Pharmacological concentrations of vitamin C inhibit hypochlorous acid generation by myeloperoxidase, and oxidized vitamin C inhibits critical thiol proteins and growth of Jurkat cells

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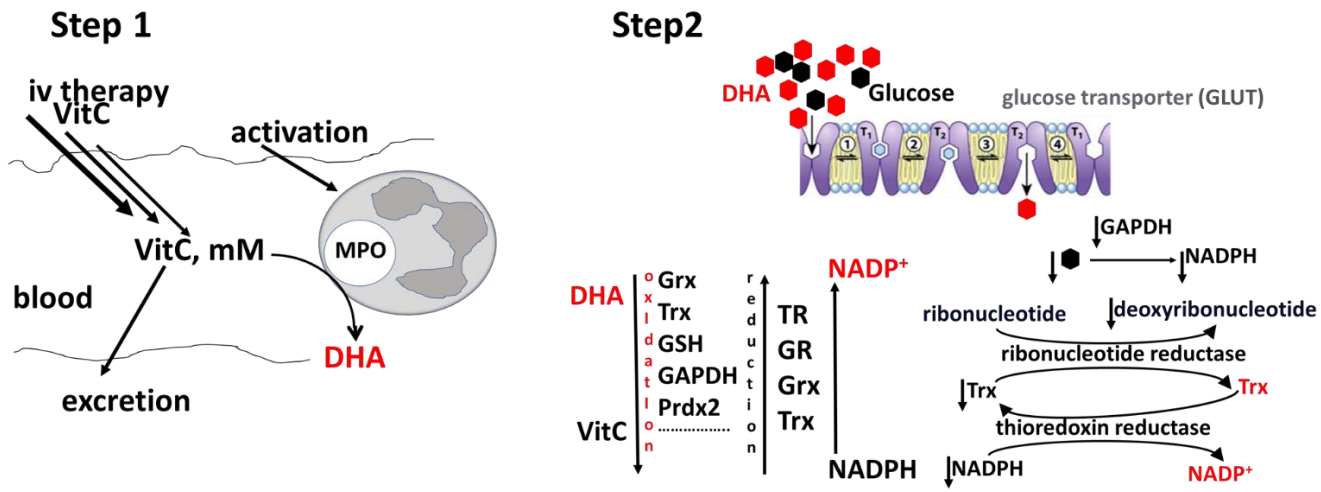
Graphical abstract:



Abstract

Vitamin C, at millimolar concentrations, directly inhibits hypochlorous acid generation by myeloperoxidase, through competition with chloride rather than by scavenging the hypochlorous acid that is formed. Products of vitamin C oxidation reacted with the thiols of peroxiredoxin 2 and GAPDH, but failed to react with the cysteine of p16^{INK4a}. The growth and viability of Jurkat cells were affected by oxidised vitamin C. These experiments were conducted in the presence of catalase, demonstrating that the biological effects were due to the products of vitamin C oxidation and not hydrogen peroxide. These findings may have practical implications for the treatment of cancer and diseases in which the deleterious effects of neutrophil activation are observed. For intravenous administration of pharmacological vitamin C to have a beneficial effect, its concentration in the blood must be maintained at millimolar levels and this can only be achieved via maintenance infusion. As a proof-of-concept, our data suggest that to enhance anticancer therapy interventions, it is crucial to implement treatments that facilitate the oxidation of vitamin C in the bloodstream.

Progress in the art of healing has always been induced by progress in basic knowledge.

Albert Szent-Gyorgyi (Woods Hole, Mass., USA, 1973)

INTRODUCTION

The seminal publication by Linus Pauling in 1976, on the effects of high-dose vitamin C (ascorbate) in cancer (1), triggered investigations aimed at understanding the biological role of vitamin C in humans. In addition to being a potent free radical scavenger, it has been shown that ascorbate is a cofactor for a family of biosynthetic and gene-regulatory monooxygenase and dioxygenase enzymes and, as such, is involved in the synthesis of collagen, carnitine, and hormones (2,3). Members of this enzyme family include the 2-oxoglutarate-dependent dioxygenases which regulate their targets through hydroxylation. In the context of cancer, these enzymes downregulate hypoxia inducible factors, key drivers of the hypoxic response and tumour cell survival. They also regulate histone and DNA demethylases, such as the ten-eleven translocase and Jumonji-C-domain-containing proteins, which are involved in the epigenetic control of oncogenes and tumour suppressors (4-6).

Despite significant advances in our understanding of the biological effects of vitamin C, there is still no answer to the question of why pharmacological levels (achieved with higher-than-normal doses for therapeutic or medical purposes) of vitamin C are necessary to observe anticancer effects. Currently, the most favored explanation attributes the anticancer effects to hydrogen peroxide (H_2O_2) formed by metal-catalyzed oxidation of high levels of ascorbate (7,8). However, doubts about the feasibility of this explanation have been raised (9,10). In this paper, we demonstrate that when catalase is present, oxidized vitamin C exhibits toxicity towards Jurkat cells and reacts with the thiols of peroxiredoxin (Prdx) 2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). H_2O_2 formed during the oxidation of vitamin C could lead to further vitamin C oxidation but is not the cause of the toxic effect of high doses of vitamin C on Jurkat cells. We investigated, and describe here, the effects of preformed oxidized ascorbate in the presence of catalase. It should be noted that the immediate product of two-electron oxidation of vitamin C is dehydroascorbate (DHA), which cannot be used in clinic due to its instability (13,14).

Our data indicate that the biological effect of pharmacological ascorbate resulted from oxidized ascorbate, therefore we propose rapid oxidation of ascorbate in the blood as a possible mechanism. Myeloperoxidase (MPO) is an appropriate candidate for this oxidation as it i) effectively generates hypochlorous acid (HOCl), which reacts quickly with ascorbate (15), and ii) can directly oxidize ascorbate (16). In this study we showed that oxidized ascorbate was able to inactivate thiol-dependent enzymes and was also cytotoxic towards Jurkat cells. In addition, we also found that millimolar levels of ascorbate inhibited HOCl generation by MPO.

We have demonstrated the oxidation of two crucial thiol-containing proteins, Prdx2 and GAPDH, which are essential for cancer cell survival, in the presence of oxidized ascorbate. Moreover, the morphology and viability of Jurkat cells were affected.

Protein $p16^{INK4a}$ is a negative regulator of cell division, inhibiting the cyclin dependent kinases 4 and 6 and preventing progression of the cell cycle. The loss of $p16^{INK4a}$ activity is widely believed to be a common and important event in the development of cancer. Oxidation of $p16^{INK4a}$ leads to the formation of disulfide-bridged dimers that subsequently form amyloid fibrils. While amyloid fibrils typically form spontaneously, $p16^{INK4a}$ is the first example of strictly oxidation-induced amyloid formation (17). However, we did not observe cysteine oxidation or dimerization of $p16^{INK4a}$ treated with oxidised ascorbate.

METHODS

Proteins

Recombinant human Prdx2 was prepared as described (18) and provided by Paul Pace. Recombinant human $p16^{INK4a}$ was a gift from Christoph Göbl (17). Human myeloperoxidase was from Tony Kettle (19). Rabbit muscle GAPDH, bovine catalase and other chemicals were from Sigma-Aldrich.

