylation and NAD^+ depletion. As described ascorbate treatment enabled the selective elimination of colorectal cancer cells with KRAS and BRAF mutation in a glutathione-dependent manner (77). It is interesting to note that according to the study glutathione oxidation and consequent cytotoxicity was accounted for GLUT1 dependent dehydroascorbate uptake which was found to be preferential as opposed to its reduced form and thus the phenomenon could be limited to colorectal cell lines. Furthermore, according to an *in vitro* study dehydroascorbate could facilitate S-thiolation mediated oxidative inactivation of proteins including glutaredoxin-1 (2).

A recent study readdressed the issue of ascorbate vs dehydroascorbate induced cytotoxicity based on a redoxome approach in a breast cancer model (8). In the given experimental conditions with 13 breast cancer cell lines ascorbate was found to be the more potent form with beyond a ten-fold factor. Meanwhile, the ascorbate sensitivity of the breast cancer cell lines was found to correlate with the expression of the antioxidant enzyme peroxiredoxin 1.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

The study also reinforced that thiol oxidation – including but not limited to proteins involved in energy metabolism and antioxidant defence – is a crucial component of pharmacologic ascorbate induced cytotoxicity. However, depending on cellular compartmentation of ascorbate a potential reducing effect is non-negligible.

The precise relation of pharmacologic ascorbate induced oxidative stress and the cellular antioxidant defence mechanisms – in focus with glutathione and glutathione-dependent antioxidant enzymes – is still under debate. According to previous studies by Park et al. ascorbate induced oxidative stress was able to increase *de novo* glutathione biosynthesis through the elevation of γ-GCS expression accompanied by increased cysteine uptake in leukaemia cells. Ascorbate also increased the expression of GST which could ultimately lead to altered redox signalling, sulfhydryl exchange or glutathionylation (58, 59).

According to a recent finding the oncogenic RAS pathway in fibroblasts is also capable of enhancing the cellular antioxidant levels by increasing the expression of the XCT cystine import protein (44).

Cysteine oxidation and consequent S-glutathionylation of proteins during oxidative stress is an emergent mechanism underlying treatments with pro-oxidant features. Mn porphyrins are functionally superoxide dismutase mimetics, often used synergistically with ascorbate as pro-oxidant agents in order to amplify oxidative stress which in turn can lead to extensive modulation of cellular processes through protein S-glutathionylation (for extensive reviews see 9, 10, 70).

Ascorbate as a part of the broad-spectrum therapeutic approach of cancer

A non-profit organization called “Getting To Know Cancer” developed the concept of broad-spectrum targeting of cancer through a complex combination of agents, emphasizing naturally occurring chemicals such as silibinin, curcumin, EGCG, kaempferol, melatonin and resveratrol. The concept was implemented within an initiative called “The Halifax Project”. In the frame of the project a series of reviews were produced on the hallmarks of cancer to collectively assess and prioritize the several existing target choices, furthermore to identify non-toxic chemicals (primarily phytochemicals or foods) for their safe combination to develop a broad-spectrum approach with the dual goal of prophylaxis

and therapy (14). Altogether eleven cancer hallmarks were reviewed by eleven teams of researchers. All of these areas belong to the most important aspects of cancer research, hence all of them are worth to consider both individually and in combination for a therapeutic approach. The project gave special attention to different phytochemicals and vitamins. However, it did not discuss the potential beneficial effects of ascorbate. We think ascorbate can target at least two of these key points. 1. One of the major hallmarks of human cancer cells is the intrinsic or acquired resistance to apoptosis (14) that can play a role in tumour development, in their progression and also in treatment resistance; since the current anticancer therapies such as chemotherapy, radio- and immunotherapy primarily act by activating cell death/apoptotic pathways in cancer cells (14). The deeper knowledge of molecular pathways standing behind the resistance of tumours to cell death can endow us for an approach to develop molecular targeted therapies. To avoid apoptosis, tumour cells overexpress anti-apoptotic molecules such as B-cell lymphoma-2 (Bcl-2) family proteins (14). Ascorbate treatment of tumour cells resulted in the formation of excess H_2O_2 that subsequently caused oxidative stress and rapid cellular ATP depletion. The depletion of ATP lead to the inhibition of mTOR activity, which in turn induced the elevated degradation of Bcl-2 (34). The reduced cellular level of Bcl-2 induced the accumulation of Beclin 1 that mediates the formation of autophagosomes. Hence ascorbate induced autophagy in cancer cells can drive an apoptosis bypassed cell death. This way autophagy can at least partly contribute to ascorbate-induced cancer cell death. This model was strengthened by the selective knockdown of Beclin 1 or by the treatment of MIA-PaCa-2 human pancreatic cancer cells with pharmacological autophagy inhibitors that resulted in the inhibition of ascorbate-induced cell death (34). 2. The second hallmark that can be touched by ascorbate is the dysregulated metabolism of cancer cells in which the uptake of glucose and the production of lactate are significantly increased. This characteristic is often called the “Warburg effect”. The observed metabolic shifts in cancer cells were associated with cellular growth, more precisely with the support of lipid-, protein- and nucleic acid biosynthesis required for tumour formation and survival (14). Beyond the biosynthetic support, the observed increased glucose consumption was also accompanied by increased pentose phosphate pathway activity in cancer cells in order to obtain reducing equivalents such as NADPH via the pentose phosphate pathway as well as