All thiol-containing proteins were reduced by the addition of 10 mM dithiothreitol (DTT) for 1 h immediately before use. Excess DTT was removed using Micro Bio-Spin 6 columns (Bio-Rad), which were prewashed with deionized water and then with 5 ml of 20 mM phosphate buffer, pH 7.4, containing 0.1 mM diethylenetriamine-penta-acetic acid (DTPA) and purged with argon. Experiments were conducted in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA.

Sources of oxidized ascorbate

1. Commercial dehydroascorbic acid (Sigma-Aldrich) was dissolved immediately before use in 20 mM phosphate buffer, containing 0.1 mM DTPA, adjusted to pH 7.4, then mixed with catalase, 10 µg/ml.

2. Sodium ascorbate was dissolved in deionized water immediately before use. HOCl was added dropwise, during vigorous mixing, to ascorbate at a molar ratio of 1:2. The immediate product of two-electron oxidation of ascorbate is DHA:
   $C_5H_5O_5CH_2ONa + HOCl \rightarrow C_5H_3O_5CH_2OH + NaCl + H_2O$, $k=6x10^6M^{-1}s^{-1}$ (20)

3. Myeloperoxidase from stock was initially diluted in H_2O and further diluted for use (to 20 or 40 nM) in phosphate buffer (20 mM, pH 7.4 containing 0.1 mM DTPA) with varying concentrations of NaCl and/or ascorbate. After mixing, H_2O_2 was added for 5 min, then catalase (10 µg/ml) was added. For analysis of methionine oxidation, protein was removed using 3 kDa centrifuge filters (Millipore) before analysis by mass spectroscopy.

GAPDH activity assay

The activity of GAPDH, either untreated or treated with oxidized ascorbate, was measured, in Tris buffer (Tris-HCl, 50 mM, pH 8) containing EDTA (0.2 mM), glyceraldehyde 3-phosphate (0.4 mM), NAD^+ (1 mM) and sodium arsenate (15 mM), by monitoring the initial reduction rate of NAD^+ as an increase in absorbance (A_{340nm}) (21) using a SpectraMax iD3 plate reader with SoftMax Pro 7.1 software (Molecular Devices, San Jose, California, USA).

Cell culture conditions and treatments

Jurkat cells (Clone E6-1, TIB-152 from ATCC) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 1% penicillin/streptomycin (Gibco, from Thermo Fisher Scientific, Auckland, NZ) and 10 % foetal bovine serum (Moregate BioTech, Hamilton, New Zealand). Cells were seeded in 24-well culture dishes, at a density of 0.25 x 10^6/ml, and, where appropriate, catalase (20 µg/ml) was added to the culture medium before treatment. Ascorbate was oxidized by reaction with HOCl immediately before addition to the cells. Phase contrast images were captured after 24 hours with an Olympus DP21 camera using a 20x magnification objective, then the cells were counted and viability measured: propidium iodide (PI) was added and the cells analysed using a Cytoflex S flow cytometer (Beckman Coulter).

Mass spectrometry

For whole protein analysis, samples containing 1 µg of protein were injected onto an Accucore-150-C4 (50 x 2.1-mm, 2.6- µm) column (60 °C) using a Dionex Ultimate 3000 HPLC system coupled to a Velos Pro mass spectrometer (Thermo Scientific, San Jose, CA). Proteins were eluted with an acetonitrile gradient from 90% solvent A (0.1% formic acid in water) and 10% solvent B (0.1% formic acid in acetonitrile) to 80% solvent B over 4.6 min at a flow rate of 400 µl/min. Mass spectra were acquired between m/z 400 and 2000 in positive ion mode. The spectra of each protein peak were averaged using Thermo Xcalibur Qual Browser 4.2.47 (Thermo Fisher Scientific) and deconvoluted to yield the molecular masses and relative intensities using ProMass for Xcalibur (version 2.8 rev 5; Novatia LLC, Monmouth Junction, NJ).

A multiple reaction monitoring (MRM) method was set up for detection of methionine and its oxidation products (Table 1), using a Sciex 6500 QTrap mass spectrometer (Framingham, MA, USA) coupled to an Infinity 1290 LC system (Agilent, Santa Clara, CA, USA). Samples were stored on the autosampler tray at 5 °C prior to injection. An Imtakt Intrada Amino Acid column (150 x 3.0 mm) was used for chromatographic separation using acetonitrile containing 0.1% formic acid (Solvent A) and 100 mM ammonium formate (Solvent B). The column temperature was set to 40 °C. The column was equilibrated with 86% Solvent A and 14% Solvent B for 6 minutes and then a linear gradient was run for 14 minutes to 100% Solvent B to achieve separation. The column was then flushed with 100% Solvent B for 2 minutes and re-equilibrated at initial conditions for 4 minutes. A flow rate of 0.25 mL/minute was used. Data were analyzed using Analyst 1.7.2 (Sciex, Framingham, MA, USA).

Table 1. The m/z values for the parent and fragment ions that were used to quantify methionine species by LC/MS.

Species (charge state)	**Parent ion (m/z)**	**Fragment ion (m/z)**
Methionine (+1)	150.06	$-C_3H_7NO_2$: 61.01
Dehydromethionine (+1)	148.04	$-CH_2S$: 102.06
Methionine sulfoxide (+1)	166.05	$-CH_4SO$: 102.06

RESULTS AND DISCUSSION

1. Effects of oxidized ascorbate on thiol-containing proteins

1.2. Peroxiredoxin 2

Reduced Prdx2 treated with oxidized ascorbate was analysed by LC/MS. Oxidized ascorbate was either in the form of commercial DHA or generated by MPO.

Prdx2 has two reactive cysteines and upon oxidation forms a covalent dimer with either one or two disulfide bonds. Prdx2 reacts rapidly with H_2O_2 and is oxidized by even trace amounts present in buffers (22). Catalase was therefore added to the oxidized ascorbate before mixing with Prdx2 to prevent oxidation of the Prdx2 cysteines by H_2O_2. After incubation of the oxidized ascorbate with the reduced Prdx2 for 10 min, samples were stored at -20 °C and analysed by LC/MS within two days. Fig. 1 and Fig. 2 show that oxidised ascorbate resulted in the loss of monomeric Prdx2 and formation of Prdx2 dimer. We also observed species with masses corresponding to a parent dimer plus 39 - 42 Da, 106 Da and 208 - 214 Da (Figs. 1C and 2C, insert).

Adducts of glutaredoxin have been shown to form in the presence of oxidized ascorbate, with an increase in molecular weight of 112 Da resulting from the addition of the 5-carbon fragment of DHA to the protein thiol (23). In our experiments the accuracy of the deconvoluted whole protein masses was within 7 Da of the theoretical masses (24). We therefore suggest that the oxidized ascorbate may form adducts on Prdx2, similar to those observed for glutaredoxin: the addition of 106 Da may be an adduct of the 5-carbon fragment of DHA, and adducts with additional masses in the range of 208 - 214 Da may represent two adducts (each of 104 - 107 Da) on the two cysteines not involved in the disulfide bond of the Prdx2 dimer.

Formation of adducts with both Prdx2 cysteines has been shown for other electrophiles (25), and the addition of 39 - 42 Da could be attributed to an acetaldehyde adduct which may be produced from the decomposition of DHA (13, 14). It will be of interest to study stability and reversibility of these observed modifications as well as the effect of decomposition of DHA on Prdx2.

1.2. GAPDH

The enzymatic activity of GAPDH depends on the presence of reduced cysteine residues and oxidation of these results in the loss of activity (Fig. 3A). As predicted, addition of oxidized ascorbate to reduced GAPDH resulted in a concentration-dependent loss of enzymatic activity (Fig. 3B).

1.3. $p16^{INK4A}$

The tumor suppressor $p16^{INK4A}$ induces cell cycle arrest and senescence in response to oncogenic transformation. Oxidation of the single cysteine of $p16^{INK4A}$ results in disulfide-dependent dimerization, which is a prerequisite of amyloid formation (17). Here, LC/MS analysis showed that incubation of the protein with oxidized ascorbate for 60 min did not result in modification of the $p16^{INK4A}$ (Fig. 4).

Effect of oxidisced ascorbate on Jurkat cells

Jurkat cells are a leukemic human T lymphocyte cell line, which we used to study the effect of oxidized ascorbate on cell morphology and viability. To do this, PI was added to label the DNA of dead cells and the number of cells was quantified by flow cytometry: the number of cells in a given volume was counted and the percentage of PI-positive cells recorded. We observed a cytotoxic effect of 2.5 mM or 5 mM ascorbate, however in the presence of catalase ascorbate was well tolerated, with no visible changes in morphology or the number of viable cells, compared to controls (Fig. 5 and Fig. 6). In contrast, when ascorbate was oxidized with HOCl before addition to the cells, there were drastic changes in cell morphology, especially at 5 mM (Fig. 5). Notably, cell viability was greatly decreased in the presence of oxidized ascorbate, and this was only partly ameliorated by catalase (Fig. 6). It will be of interest to study the state of Prdx2 and GAPDH in cells after treatment to see whether they are inactivated in treated cells.

3. Inhibition of myeloperoxidase by pharmacological levels of ascorbate

MPO is a heme-containing peroxidase most abundantly expressed by neutrophils. It uses H_2O_2 formed by activated neutrophils to generate HOCl which, as well as being bactericidal, can have unwanted side effects. In its basal state MPO reacts with H_2O_2 and is converted to Compound I. Compound I subsequently reacts with Cl^-, with a second-order rate constant of ~10^4 $M^{-1}s^{-1}$, to produce HOCl and return to its basal state (26). Compound I may alternatively react with ascorbate to form Compound II, which reacts with another molecule of ascorbate before converting back to MPO in its basal state. In comparison to Cl^-, the second-order rate constant for the reaction of ascorbate with Compound I is much faster, ~10^6 $M^{-1}s^{-1}$ (16). This suggests that pharmacological levels of ascorbate, which can exceed 10 mM in the blood (27), would outcompete physiological concentrations of Cl^-, and therefore MPO would produce oxidized ascorbate rather than HOCl under these conditions. To test this hypothesis, we used purified MPO and mass spectrometry to analyse methionine oxidation by MPO-generated HOCl, in the presence and absence of ascorbate.

Measuring the inhibition of HOCl generation by MPO in the presence of ascorbate is complicated by the very fast reaction of HOCl with ascorbate, which has a second order rate constant of $6x10^6$ $M^{-1}s^{-1}$(16). It is therefore necessary to distinguish between the genuine inhibition of HOCl generation by MPO, and scavenging of the

formed HOCl by ascorbate. To address this issue, we introduced methionine to the reaction mixture: the reaction of methionine with HOCl has a second order rate constant of $3.8x10^7$ $M^{-1}s^{-1}$ (28) and therefore, at equimolar with ascorbate concentrations, methionine should scavenge most of the HOCl.

Hydrogen peroxide oxidizes methionine to form methionine sulfoxide while HOCl oxidizes methionine to a mixture of dehydromethionine (DHM) and methionine sulfoxide (29), both of which can be measured using mass spectrometry. We measured the intensity of signals corresponding to methionine-related species, generated by MPO, when it was incubated with methionine in the presence of 100 mM NaCl after the addition of H_2O_2. Fig. 7 shows chromatograms of the species detected in the model mixture of methionine with HOCl, and these include methionine, two isomers of dehydromethionine, and methionine sulfoxide. With MPO activated by H_2O_2 in the presence of NaCl we observed the formation of DHM and methionine sulfoxide, which were decreased by half when ascorbate was present (Fig. 8). The degree of inhibition of methionine oxidation was not affected by lowering the concentration of NaCl to 10 mM. The fact that inhibition of methionine oxidation was independent of the NaCl concentration may be explained by the direct oxidation of ascorbate by MPO and subsequent oxidation of methionine by oxidized ascorbate.

To assess whether direct oxidation of ascorbate by MPO leads to methionine oxidation, we conducted two sets of experiments. First, we delayed by 3 min the addition of methionine to the mixture containing MPO, NaCl, ascorbate and H_2O_2, to which catalase was added after 5 min. In this experimental setup, all of the HOCl would be scavenged by ascorbate before the addition methionine. In this case we observed the formation of DHM and methionine sulfoxide (Table 2). Second, we excluded NaCl from the mixture and looked for methionine oxidation at various concentrations of H_2O_2 (Table 3). Methionine oxidation occurred in the absence of NaCl (Table 3) and the formation of DHM showed a linear dependence on the concentration of H_2O_2 (Fig.7).

In contrast, the signal of methionine sulfoxide remained unchanged (Table 3), suggesting that it corresponds to the methionine sulfoxide present in the methionine stock solution.

We analysed commercially sourced methionine and methionine sulfoxide and, after equal loading, detected the following: the methionine, showed signal intensity of $4.57x10^7$ and $1.77x10^5$ for methionine and methionine sulfoxide, respectively; and in the methionine sulfoxide, these were $5.41x10^7$ and $1.78x10^5$ signals for methionine sulfoxide and methionine, respectively. In both chemicals the signal corresponding to DHM was in the range of 10^4.

It should be noted that the reaction of Compound I with Cl^- is complex and dependent on the concentration of Cl^-. Moreover, the efficacy of Compound I formation has a dependence on the amount of H_2O_2 available (26). Therefore, characterisation of the detailed mechanism behind the inhibition of HOCl generation by ascorbate is warranted.

Collectively, these data indicate that pharmacological levels of ascorbate can inhibit HOCl production by MPO and that ascorbate can react with MPO to produce species able to oxidize methionine. Little is known about the ability of the products of ascorbate oxidation to react with methionine, with a single paper reporting the observation of free methionine oxidation in solutions containing ascorbic acid where methionine sulfoxide and other unidentified products were detected (30

Further investigation into the mechanism of methionine oxidation by the products of ascorbate oxidation is warranted to determine whether DHM is a characteristic feature of the reaction.

GENERAL DISCUSSION

Pharmacological treatments of cancer patients can be successful but are often confounded by i) a lack of selectivity, ii) the severity of side effects, iii) the development of tumour resistance to treatments, and/or iv) the appearance of secondary tumours due to carcinogenic effects on normal cells. In recent years there has been an interest in using high doses of vitamin C for cancer treatment. A growing body of evidence suggests that pharmacological vitamin C can be effective against tumour cells and generally no negative side effects have been reported. However, the anti-cancer mechanism of high doses of vitamin C is not fully understood and there is speculation that the full potential of vitamin C could be achieved in combination with other treatments. Currently, the anti-cancer mechanism of high doses of vitamin C is explained through pro-oxidant chemistry involving redox-active labile iron and H_2O_2 (7,8). Well-founded doubts remain however, that H_2O_2 generated by high doses of vitamin C could fully explain the anti-cancer effect, but no other feasible explanation has been suggested (9,10).