pyruvate from glycolysis to detoxify the elevated level of H_2O_2 and other hydroperoxides observed in cancer cells (5, 72). This way the enhanced glucose consumption of cancer cells can be explained at least partly by the enhanced GSH consumption and the consequent enhanced NADPH requirement due to the increased steady-state ROS levels (72). In strong support of this hypothesis cancer cells were more susceptible to glucose deprivation or the inhibition of glucose transporters (5). Hence the utilization of glucose by the enhanced NADPH requirement due to pharmacologic ascorbate induced oxidative stress may provide a biochemical target for selectively enhancing oxidative stress and consequent cytotoxicity in human cancer cells.

It seems that ascorbate can invade cancer cells via the Warburg effect in multiple ways. Oncogenic KRAS plays an important role in the disruption of the metabolic homeostasis through the alteration of glucose uptake and glycolytic flux in colon and pancreatic tumours that often display very high resilience to chemotherapy (1). Since pharmacologic ascorbate treatment induced the detachment of oncogenic RAS from the plasma membrane, and the constitutive KRAS signalling activity depends on its level of enrichment on the plasma membrane, the EGFR/MAPK phosphorylation cascade could be aborted. This way the ERK1/2 dependent phosphorylation of pyruvate kinase M2 (PKM2) was also inhibited, that resulted in the inhibition of GLUT-1. Since GLUT-1 is considered as a major regulator of the Warburg effect, the downregulation of GLUT-1 expression and PKM2 expression and its phosphorylation at ser37 suggest that ascorbate can also exert its anti-tumour activity by targeting enzymes and signalling pathways involved in the Warburg effect (1).

Unfortunately, tumours harbouring mutated KRAS can be characterized by anti-EGFR monoclonal antibody cetuximab (Erbitux) resistance. According to this observation, the LoVo and SW480 colon carcinoma cells (both displaying different KRAS mutations) did not exhibit any sensitivity to cetuximab, but the cell growth of HT29 line with wild type KRAS was clearly inhibited by cetuximab (1). However, the treatment of all the three cell lines with both cetuximab (0.4 μM) and ascorbate (5mM) abolished cell growth in all lines. The combined treatment of the cells with cetuximab and ascorbate was more effective than ascorbate alone, suggesting that vitamin C may synergize with cetuximab. Furthermore,

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

tumour growth of SW480 xenografts harbouring KRAS mutation could be strongly reduced by ascorbate treatment at pharmacological concentration (4g/kg body weight) (1). Both *KRAS*/*BRAF* mutation and ERK1/2 activation have been considered recently as biomarkers for chemoresistance in gastrointestinal cancer to agents such as 5-Fu, SN38 and Oxaliplatin (37) giving special importance to the effect of ascorbate on the cells with RAS mutation. Furthermore, the activation of ERK1/2 was also reported to be responsible for chemoresistance of glioma cells to Temozolomide (38). Unfortunately, no information was published on the possible presence of *KRAS*/*BRAF* mutation or ERK1/2 activation in the first human phase I clinical trial of pharmacologic ascorbate combined with radiation and temozolomide in newly diagnosed glioblastoma patients (3). However, the trial closed with quite promising results since the median progression-free survival was 9.4 months and the median overall survival was 18 months while glioblastoma patients treated with radiation and temozolomide therapy alone could only be characterized by a median overall survival of 14.6 months and progression-free survival of 7 months (3). Furthermore, no dose-limiting toxicities occurred and the adverse events related to pharmacologic ascorbate were quite mild, including dry mouth and chills. Summarily, high dose ascorbate (20 mM) was found to be safe when it was combined with radiation and temozolomide therapy and a phase II clinical trial was initiated to assess the efficacy of pharmacologic ascorbate in glioblastoma patients treated with radio- and temozolomide therapy (3).