We have demonstrated that the viability of Jurkat cells was decreased by the presence of millimolar concentrations of ascorbate, and that catalase effectively protected cells. By removing H_2O_2, catalase, prevents the formation of DHA and thus inhibits the toxic effect. The toxicity of high doses of ascorbate could therefore be attributed to the generation of H_2O_2, followed by the formation of oxidized ascorbate, which was the bona fide cause of toxicity. Indeed, we observed cytotoxicity when a mixture of ascorbate and preformed oxidised ascorbate was added to cells in the presence of catalase.

We propose that the maximum anti-cancer effect of pharmacological levels of ascorbate could be achieved by maintaining high concentrations of the products of ascorbate oxidation. This would affect the availability of critical thiol-dependent proteins through exhaustion of the reduction machinery involved in recycling DHA to ascorbate, rather than through ascorbate-driven H_2O_2 production. Further work is needed to confirm this in the physiological setting and employing the Gulo(−/−) knockout mouse model, bearing tumours, would offer a valuable method to test the hypothesis in vivo.

Recent publications highlight the targeting of thiol metabolism as a promising approach to anti-cancer therapy (31,32) and these are supported by the findings that

peroxiredoxins and thiol metabolism are upregulated in tumours (33,34). Peroxiredoxins are thiol-containing proteins that cycle between oxidised and reduced forms, and a growing body of evidence indicates their critical role in cell signalling (35-37). It has been reported that inhibition of peroxiredoxins results in leukemic cell differentiation (38). Prdx2 is essential for proliferation and S-phase progression, and it promotes colorectal cancer cell-cycle progression through activation of the p38 MAPK pathway (39). Thiol oxidation in GAPDH inhibits its enzymatic activity (40), which is essential for glucose metabolism, and tumour growth in vivo is suppressed when the GAPDH redox switch is disabled in tumour cells (41). Our observed inactivation of two thiol-dependent proteins, Prdx2 and GAPDH, which are critical for the survival of cancer cells, warrants further study of these proteins in cells treated with oxidized ascorbate.

High doses of ascorbate are known to be toxic to cells in culture, and the protective effect of catalase appears to support the involvement of H_2O_2 as a culprit (42). Ascorbate is known to generate H_2O_2 in reaction with oxygen and also itself reacts with H_2O_2 (43,44). Therefore, catalase can effectively inhibit the H_2O_2-dependent formation of oxidized ascorbate. In our experiments, it was found that 2.5 mM and 5 mM ascorbate, in the presence of catalase, were indeed harmless for Jurkat cells, while it had a devastating effect when half of it was oxidized immediately before addition to cells. There are several potential mechanisms involved. Modification of Prdx2 and GAPDH may lead to disruptions in cell signalling and glucose metabolism, respectively. In addition, glucose metabolism can be suppressed through competition between glucose and DHA, the initial product of the two-electron oxidation of ascorbate, as both are taken up into cells by the GLUT family of transporters, which are overexpressed in tumors (45). The glucose concentration in blood is approximately 5 mM. Thus, by maintaining vitamin C at 10 mM or higher, one may induce competition for glucose transport into cancer cells. Combined with the inhibition of GAPDH by DHA, this approach could downregulate glucose metabolism. DHA can be effectively reduced by intracellular thiols, e.g. GSH (11), thioredoxin (Trx) (12) or Prdx (this paper). This could result in an elevated rate of thiol oxidation and increased oxidation of NADPH as it is consumed in reactions to reduce the disulfides formed. This might diminish the availability of Trx to ribonucleotide reductase, with a knock-on effect of lowering the production of deoxyribonucleoside precursors necessary for DNA synthesis, and resulting in decreased cell proliferation. Disulfidptosis, a new form of cell death triggered by disulfide stress, is characterized by collapse of the cytoskeleton, F-actin contraction and detachment from the plasma membrane, due to the intracellular accumulation of disulfides (46).

In this study we have demonstrated that oxidized ascorbate inactivated Prdx2 and GAPDH, while no effect was observed for $p16^{INK4A}$. The reactivity of various thiols depends on the nature of the reagent, the protonation state of the thiol, and the surrounding amino acid composition (24, 47-50). The fact that $p16^{INK4A}$ thiol is not susceptible to oxidation by the products of ascorbate oxidation indicates the selectivity of the anti-cancer effect of pharmacological ascorbate.

Direct use of DHA for patient treatment would not be possible, due to its fast rate of decomposition (13,14). More promising would be treatment with ascorbate followed by its oxidation in situ. The oxidation could be performed by MPO from activated neutrophils: ascorbate could be effectively oxidized by HOCl, formed by Compound II of activated MPO, (20) as well as directly by Compounds II and III (16,51).

Here, we have demonstrated that millimolar levels of ascorbate effectively competed with physiological levels of chloride, leading to the generation of oxidized ascorbate and simultaneous inhibition of HOCl generation by MPO.

The observed inhibition of HOCl generation by MPO, in the presence of millimolar ascorbate, may be a promising strategy for the treatment of various conditions in which the side effects of activated MPO are implicated (52). Although pharmacological companies are investing in the search for MPO inhibitors (53-55), a potent inhibitor of MPO, with demonstrated efficacy in human studies, has not yet been identified.

Thus far the results of clinical studies using high doses of vitamin C in the treatment of sepsis have been controversial (56). The underlying problem which may inhibit a positive clinical outcome could lie in the fast clearance of vitamin C. With a half-life of approximately 2 hours, and the elimination of vitamin C from plasma following an exponential pattern, high plasma levels would be short-lived after a single intravenous injection (27). A continuous infusion aimed at maintaining blood ascorbate concentrations in the millimolar range could prove beneficial for inhibiting MPO activity in pathological conditions such as sepsis, where excessive neutrophil activity poses a concern.

Based on our results, we hypothesise that intravenous infusion to maintain a plasma vitamin C concentration in the millimolar range, along with activation of MPO to allow vitamin C oxidation, could be beneficial for anti-cancer therapy (Fig.10).

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Table 2. Oxidation of methionine by the products of ascorbate oxidation.

	Signal intensity		
	Methionine	Dehydromethionine	Methionine sulfoxide
Full system 100 mM NaCl	1.64E+08	5.62E+06	4.32E+05
Full system minus methionine plus ascorbate 100 mM NaCl + methionine after 3 min	1.56E+08 (95%)	1.25E+06 (22%)	2.33E+05(54%)

Full system – MPO, methionine, H_2O_2. Each species was quantified by LC/MS using an MRM method (Table 1).

Table 3. Oxidation of methionine by MPO in reaction with H_2O_2 and ascorbate in the absence of NaCl. Each species was quantified by LC/MS using an MRM method (Table 1).