Conclusions

Transition metals such as copper and iron in their reduced state are capable of reacting with oxygen forming superoxide anion and then H_2O_2. H_2O_2 can readily react with further transition metal ions in the Fenton reaction to form the highly reactive, cytotoxic hydroxyl radical. The continuous regeneration e.g. reduction of transition metals is the prerequisite of the continuous formation of hydroxyl radical. Ascorbate as an excellent reducing agent is suitable for the continuous regeneration of transition metals. While ascorbate donates an electron to the metals, it gets oxidized resulting in continuous consumption of ascorbate. Hence a suspended level of hydroxyl radical formation can only be reached at higher, so called pharmacological concentration of ascorbate.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Although there are conflicting results on the reprogramming of antioxidant defence in tumour cells, the persistent oxidative stress is not debated in cancer cells and it is also evidenced in various tumours (15). It seems that cancer cells are able to adjust oxidative stress to a level which is sufficient to maintain their survival but not to initiate their elimination (15). This adaptation of cancer cells to elevated ROS level treads a very fine line between their proliferation and cell death. Therefore, increased ROS levels are frequently used as cytotoxins in cancer patients (57).

Pharmacologic ascorbate can induce the death of cancer cells which is clearly related to its above drafted ROS generating property and depends on its H_2O_2 and hydroxyl radical production ability. Since the cytotoxicity induced by pharmacologic ascorbate was unequivocally oxidative (72), it seemed obvious, that ferroptosis, the only clearly known oxidative stress and lipid peroxidation driven cell death (28), was involved in high dose ascorbate induced cancer cell death. This hypothesis was also supported by the similar features of the cell deaths such as iron dependency, caspase independency, and the possible involvement of autophagy (28, 35, 72). Interestingly, ferroptosis inhibitors were unable to elevate the viability of high dose ascorbate treated cancer cell lines. Furthermore, treatment with pharmacologic ascorbate and with the ferroptosis inducer RSL3 resulted in cell deaths with different morphology. The lipophilic antioxidant α-tocopherol could not save the ascorbate treated HT-1080 cells, while it could effectively counter the fall in viability initiated by erastin and RSL3 (28, 48). All these observations suggest that ferroptosis is not involved in pharmacologic ascorbate induced cell death. Moreover, at low and moderate concentrations ascorbate behaved as an effective inhibitor of both RSL3 and erastin induced ferroptosis (47) (Fig. 3). This observation draws attention to the anti-ferroptotic effect of ascorbate that may have crucial importance in the possible clinical application of ferroptosis inducers.

The caspase independency of high dose ascorbate induced cell death also proposed the possible involvement of necroptosis and autophagy. The inhibitory effect of the necroptosis inhibitor necrostatin-1 and the increase of the necroptosis marker, RIPK1 suggested the possible role of necroptosis in moderate concentration ascorbate induced cell death (47) (Fig. 4).

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Similarly, ascorbate also promoted autophagy in a dose-dependent manner. The formation of autophagosome was reinforced by the increased ratio of LC3II/LC3I due to ascorbate treatment. Furthermore, ascorbic acid-dependent cell death was suppressed by the knock-down of Beclin1 a key inducer of autophagosome formation (45). The pharmacologic inhibition of autophagy by either wortmannin or bafilomycin A1 resulted in the inhibition of cell death due to moderate ascorbate treatment, however, their protective role was lost at higher ascorbate concentrations (47).