	Signal intensity		
	Methionine	Dehydromethionine	Methionine sulfoxide
MPO, Asc, Meth + 20 μM H_2O_2	1.16E+08	3.38E+05	4.46E+05
MPO, Asc, Meth + 40 μM H_2O_2	1.11E+08	5.94E+05	4.92E+05
MPO, Asc, Meth + 100 μM H_2O_2	1.11E+08	1.20E+06	5.54E+05
MPO, Asc, Meth + 200 μM H_2O_2	1.16E+08	2.35E+06	4.95E+05

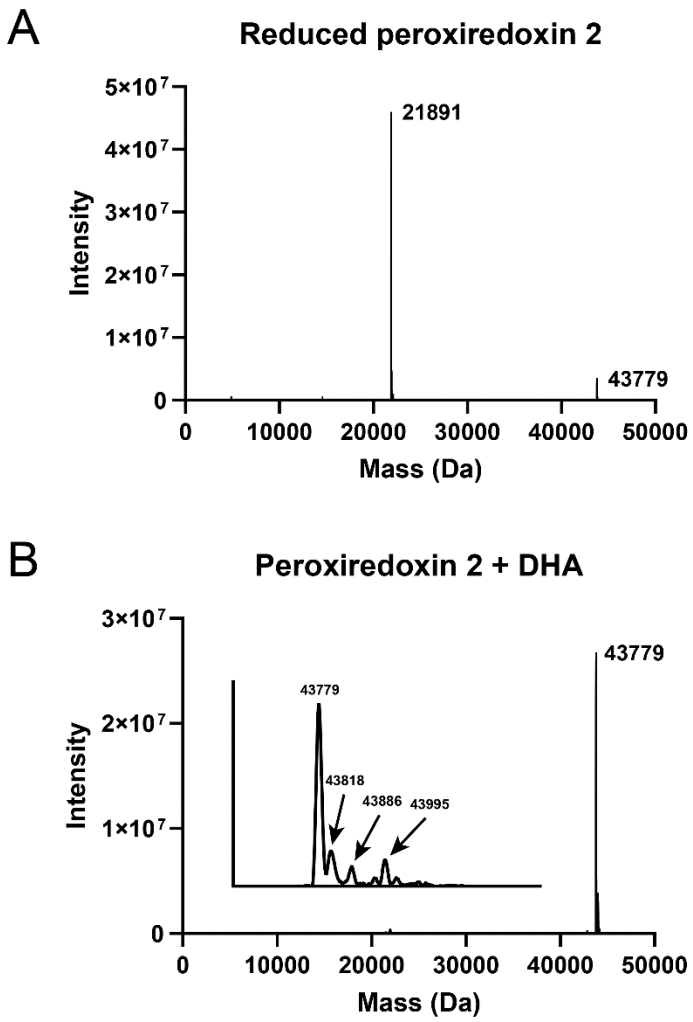
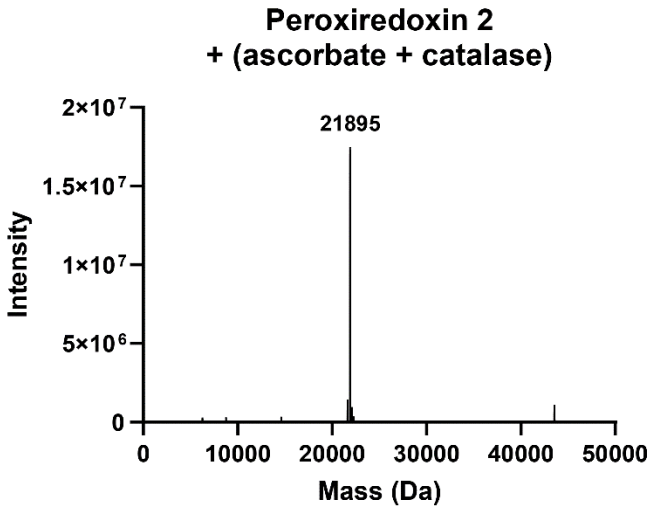


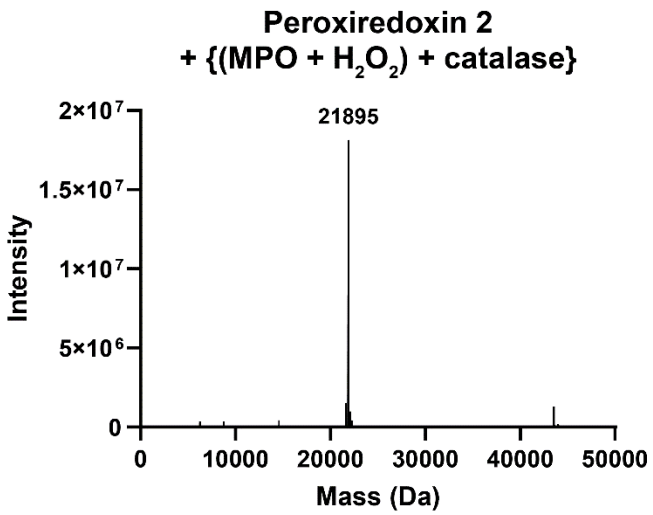
Figure.1. Oxidation of Prdx2 by DHA.

LC/MS analysis of whole protein. Deconvoluted data are shown for reduced Prdx2, 5 µM (A) and Pdx2 after the mixture of DHA, 500 µM with catalase, 10 µg/ml was added to protein (B,C).

A


Peroxiredoxin 2
+ (ascorbate + catalase)
21895
Intensity
2×10⁷
1.5×10⁷
1×10⁷
5×10⁶
0
0
10000
20000
30000
40000
50000
Mass (Da)

B


Peroxiredoxin 2
+ {(MPO + H2O2) + catalase}
21895
Intensity
2×10⁷
1.5×10⁷
1×10⁷
5×10⁶
0
0
10000
20000
30000
40000
50000
Mass (Da)

C

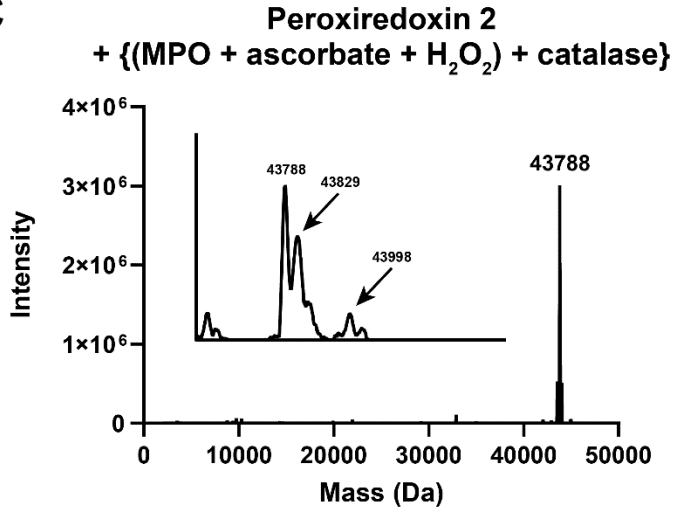

Peroxiredoxin 2
+ {(MPO + ascorbate + H2O2) + catalase}
43788
43829
43998
43788
Intensity
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
0
10000
20000
30000
40000
50000
Mass (Da)

Figure 2. Oxidation of Prdx2 by the products of ascorbate oxidation pre-formed by HOCl.

Whole protein was analysed by LC/MS.Ingredients: Prdx2, 5 µM; MPO, 20 nM; ascorbate, 200 µM; H_2O_2, 40 µM; catalase, 10 µg/ml. A - Prdx2 was added to the mixture of ascorbate with catalase; B - Prdx2 added to MPO; C – H_2O_2 was added to the mixture of MPO with ascorbate, after 5 min catalase was added followed by Prdx2.

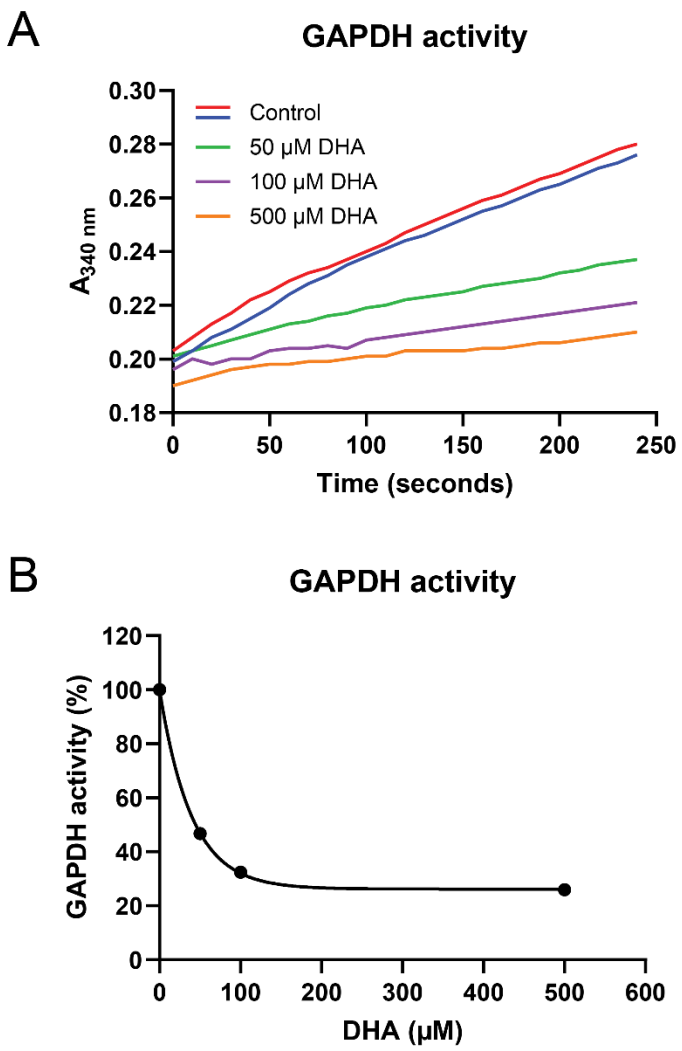


Figure 3. Inhibition of GAPDH by the products of ascorbate oxidation.
Ascorbate was mixed with HOCl at a 2:1 molar ratio, followed by the addition of catalase (10 µg/ml). This mixture was then immediately added to GAPDH. After 30 min, enzymatic activity was measured (see Methods). (A). The indicated concentration of dehydroascorbic acid (DHA) was based on the assumption that half of the ascorbate was oxidized by HOCl to form DHA (B). Control was without DHA.

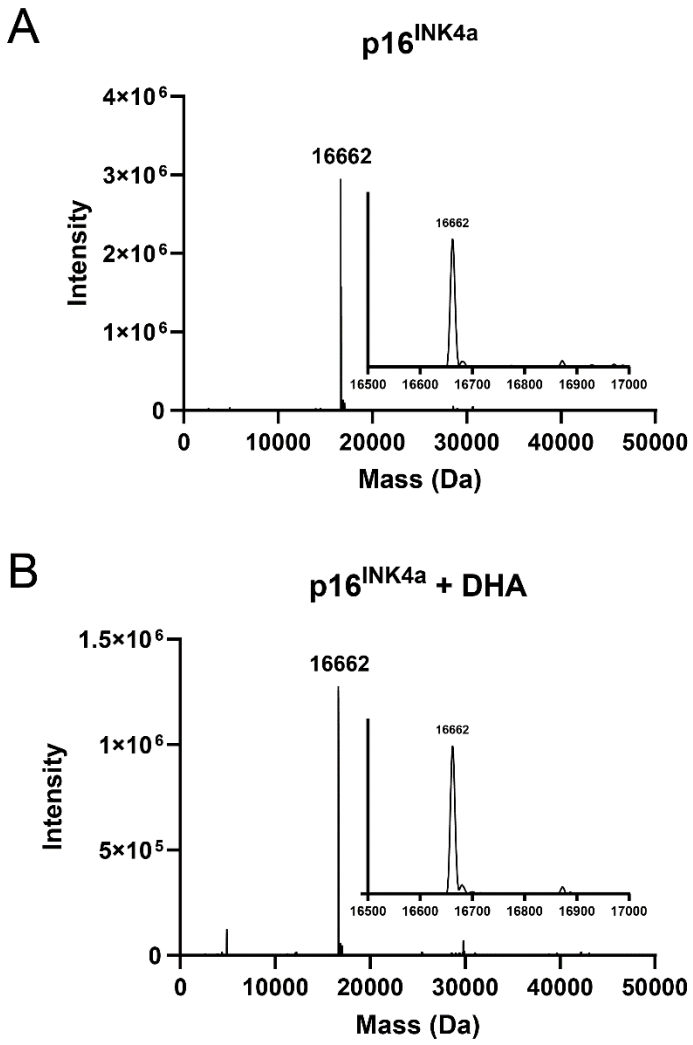


Figure 4. LC/MS analysis of the interaction of products of ascorbate oxidation with $p16^{INK4a}$. 10 µM $p16^{INK4a}$ (A) was mixed with HOCl (2:1 molar ratio) and immediately added to $p16^{INK4a}$ (B) to give 500 µM DHA in reaction mixture. After 60 min incubation the mixture was applied on Micro Bio-Spin 6 columns (Bio-Rad) to remove unreacted low molecular mass molecules.