All these results suppose a possible alteration in the type of cell death by the rising concentration of ascorbate. Autophagy and necroptosis may play a role at intermediate concentrations of ascorbate, but caspase independent apoptosis may become dominant at higher concentrations. This is supported by the observation that hydroxyl radical induced cell death was found to be caspase independent but it could be characterized by the typical morphological and biochemical changes of apoptosis (61). Furthermore, DNA double-strand breaks could be generated via the production of hydroxyl radical in pharmacologic ascorbate treated cancer cell lines (24, 51). Albeit DNA damage triggers the extended activation of PARP1 resulting in intensive NAD^+ consumption and in the consequent depletion of ATP. Both the pharmacologic inhibition and the genetic deletion of PARP1 prevents the depletion of NAD^+ and ATP but did not prevent cell death. Moreover the rate of cell death due to high dose ascorbate treatment was enhanced in PARP1 knockout cells or in PARP1 inhibitor pre-treated cells (16). It is presumed that DNA damage could be the primary contributor of the anti-cancer activity of pharmacologic ascorbate, as opposed to the rupture of bioenergetics.

The deeper knowledge of molecular pathways of different cell death mechanisms and those standing behind the resistance of tumours to cell death can endow us for an approach to develop molecular targeted therapies. On the base of the above observations at least two of the key features of cancer cells can be targeted by ascorbate individually or rather in a combinational therapeutic approach. These two targets are the intrinsic or acquired resistance to apoptosis and the dysregulated metabolism of cancer cells. This train of thought seems to be relevant since the first human phase I clinical trial of pharmacologic ascorbate combined with radiation and temozolomide therapy of newly

diagnosed glioblastoma patients closed with quite promising results. Both the median progression-free survival and the median overall survival were elongated due to the ascorbate co-therapy (3). Furthermore, no dose-limiting toxicities occurred and adverse events related to pharmacologic ascorbate were quite mild. We believe, that these results are just the beginning and the phase II clinical trial will result in more success.

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Author Disclosure Statement

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Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Abbreviations Used

DSB, double stand break

EC50, half maximal effective concentration

GPX, Glutathione peroxidase

GSH, reduced glutathione

GSSG, glutathione disulphide or oxidised glutathione

H_2O_2, hydrogen peroxide

$HO^{\cdot}$, hydroxyl radical

IC50, half maximal inhibitory concentration

KO, gene knockout

LOOX, lipid peroxide

mtDNA, mitochondrial DNA

nDNA, nuclear DNA

O_2, molecular oxygen

$O_2^{\cdot -}$, superoxide radical

PARP, poly (ADP-ribose) polymerase

RIPK, receptor interacting protein kinase

ROS, reactive oxygen species

SVCT, sodium dependent vitamin C transporter

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

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Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

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Figure legends

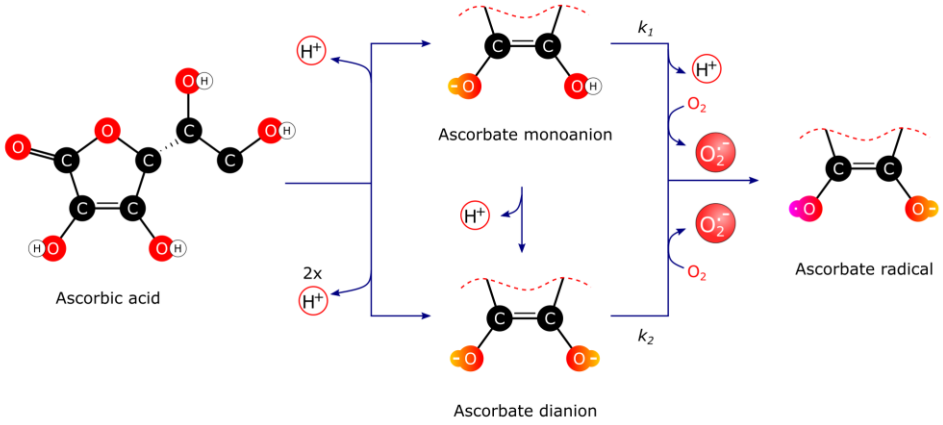

Fig 1. The autoxidation of different forms of ascorbate

The autoxidation of ascorbate results in the formation of ascorbyl radical and superoxide anion. At physiological pH the relatively rare dianion form of ascorbate autoxidizes approximately six orders of magnitude faster ($k_2=3*10^2$) than the much more abundant monoanion form ($k_1=3*10^{-4}$). This way in biological systems, in the course of the formation of ascorbyl radical, ascorbate can donate an electron to a transition metal such as iron or copper. The reduced metal is capable of reacting with oxygen forming further superoxide anions.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

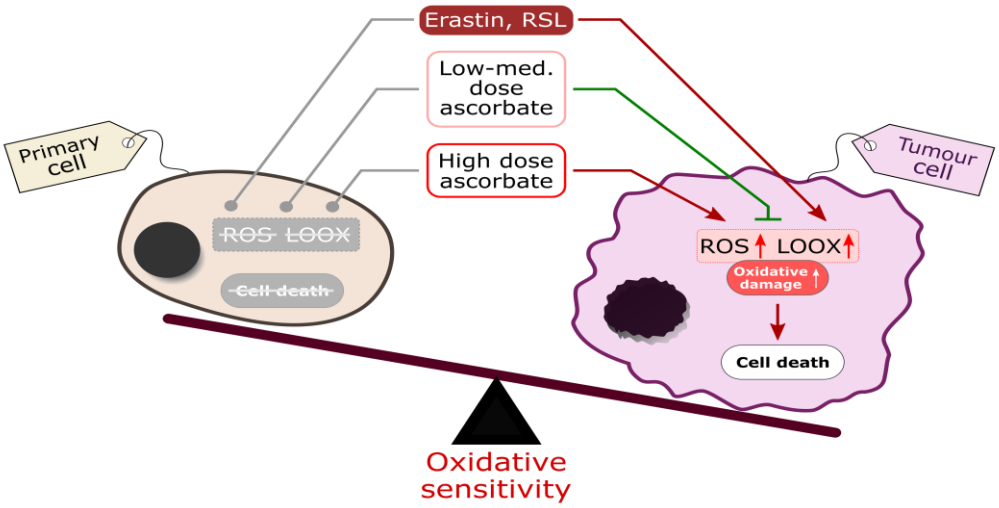

Fig 2. The different behaviour of primary and tumour cells to pharmacologic ascorbate and ferroptosis inducers.

Cancer cells can be characterized by elevated oxidative stress. On one hand the generation of ROS is increased in them due to the enhanced metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signalling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase or due to the crosstalk with infiltrating immune cells. On the other hand the elimination of different ROS types is compromised in cancer cells due to the significantly lower level and activity of catalase and glutathione peroxidase (GPX). Interestingly their activity is one to two orders of magnitude lower in cancer cell lines than in primary cells and tissues. In addition cancer cells can be characterized by elevated intracellular labile iron levels that leads to enhanced formation of ROS, especially the formation of the highly reactive hydroxyl radical. This difference between tumour and non-tumour cells gives the possibility to selectively kill them by elevating their oxidative pressure treating them by ferroptosis inducers (erastin or RSL3) or by pharmacologic ascorbate.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