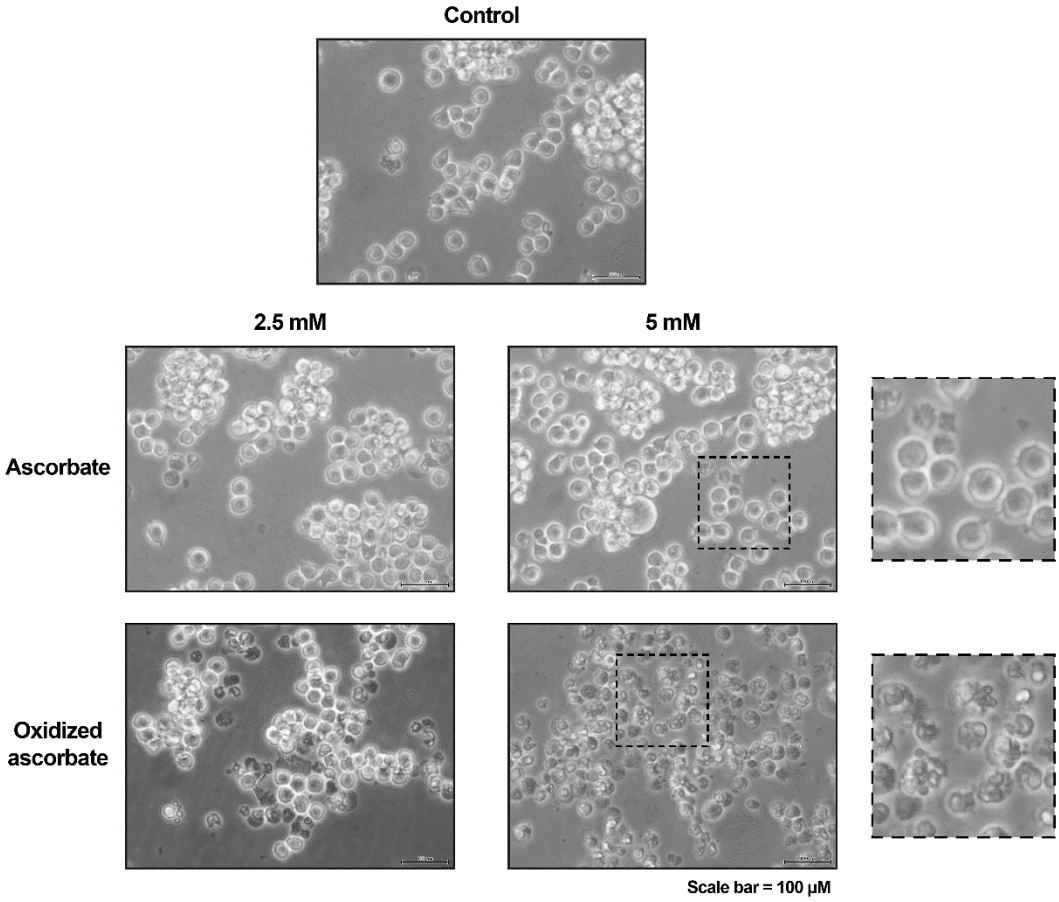


Figure 5. Morphological changes in Jurkat cells incubated in the presence of reduced or oxidized ascorbate. Cells were seeded at a density of 0.25 x 10^6/ml before the addition of either ascorbate or oxidized ascorbate (generated by reaction with HOCl immediately before use) at 2.5 or 5 mM, in the presence of catalase (20 µg/ml). Catalase was also added to control cells. After incubation for 24 h the cells were imaged using a 20x magnification objective, scale bar = 100 µm.

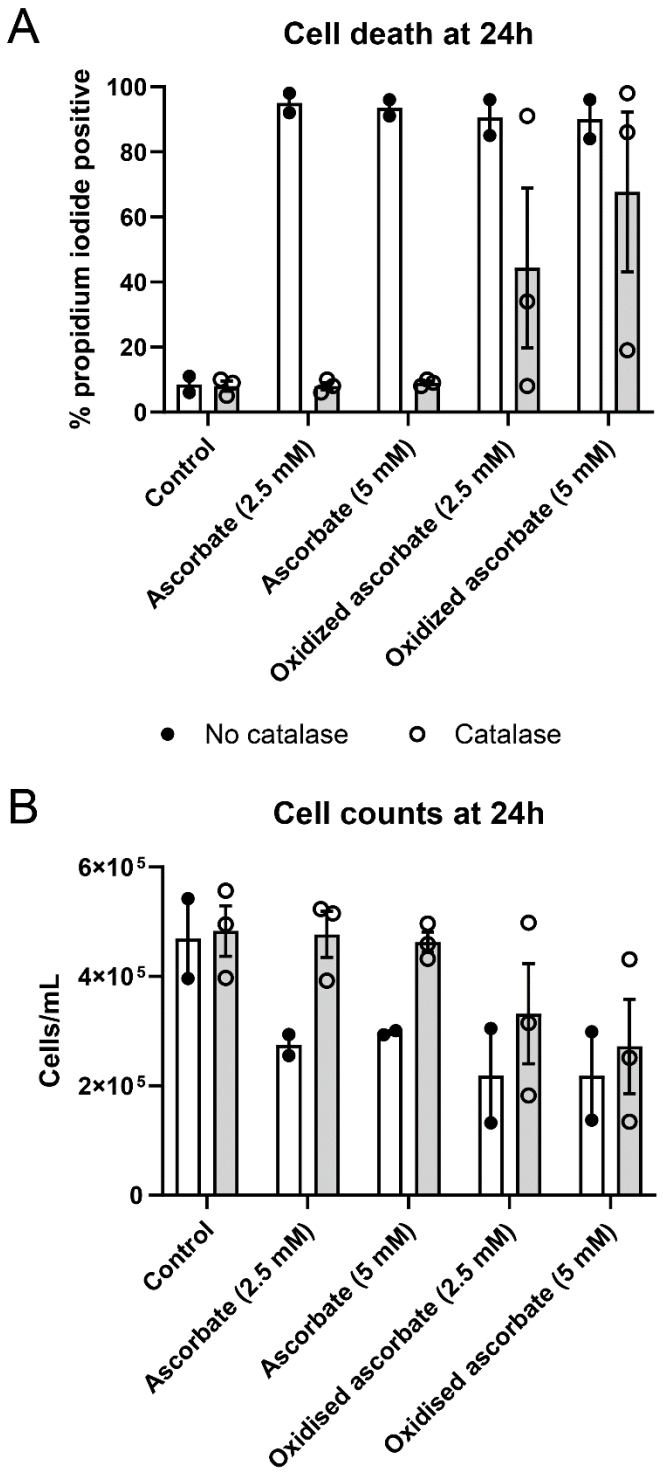


Figure 6. Viability of Jurkat cells after treatment with oxidized ascorbate for 24 h. Ascorbate (0.5 M) was mixed with HOCl (0.25 M), and catalase (20 µg/ml) was added immediately before adding to cells. After 24 h cells were analysed by propidium iodide-dependent fluorescence using flow cytometry. (A) shows % dead cells and (B) the total number of cells.