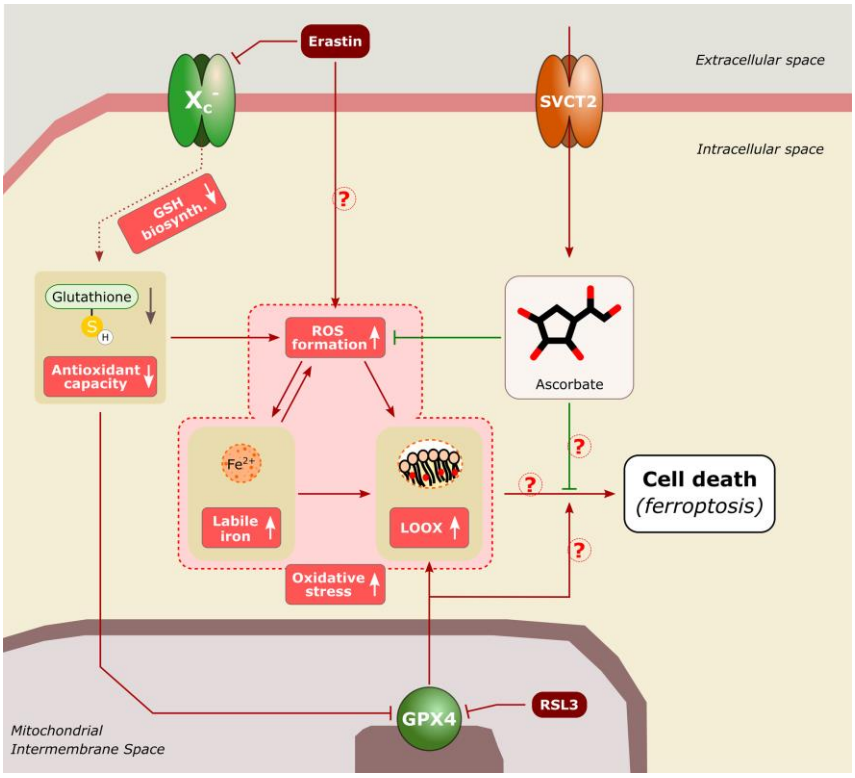

Fig 3. The effect of ascorbate on the ferroptotic pathway in cancer cells

The induction of the novel described iron dependent cell death, ferroptosis accompanied by the accumulation of ROS and lipid hydroperoxides. Its specific inhibitors such as ferrostatin-1 or liproxstatin behave as radical trapping antioxidants. Albeit pharmacologic ascorbate similar to RSL3 treatment induced ROS and lipid peroxide formation, the co-treatment of RSL3 treated cells with low dose ascorbate significantly decreased the level of both ROS and lipid peroxide formation and consequently inhibited the initiation of ferroptosis.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

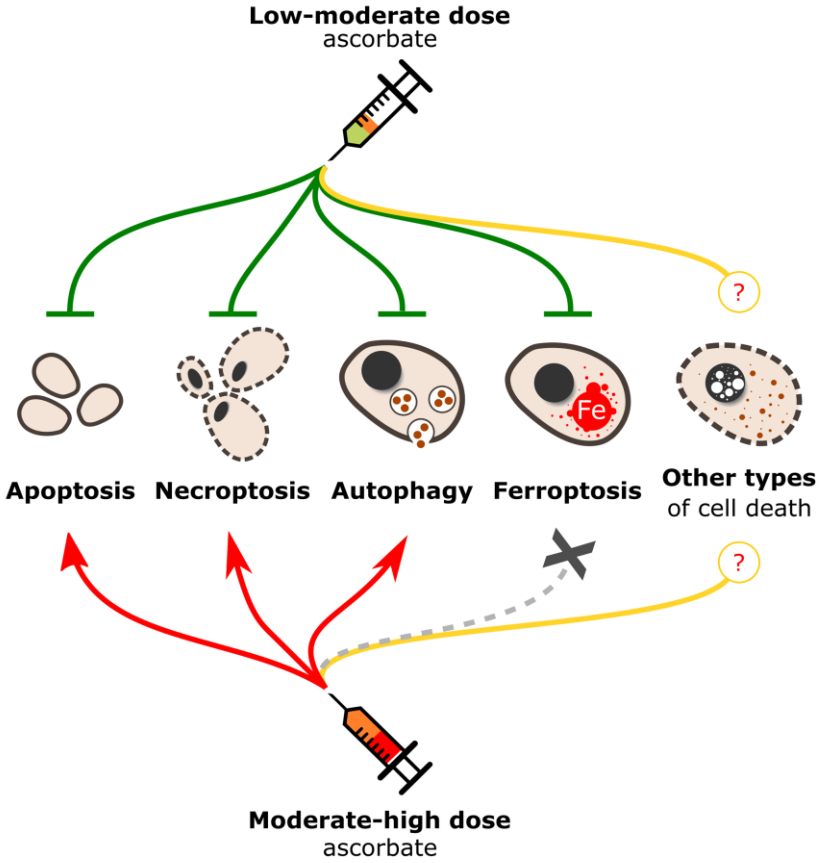

Fig 4. The interrelationship of different cell death mechanisms and high/low dose of ascorbate treatments

The oxidative nature of pharmacologic ascorbate induced cytotoxicity and the similar features of the ROS driven cell death, ferroptosis, raised the possibility of the tight connection of these two processes, however recent results showed that ferroptosis inhibitors (ferrostatin-1 and liproxstatin-1) were unable to elevate the viability of high dose ascorbate treated cancer cell lines. Moreover it was uncovered that at low to moderate concentrations ascorbate behaved as an inhibitor of both RSL3 and erastin induced ferroptosis.

The caspase independency of high dose ascorbate induced cell death proposed the possible involvement of necroptosis and autophagy. The inhibitory effect of the necroptosis inhibitor necrostatin-1 and the increase of the necroptosis marker, RIPK1 at moderate concentration of ascorbate suggested the possible role of necroptosis by moderately high concentration ascorbate induced cell death. Ascorbate also promoted the

caspase-independent self-cannibalism in a dose-dependent manner. The formation of autophagosome was reinforced by the increased ratio of LC3II/LC3I due to ascorbate treatment. Furthermore, ascorbic acid-dependent cell death was suppressed by the knock-down of Beclin1 a key inducer of autophagosome formation. The pharmacologic inhibition of autophagy by wortmannin and bafilomycin A1 resulted in the inhibition of cell death due to moderate ascorbate treatment, however their protective role was lost at higher ascorbate concentrations. All these results suppose the presence of autophagic response during ascorbate treatment.

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

Antioxidants and Redox Signaling
Vitamin C and cell death (DOI: 10.1089/ars.2019.7897)

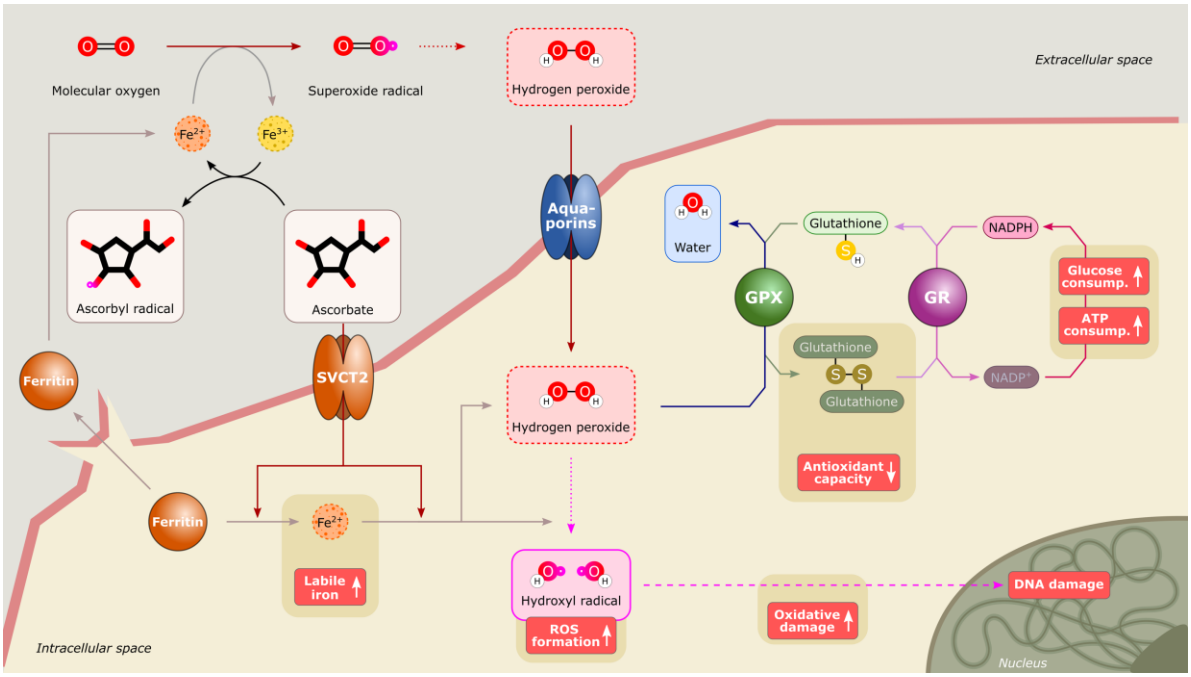

Fig. 5. The generation of ROS by pharmacologic ascorbate and their potential role in cell death mechanisms.

In biological systems, through the course of ascorbyl radical formation, ascorbate can donate an electron to a transition metal such as iron. The reduced metal is capable of reacting with oxygen forming superoxide anion and then H_2O_2. The nascent H_2O_2 then passes through the cell membrane by the mediation of specific aquaporins. Binding of iron to proteins, such as ferritin strictly controls the level of its free or labile form. ROS and the high dose of ascorbate can disrupt cellular iron metabolism that leads to an increased labile iron pool. The high concentration of H_2O_2 in the presence of high-labile iron facilitates the Fenton reaction that generates the highly toxic hydroxyl radical. GPX or catalase by scavenging the H_2O_2 can interrupt this harmful pathway and prevent the elevation of labile iron pool and the cytotoxicity of high-dose ascorbate. DNA is damaged by the very reactive hydroxyl radical. More precisely DNA double-strand breaks could be generated via the production of H_2O_2 and hydroxyl radical in pharmacologic ascorbate treated cancer cells. The potential role of intracellular ascorbate was underline by the observation that the knockdown of the ascorbate transporter SVCT2 dramatically alleviated DNA damage and ATP depletion.