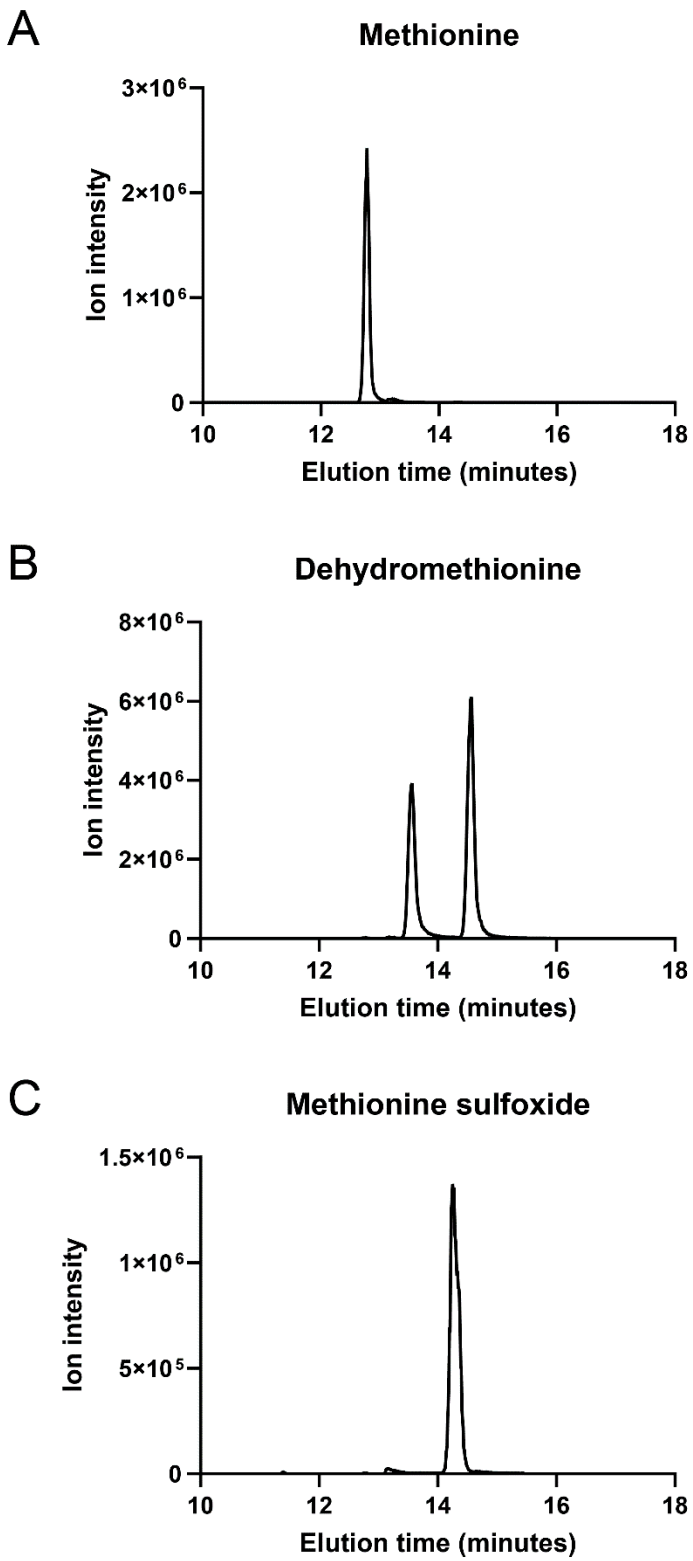


Figure 7. Chromatograms of methionine, dehydromethionine, and methionine sulfoxide. Methionine was mixed with HOCl at the molar ratio 2:1.

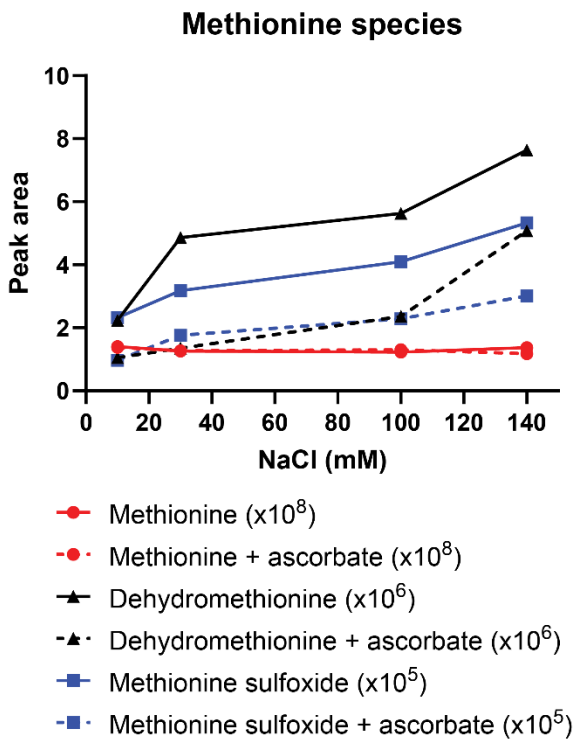


Figure 8. Inhibition of HOCl generation by myeloperoxidase in the presence of ascorbate. The reaction mix contained MPO (40 nM) and methionine (10 mM) and the reaction was started by the additon of H_2O_2 (40 µM). Where present, the ascorbate concentration was 10 mM and catalase (10 µg/ml) was added after 5 min. Protein was removed from samples before analysis by mass spectrometry (see Methods). Recorded species: methionine (red), methionine sulfoxide (blue), dehydromethionine (black), in the absence (solid lines) and presence (dashed lines) of ascorbate. The intensity of the signals were $x10^8$ for methionine, $x10^5$ for methionine sulfoxide and $x10^6$ for dehydromethionine.

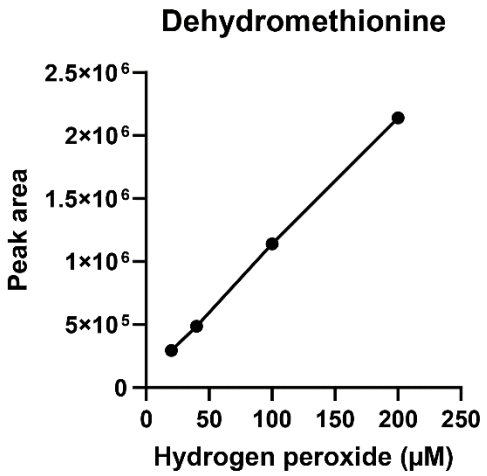


Figure 9. Dehydromethionine formation by MPO in the presence of ascorbate and absence of NaCl.

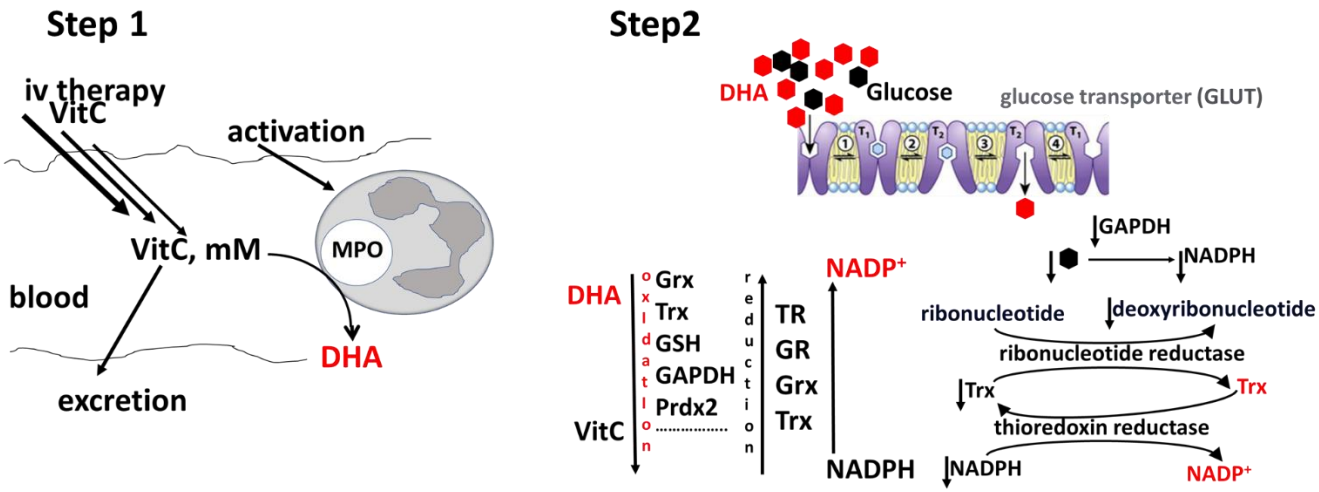


Fig.10. Administering a maintenance infusion of vitamin C to sustain a concentration in the millimolar range can effectively inhibit the generation of hypochlorous acid by activated myeloperoxidase (MPO) and promote the oxidation of vitamin C to its oxidized form, DHA (Step 1). DHA competes with glucose to enter cancer cells, leading to intracellular thiol oxidation and inactivation of critical proteins, subsequent downregulation of reducing power, and a depletion of precursors necessary for DNA synthesis (Step 2).